THE UNIVERSITY OF CHICAGO

# MECHANISMS UNDERLYING ROBUST TRANSPLANTATION TOLERANCE

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# DEDICATION

I would like to dedicate my thesis to my family.

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AICD: activation-induced cell death
ANOVA: analysis of variance
APC: antigen-presenting cell
AR: acute rejection
BAFF: B-cell activating factor
BCR: B cell receptor
Breg: regulatory B cell
BTLA: B and T lymphocyte attenuator
CFSE: carboxyfluorescein succinimidyl ester
CFU: colony forming unit
ChIP: chromatin immunoprecipitation
CTLA-4: cytotoxic T-lymphocyte antigen 4
DAMPs: damage-associated molecular patterns
DC: dendritic cell
DMEM: Dulbecco modified Eagle medium
DSA: Donor specific antibody
DST: donor splenocyte transfusion
ECR: evolutionarily conserved region
EMSA: electrophoretic mobility shift assay
GVHD: graft-versus-host disease
HEL: Hen egg lysozyme
HBSS: Hanks balanced salt solution
HEPES: N-2-hydroxyethylpiperazine-N′-2- ethanesulfonic acid
HVEM: herpesvirus entry mediator
IDO: indoleamine 2,3-dioxygenase
IFNγ: Interferon gamma
Lm: Listeria monocytogenes
LPS: lipopolysaccharide
mAb: monoclonal antibody
MAMPs: microbial-associated molecular patterns
MDSCs: myeloid-derived suppressor cells
MFI: mean fluorescence intensity
MHC: Major Histocompatibility Complex
MLR: mixed lymphocyte reaction
Mreg: regulatory macrophage
MSCs: mesenchymal stromal cells
MST: median survival time
n: number in group
NA: not applicable
N.D.: not determined
ns: not significant
OVA: ovalbumin

P: probability
PBS: phosphate-buffered saline
PD-1: Programmed cell death protein 1
PD-L1: Programmed death ligand-1
PMA: phorbol myristate acetate
PML: progressive multifocal leukoencephalopathy
PTLD: post-transplant lymphoproliferative disorder
SD: standard deviation
SEM: standard error of the mean
TAICs: Transplant acceptance-inducing cells
TCR-Tg: T cell receptor transgenic
Tfh: T follicular helper
Th: T helper [cell]
TLR: Toll-like receptor
TNF$\alpha$: Tumor necrosis factor alpha
Tol: tolerant
Tregs: regulatory T cells
TSDR: Treg cell-specific demethylated region
TSS: transcriptional start site

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ABSTRACT

Since the beginning of vascularized solid organ transplantation in the last century, the goal after successful surgery has been to prevent the new organ from being rejected by the recipient's immune system. To prevent transplant rejection, immunosuppressive drugs have been used, albeit suboptimally and with many side effects. Transplantation tolerance, if achieved, would allow the recipient to accept the transplant long-term following a short-term treatment in the absence of any long-term immunosuppression and is a long-standing goal of the transplantation field. To date, transplantation tolerance has been prospectively induced in a limited number of patients through regimens combining donor bone marrow and solid organ transplantation, and has also developed spontaneously in rare individuals initially treated with conventional immunosuppression. To broaden the applicability of tolerance to all patients, a better understanding of tolerance mechanisms is required. In this thesis, we investigated a mouse model of costimulation-blockade-induced tolerance to understand which mechanisms of tolerance were actively engaged to maintain and define robust tolerance. Tolerance exists on a gradient rather than as an all-or-none state—multiple mechanisms need to be simultaneously engaged in order to maintain robust tolerance. A loss of tolerance can occur if enough mechanisms are simultaneously disrupted, but as these re-engage, tolerance can spontaneously reemerge. Two additional elements of T cell tolerance have been newly identified to occur in this model. Together, these data broaden our understanding of tolerance and afford new avenues of research to investigate with the hope of ultimately achieving transplantation tolerance in the clinic.

# I. INTRODUCTION[1]

## A. Introduction

Transplantation is a cure for end-stage organ failure. However, the transplanted organs are recognized by the recipient immune system as non-self and succumb to rejection unless the transplant recipients are maintained on lifelong immunosuppression. A goal in transplantation is to achieve donor-specific tolerance to the transplanted graft, which is defined as indefinite graft acceptance in the absence of ongoing immunosuppression while the recipient's immune response to infections and malignancies is retained. This state has been successfully achieved in animal models but much more rarely in patients. The mechanisms underlying transplantation tolerance

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[1] **Note:** The following section contains material that was published previously and has been minimally edited for this thesis. All sections of the Introduction were initially published as part of an article titled "Mechanisms Underlying Transplantation Tolerance" for Elsevier's Reference Module in Biomedical Sciences (Miller et al., 2014a) with the following exceptions. Detailed/updated references have been included. Additional material has been added to the subtopic on infections as barriers to transplantation tolerance, which was submitted as "The Impact of Infections on Transplantation Tolerance" to the American Society for Histocompatibility and Immunogenetics for a quarterly newsletter, and to the subtopic on spontaneous transplantation tolerance, which was published previously as "Lessons and Limits of Mouse Models" for the Cold Spring Harbor Perspectives in Medicine (Chong et al., 2013). The summary has been expanded for the thesis.

Mechanisms Underlying Transplantation Tolerance
Authors: Michelle L. Miller, Anita S. Chong, Maria-Luisa Alegre

The Impact of Infections on Transplantation Tolerance
Authors: Michelle L. Miller, Anita S. Chong, Maria-Luisa Alegre

Lessons and Limits of Mouse Models
Authors: Anita S. Chong, Maria-Luisa Alegre, Michelle L. Miller, Robert L. Fairchild

Contributions:

- Co-wrote these three manuscripts with Maria-Luisa Alegre and Anita S. Chong
- Figure 1.3, Figure 1.4, compiled Table 1.2

derive from pathways of central and peripheral immunological tolerance that prevent autoimmunity.

The study of transplantation tolerance stems from observations by Owen in 1945, studying Freemartin cattle (fraternal or dizygotic twins), whose placentas fuse *in utero* thereby allowing cross-circulation and permanent hematopoietic chimerism (Owen, 1945, Figure 1.1, and reviewed in Starzl, 1962). Hematopoietic chimerism refers to the state in which hematopoietic, or immune cells, coexist in the same host in a state of immunological tolerance. This state of tolerance permits the reciprocal acceptance of skin grafts from Freemartin cattle.

This observation was followed by another major discovery in 1953 by Billingham, Brent, and Medawar who demonstrated that antigen-specific tolerance could be therapeutically induced (Billingham et al., 1953). They observed that mice receiving donor hematopoietic cells as fetuses and sometimes as neonates before their own immune systems were fully developed not only accepted the donor hematopoietic cells but could, as adults, also accept skin grafts from donors of the same genetic background as the hematopoietic cells (Billingham et al., 1953). This experiment was fortuitously performed in animals of sufficiently similar genetic background, such that graft-versus-host disease (GVHD) was not at first noticed. GVHD occurs when the donor hematopoietic cells recognize and attack recipient's tissues and is a serious risk of this tolerance induction approach.

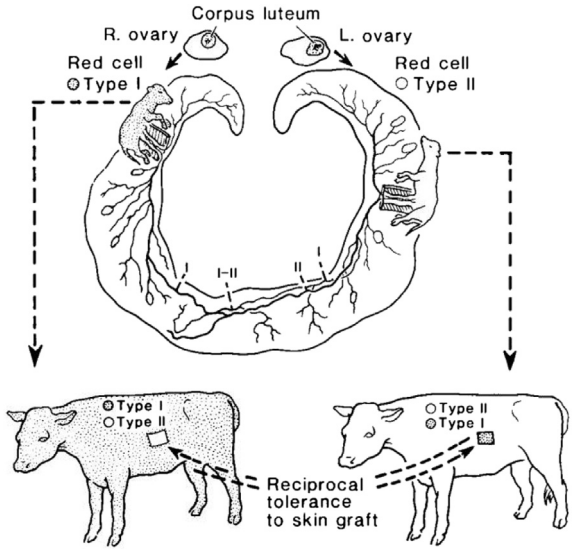


**Figure 1.1: Hematopoietic chimerism in Freemartin (dizygotic twins) cattle described by Owen (1945)**
Reciprocal tolerance formed to hematopoietic cells following intrauterine circulatory exchange after placental fusion in dizygotic twins. These animals then were shown to have mutual tolerance to skin grafts as adults. Reproduced from Starzl and Butz, (1962), *Surgical Clinics of North America,* **42***,* 55–67.

B. Central tolerance

Central tolerance refers to the deletion in the thymus of immature thymocytes expressing T cell receptors (TCR) recognizing self-antigens with high affinity and the development of natural regulatory T cells (nTregs) that express the transcription factor Foxp3 and can suppress the activity of other immune effector cells (Hori et al., 2003; Hsieh et al., 2004). Thymocytes entering the thymus first undergo positive selection, ensuring they can recognize self-Major Histocompatibility Complex (MHC) molecules, followed by negative selection to purge autoreactive thymocytes that recognize self-MHC with high affinity. In addition, a specific subset of antigen-presenting cells (APCs) in the thymus presents antigens normally expressed only in peripheral organs.

Expression of these self-antigens in the thymus is controlled by the transcription factors AIRE (Anderson et al., 2002, 2005) and Fezf2 (Takaba et al., 2015) and is thought to be critical for the deletion of T cells recognizing these tissue antigens as well as for the development of self-reactive Tregs (Malchow et al., 2013). Patients with hypomorphic AIRE mutations develop profound autoimmunity. After passing both positive and negative selection, thymocytes exit the thymus and enter the rest of the body–often referred to as the periphery—as mature T cells. Some T cells that are self-reactive but survive the negative selection process can become anergic (hyporesponsive). Other T cells with self-reactivity may escape negative selection and circulate in the periphery as potential effector T cells (Taniguchi et al., 2012). These cells pose an autoimmunity threat unless they are controlled by peripheral mechanisms of tolerance that will be described in later sections.

B cells undergo selection in the bone marrow, and this process is based also on the strength of B cell receptor (BCR) signaling. B cells with a self-reactive BCR undergo further rearrangement of their BCR genes through a process called receptor editing to generate B cells that are no longer self-reactive (Casellas et al., 2001; Gay et al., 1993; Tiegs et al., 1993). Autoreactive B cells that escape negative selection in the bone marrow can still becoming hyporesponsive or anergic in the periphery so as to prevent their effector function and ensure self-tolerance (Goodnow et al., 1988; Merrell et al., 2006).

Though only peripheral tolerance mechanisms were examined in this thesis, it is important to understand how peripherally programmed deletion, regulation and

hyporesponsiveness maintain tolerance in relation to deletional and regulatory central tolerance mechanisms as there is substantial overlap in the mechanisms programmed in these two manners. Two strategies to manipulate central tolerance mechanisms to induce tolerance to transplant antigens are outlined below.

1. Intra-thymic delivery of donor antigens

The process of central tolerance to avoid autoimmunity has been harnessed experimentally to induce tolerance to transplanted organs (Chen et al., 1997; Stadlbauer et al., 1997). Donor antigens have been injected intra-thymically into recipients to be processed and presented to developing thymocytes thus inducing their deletion through negative selection, as well as promoting the anergy or unresponsiveness of cells that escape death (Sayegh et al., 1994). Injecting donor antigen in the form of peptides in the thymus has been shown to produce nTregs that are important for maintaining tolerance to islet allografts in rats (Saborio et al., 1999). In a similar approach, donor splenocytes that were injected intra-thymically in combination with transient peripheral T cell depletion induced tolerance to donor cardiac allografts in mice (Jones et al., 1998). When donor cells persisted in the thymus, prolonged deletion of donor-reactive thymocytes was observed and donor-specific tolerance was induced. However, when this approach was attempted in a limited number of human heart transplant patients, it did not prevent rejection (Remuzzi et al., 1995).

2. Chimerism

a. Total hematopoietic chimerism

The success of hematopoietic chimerism at inducing transplantation tolerance has prompted investigations into developing clinically applicable protocols. Early protocols aimed at complete donor hematopoietic chimerism in patients involved total body irradiation of recipients and an allogeneic bone marrow transplant from the same donor as a kidney graft (Sayegh et al., 1991). Central tolerance in this case is induced by donor hematopoietic cells that give rise to APCs that repopulate the recipient thymus and facilitate negative selection or anergy of donor-reactive T cells. However, total body irradiation incurs great morbidity while the complete replacement of recipient APCs with donor APCs results in sub-optimal protective immune responses against infections, as T cells activated via recognition of microbe-derived antigens on donor MHC expressed by peripheral donor APCs will likely fail to recognize infected host cells that express recipient MHC. In addition, GVHD frequently results from this procedure, and thus less myeloablative strategies were developed to circumvent these morbidities.

b. Mixed hematopoietic chimerism

Mixed hematopoietic chimerism is the state in which both recipient and donor hematopoietic cells co-exist, resulting in immune competence and reduced vulnerability to GVHD. Protocols for inducing mixed hematopoietic chimerism combine immunosuppressive drugs with low-level of total body irradiation or irradiation localized to lymphoid organs (to prevent host cells from destroying the donor hematopoietic cells)

as a way of immune pre-conditioning the host (reviewed in Pilat and Wekerle, 2010, Figure 1.2). Combined bone marrow and solid organ transplants have been shown to result in prolonged graft survival in pre-clinical models and in humans. The first studies in humans were in multiple myeloma patients who received combined HLA-matched kidney and bone marrow transplants (Fudaba et al., 2006). Later trials enrolled recipients of HLA-mismatched kidney and bone marrow transplants to induce mixed chimerism (Kawai et al., 2008; Scandling et al., 2012). Despite the fact that all the HLA-mismatched patients rapidly lost donor chimerism, the majority were successfully weaned from their immunosuppression and graft survival was maintained for many years, demonstrating that this approach can induce transplantation tolerance in humans (Spitzer et al., 2011) (Table 1.1).

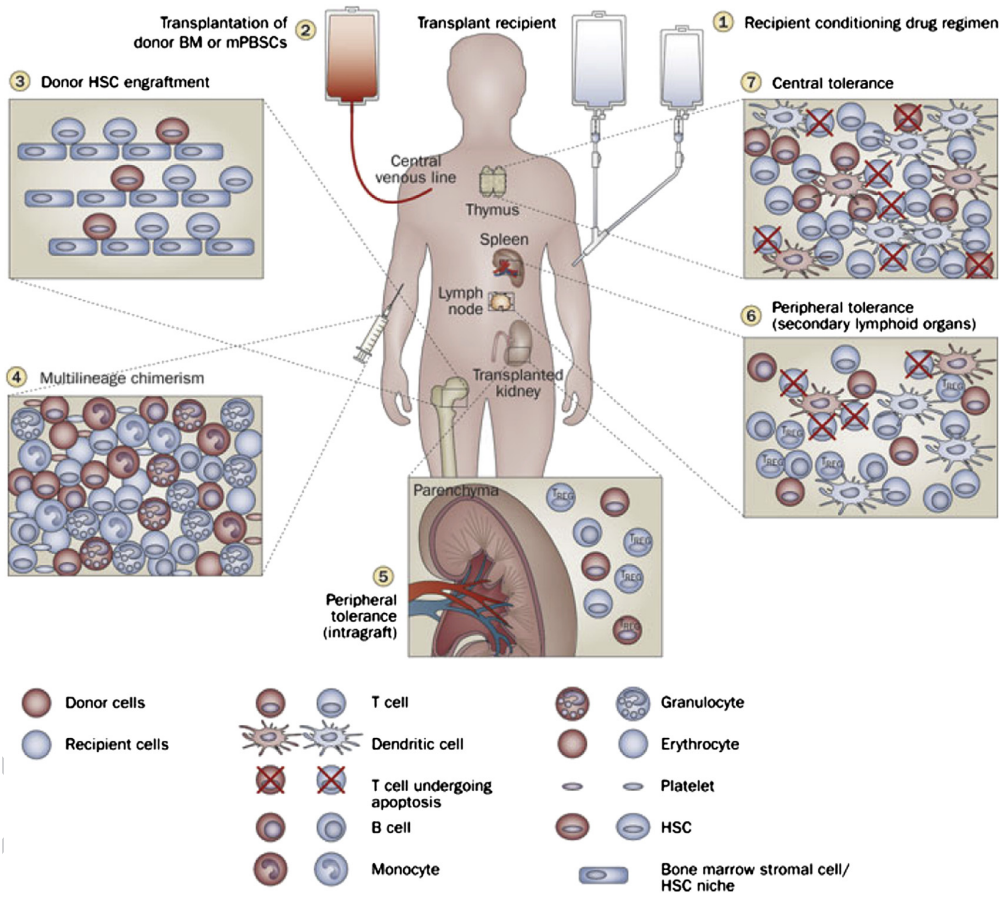


**Figure 1.2: Tolerance through mixed chimerism**
(1) In order to allow engraftment of the allogeneic bone marrow (BM) that is to be transplanted, a combination of drugs is given (in some cases along with irradiation) to condition the recipient. (2) Infusion of donor BM or mobilized peripheral blood stem cells (mPBSCs). (3) Donor hematopoietic stem cells within the bone marrow transplant engraft in the recipient's bone marrow stem cell niches where they co-exist with recipient hematopoietic stem cells (HSCs). (4) Donor and recipient HSCs are a continual, long lasting, self-renewing source of donor and recipient hematopoietic cells of all lineages, leading to multilineage mixed chimerism (conveniently detected in peripheral blood). Pre-existing mature T cells that are donor-reactive (and presumably newly developing donor-reactive T cells escaping negative selection in the thymus) are tolerized in the periphery through both deletional and non-deletional mechanisms likely occurring in secondary lymphoid organs (6) and in the graft itself (5). (6) Donor antigen presenting cells (APCs, in particular B cells) and recipient APCs (dendritic cells and B cells) facilitate progressive clonal deletion and regulation of donor-reactive T cells. (7) Critically, donor dendritic cells seed the thymus where they mediate central tolerance through negative selection of newly developing donor-reactive thymocytes, creating a T-cell repertoire tolerant to both self and donor (that is, 'new self'). Figure originally appeared in Pilat N and Wekerle T. (2010). Transplantation tolerance through mixed chimerism. *Nature Reviews Nephrology* **6**, 594-605.

| Organ | Conditioning[a] | Immuno-suppression[a] | Hematopoietic chimerism[b] | Allograft survival[c] | Reference |
|---|---|---|---|---|---|
| Kidney (HLA-matched) | ALG; CP | Maintenance CsA azathioprine; prednisone | N/A | 41/54 pts (Median follow-up: 16 mo) | (Barber et al., 1991) |
| | ALG;CP;donor BM | Maintenance CsA azathioprine; prednisone | N/A | 54/57 pts (Median follow-p: 16 mo) | |
| Kidney (HLA-matched) | CP; ATG; TI; donor BM | 2 months CsA | Transient/ persistent | >87, >63, >51, >42, >33, >24 mo | (Bühler et al., 2002; Fudaba et al., 2006; Spitzer et al., 1999) |
| Kidney (HLA-matched) | ATG; TLI; donor PBSC | 6 months CsA | Persistent | >28 mo avg (16 pts) | (Scandling et al., 2012) |
| Kidney (haplo-compatible) | TBI, ARA-C, CP, ATG (splenectomy) | 10 months CsA, Pred | Persistent | >15 mo | (Sorof et al., 1995) |
| Kidney (related donors) | Not specified, prior BMTx to treat hematological disorders | None | Persistent | >30, >15, >3 mo | (Butcher et al., 1999) |
| Kidney (at least HLA-DR mismatched) | Donor BM | 0.5 month ALG + maintenance | Persistent microchimerism | 21/23 pts at 12 mo (but "chronic rejection") | (McDaniel et al., 1994) |
| | | | Transient/ND | 1/7 patients at 12 mo | |
| Kidney (HLA-mismatched) | CP; αCD2; TI; donor BM; pts 4&5: +rituximab +Pred | ≤14 months CsA/Rapa | Transient | >76, >70, >43, >36, **0.3** mo; donor-specific antibodies | (Kawai et al., 2008; Porcheray et al., 2009) |
| Kidney (HLA-mismatched) | TLI; ATG; rituximab; CP; PBSC + HSCT +/- MSC | First 3 months: CsA, Pred After 3 months: azathioprine; prednisone | Transient/ND | 96/100 at 12 mo +MSC | (Vanikar et al., 2011) |
| | | | | 85/100 at 12 mo -MSC | |
| Kidney (HLA-mismatched) | Fludarabine; TBI; CP; donor HSC + FC | FK506, mycophenolate mofetil | Transient/persist ent | >32, >24 (2 pts), >12 (4 pts), **3** mo | (Leventhal et al., 2012) |
| Pancreatic islet (HLA-mismatched) | High dose HSC | 12 months "Edmonton" (FK506, Rapa) | Transient | **17, 16, 15.7, 15, 6, 5.3** mo | (Mineo et al., 2008) |
| Liver | ATG; CP; donor HSC | 1-3 months FK506, Rapa | Transient/ND | >12.3, >9 mo | (Donckier et al., 2004) |

**Table 1.1: Combined bone marrow and organ transplantation in humans**

**Table 1.1, continued:** [a] α, antibody to; ALG, anti-lymphocyte globulin; ARA-C, arabinofuranosyl cytidine; ATG, anti-thymocyte globulin; BM, bone marrow; CsA, cyclosporin A; CP, cyclophosphamide; FC, facilitating cells; HSC, CD34 + hematopoietic stem cells; mo, months; MSC, adipose-derived mesenchymal stem cell; PBSC, peripheral blood stem cells; Pred, prednisone; pts, patients; Rapa, Rapamycin; TBI, total body irradiation; TI, thymic irradiation; TLI, total lymphoid irradiation. [b] N/A, not analyzed; ND, not detected. [c] Patients that rejected their allografts are indicated in bold. All times are listed in months post-transplantation. Reproduced with modification from Pasquet, L. et al., (2011). Hematopoietic chimerism and transplantation tolerance: a role for regulatory T cells. *Frontiers in Immunology*, **2**, p.80.

Several models suggest that mixed chimerism as a tolerance induction protocol is not sufficient to prevent long-term chronic rejection if chimerism is lost (Pasquet et al., 2011). However, it has been a challenge to achieve stable mixed chimerism in humans. Suzanne Ildstad in 1994, reported that "facilitating cells" were required for optimal donor bone marrow engraftment with minimal GVHD (Kaufman et al., 1994). A protocol with non-myeloablative conditioning and transplantation of facilitating cells was reported to successfully induce tolerance in recipients of HLA-mismatched kidney transplants (Leventhal et al., 2012).

Other approaches to achieve stable mixed chimerism have included the administration of *in vitro* generated Tregs. In a couple of reports, Tregs were combined with donor umbilical cord blood cells to reduce GVHD in patients receiving unrelated (HLA-mismatched) umbilical cord blood cells as a source of hematopoietic stem cells for transplantation (Brunstein et al., 2011, 2015). These studies reported on a moderate reduction in the rates of GVHD.

Though mixed hematopoietic chimerism was driven conceptually as a means to induce central tolerance, it has been shown to also induce peripheral mechanisms of

tolerance, involving peripheral T cell deletion and anergy as well as regulation by Tregs (Bigenzahn et al., 2005; Domenig et al., 2005; Kurtz et al., 2004; Lucas et al., 2011; Wekerle et al., 2003). While mixed chimerism was not examined in this thesis it is the only strategy that has been able to induce tolerance prospectively in the clinic and understanding the mechanisms of tolerance that it imposes should inform future strategies of tolerance induction.

## C. Peripheral tolerance

T cells and B cells with self-reactivity can escape negative selection and populate the periphery. To prevent autoimmunity, peripheral mechanisms of tolerance control self-reactive T and B cells by inducing their deletion, rendering them intrinsically anergic, or extrinsically suppressing them. Many strategies to induce tolerance to transplant antigens attempt to exploit these peripheral mechanisms of tolerance, though they have failed so far to induce tolerance to transplants in humans despite promising results in animal models.

### 1. Anergy

B cells and T cells can both become anergic in the periphery. T cell anergy is a state of intrinsic hyporesponsiveness in which cells produce less IL-2 and show decreased proliferation when they are restimulated (Jenkins and Schwartz, 1987; Schwartz et al., 1989). T cell anergy can be induced by TCR stimulation in the absence of costimulation, suboptimal TCR engagement by altered peptide ligands, high calcium

flux with ionomycin, and some bacterial superantigens (Chai and Lechler, 1997; Macián et al., 2002; Miller et al., 1999; Sloan-Lancaster et al., 1993). Production of other cytokines such as IFNγ and other functions such as cytotoxicity may or may not be reduced (Sundstedt et al., 1994). Molecularly, the anergic state is often observed to correlate with NFAT signaling without AP-1, and diminished Ras/MAPK signaling (Fields et al., 1996; Hsiao et al., 2009; Sundstedt and Dohlsten, 1998; Sundstedt et al., 1996). The anergic state is enforced by ubiquitin ligases, which degrade or inactivate downstream TCR signaling molecules (Anandasabapathy et al., 2003; Jeon et al., 2004).

B cells also acquire a state of cell-intrinsic hyporesponsiveness or anergy that can be induced by BCR recognition of antigen in the absence of secondary signals, and this event leads to a downregulation of BCR expression and partial blockade of downstream BCR signaling pathways (Cooke et al., 1994; Healy et al., 1997). Subsequent encounter with antigen by these anergic B cells then results in blunted B cell responses.

a. Costimulation blockade

T cells integrate multiple secondary signals at the time of TCR engagement with cognate antigen in order to mount an appropriate inflammatory response, or to enter a hyporesponsive state. These secondary signals are delivered by ligands on APCs engaging costimulatory receptors on the surface of T cells, and can either be activating or inhibitory (reviewed in McGrath and Najafian, 2012). T cells that receive a signal

through their TCR in the absence of positive costimulation or in the presence of negative costimulation can become hyporesponsive or die. Costimulation blockade that interferes with positive costimulatory signals can induce T cell hyporesponsiveness or deletion and affect Treg function. Therapies blocking CD154/CD40 signaling (used in this thesis) as well as other costimulatory targets are outlined below.

i. Anti-CD154/anti-CD40

Anti-CD154 (CD40L) is a monoclonal antibody developed to block interactions between CD154 on activated T cells and CD40 on APCs. In various mouse models, anti-CD154 induced long-term donor-specific tolerance through several mechanisms of peripheral tolerance, including the deletion of graft-reactive T cells, an increased percentage of regulatory cells and the induction of hyporesponsiveness in the remaining graft-reactive cells (Iwakoshi et al., 2000; Pinelli et al., 2013; Quezada et al., 2005). This treatment also promoted the upregulation of several negative costimulatory receptors on T cells such as PD-1 and CTLA-4 (Haspot et al., 2008).

In non-human primate models, this antibody was efficacious at inducing long-term graft acceptance (Kirk et al., 1999). Unfortunately, when anti-CD154 was used in early clinical trials, several adverse events involving thromboemboli resulted in the early termination of the trials (Kawai et al., 2000). It was subsequently determined that CD154 was expressed on human platelets (Henn et al., 1998; Koyama et al., 2004). Because of the efficacy of anti-CD154 at inducing transplantation tolerance, there is still interest in developing monoclonal antibodies that block this pathway and several groups

are developing blocking antibodies that target the costimulatory receptor CD40 itself. So far, anti-CD40 antagonistic antibodies have been reported in combination with CTLA-4-Ig to induce tolerance to skin grafts following bone marrow transplantation in mouse models and to prolong islet and kidney allograft survival in two different non-human primate models (Adams et al., 2005; Badell et al., 2012; Gilson et al., 2009).

ii. CTLA4-Ig

One costimulatory pathway that has been used to prolong graft survival in animal models and human transplant patients is the membrane-bound receptor cytotoxic T-lymphocyte antigen 4, or CTLA-4. CTLA-4 is a negative costimulatory molecule that has homology to CD28, an important activating costimulatory receptor on T cells. CD28 binds to its ligands CD80 (B7-1) and CD86 (B7-2) on APCs, but CTLA-4 has much higher affinity for CD80 and CD86 than CD28, thus competing out their ability to bind to CD28 and give a positive signal to T cells (van der Merwe et al., 1997). CTLA-4 can also pull CD80 and CD86 from the APC surface and internalize them into the T cell by a mechanism called trogocytosis that leaves the APC unable to engage CD28 molecules on T cells (Qureshi et al., 2011). The cytoplasmic tail of CTLA-4 can also stimulate phosphatases that inhibit activating signals from the TCR (Bradshaw et al., 1997). Finally, CTLA-4 binding to CD80 and CD86 can induce these 2 molecules to activate the enzyme indoleamine 2,3-dioxygenase (IDO) that degrades tryptophan, an essential amino acid required by T cells to proliferate (Mellor et al., 2004) (Figure 1.3). These T cell-inhibitory properties of CTLA-4 prompted the generation of the immunosuppressive

drug CTLA-4-Immunoglobulin (CTLA-4-Ig). CTLA-4-Ig is a soluble form of the extracellular domain of CTLA-4 fused to a human IgG tail to increase its half-life. In mouse models, CTLA-4-Ig has been shown to induce long-term acceptance of cardiac allografts and, in combination with anti-CD154, of skin allografts (Larsen et al., 1996).

Abatacept is a CTLA-4-Ig protein that has been approved in 2005 for use in human patients with rheumatoid arthritis and, in 2011, belatacept, a second-generation CTLA-4-Ig, was approved by the FDA for use in kidney transplant recipients. Belatacept differs from CTLA-4-Ig by 2 amino acids in the CD80- and CD86-binding domains resulting in higher binding affinity (Larsen et al., 2005). In a human Phase III clinical study, belatacept treatment resulted in long-term graft survival and better long-term renal function (as measured by glomerular filtration rate) despite increased episodes of acute rejection compared to cyclosporine, a commonly used immunosuppressant that has renal toxicity that shortens the lifespan of kidney allografts (Vincenti et al., 2010, 2016). One potential caveat with the high affinity belatacept is that it not only blocks CD28 engagement but also that of T cell endogenous CTLA-4. CTLA-4 is an important negative regulatory signal for effector T cells and plays a role in Treg suppressive function (Read et al., 2000; Wing et al., 2008). Therefore belatacept may block or diminish these negative signals resulting in increased incidence of acute rejection. Use of single chain anti-CD28 antibodies that lack agonistic activity and are able to prevent CD28 but not CTLA-4 engagement may become a better strategy and is under investigation (Suchard et al., 2013).

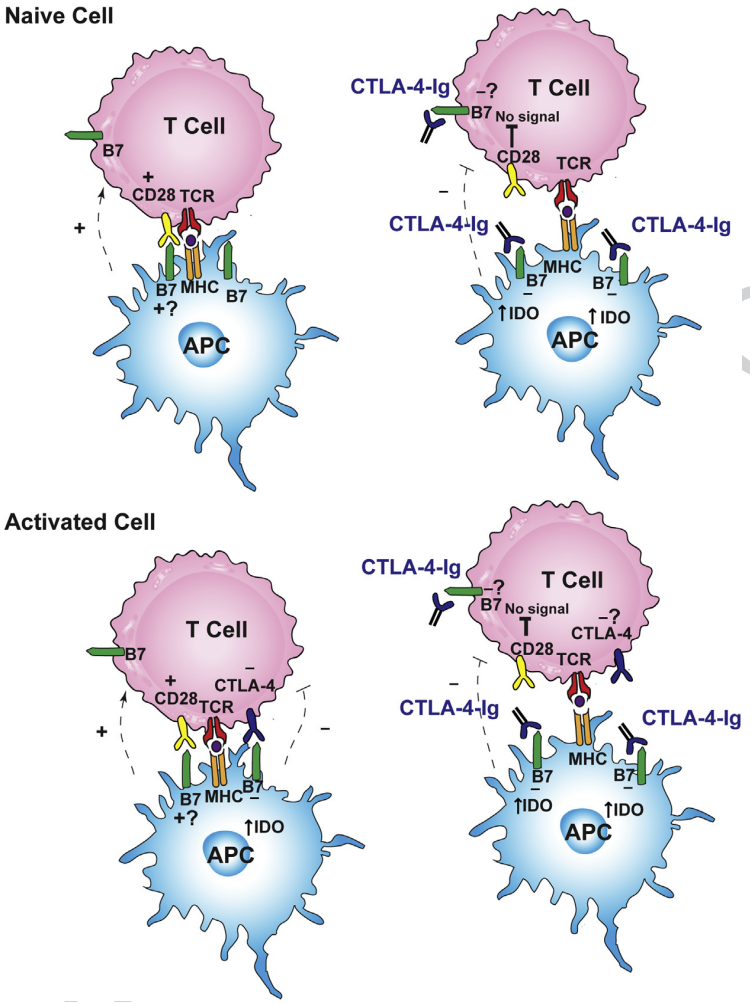


**Figure 1.3: CTLA-4-Ig affects signaling on naïve and activated T cells**
A naïve (top) and previously activated (bottom) T cell interaction with an APC in the absence (left) and in the presence (right) of CTLA-4-Ig. "+": activating signals, "-" inhibitory signals, and "?" suggests putative signaling.

iii. Other potential therapeutic targets

1. PD-1 and PD-L1 and PD-L2

PD-1 is a negative costimulatory molecule that is upregulated on activated T cells, B cells and myeloid cells to dampen their activation in a negative feedback loop (reviewed in McGrath and Najafian, 2012). It is also a marker of chronic antigen exposure in exhausted T cells such as following chronic viral infections (Barber et al.,

2006). PD-1 is often expressed on graft-infiltrating cells in tolerant hosts. The two ligands for PD-1 are expressed on different subsets of cells. PD-L1 is expressed on many cell types of hematopoietic and non-hematopoietic origin, while PD-L2 is inducibly expressed on macrophages and dendritic cells. Blocking PD-1/PD-L1 interactions with an antagonistic anti-PD-L1 antibody has been shown to precipitate rejection in several animal models, suggesting an intact PD-1:PD-L1 pathway is important for long-term graft acceptance and tolerance (Tanaka et al., 2007). PD-1 is also expressed by regulatory T cells and may be important for their suppressive capabilities (Raimondi et al., 2006). PD-L1-Ig is an agonistic reagent for PD-1 that has been shown in combination with cyclosporine to prolong cardiac allograft survival in a mouse model (Ozkaynak et al., 2002). Combining PD-L1-Ig with anti-CD154 showed a synergistic prolongation of islet allograft survival in an animal model (Gao et al., 2003). PD-L1 can also bind to B7-1 and it is unknown how agents such as belatacept may interfere with the interaction between PD-L1 and B7-1 (McGrath and Najafian, 2012). Blocking anti-PD-1 and anti-PD-L1 antibodies are being used in clinical trials to enhance immune responses to cancers (Chen and Han, 2015), but agonistic reagents for clinical transplantation have not yet been reported.

2. BTLA

B and T Lymphocyte Attenuator, BTLA, has structural similarity to PD-1 and CTLA-4, as all three are members of the immunoglobulin superfamily. BTLA is a negative costimulatory molecule when it binds to its ligand, herpesvirus entry mediator

(HVEM). BTLA and HVEM are expressed on T, B, and NK cells as well as on dendritic cells and endothelial cells, and BTLA is highly expressed on anergic T cells (Hurchla et al., 2005). In transplantation, depleting and non-depleting anti-BTLA antibodies have been used to prolong islet allograft survival, and have been shown in combination with CTLA-4-Ig to enhance graft-infiltrating Tregs and to increase PD-1 expression on $CD4^+$ T cells (Truong et al., 2007). In other studies, blocking BTLA led to accelerated rejection of partially MHC-mismatched cardiac allografts in mouse models but played a less critical role in the rejection of fully MHC-mismatched transplants (Tao et al., 2005). The difference in outcomes may be due to different monoclonal antibodies used in those studies (McGrath and Najafian, 2012).

3. Tim-3

T cell immunoglobulin-3, Tim-3, is important for the function of Tregs, and blocking its interaction with its ligand galectin-9 leads to reduced suppressive capacity of Tregs (Sabatos et al., 2003). In transplant models, treatment with anti-Tim-3 led to rejection of allografts in a Treg-dependent model of islet transplantation tolerance induced with anti-CD154 plus donor-specific transfusion (DST) and in a murine fully-mismatched cardiac allograft model (Boenisch et al., 2010; Sánchez-Fueyo et al., 2003). Galectin-9 is selectively expressed on Tregs and not other T cells, and its addition promoted longer survival of skin and cardiac allografts (He et al., 2009; Wang et al., 2008a, 2009a).

2. Deletion

Deletion of lymphocytes in the periphery can be due to “passive” cell death or activation-induced cell death (AICD) (Wells et al., 1999). Passive cell death results from deprivation of pro-survival cytokines and decreased expression of anti-apoptotic proteins such as Bcl-$x_L$. AICD results from repeated stimulation through the TCR and is mediated by signals from the tumor necrosis factor receptor, TNFR, or Fas receptor and can occur even in the presence of Bcl-$x_L$ or its related family member Bcl-2 (Guerrero et al., 2013). Absence of peripheral deletion in Fas-deficient (Fas/lpr) mice or in mice deficient for the pro-apoptotic Bcl-2 family member Bim leads to autoimmunity (Bouillet et al., 1999; Hughes et al., 2008; Hutcheson et al., 2008). Peripheral T cell deletion has been shown to play a role in many strategies of transplantation tolerance induction (Haspot et al., 2008; Wekerle et al., 2001; Wells et al., 1999). However, another study did not identify a role for passive cell death pathways in tolerance induction (Lehnert et al., 2007). Certain instances of peripheral “deletion” may be masked by expansion of other subsets of T cells as has been observed for the disappearance of low avidity T cells following peptide immunization without a detectable increase in apoptosis (Baumgartner et al., 2012). More research needs to be done to determine which cell death mechanisms play a role in peripheral deletion during tolerance induction and to determine whether deletion affects all alloreactive T cells equally or only certain subsets.

*a.* i. *In vivo* depletion

Peripheral depletion of all T cells is used for tolerance induction in animal models and as immunosuppression in patients, using global lymphodepleting agents such as CAMPATH-1 (also known as alemtuzumab or anti-CD52), anti-TCR$\alpha\beta$, OKT3 (anti-CD3), MEDI-507 (anti-CD2), and rabbit anti-thymocyte globulin (ATG) (Abramowicz et al., 1992; Chatenoud et al., 1990; Chavin et al., 1992; Kirk et al., 2003; Shapiro et al., 2005; Soulillou et al., 1990). Daclizumab (discontinued) and basiliximab are two anti-CD25 monoclonal antibodies used to selectively deplete activated T cells which express CD25 (IL-2R$\alpha$); however, this marker is also expressed on Tregs and this treatment could also deplete these cells (Page et al., 2012). There are several risks associated with the use of these global lymphodepleting agents. First, severe lymphodepletion leaves patients susceptible to infections and malignancies until the immune system recovers. Second, the remaining T cells and new thymic emigrants expand dramatically in a lymphopenic environment and differentiate into memory-like T cells that are more difficult to immunosuppress (Wu et al., 2004). Lymphopenia-induced proliferation has also been shown to revert cells from an anergic phenotype into one of regained responsiveness and can break tolerance (Brown et al., 2006; Iida et al., 2013; Schietinger et al., 2012). Thus, depleting agents may paradoxically be detrimental for tolerance induction.

Alloantibodies can lead to allograft rejection and prevent the induction of tolerance, thus depletion of B cells and/or antibody-producing plasma cells may aid the induction of tolerance. A number of approaches have been developed to deplete B

cells. The monoclonal antibody against CD20, rituximab, has been used in transplant patients to deplete B cells while the proteasome inhibitor bortezomib has been used to target plasma cells (van den Hoogen et al., 2015; Wang et al., 2015). Belimumab is a monoclonal antibody that blocks B cell activating factor (BAFF) signaling to B cells. BAFF promotes the survival and proliferation of B cells, and its blockade in mouse models of cardiac and islet transplantation has promoted tolerance through follicular and alloreactive B cell depletion (Parsons et al., 2012). However, a phase II clinical trial in patients with high levels of alloantibodies awaiting kidney transplants was not deemed effective because the study did not meet its primary efficacy goal to reduce circulating alloantibody levels. Atacicept and BR3-Fc are two other BAFF-blocking agents that have shown reduction in alloantibodies and peripheral B cells in non-human primate transplant models although they have not yet been tested in humans (Vugmeyster et al., 2006). The new discovery of a subset of B cells with inhibitory function often referred to as Bregs (see below) should urge some caution for global B cell-depleting strategies. Indeed, IL-10-producing Bregs have been shown to be prolong islet transplant survival (Ding et al., 2011) and increases in transitional B cells have been correlated with tolerance in patients who retained their grafts after withdrawal of immunosuppression for various reasons (Newell et al., 2010; Sagoo et al., 2010).

b. ii. *Ex vivo* depletion

To avoid some of the detrimental consequences of using global lymphodepleting agents, strategies to selectively deplete alloreactive cells have been developed. For

many of these strategies, T cells are isolated from the recipient and stimulated *ex vivo* with donor cells in an assay called a mixed lymphocyte reaction (MLR). Host cells can be labeled with a membrane dye that is diluted in half with each cell division. Selective transfer into the recipient of cells that retain high levels of the dye, indicative of their having remained undivided, has been termed alloreactive T cell "pruning" (Hu et al., 2008; Watson et al., 2004). Other strategies involve the use of a phototoxic dye that is selectively taken up by activated cells and then exposing the cells to light to cause photodepletion (Mielke et al., 2008), or using chemotherapeutic drugs such as methotrexate to kill actively dividing cells in the MLR before returning the non-alloreactive cells to the transplant recipient (Sathe et al., 2007). Most of these strategies have been tested only in animal models, but photodepletion is already being used in clinical trials for human bone marrow transplant recipients (Mielke et al., 2008).

c. Inhibition of T-cell activation signaling

Another strategy to induce transplantation tolerance is to target signaling pathways important for T cell activation, such as the kinase JAK3 or the transcription factor, NF-κB. JAK3 is an important downstream signaling molecule that is activated upon cytokine receptor stimulation, and inhibition of JAK3 in rodent models has been shown to induce T cell deletion and to prolong allograft acceptance (Wen et al., 2001). A JAK3 inhibitor has been used successfully in non-human primate studies and is currently being investigated in clinical trials in transplant recipients (Vincenti et al., 2015). Inhibition of NF-κB in T cells leads to tolerance of cardiac allografts and

prolonged islet allograft survival because NF-κB-deficient alloreactive T cells are more susceptible to passive and active cell death (Molinero et al., 2009; Porras et al., 2012). Overexpression of Bcl-$x_L$ was able to restore alloreactive T cell survival and precipitate cardiac and islet allograft rejection in mice. Similarly, genetic deficiency or blockade of Fas signaling with monoclonal antibody treatments restored cardiac allograft rejection (Molinero et al., 2009; Zhou et al., 2005). Genetic T cell deficiency in PKC-θ, a signaling molecule downstream of the TCR and upstream of NF-κB, also led to prolonged cardiac allograft survival in mice (Manicassamy et al., 2008). PKC-θ-deficient T cells underwent more apoptosis due to reduced Bcl-$x_L$ expression. While not yet studied in larger animal models or in humans, T-cell-specific inhibition of NF-κB may be a useful strategy to induce transplantation tolerance.

3. Regulation

Tolerant T cells do not exist in isolation and are impacted extrinsically through direct and indirect interactions with other tolerogenic cells in the transplant recipient. Cellular therapies have been used to exploit the capacity of tolerogenic cells to extrinsically regulate their environment.

a. Cellular therapies

i. Regulatory T cells

1. Types

Regulatory T cells are a subset of T cells that can secrete tolerogenic cytokines such as IL-10 and suppress the pro-inflammatory functions of effector T cells such as

their cytotoxicity, cytokine production and proliferation. There are several types of regulatory T cells: nTregs are $Foxp3^+CD4^+$ T cells that differentiate in the thymus while *in vivo* induced regulatory T cells (iTregs) differentiate from naïve $CD4^+$ T cells into $Foxp3^+$ cells in the periphery (Fontenot et al., 2003; Hori et al., 2003; Zheng et al., 2008). Naïve T cells can be induced to differentiate into iTregs *in vitro* upon stimulation in the presence of cytokines such as TGF-β and IL-2 (Chen et al., 2003). Deficiency in Foxp3 leads to immune dysregulation, polyendocrinopathy, enteropathy, and X-linked inheritance (IPEX) syndrome in humans resulting in lethal autoimmunity (Bennett et al., 2001). Other types of regulatory T cells that do not express Foxp3 include Tr1 cells, CD8 suppressive cells, double negative ($CD4^-CD8^-$) T cells and NKT cells (reviewed in Wood et al., 2012).

2. Mechanisms of action

Regulatory T cells can suppress T cells, B cells, DCs and other immune effector cells via secretion of the cytokines IL-10, TGFβ or IL-35 (Chen et al., 1998; Collison et al., 2007; Hara et al., 2001). They can exert contact-dependent suppression through the delivery of granzyme B to induce cell death, or via gap junction transfer of cAMP to inhibit proliferation and differentiation in target cells (Bopp et al., 2007; Gondek et al., 2005). The high expression of the IL-2Rα, CD25, on their surface is thought to enable them to better compete for IL-2 than other effector cells (de la Rosa et al., 2004). CTLA-4 on Tregs has also been shown to be important for inhibiting DC function by activating

IDO or reducing positive costimulatory ligands on the DC surface (Mahnke et al., 2007; Mellor et al., 2004).

3. Strategies for *in vitro* expansion

Strategies for expanding Tregs include isolation of Tregs from peripheral blood or umbilical cord blood cells and culture *in vitro* before returning the cells to the patient. This technique has been limited by the lack of a set of surface markers that specifically identify Tregs. Commitment to a stable Treg phenotype appears to depend on the Treg cell-specific demethylated region (TSDR), a marker of open chromatin and active gene transcription (Polansky et al., 2008). Some studies have shown $Foxp3^+$ cells reverting to $Foxp3^-$ effectors (Duarte et al., 2009; Zhou et al., 2009), which may lead to adverse effects for human transplant patients, so the identification of stable Tregs for adoptive transfer is important to avoid this risk. $CD127^{lo}$, LAP and CD45RA have been shown to be markers of Tregs that are highly suppressive when transferred *in vivo* (reviewed in Issa and Wood, 2012). Anti-CD3- and anti-CD28-bound microbeads in combination with human IL-2 have been used to polyclonally expand Tregs *ex vivo* (Sagoo et al., 2008), while the stimulation of Tregs with donor APCs has been used to generate donor-specific Tregs (Sagoo et al., 2011). Donor-specific Tregs have been shown to be more potent than polyclonal Tregs. Infusion of *in vitro* expanded Tregs has already been used in patients receiving bone marrow transplants, resulting in reduced GVHD and prolonged graft survival (Brunstein et al., 2011, 2015).

4. Strategies for *in vivo* expansion or induction

a. Anti-CD3

CD3 is a component of the TCR complex. The first mouse antibody to human CD3 used in transplant patients, OKT3, was developed in 1979. Its use was limited because of side effects from its immune activating and mitogenic properties (van Wauwe et al., 1980). New antibodies with modified Fc portions were generated to circumvent some of these side effects. Anti-CD3 has been used in young Type 1 diabetic patients to preserve tolerance to transplanted insulin-producing β-cells (Herold et al., 2002). In addition to promoting T cell deletion, it was shown in mouse models to increase the ratio of regulatory to Teffector cells (reviewed in Chatenoud, 2003).

b. Anti-CD4

CD4 is a TCR co-receptor expressed on helper T cells and at lower levels on some APCs. Non-depleting CD4 antibodies have been used in small and large animal models of transplantation for tolerance induction (Waldmann et al., 2008). These antibodies are thought to predominantly promote the generation of iTreg or activate nTregs but may also function to render effector T cells anergic. This treatment has also been shown to confer infectious tolerance whereby Tregs infiltrating tolerated skin grafts can generate iTregs from other T cells through the modification of APCs (Kendal et al., 2011; Qin et al., 1993). Human studies using anti-CD4 monoclonal antibodies have not yet examined the role of Tregs in transplant recipients.

c. T cell depletion agents: anti-TCR$\alpha\beta$, anti-CD52 (alemtuzumab) and anti-thymocyte globulin (ATG)

Though not the primary mechanism for their tolerance induction, T cell depletion agents have also increased regulatory cell populations in some settings. Anti-TCR$\alpha\beta$ has been shown to increase regulatory NKT cells in a murine model of autoimmune disease (Lavasani et al., 2007). Alemtuzumab may induce regulatory B cells, Bregs, in kidney transplant patients (Heidt et al., 2012). Rabbit anti-murine thymocyte globulin, a polyclonal antibody used to deplete T cells has also been shown to promote Treg generation (Lopez et al., 2006).

d. Rapamycin

Rapamycin is a selective inhibitor of the mammalian target of rapamycin (mTOR) complex, which senses environmental cues and is important for cell growth, proliferation and metabolism. In addition to its effects on DCs and other APCs, rapamycin has been shown to increase Treg to effector T cell ratios in mouse models and in patients, whereas calcineurin inhibitors can block this effect (Battaglia et al., 2005; Lange et al., 2010; Ma et al., 2009a). Rapamycin has also been shown to upregulate Foxp3 and may promote the generation of iTregs (Long and Buckner, 2008). Rapamycin has been used with IL-2 to expand Tregs *in vitro* for patients with autoimmune diseases (Long et al., 2012). However, at low doses, rapamycin can also promote memory $CD8^+$ T cells highlighting context-dependent dual potential functional outcomes (Araki et al., 2009).

e. IL-2 plus anti-IL-2

When administered with a non-neutralizing anti-IL-2 monoclonal antibody, IL-2 acquires an increased half-life *in vivo* and more potent function as a pro-survival cytokine (Courtney et al., 1994). As Tregs express CD25, they benefit from this treatment. This strategy has been used in patients to expand Tregs *in vivo* more than 10-fold. Low-dose IL-2 is being used in a clinical trial to reduce chronic GVHD (Koreth et al., 2011).

ii. Tolerogenic dendritic cells

DCs are professional APCs that are derived from either myeloid or lymphoid progenitors and can be broadly classified into conventional and plasmacytoid DCs. DCs instruct T cells to differentiate into distinct effector or regulatory subtypes based on their cytokine production and expression of costimulatory ligands. DCs are important for self-tolerance because their depletion can result in autoimmunity (reviewed in Ganguly et al., 2013). Tolerogenic characteristics of DCs include the ability to promote nTreg function, iTreg formation, the expression of lower levels of MHC and costimulatory ligands, and the reduced production of pro-inflammatory cytokines. Additionally, tolerogenic DCs can resist being killed by T cells and can in turn kill alloreactive effector T cells (reviewed in Morelli and Thomson, 2007).

Tolerogenic DCs have been generated *in vitro* for adoptive transfer into transplant recipients in animal models and, more recently, in human patients (Moreau et al., 2012). Unexpectedly, host DCs have been shown to be essential for the beneficial

effects of adoptively transferred tolerogenic DCs as host DC depletion prevented the transferred tolerogenic DCs from inhibiting alloreactive T cells (Divito et al., 2010; Wang et al., 2012). Tolerogenic DCs are predominantly immature dendritic cells and can be generated by treating DCs with rapamycin, dexamethasone, vitamin D3, IL-10, TGF-β, or transfer of genes encoding pro-tolerogenic molecules such as IL-10, TGF-β, CTLA-4-Ig, IDO, PD-L1, CD95L, or TRAIL (reviewed in Morelli and Thomson, 2007). Rapamycin-treated DCs were able to mediate indefinite cardiac allograft survival in mice, and costimulation blockade may also increase tolerogenic DCs *in vivo* (Turnquist et al., 2007). One risk associated with transferring donor DCs in human patients is that they may immunize rather than tolerize patients to alloantigens if the DCs' tolerogenic phenotype is not stable.

ii. Tolerogenic macrophages

Regulatory macrophages (Mregs) are a subset of macrophages producing high levels of IL-10 that have the ability to suppress T cell proliferation *in vitro* (Fleming and Mosser, 2011). Transplant acceptance-inducing cells, TAICs, are macrophages contaminated with other leukocytes, which have been used in two clinical trials to test whether patients could be weaned from immunosuppression (Hutchinson et al., 2008a, 2008b, 2011). Despite a role for TAICs in tolerance to allografts in animal models, they did not promote tolerance in human kidney transplant recipients, though graft-survival could be maintained with low dose immunosuppression. Purified Mregs have led to better outcomes but studies are still preliminary (Riquelme et al., 2012). The *ONE*

*Study* is a European multi-center clinical trial that is comparing the safety and feasibility of using expanded Mregs, nTregs, Tr1 cells or tolerogenic DCs in kidney transplant recipients treated with the same adjunctive immunosuppression protocol.

iv. Mesenchymal stromal cells

Derived from the bone marrow, mesenchymal stromal cells (MSCs) have been shown to increase Treg populations and to produce nitric oxide and IDO to inhibit T cell activation and proliferation (reviewed in Singer and Caplan, 2011). These cells can decrease proinflammatory cytokine production from other cells and increase pro-tolerogenic cytokines such as IL-10 and IL-4. Transfer of MSCs at the time of transplantation enhanced skin graft and islet allograft survival in rats (Aksu et al., 2008; Solari et al., 2009). MSC transfer prolonged survival in a majority of patients with GVHD in one clinical trial (Le Blanc et al., 2008).

b. Other cell types Involved in tolerance

i. Mast cells

Mast cells have been shown to be linked to Treg-mediated tolerance. Tolerance (to heart and skin allografts) could not be induced in mice deficient in mast cells, and IL-9 produced by mast cells was shown to be important for maintaining tolerance (Lu et al., 2006). Conversely, Tregs need to suppress mast cell degranulation in order to maintain tolerance. Mast cell degranulation can result in Treg egress from a skin graft and loss of expression of suppressor molecules (de Vries et al., 2009).

ii. Natural killer cells

Despite the role for natural killer (NK) cells in allograft rejection, in mouse models of tolerance induction with anti-CD154, NK cells have also been shown to be important for tolerance to islet and cardiac allografts (Beilke et al., 2005; van der Touw et al., 2012). Perforin production by NK cells was found to be important for NK cells to limit T cell responses in the presence of anti-CD154 (Beilke et al., 2005). Depletion of NK cells led to acute rejection of cardiac allografts in mice receiving anti-CD154, suggesting their involvement in tolerance induction (van der Touw et al., 2012). One mechanism by which NK cells may promote tolerance in a murine skin allograft model is the killing of donor APCs, which then prevents the APCs from stimulating recipient T cells (Yu et al., 2006).

ii. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of cells that can differentiate into macrophages, DCs, and granulocytes. MDSCs in a mouse model of cardiac allograft transplantation were induced by costimulation blockade and accumulated in the graft where they promoted the development of Tregs (Garcia et al., 2010). MDSCs can also inhibit T cell proliferation *in vitro* through the production of iNOS (Mazzoni et al., 2002). More recently, they have been shown to function via expression of DC-SIGN (Conde et al., 2015).

iv. Regulatory B cells

Regulatory B cells (Bregs) are a subset of B cells that are tolerogenic. T cell Ig domain and mucin domain protein 1, Tim-1, has recently been identified as a costimulatory molecule expressed on IL-10-producing Bregs (Ding et al., 2011). Naïve B cells may also fall into the category of Bregs as they have been shown to present antigen to T cells in a manner that stimulates naïve T cells to become regulatory cells instead of effectors. Immature transitional B cells have also been shown to play a regulatory role in murine models (Kirk et al., 2010). Increased numbers of naïve, transitional, and unswitched memory B cell subsets were identified in tolerant kidney patients who discontinued their immunosuppression, though whether these are a cause or a consequence of tolerance is not yet known (Turka and Lechler, 2009).

4. Other strategies of tolerance induction

a. Oral tolerance

Oral tolerance occurs naturally to food-borne antigens and to gut microorganisms. High doses of antigen administered orally lead to anergy/deletion of antigen-specific T cells whereas low doses can induce Tregs (Weiner et al., 1994). The highest concentration of iTregs identified to date is in the intestine. Donor MHC-binding peptides have been administered orally to prevent rejection in a model of cardiac allograft transplantation in mice (Niimi et al., 1999). Donor cells such as splenocytes have also been administered orally in order to induce tolerance (Niimi et al., 2000).

b. Combination therapies

As there are numerous mechanisms identified for transplantation tolerance, more than any single mechanism will likely need to be induced to be effective in patients. Already, studies combining costimulation blockade with hematopoietic chimerism induction have resulted in better graft survival without evidence for chronic rejection in animal models (Shirasugi et al., 2002). In many cases, dominant infectious tolerance was present in addition to deletional tolerance, suggesting a critical role for regulatory cells. Strategies to combine agents that will induce multiple mechanisms of tolerance in the clinic may ultimately be the optimal strategy for achieving robust transplantation tolerance in patients.

D. Barriers to tolerance induction

1. Memory T cells

Memory T cells are antigen-experienced cells that mediate a faster and more potent response upon repeat encounter with antigen. These cells are long-lived and when developed following an infection, can protect against subsequent infections with the same pathogen. The presence of memory cells that are reactive against a transplanted organ, however, is a barrier to transplantation tolerance (Adams et al., 2003; Valujskikh and Li, 2007). There are several attributes of memory cells that may explain the positive correlation between higher frequencies of graft-reactive memory T cells and graft rejection. Memory T cells are less reliant on costimulation for their activation, and thus are resistant to therapies that involve costimulation blockade (Floyd

et al., 2011). Humans have many more memory T cells than mice kept in specific pathogen-free conditions and some of these T cells lack CD28. This may explain in part why therapies targeting CD28 have been less successful in patients. Memory T cells tend to be enriched for higher affinity cells compared to naïve T cells as higher affinity T cells can survive better following a primary response (Baumgartner et al., 2012; Day et al., 2007). These higher-affinity memory T cells may be activated by lower stimuli and may be more resistant to depletion and AICD than naïve T cells.

Graft-reactive memory T cells can arise following previous exposure to donor antigens during prior transplants, blood transfusions or pregnancies. T cells specific to microbial antigens that have acquired memory to a prior infection may be cross-reactive to alloantigens, a phenomenon termed heterologous immunity (Adams et al., 2003). In addition, some T cells can express TCRs of 2 distinct specificities such that if a T cell becomes memory after being activated by a microbial antigen recognized by its first TCR, it can then act as a memory T cell when the cell's second graft-reactive TCR is engaged, even though the graft antigen had never been encountered before (Morris and Allen, 2009). Finally, T cells bearing similar phenotypes to memory cells can arise in an antigen-independent fashion after homeostatic proliferation. Homeostatic proliferation occurs when a small number of remaining T cells proliferate in response to growth factors to fill the niche left open following T cell loss. After T cell depletion, the remaining graft-reactive cells or new thymic emigrants can undergo homeostatic proliferation and acquire memory-like function, thus becoming resistant to tolerance induction (Wu et al., 2004).

a. Blocking memory T cell adhesion

Efforts to selectively target memory cells include antibodies that block adhesion through the integrin lymphocyte function associated antigen 1, LFA-1, anti-VLA4 antibodies and an LFA-3-Ig fusion protein (Kitchens et al., 2012; Weaver et al., 2009). These reagents have been shown to effectively control memory T cells and prevent rejection of kidney or islet grafts in mice and non-human primates (Badell et al., 2010; Kitchens et al., 2012). One drawback to these treatments for tolerance induction is that they also target memory cells with reactivity to infectious agents, leaving the transplant recipient vulnerable to more infections. In addition, low incidence of progressive multifocal leukoencephalopathy (PML) in clinical trials using similar agents in autoimmune settings has decreased enthusiasm for clinical transplantation (Carson et al., 2009).

b. Blocking costimulatory molecules on memory T cells

Another strategy to target memory T cells is to block costimulatory molecules, OX40, ICOS, 4-1BB, expressed preferentially on memory but not naive T cells. OX40 (CD134) is a member of the TNFR family that facilitates effector cells becoming memory cells and promotes their survival and proliferation (Gramaglia et al., 2000; Rogers et al., 2001; Song et al., 2008). It also prevents Treg suppression and new Tregs from being differentiated (Vu et al., 2007). OX40 blockade has been shown to prolong survival of cardiac allografts in a minor-mismatch mouse cardiac allograft model (Curry et al.,

2004) while agonistic OX40 is currently being used in clinical trials to restore T cell responsiveness in patients with tumors (Aspeslagh et al., 2016).

ICOS, or inducible costimulator of T cells, is a member of the CD28 family of proteins that is expressed on activated T cells and is a positive costimulatory receptor. Its ligand, ICOSL, is expressed on APCs. Combined blockade of ICOS:ICOSL and CD40:CD154 interactions has been shown in animal models to prolong heart and islet allograft survival (Guo et al., 2004; Nanji et al., 2004; Zhang et al., 2008). Blockade of ICOS:ICOSL and CD40:CD154 interactions in combination with DST has been used to prolong murine skin and liver transplants (Guo et al., 2002; Xu et al., 2008). Blockade of ICOS has not yet been translated to patients.

4-1BB (CD137) is a member of the TNFR family that is expressed on activated T cells as well as other cell types. Its ligand 4-1BBL is also expressed on activated T cells. Similar to OX40, blockade of 4-1BB has shown prolonged allograft survival in animal models but has not yet been used in human transplant patients (Cho et al., 2004). Also similar to OX40, agonistic 4-1BB treatments are being used in clinical trials for tumor-bearing patients.

CD27/CD70 is another costimulatory molecule pair that could serve as a potential target for memory T cells but studies have only been reported in mouse transplantation models (McGrath and Najafian, 2012). Anti-CD70 treatment was able to block memory cell-mediated cardiac allograft rejection in mice without lymph nodes (Yamada et al., 2005). LIGHT/HVEM may also be a target for memory cells in transplantation, but so far studies disrupting HVEM or its other binding partner BTLA have had both positive and

negative results for transplantation outcomes in mouse models (reviewed in McGrath and Najafian, 2012). HVEM can additionally bind CD160 and can have positive or negative costimulatory effects depending on which ligand HVEM binds. Its binding to LIGHT confers positive costimulation and may be the most desirable to interrupt in this pathway.

Pre-transplant memory T cells can act as barriers to transplantation tolerance, but whether memory cells can develop following exposure to alloantigen in the presence of costimulation blockade or other immunosuppressive therapies and progressively become barriers to the maintenance of tolerance is unknown and was explored in this thesis.

2. Allospecific B cells

Allospecific B cells are a barrier to transplantation tolerance because they can secrete donor-specific antibodies that can bind graft tissues and lead to antibody-mediated rejection (AMR) by both complement fixation-dependent and -independent mechanisms. Alloreactive B cells can also present alloantigens to T cells by having their BCR bind and internalize graft antigens, whose protein components are processed into peptides that are presented on MHC molecules, and can also promote T cell memory (Whitmire et al., 2009). Like memory T cells, memory B cells as well as pre-formed antibodies have been shown to be a barrier to transplantation tolerance (Burns and Chong, 2011). While the role of allospecific B cells was not directly investigated in this thesis, allospecific B cells are present as antigen presenting cells and costimulation

blockade disrupts their interaction with allospecific T cells during the induction phase, potentially impacting the tolerance mechanisms able to be programmed in these T cells.

a. Depleting B cells and plasma cells

See *In vivo* depletion above.

3. Infections

Infections in human transplant recipients are associated with increased episodes of acute rejection, but a direct causal relationship has not been shown. The use of antibiotics, combined with a temporary reduction in the levels of immunosuppression to combat infections, have made it especially difficult to demonstrate cause. In animal models, infections or exposure to microbial products at the time of transplantation can prevent the induction of tolerance and lead to graft rejection (Table 1.2).

| Infection | Transplant Model | Prevents Tolerance Induction? | Breaks Established Tolerance? | References |
|---|---|---|---|---|
| LCMV | Skin, anti-CD154+DST | Yes | No | (Welsh et al., 2000) |
| Pichinde virus | Skin, anti-CD154+DST | Yes | N.D. | (Welsh et al., 2000) |
| *Murine cytomegalovirus* | Skin, anti-CD154+DST | No | N.D. | (Welsh et al., 2000) |
| *Vaccinia virus* (VV) | Skin, anti-CD154+DST | No | N.D. | (Welsh et al., 2000) |
| LCMV→VV or LCMV→VV→VSV | Skin, anti-CD154, CTLA4-Ig and BM Tx | Yes | N.D. | (Adams et al., 2003) |
| *Leishmania major* | Skin, anti-CD154+DST | Yes | N.D. | (Pantenburg et al., 2002) |
| *Listeria monocytogenes* | Heart, anti-CD154+DST | Yes | Yes | (Wang et al., 2008b, 2010) |
| *Pseudomonas aeruginosa* | Skin, anti-CD154+DST | No | N.D. | (Ahmed et al., 2011a) |
| *Staphylococcus aureus* | Skin, anti-CD154+DST | Yes | No | (Ahmed et al., 2011a) |

**Table 1.2: Infections that have been tested in animal models using costimulation blockade-induced tolerance**
Abbreviations: bone marrow transplant (BM tx), donor splenocyte transfusion (DST), Lymphocytic choriomeningitis virus (LCMV), not determined (N.D.), Vesicular stomatitis virus (VSV)

a. TLR ligands are able to prevent the induction of tolerance

Organs with a higher microbial load such as lungs and intestinal transplants tend to have poorer graft outcomes than more “sterile” organs such as hearts and kidneys (UNOS 2015: transplants performed 1997-2004). One potential explanation for these differences in outcome is that signals from colonizing microbes could be an environmental factor contributing to graft rejection. To test the hypothesis that microbial

signals could negatively impact tolerance induction, Toll like receptor (TLR) ligands were administered at the time of transplantation in fully MHC-mismatched allograft mouse models using costimulation blockade for tolerance induction (Chen et al., 2006; Thornley et al., 2006). Engagement of a single TLR with one of its ligands was sufficient to prevent tolerance induction of skin grafts and even of heart grafts that normally have a low microbial burden (Chen et al., 2006; Thornley et al., 2006). Administration of TLR-ligands prevented the induction of tolerance by enhancing Th1 and Th17 differentiation and suppressing natural Treg function, as well as increasing intra-graft accumulation of T effector cells and decreasing the percentage of $CD4^+$ $Foxp3^+$ regulatory T cells (Chen et al., 2006, 2009; Porrett et al., 2008). Furthermore, TLR signaling through the downstream adaptor molecule MyD88 was important for the long-term acceptance of minor-antigen mismatched skin grafts (Chen et al., 2006; Goldstein et al., 2003), and better long-term graft survival was observed when both the donor and the recipient were deficient in MyD88 (Goldstein et al., 2003). It is important to note that in addition to sensing microbial-associated molecular patterns (MAMPs), TLRs can also recognize host molecules during cell damage, called damage-associated molecular patterns (DAMPs). Thus the fact that TLR- and MyD88-dependent signals prevent transplantation tolerance induction does not necessarily imply microbial molecules as the ligands and does not exclude the contribution of DAMPs. Therefore, data from these studies suggest that either MAMPS and/or DAMPs from the transplant are sensed by the TLR-MyD88 pathway and can contribute to how the immune system responds to the allograft.

b. Infections and the induction of tolerance

It is already appreciated from clinical data that infections occurring peri-operatively are poor prognostic indicators for graft outcome in transplant recipients receiving conventional immunosuppression (reviewed in Chong and Alegre, 2012). But what impact, if any, do infections have for tolerance induction? In animal models using costimulation blockade for tolerance induction, viral infections at the time of transplantation, such as LCMV and Pichinde virus, were the first to be shown to prevent long-term graft acceptance (Welsh et al., 2000). Subsequently, infection with the parasite *Leishmania major*, the bacterium *Listeria monocytogenes*, or the bacterium *Staphylococcus aureus* was also found to prevent tolerance induction to allografts (Ahmed et al., 2011a; Pantenburg et al., 2002; Wang et al., 2008b). The mechanism by which each infection prevents tolerance induction may be unique, and not every pathogen that has been used to infect mice has precluded transplantation tolerance—infections with murine cytomegalovirus, *Vaccinia virus* or *Pseudomonas aeruginosa* all failed to impact graft survival (Ahmed et al., 2011a; Welsh et al., 2000). Infection with either the LCMV virus or Leishmania were both found to activate and increase a population of T cells that were cross-reactive with antigens in the donor allograft providing two examples of heterologous immunity (Brehm et al., 2003; Pantenburg et al., 2002). Prior infection with viruses that activated heterologous T cells was also detrimental to tolerance induction two months later (Adams et al., 2003). With increased precursor frequencies of alloreactive T cells in these three studies, the recipients were made refractory to tolerance induction. This was in contrast to the study

using infection with *L. monocytogenes,* where cross-reactivity between bacterial antigens and alloantigens was not detected. Instead, the *L. monocytogenes* and also *S. aureus* prevented tolerance induction through the cytokines produced by the host to eliminate them (Ahmed et al., 2011a; Wang et al., 2008b). In the absence of signaling by certain cytokines such as type I interferons, or upon IL-6 neutralization, infection with *L. monocytogenes* and *S. aureus*, respectively, were no longer able to prevent tolerance induction (Ahmed et al., 2011a; Wang et al., 2008b). These cytokines may act in part to activate alloreactive T cells in a bystander (non-cognate antigen) manner, by allowing effector T cells to overcome suppression by regulatory cells, and to mature into memory T cells, as has been shown for IL-6 (Nish et al., 2014; Pasare and Medzhitov, 2003). In summary, several different infections including, viral, parasitic, and bacterial (both intracellular and extracellular), can prevent induction of transplantation tolerance.

c. Infections and the maintenance of tolerance

Recent reports on small numbers of patients have demonstrated, as a proof of principle, that tolerance can be induced prospectively in the clinic (reviewed in Sachs et al., 2014). In addition, rare patients have developed spontaneous tolerance to their grafts after stopping treatment following a long period of immunosuppression. A study of these exceptionally rare, spontaneously tolerant kidney transplant individuals has been published (Brouard et al., 2012). Although the kidney grafts were maintained for a median of nine years without any immunosuppression, several of the patients lost graft function over the course of the subsequent follow-up. While the sample size was small,

5/8 of the patients who lost graft function had experienced a bacterial or viral infection preceding their graft loss, whereas this happened more rarely (2/19) in the patients who maintained their tolerance (Brouard et al., 2012). This study prompts the hypothesis that infections may have an impact not only during the peri-transplant period but also later on, and therefore may compromise a host's ability to maintain tolerance.

The causal relationship between infections and the loss of transplantation tolerance during its maintenance period has been examined in mouse models. Administration of TLR ligands, LCMV infection, and *S. aureus* infection that all prevented the induction of transplantation tolerance all failed to break tolerance once it had been stably established (de Vries et al., 2009; Welsh et al., 2000). Listeria infection, however, is one infection that is sufficient both to prevent tolerance and to break it in its maintenance phase. Listeria infection caused an acute rejection event in a majority of tolerant recipients, characterized by an increase in graft-infiltrating cells, and an increase in splenic alloreactivity (Wang et al., 2010). This rejection was dependent on both $CD4^+$ and $CD8^+$ T cells, yet no cross-reactivity was identified between T cells from Listeria-infected mice and donor antigens (Wang et al., 2010). Cytokine production was again essential to Listeria's ability to cause rejection, as both IL-6 and IFN$\beta$ were each necessary in order for Listeria to precipitate rejection and the combination of the two cytokines was sufficient to trigger a loss of tolerance in uninfected animals (Wang et al., 2010). High levels of IL-6 and IFN$\beta$ in combination may be unlikely to co-occur with many single infections, which may explain the uniqueness of Listeria thus far as a pathogen capable of abrogating tolerance during its maintenance phase. In tolerant

patients, concurrent infections with a bacterium and a virus could result in a similar cytokine environment and be detrimental to maintaining graft long-term graft survival. The pro-inflammatory cytokines produced during an infection can increase costimulatory ligands on APCs and antigen presentation, leading to increased priming of alloreactive T cells. These cytokines can also promote the differentiation of alloreactive T cells into pro-inflammatory effector subsets. Cytokines produced during the infection can also themselves be damaging to the graft.

Whether bystander activation of tolerant T cells during an infection can permanently abrogate donor-specific tolerance and promote the development of memory to the rejection event was investigated in this thesis.

## E. Spontaneous transplantation tolerance

### 1. Liver transplantation

Liver allografts are accepted in many rat and mouse strain combinations in the absence of immunosuppression. The acceptance of these allografts has been shown to be dependent upon $CD25^+$ regulatory cells (Li et al., 2008), invariant natural killer cells (Morita et al., 2007), Fas-FasL (Uchiyama et al., 2002), IFN$\gamma$ (Mele et al., 2003), CTLA-4 (Li et al., 2005) and PD-1 signaling (Morita et al., 2010), and low levels of NF-$\kappa$B in liver dendritic cells (Ma et al., 2009b). The broad spectrum of peripheral tolerance mechanisms, including deletion, anergy, and regulation, implicated in the liver allograft model underscores the critical role of the transplanted organ in facilitating the development of immunological tolerance, and also the complexity of the tolerant state.

The tolerogenicity of the allogeneic liver is recapitulated in clinical transplantation, and thus further investigation with this model of tolerance is warranted, despite the rather enormous technical challenges of performing successful liver transplantation in mice.

Patients who are recipients of liver transplants most commonly develop spontaneous tolerance to their allograft and can be weaned from immunosuppression, with estimates of this operational tolerance occurring in up to 20% of adult recipients (Benítez et al., 2011). Studies of biomarkers in liver transplant patients with operational tolerance show an enrichment of genes related to NK cells and in the regulation of iron metabolism (Bohne et al., 2012).

The liver may be a more tolerogenic organ because it encounters many foreign food and microbial antigens, and because of its regenerative properties when partially damaged. Kupffer cells, which are specialized liver macrophages, may also play a role in tolerance to liver transplants because their depletion leads to graft rejection (Callery et al., 1989). Kupffer cells have the unusual property of making the pro-tolerogenic cytokine IL-10 in response to the normally pro-inflammatory bacterial product lipopolysaccharide (LPS) (Knolle et al., 1995). Hepatic stellate cells, which are fat-storing pericytes in the liver, have also been shown to be pro-tolerogenic through the induction of effector T cell apoptosis and the promotion of increased Tregs and MDSCs (Dangi et al., 2012; Jiang et al., 2013). Additionally, liver DCs tend to be of the immature or tolerogenic phenotype (Pillarisetty et al., 2004).

The complexity of the tolerant state in liver transplantation combined with the clinical data implicating the liver as the most susceptible organ to operational tolerance

suggest that the cooperativity of multiple mechanisms of tolerance may favor a robust tolerance.

2. Kidney transplantation

In certain mouse strain combinations of donors and recipients, the kidney allografts are spontaneously accepted without immunosuppression or tolerogenic therapies despite MHC and minor antigen mismatches. While this outcome does not recapitulate the vast majority of clinical data, it has been used to shed light into the immunological pathways that can achieve tolerance in a tissue-specific manner. The long-term survival (> 60 days) of kidneys from A/J (H-$2^a$) donors → C57BL/6 (H-$2^b$) recipients was originally reported by Russell and colleagues (Russell et al., 1978). Spontaneous acceptance of complete MHC-mismatched renal allografts has also been reported in several other strain combinations including DBA/2 ($H2^d$) → C57BL/6 (Bickerstaff et al., 2001). Long-term survival was accompanied by an absence of renal allograft injury by histological analysis and without a rise in recipient serum creatinine levels. Essential roles for TGFβ signaling and regulatory T cells, as well as IDO signaling, which increased over time in graft-resident dendritic cells, have been reported (Cook et al., 2008). Thirty percent of these kidney transplant recipient mice with detectable IDO-producing dendritic cells accepted donor skin grafts without any immunosuppression, thus formally demonstrating tolerance (Cook et al., 2008). In another strain combination, C57BL/6 kidney allografts into B10.BR recipients also showed spontaneous graft acceptance and tolerance to subsequent skin allografts

(Wang et al., 2011). Increased Foxp3 mRNA expression was observed in those kidney allografts, suggesting a role for Tregs. Thus these kidney models, though not reflective of the clinical situation, provide a unique opportunity to study the pro-tolerogenic environment of the allograft. Persistent antigen contributing to T cell exhaustion is another way that an allograft may promote a pro-tolerogenic environment and one investigated in this thesis.

Kidney transplant patients who stop their immunosuppression due to noncompliance or other causes and retain their graft are deemed operationally tolerant. A few individuals from this rare patient population were studied to assess if biomarkers of tolerance could be found in these patients and could be used to predict patients who can be successfully weaned from immunosuppression (Roussey-Kesler et al., 2006). Gene arrays of peripheral blood cells from two groups of patients that met these criteria identified an enrichment of genes expressed by naïve/immature B cells in the tolerant patients compared to non-tolerant patients (Newell et al., 2010; Sagoo et al., 2010). Research is ongoing to understand the role this B cell signature plays in the state of human transplantation tolerance.

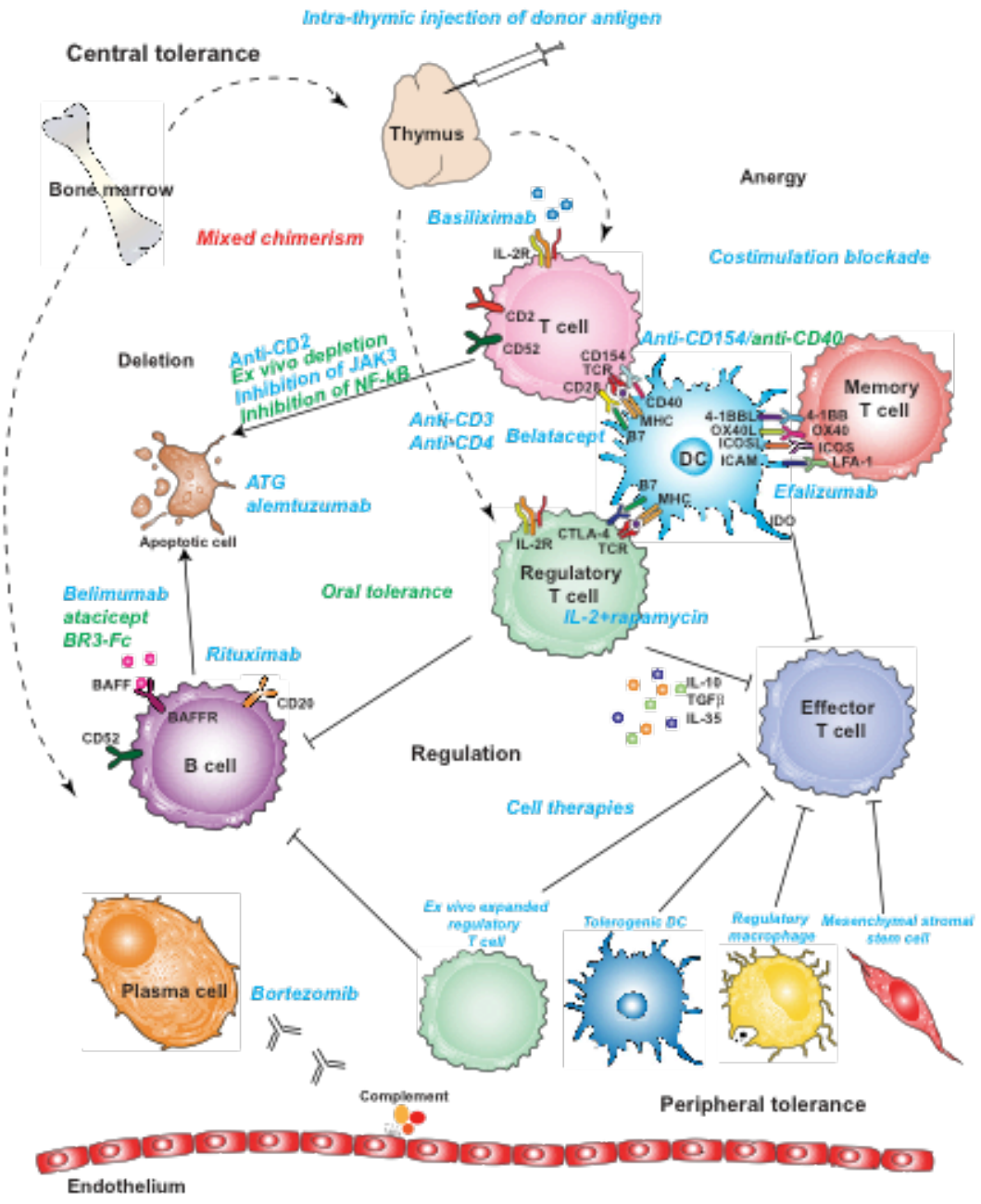


**Figure 1.4: Therapeutic strategies for transplant tolerance induction**
Central tolerance mechanisms (Top left) include mixed chimerism and intrathymic injection of donor antigens. The dotted arrows indicate cell types originating from the bone marrow, unrelated to mixed chimerism. Peripheral mechanisms of tolerance include anergy (Top right) such as costimulation blockade, deletion (center) such as through depleting antibodies and inhibition of T cell activation pathways, and regulation (Bottom right) such as through the transfer of tolerogenic cell types including tolerogenic DCs, regulatory T cells, macrophages, and mesenchymal stromal cells, which can inhibit effector T cells.

**Figure 1.4, continued:** (Bottom left) The humoral response can be inhibited through depletion of B cells and blockade of survival factors (such as BAFF) and depletion of plasma cells. Successful therapies in humans are in red. Therapies that have been used in humans but have either been shown to not be effective for transplantation or the efficacy is still undetermined are in blue. Therapies that have only been used so far in animal models are in green. Adapted from Page, E.K., Dar, W.A. & Knechtle, S.J. (2012). Tolerogenic therapies in transplantation. *Frontiers in Immunology*, **3**, p.198.

F. Summary

Immunological tolerance to self has evolved to protect against autoimmunity. In order to induce transplantation tolerance, many strategies have been developed to mimic both central and peripheral mechanisms of self-tolerance (Figure 1.4, Table 1.3), including mixed chimerism, costimulation blockade, and the adoptive transfer of tolerogenic cells. Many strategies that have been successful in animal models have not been translated as effectively into human transplant patients, suggesting pre-existing barriers to transplantation tolerance in humans or a lack of a full understanding of transplantation tolerance. The study of rare patients retaining their grafts after cessation of immunosuppression may provide insights into new strategies for achieving donor-specific transplantation tolerance for all patients.

In addition, having a better understanding of the mechanisms that induce and maintain tolerance successfully in animal models may also lead to improved therapies for transplant patients. For this thesis, we used a robust model of costimulation blockade-induced transplantation tolerance in mice, along with tracking of graft-specific T cells, to investigate how known mechanisms of peripheral tolerance cooperate to maintain graft acceptance and whether additional T cell mechanisms of tolerance operate in transplantation. We found that robust transplantation tolerance is resistant to

pro-inflammatory stimuli and can be spontaneously restored after it is transiently lost. Its robustness and resilience are likely due to the fact that it is comprised of a constellation of T cell mechanisms of tolerance that includes cell-extrinsic regulation, expression of cell-intrinsic inhibitors, limits to the size of the alloreactive T cell pool, population-level prevention of clonal expansion of high avidity cells, and T cell hyporesponsiveness.

| Category | Therapeutic Strategy | Mechanism |
|---|---|---|
| Central Tolerance | Mixed chimerism | Infusion of donor bone marrow into myeloablated/immune-conditioned recipient, to produce co-existence of donor and recipient cells |
| | Intra-thymic injection of donor antigen | Intra-thymic injection of donor antigens or donor cells to induce deletion as well as anergy and to promote the development of donor-specific nTregs in the thymus |
| Anergy | Abatacept | CTLA-4 Ig, blockade of CD28:CD80/86 costimulatory pathway |
| | Belatacept | High affinity variant of CTLA-4 Ig (LEA29Y), blockade of CD28:CD80/86 costimulatory pathway |
| | Anti-CD154 | Blockade of CD40:CD154 costimulatory pathway |
| Deletion | Anti-thymocyte globulin (ATG) | Depleting polyclonal antibodies to thymocytes that express multiple target antigens; possible induction of regulatory T cells |
| | Alemtuzumab (CAMPATH-1, anti-CD52) | Depleting mAb to CD52, on T, B, NK cells, some monocytes |
| | OKT3 | Depleting mAb to CD3 on T cells, also induces regulatory T cells |
| | *Ex vivo* depletion | Selective removal (pruning) of donor-reactive cells though cell labeling and depletion of those that proliferate |
| | JAK3 inhibition | Inhibition of signaling downstream of cytokine stimulation to prevent T cell activation |
| | Rituximab | Depleting mAb to CD20 expressed on B cells |
| | Belimumab | Blockade of B cell activating factor (BAFF), causing depletion of follicular and alloreactive B cells, decrease in alloantibody response, and promotion of immature/transitional B cell phenotype and a regulatory cytokine environment |
| | Atacicept | Blockade of BAFF and APRIL |
| | BR3-Fc | Blockade of BAFF, causing decrease in peripheral, marginal zone, and follicular B cells |
| | Bortezomib | Proteasome inhibitor, causing apoptosis of mature plasma cells |
| Other T cell therapies | Basiliximab, | Blockade of CD25 (interleukin 2 receptor α chain) |
| | Daclizumab | Blockade of CD25 (interleukin 2 receptor α chain) |
| | Efalizumab | Blockade of LFA-1:ICAM-1 costimulatory pathway to inhibit memory T cell adhesion |
| Regulation | Anti-IL-2 + rapamycin | Interleukin 2 + rapamycin, to increase regulatory T cell proliferation and survival, and stabilize the expression of Forkhead box P3 (Foxp3) |
| | Regulatory T cells | Infusion of *in vitro* expanded regulatory T cells, to inhibit inflammatory cytokine production, downregulate costimulatory and adhesion molecules, promote anergy and cell death, convert effector T cells to a regulatory phenotype, and produce suppressive cytokines IL-10, TGFβ, and IL-35 |
| | Regulatory T cells + IL-2 | As above, plus the addition of IL-2 to promote Treg survival, development, and expansion |
| | Tolerogenic dendritic cells | Immunomodulatory effects include their ability to acquire and present antigen, expand and respond to antigen-specific Tregs, constitutively express low levels of MHC and costimulatory molecules, produce high IL-10 and TGFβ and low IL-12, resist activation by danger signals and CD40 ligation, resist killing by natural killer or T cells, and promote apoptosis of effector T cells |
| | Regulatory Macrophages | Immune suppression mediated through the enrichment of $CD4^+$ $CD25^+$ Foxp3 cells and cell contact- and caspase-dependent depletion of activated T cells |
| | Mesenchymal Stromal Cells | Inhibition of T cell activation and proliferation, potentially due to production of IL-10, nitric oxide (NO), and IDO, and suppression of IFNγ and IL-17 |

**Table 1.3: Strategies for tolerance induction in humans**

**Table 1.3 continued:** This table outlines the pharmacologic, biologic, and cellular therapies discussed in this article, categorized by underlying tolerance mechanism. Reproduced with modification from Page, E.K., Dar, W.A. & Knechtle, S.J., (2012). Tolerogenic therapies in transplantation. *Frontiers in Immunology*, **3**, p.198.

## II. RESULTS – SPONTANEOUS RESTORATION OF TRANSPLANTATION TOLERANCE AFTER ACUTE REJECTION[1]

### A. Abstract

Transplantation is a cure for end-stage organ failure but, in the absence of pharmacological immunosuppression, allogeneic organs are acutely rejected. Such rejection invariably results in allosensitization and accelerated rejection of secondary donor-matched grafts. Transplantation tolerance can be induced in animals and a subset of humans, and enables long-term acceptance of allografts without maintenance immunosuppression. However, graft rejection can occur long after a state of transplantation tolerance has been acquired. When such an allograft is rejected, it has been assumed that the same rules of allosensitization apply as to non-tolerant hosts and that immunological tolerance is permanently lost. Using a mouse model of cardiac transplantation, we show that when *Listeria monocytogenes* infection precipitates acute rejection, thus abrogating transplantation tolerance, the donor-specific tolerant state re-emerges, allowing spontaneous acceptance of a donor-matched second transplant.

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[1] **Note:** The following section titled "Spontaneous restoration of transplantation tolerance after acute rejection" is reproduced with minor editing, and with figure renumbering, from reference (Miller et al., 2015) as allowed by copyright under the Creative Commons license, Nature Publishing Group.
Authors: Michelle L. Miller, Melvin D. Daniels, Tongmin Wang, Jianjun Chen, James Young, Jing Xu, Ying Wang, Dengping Yin, Vihn Vu, Aliya N. Husain, Maria-Luisa Alegre and Anita S. Chong

Contributions:
- Assisted Melvin Daniels for the experiments involved in Figure 2.1b, 2.2a Figure 2.3
- Assisted with characterization of the phenotype of graft-infiltrating cells in Figure 2.5 (with Melvin Daniels, Tongmin Wang)
- Quantification of bacterial CFU in Figure 2.6a (with Melvin Daniels)
- Enumeration of Listeria-specific T cells in Figure 2.6b
- Enumeration of graft-infiltrating cells in Figure 2.6c
- Design of experiments and statistical analyses

Co-prepared manuscript with Melvin Daniels, Anita Chong and Maria-Luisa Alegre

These data demonstrate a setting in which the memory of allograft tolerance dominates over the memory of transplant rejection.

## B. Introduction

Solid organ transplantation is the therapy of last resort for end-stage organ failure, but, in the absence of immunosuppression, T cell-dependent acute rejection of allografts invariably ensues. Rejection is accompanied by allosensitization and the accelerated rejection of a second donor-matched transplant, as first reported for humans and rabbits by Medawar and colleagues (Gibson and Medawar, 1943; Medawar, 1944). To prevent rejection, current immunosuppressive therapies that target T cells non-specifically have to be taken life-long, leaving patients more susceptible to infections and tumors, in addition to having off-target side-effects. Donor-specific transplantation tolerance, in which alloreactive T cells are specifically incapacitated while leaving the rest of the immune responses intact, has long been the goal for clinical transplantation. Robust peripheral tolerance to allografts can be achieved in mice by administration of anti-CD154 (anti-CD40L) mAb and donor-specific transfusion (DST) (Niimi et al., 1998). Such treatment at the time of transplantation results in long-term acceptance of a first cardiac allograft and subsequent acceptance of a second donor-matched heart, while allowing normal rejection of a second genetically distinct heart (Wang et al., 2009b).

In humans, transplantation tolerance has been challenging to achieve, but in recent years several groups have been able to induce it prospectively both in HLA-

matched and HLA-mismatched donor-recipient combinations (Kawai et al., 2008; Leventhal et al., 2012; Scandling et al., 2008). In addition, a proportion of liver and renal transplant recipients treated with conventional immunosuppression and who subsequently discontinued treatment, achieved a state of operational tolerance in which the transplanted organ remained stably functional for years with minimal histological signs of graft pathology (Feng et al., 2012; Martinez-Llordella et al., 2007; Newell et al., 2010; Sagoo et al., 2010). Notably, some of these tolerated transplants eventually succumbed to rejection, which manifested as a slow deterioration in graft function (Brouard et al., 2012). The underlying basis for allograft rejection after long-periods of operational tolerance is not known, although in some instances bacterial infections have been described to precede graft loss (Ahmed et al., 2011b; Brouard et al., 2012). These observations of a potential link between infection and graft loss are reminiscent of our previous report showing, in mice, that infection with the intracellular Gram-positive bacillus *Listeria monocytogenes* (Lm) ≥60 days after stable heart allograft tolerance precipitated rejection in a fraction of infected hosts (Wang et al., 2010). Lm-triggered rejection was T cell-dependent, correlated with increased intra-graft donor-specific T cell alloreactivity in the absence of detectable cross-reactivity between bacterial and donor antigens (Wang et al., 2008b), and was dependent on the production of IL-6 and signaling through the type I IFNR (Wang et al., 2010). Furthermore, acute rejection of tolerant allografts in uninfected recipients was recapitulated by the combined induction of IL-6 and IFNβ *in vivo* (Wang et al., 2010).

It is assumed that the loss of operational tolerance in patients or animals is permanent such that re-challenge with a donor-matched graft would result in accelerated rejection, paralleling the consequences of acute rejection in a naïve host. Using a fully allogeneic cardiac allograft mouse model, we here report that the loss of transplantation tolerance following Lm infection-triggered allograft rejection is unexpectedly transient, as a second donor-matched transplant is spontaneously accepted after the temporary period of alloreactivity triggered by the infection waned. We show that this restored state of tolerance that dominates over a memory of rejection is mediated by Tregs and exhibits exquisite specificity, as these recipients are able to generate protective immunity against the infectious agent and remain capable of rejecting third party cardiac allografts.

## C. Materials and Methods

### Study design

To calculate the sample size for the comparison of means of two independent samples, utilizing the online sample size calculator from Rollin Brant (University of British Columbia), with at least n=3 per group, there is 80% power to detect 3.5 fold differences or greater even if the average standard deviation is as large as the smaller group's mean. With at least n=4 per group and alpha=0.05, there is 80% power to detect 3 fold differences or greater in population means. With these same parameters, to have 80% power to detect a 2.5 fold change, at least 7 individuals per group were needed and to detect a 2-fold change, at least 16 individuals per group were needed.

If the standard deviation is half the smaller group's mean, then n=4 is sufficient at 80% power to see 2-fold changes or greater, and n=3 is sufficient at 80% power to see 2.25-fold changes or greater. When results were not Gaussian, non-parametric statistics were used in the final analysis with often twice the number of samples than would be required to see a difference if the data were Gaussian once multiple experiments are pooled. Multiple comparisons were corrected by Bonferroni (parametric) or Dunn's (non-parametric) tests.

For graft survival analyses, using the PS program (Power and Sample size version 3.1.2), with alpha=0.05, a follow-up time of 60 days and the control group rejecting with a MST = 7 days, an average of n=4 animals were needed per group to detect hazard ratio of 0.137 or lower. This shifts to n=5 for hazard ratios 0.17 or below, and to n=6 for hazard ratios of 0.198 or below. These numbers remain similar if the shorter median survival time of two groups is 23 days provided there is a longer follow-up time of 120 days. For animal survival analyses, the PS program was also used. With alpha=0.05, a follow-up time of 20 days, and the primary lethal infection group succumbing to infection with a MST of 3 days, n=4 animals were needed per group to detect a hazard ratio of 0.164 or lower.

No outliers were excluded and each experiment was repeated at least twice. The number of replicate experiments and whether the results were pooled from multiple experiments is listed in each figure legend.

Mouse transplantation and Lm infection

6-8 week-old female C57BL/6 (B6, H-$2^b$) mice as recipients and BALB/c (B/c, H-$2^d$) and C3H/HEN (C3H, H-$2^k$) mice as donors were purchased from Jackson or Harlan Laboratories. Mouse abdominal heterotopic cardiac transplantation was performed using a modified technique from Corry and colleagues (Wang et al., 2008b) in which the aorta and pulmonary artery of the graft end-to-side was anastomosed to the recipient's aorta and vena cava, respectively, while cervical area heterotopic cardiac transplantation was performed with the aorta and pulmonary artery of the graft anastomosed end-to-end to the recipient's common carotid artery and jugular vein, respectively. Transplantation of secondary hearts in the abdominal cavity was performed with vascular anastomoses similar to (aorta and pulmonary artery of the graft to the abdominal aorta and inferior vena cava of the host) but immediately adjacent (above or below) to those of the primary allograft. The rejected primary allograft was left in place and recipients did not receive antibiotics. Tolerance was induced by administration of anti-CD154 (BioXCell; 0.3-0.5mg/mouse, i.v. on d0, and i.p. on d7, and d14 post-transplantation) and DST ($10^7$ donor splenocytes on the day of transplantation) to recipient mice. Cardiac grafts were checked by palpation and the day of rejection was defined as the last day of detectable heartbeat. *Listeria monocytogenes* (Lm) engineered to express the model antigen ovalbumin (OVA) was grown overnight and then re-diluted 1:50 the next day and regrown for 1.5 hours to obtain cultures in early log phase growth before enumeration of CFU by measurement of the absorbance at $OD_{600}$ with a spectrophotometer (Wang et al., 2008b). Doses of 3-5 x $10^6$ CFU (i.p.)

were chosen for infection experiments to obtain highest rejection rate with minimal lethality. In some experiments, Lm ($10^5$ CFU) was administered on the day of transplantation to mice that did not receive immunosuppression (acute rejection group), as a control. Allograft rejection was determined by heartbeat cessation using manual palpation of the closed abdomen. In addition, abdominal hearts were visually inspected prior to sacrifice to ensure that the grafts never regained a heartbeat following Lm-dependent rejection. For lethal Lm challenge, mice were infected with $1.2 \times 10^7$ CFU (i.p.). All Lm-infected mice were kept in biosafety facilities. All animals were used in agreement with the University of Chicago Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Histopathology

Grafts were removed and placed in 10% formalin. Sections were stained with H&E and the slides were scanned via CRi Pannoramic Scan Whole Slide Scanner at 40x, and then patched together to form one single image of the tissue. Pannoramic Viewer (a free downloadable software from 3DHISTECH) was used to view these image files. Images were examined by two independent investigators in a blinded manner. Histological sections were scored based on the percentage of infiltration observed in the interstitial tissue and as follows: 0% = 0, 1-25% =1, 25-50% = 2, 50-75% = 3, and 75-100% = 4.

IFNγ ELISpot assays

T cells from spleen were purified by negative selection using cell isolation kits (Miltenyi Biotec Inc.), and cell purity was confirmed to be >93% by flow cytometry. T cells ($2x10^5$/well in triplicate) were co-cultured for 24 hours with T cell-depleted irradiated (3000 rads) C57BL/6xBALB/c (F1) or BALB/c splenocytes, or with syngeneic (B6) splenocytes incubated with or without heat-killed Lm ($5\times10^5$/well). An IFNγ ELISpot kit was used according to manufacturer's instructions (BD Biosciences) and the numbers of spots per well were enumerated using the ImmunoSpot Analyzer (CTL Analyzers LLC).

Isolation of graft infiltrating cells

Graft-infiltrating cells were isolated from cardiac grafts that were extensively rinsed with heparin/1xHBSS solution (Cellgro), cut into small pieces and digested with 400 U/ml collagenase IV (Sigma), 0.01% DNase I (MP Biomedicals), 10 mM HEPES (Cellgro) in HBSS. The cells were then washed, stained, and analyzed by flow cytometry.

Enumeration of Listeria Colony Forming Units

Spleens, livers, transplanted hearts and native hearts were isolated from each animal at 48 hours post Lm infection. Tissues were homogenized first with a surgical blade and then with a Tissue Tearor hand-held homogenizer (Biospec Products) in 0.05%

Tween in water. Serial dilutions of the homogenates were plated on Brain Heart Infusion agar (BD) and incubated at 37ºC for 24 hours before colonies were counted.

Flow cytometry analysis

Splenocytes and total graft-infiltrating cells were stained using monoclonal antibodies specific for mouse CD90.2 (53-2.1, cat# 0902), CD4 (GK1.5, cat# 0041), CD8 (53-6.7, cat# 0081), CD44 (IM7, cat# 0441), PD-1 (J43, cat# 9985), CTLA-4 (UC10-4B9, cat# 1522), IFNγ (XMG1.2, cat# 7311) and Foxp3 (FJK-16s, cat# 5773) (eBioscience), and in some experiments, OVA:$K^b$ OVA pentamers (ProImmune) to identify Lm-OVA-specific T cells, and analyzed by flow cytometry (LSRII, BD). Pentamer staining was performed using one test (10 μl) to stain 5 million cells in 100 μl. For intracellular cytokine analysis, whole lymph node cells (cervical, axillary, brachial and inguinal) or splenocytes were stimulated for 24 h with T-cell-depleted BALB/c x C57BL/6 F1 or C57BL/6 splenocytes with or without heat-killed Lm, and all samples were incubated with GolgiPlug (BD Biosciences) for the last 6 h. Cells were resuspended at $10^6$ cells/20 μl and the following dilutions of 0.2 mg/ml of antibodies to CD90.2 (1:400), CD8 (1:100), CD4 (1:100), PD-1 (1:100), CTLA-4 (1:00), and IFNγ (1:100) were used. CD44 and Foxp3 antibodies were diluted 1:100 from a 0.5 mg/ml stock. Cells were fixed and permeabilized for intracellular staining using a Foxp3 buffer staining kit (eBioscience). Viability of the cells was determined by using Viability Dye (for fixed samples) or DAPI (for unfixed samples) (Invitrogen). Data were analyzed using FlowJo software (TreeStar).

In vivo depletion and blockade with monoclonal antibodies

$CD25^+$ T cells were depleted with a single i.v. dose of anti-CD25 (PC61, 0.4 mg/mouse, BioXCell) on the day prior to second heart transplantation. Depletion was confirmed by loss of $Foxp3^+CD4^+$ cells as determined by flow cytometry of peripheral blood mononuclear cells 1 week post antibody administration.

Statistical methods

The PS program was used to calculate survival analyses (Dupont and Plummer, 1990) and the online power calculator from Rollin Brant (University of British Columbia) was used for sample size calculations for comparison of two means. The two-tailed Student's t test or one or two-way ANOVA and post-hoc Bonferroni test for multiple comparisons, or, where appropriate, non-parametric Mann-Whitney or Kruskal-Wallis with Dunn's post-hoc was performed to determine statistical differences between groups. Graft mean survival time (MST) and p-values were calculated using the Kaplan-Meier/log rank test (Prism5; GraphPad Software Inc.).

D. Results

1. Return of tolerance after infection-induced graft rejection

Naïve hosts who acutely reject an allograft after transplantation develop allosensitization that results in the accelerated rejection of second donor-matched allografts transplanted at distal locations (Gibson and Medawar, 1943; Medawar, 1944, 1945). We tested whether tolerant hosts that reject an allograft following a bacterial

infection become similarly allosensitized. To this end, we used an experimental model of cardiac transplantation, where fully-mismatched BALB/c (H-$2^d$) grafts vascularized in the abdominal cavity were accepted long-term by C57BL/6 (H-$2^b$) recipients following a transient treatment at the time of transplantation with anti-CD154 and DST. Intraperitoneal (i.p.) infection with Lm 60 days post-transplantation overcame tolerance and induced a T cell-dependent rejection in approximately half of the recipients (Fig. 2.1a and (Wang et al., 2010)). This rejection was unlikely to be due to non-tolerant anti-Lm T cells that cross-reacted with BALB/c antigens, as untransplanted C57BL/6 mice that had been pre-sensitized with Lm did not display enhanced responsiveness to BALB/c stimulators *in vitro* when compared to unsensitized controls (Fig. 2.1b), or increased cytotoxicity to BALB/c targets *in vivo* (Wang et al., 2008b), or acquired resistance to costimulation blockade upon subsequent transplantation with BALB/c hearts in contrast to BALB/c-sensitized recipients (Wang et al., 2008b). We further confirmed that the rejection-triggering effect of Lm was not limited to the BALB/c to C57BL/6 strain combination, as a subset of C57BL/6 mice made tolerant to C3H/HEN (H-$2^k$) cardiac allografts also rejected their transplant acutely following Lm infection (Fig. 2.1c).

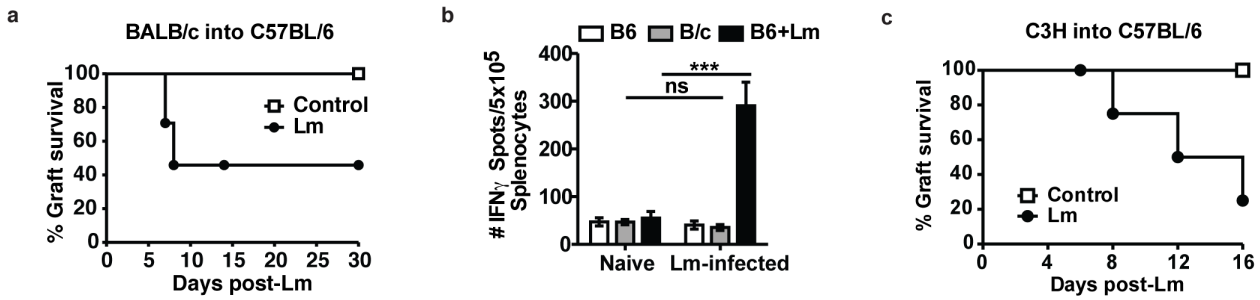


**Figure 2.1: Lm infection of transplant tolerant mice triggers acute allograft rejection in a subset of animals**

B/c (a) or C3H/HEN (c) hearts were transplanted into B6 recipients treated with anti-CD154+DST at the time of transplantation (a, n=29; c, n=7). On day 60 post-transplantation, a subset of tolerant mice (a, n=24; c, n=5) was infected with Lm i.p. and graft survival was assessed in these mice and in uninfected control tolerant mice. $p<0.05$ by Log-rank test. (b) Naïve C57BL/6 mice were infected with Lm. Mice were sacrificed 7 weeks later and splenocytes from uninfected and Lm-infected mice were stimulated with T-depleted C57BL/6xBALB/c F1 (B/c) or syngeneic B6 splenocytes and analyzed by ELISpot (n=3 per group, experiment repeated twice). Data are presented as mean ± SEM. The syngeneic response was subtracted from the alloreactive response.

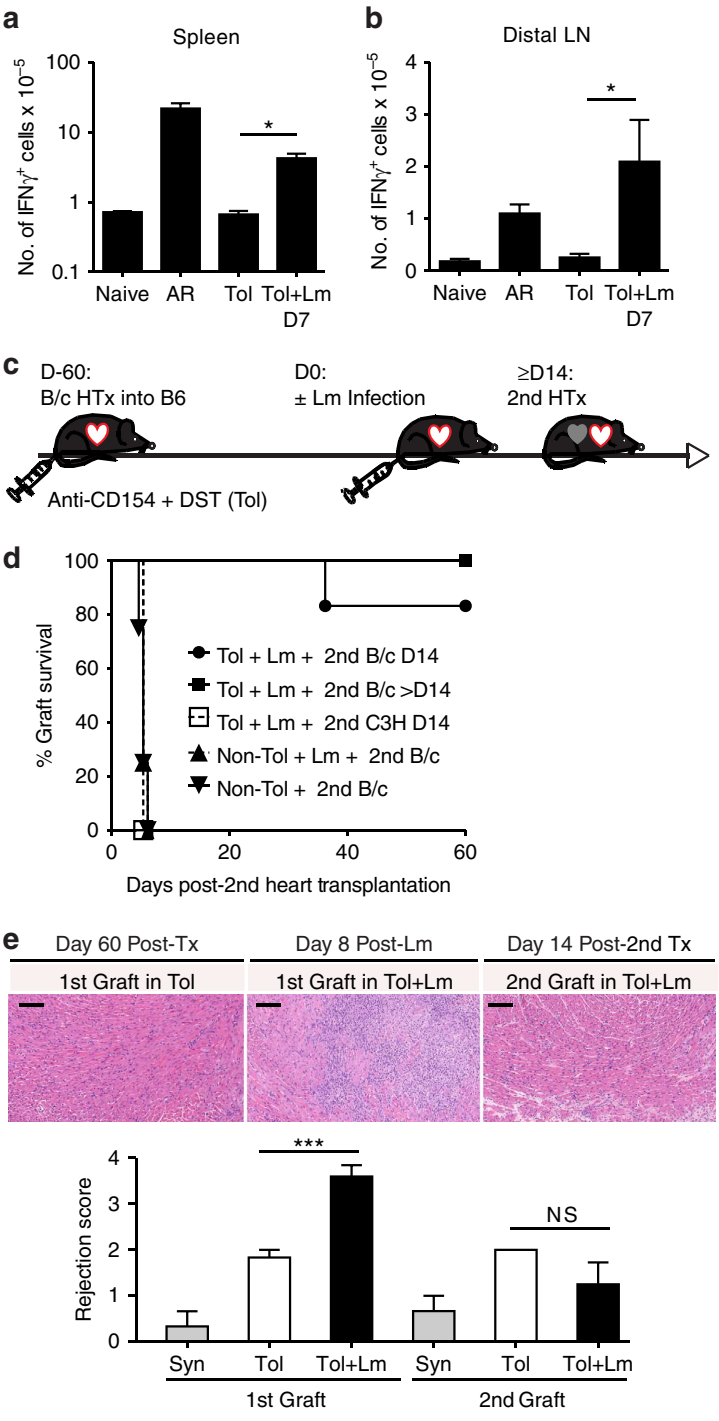


**Figure 2.2: Absence of memory of transplant rejection following acute rejection of tolerated allografts**

**(a)** and **(b)** BALB/c (B/c) hearts were transplanted into C57BL/6 (B6) recipients untreated (acute rejection, AR group) or treated with anti-CD154+DST at the time of transplantation (Tol group).

**Figure 2.2, continued:** On day 60 post-transplantation, a subset of tolerant mice were infected with Lm i.p. (Tol+Lm group). Untransplanted mice (naïve group) were used as controls. Splenocytes (**a**) and distal peripheral lymph nodes (Distal LN) including cervical, brachial and axillary populations (**b**) from naïve, AR (D7 post-HTx), Tol (D60 post HTx), and Tol+Lm (D7 post-Lm) mice were stimulated with T-depleted B6 (syngeneic) or B6xB/c F1 (allogeneic) splenocytes. IFNγ-producing cells were analyzed by intracellular flow cytometry on $CD44^+CD90^+$-gated events (Naïve, Tol, Tol+Lm n=8 per group, AR n=4, experiment repeated twice, results pooled). The percentage of T cells specific for alloantigen (%allo-%syn) was multiplied by the number of live cells counted by the Trypan blue exclusion method to obtain the total number of $IFN\gamma^+$ alloreactive T cells. Statistical significance at * $p<0.05$ by Kruskal-Wallis test with Dunn's. (**c**) Experimental design for the transplantation of second allografts. (**d**) B6 mice treated with anti-CD154+DST at the time of B/c abdominal heart transplantation (on day minus 60) and that had rejected their allografts following Lm infection ($3\text{-}5x10^6$ CFU i.p. on day 0) (Tol+Lm) were re-transplanted in the neck on day 14 with B/c (n=6) or C3H (n=4) allografts, or on days >14 (21-42) with B/c allografts (n=4). As controls, animals transplanted with B/c hearts ± Lm infection at the time of transplantation (Non-Tol +/-Lm + $2^{nd}$ B/c) received a $2^{nd}$ B/c heart 2 weeks later (n=4 each) in the neck. (**e**) Histology of $1^{st}$ Tol B/c graft 60 days post-transplantation (left), $1^{st}$ Tol+Lm B/c graft at the time of rejection 8 days post-infection (middle), and $2^{nd}$ B/c cervical graft day 14 post-$2^{nd}$ transplantation into mice that had rejected their first tolerated B/c heart following Lm infection (Tol+Lm, right), and composite histological scores. Scale bar=100 μm. Data are presented as mean ± SEM of 3-6 hearts/group, repeated at least twice. Statistical significance by Student's t-test at ***$p\leq0.001$ or not significant (ns).

Consistent with allosensitization, we detected an increase in the number of IFNγ-producing alloreactive T cells at the time of graft rejection in the spleens of tolerant mice following intra-peritoneal Lm infection. We similarly observed an increase in the number of IFNγ-producing alloreactive T cells in peripheral lymph nodes (Fig. 2.2a,b) distal to the secondary lymphoid organs where T cells are primed following cardiac transplantation (Reed et al., 2003). The presence of alloreactive T cells in both the spleen and distal lymph nodes supports their systemic recirculation from the sites of T cell priming during the process of Lm-mediated loss of tolerance.

To assess if this infection-triggered allograft rejection and increase in alloreactivity in formerly tolerant recipients would result in accelerated rejection of a second transplant, animals that had completely rejected their abdominal allografts following Lm infection were re-transplanted in the cervical area with either donor-matched (BALB/c) or third-party (C3H/HEN) cardiac allografts 14 days after Lm infection (Fig. 2.2c). Instead of undergoing rejection in an accelerated fashion, the second donor-matched hearts were spontaneously accepted long-term in the absence of immunosuppressive therapy, while third party C3H/HEN transplants were promptly rejected (Fig. 2.2d). Similar spontaneous acceptance of second BALB/c hearts was observed with grafts transplanted on day 42 rather than day 14 post-infection of tolerant recipients, to allow more time for potential memory responses to develop. Histology of second donor-matched cervical hearts at day 14 post-transplantation confirmed an absence of acute cellular rejection, with similar low rejection scores in second hearts transplanted into previously infected or uninfected tolerant recipients and in contrast to high scores in the first hearts undergoing Lm-dependent acute rejection (Fig. 2.2e). Thus, unlike naïve animals that acutely reject second distal donor-matched allografts, tolerant animals that undergo infection-triggered acute rejection spontaneously accepted second distal donor-matched allografts.

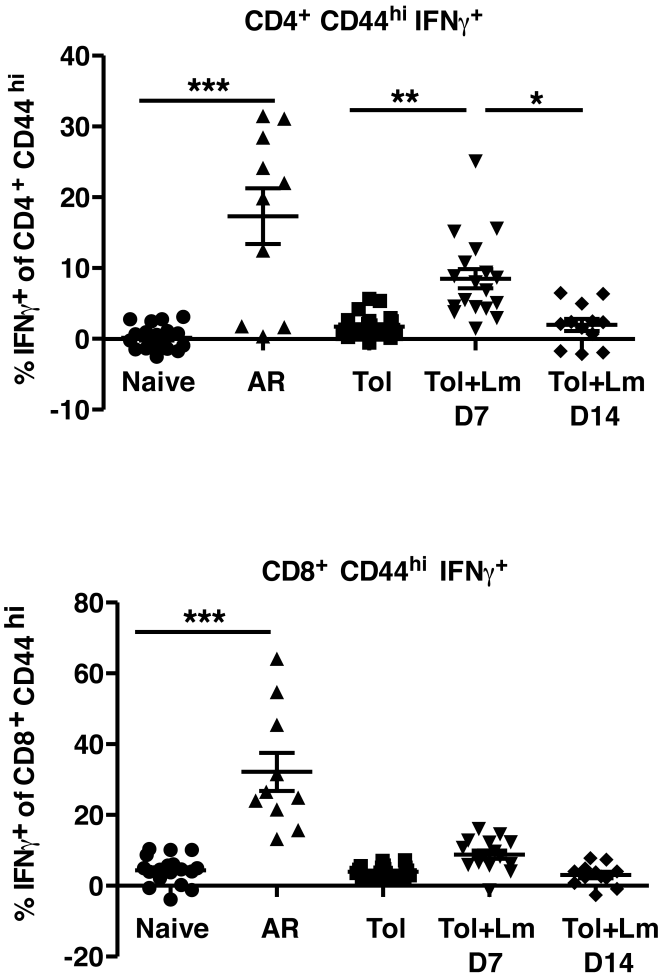


**Figure 2.3: Lm infection in transplant tolerant hosts results in transient increase in alloreactivity**

B/c hearts were transplanted into B6 recipients either untreated (AR group) or treated with anti-CD154 at the time of transplantation (Tol group). Some tolerant animals were infected with Lm at day 60 post-transplantation (Tol+Lm group). Unmanipulated naïve mice were used as controls (naïve group, n=20). Animals were sacrificed 7 days after transplantation for the AR group (n=10), >60 days for the Tol group (n=20), and day 7 (n=15) or day 14 (n=12) for the Tol+Lm groups. Splenocytes were restimulated overnight with T-depleted B6 syngeneic, or B/cxB/6 F1 allogeneic splenocytes in the presence of golgi plug, permeabilized and stained with anti-CD4, -CD8, -CD44 and –IFNγ and analyzed by flow cytometry, gating on $CD44^{hi}$ cells. The percentage of $IFN\gamma^+$ $CD4^+$ and $CD8^+$ cells specific for alloantigen (%allo-%syn) is plotted. Results are pooled from five independent experiments and are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with Bonferroni post-test.

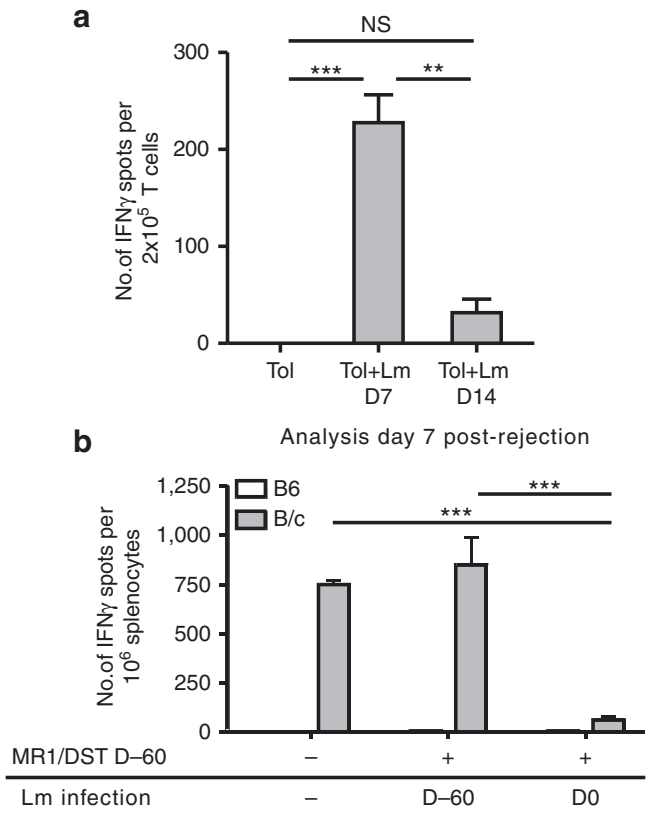


**Figure 2.4: The detection of allosensitization after Lm is transient**
(**a**) Splenic enriched T cell populations from Tol+Lm mice were stimulated with T-depleted B6xB/c F1 and syngeneic B6 splenocytes and analyzed by IFNγ ELISpot (n=3 per group, experiment repeated twice; syngeneic response subtracted from allogeneic response). (**b**) Frequencies of alloreactive IFNγ-producing cells analyzed by IFNγ ELISpot on day 7 after unmodified acute rejection (AR) or rejection in anti-CD154+DST-treated recipients infected with Lm on the day of transplantation (Lm D -60), versus following Lm-mediated rejection in tolerant mice (Lm D 0). N=3-5/group and experiments repeated at least twice. Data are presented as mean ± SEM. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns, not significant by one-way ANOVA with Bonferroni or Kruskal-Wallis with Dunn's for multiple comparisons.

2. Donor reactivity following Lm infection is transient

To investigate the mechanism underlying the spontaneous acceptance of a second donor-matched allograft, anti-donor responses were measured at the time of second transplantation. Whereas the frequency (Fig. 2.3 and Fig. 2.4a) of detected IFNγ-producing splenic alloreactive T cells was increased at day 7 post-Lm infection,

coincident with the rejection of the established first allograft, this frequency was significantly reduced at the time of transplantation of the second allograft (14 days after Lm infection and 7 days after rejection of the established grafts) and was comparable to tolerant non-infected recipients. This was different from non-tolerant mice that had acutely rejected their grafts, and where significantly increased frequencies of IFNγ-producing alloreactive cells remained detectable 7 days after rejection (Fig 2.4b). Furthermore, when Lm infection was on the day of transplantation, a time at which it prevents the induction of tolerance by anti-CD154/DST and triggers rejection 7-8 days after transplantation (Wang et al., 2008b), elevated frequencies of IFNγ-producing alloreactive T cells were also observed 7 days after acute rejection (Fig 2.4b), indicating that Lm infection *per se* does not trigger the rapid reduction in the frequency of IFNγ-producing alloreactive T cells.

3. The return of transplantation tolerance is Treg-dependent

The transiency of the measurable alloresponse after Lm infection of tolerant mice could theoretically be due to impaired survival or function of the alloreactive T cells, and/or regulation by $Foxp3^+$ T cells. Indeed, we had previously shown that tolerated allografts contained a high percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells among $CD4^+$ cells (Chen et al., 2009; Wang et al., 2010). We confirmed here that the high percentage of $CD4^+Foxp3^+$ cells among $CD4^+$ events in the first accepted allografts was significantly reduced during Lm-dependent acute rejection of the first allografts. This reduction in percentage of $Foxp3^+$ cells was due to an influx of effector T cells as the

total number of Tregs remained unchanged with Lm infection ((Wang et al., 2010) and see Fig. 2.6c). Of interest, the percentage of $Foxp3^+$ cells was re-established in the donor-matched second allografts transplanted 14 days after Lm infection (Fig. 2.5a), and was in fact, similar to the percentage of $CD4^+Foxp3^+$ T cells in second donor-matched allografts transplanted into non-infected tolerant recipients. This Treg percentage was significantly higher than that detected in syngeneic grafts, consistent with the tolerance to allografts being actively maintained by Tregs. Finally, a greater percentage of Tregs from tolerated allografts expressed a PD-$1^{high}$ and CTLA-$4^{high}$ phenotype of activated Tregs (Rosenblum et al., 2011), compared to Tregs from the spleen of naïve or tolerant animals (Fig. 2.5b,c).

To determine whether functional alloreactive T cells persisted at the time of second transplantation and whether they were suppressed by regulatory cells after the infection subsided, previously tolerant animals that had rejected their abdominal graft following Lm infection were depleted of Tregs by treatment with the anti-CD25 mAb PC61 one day before the transplantation of a second cervical BALB/c heart. Anti-CD25 administration prevented the acceptance of the second cervical transplants in animals that had rejected their first abdominal allografts after Lm infection (Fig. 2.5d). Thus, these data confirm that alloreactive T cells capable of rejecting a transplant had been induced following Lm infection, and persisted systemically at the time of second transplantation but their function was dominantly suppressed by $CD25^+$ regulatory cells. In contrast, anti-CD25 administration did not trigger rejection of second allografts transplanted into tolerant uninfected recipients.

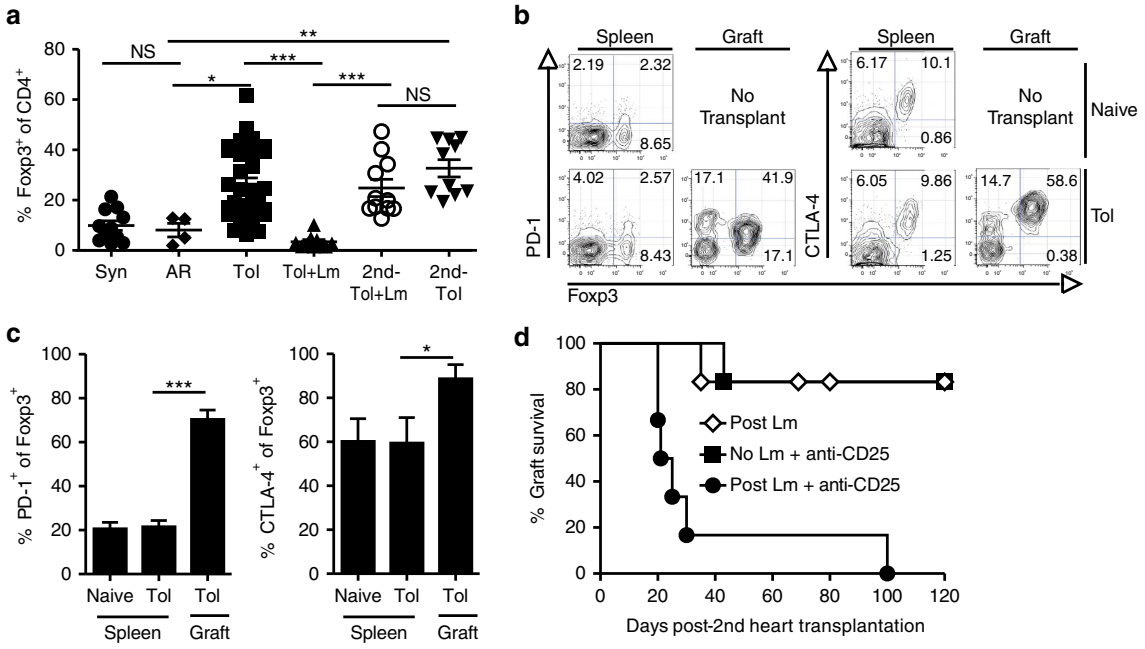


**Figure 2.5: The return of tolerance is dependent on Tregs**

(**a**) Cardiac allografts were harvested >60 days post transplantation for syngeneic grafts (Syn, n=11) or allogeneic tolerated grafts (Tol, n=34). AR allografts (n=4) were harvested 1 week post transplantation. Loss of tolerance hearts were harvested 1 week post Lm (with infection at 60 days post-transplant) (Tol+Lm, n=10). For the restoration of tolerance, second donor cardiac allografts were transplanted 14 days after Lm infection of tolerant recipients, i.e. 1 week post-rejection of the first allograft ($2^{nd}$-Tol+Lm, n=11). Control second donor allografts were transplanted in tolerant non-infected mice ($2^{nd}$-Tol, n=10) at a comparable time (74 days post $1^{st}$ heart transplant). Second grafts were harvested 2 weeks after transplantation. Graft-infiltrating leukocytes were analyzed by flow cytometry, gated on $CD4^+$ events and displayed as % $Foxp3^+$ of CD4 cells. Data are presented as mean ± SEM and are pooled from 2-17 replicate experiments. (**b**) and (**c**) Representative flow cytometry plots of PD-1 and CTLA-4 expression on $Foxp3^+$ Tregs in the spleen and grafts of naïve, untransplanted and tolerant mice (**b**) and quantification (**c**). Naïve spleen n=12, Tol spleen n=9, Tol graft n=12. Data are presented as mean ± SEM and are pooled from 7-8 experiments. *p<0.05; **p<0.01; ***p<0.001; ns, not significant by one-way ANOVA with Bonferroni or Kruskal-Wallis with Dunn's for multiple comparisons. (**d**) Fourteen days after Lm infection, recipients with rejected hearts and uninfected tolerant mice were transplanted with donor-matched heart grafts. Anti-CD25 treatment was administered once, the day before second heart transplantation (n=6 each). Controls (Post-Lm, n=6) are the same as shown in Figure 2.1d. Data are presented as percent graft survival of the second B/c hearts with or without anti-CD25 therapy (p<0.01 by Log-rank test) and are pooled from 3 experiments.

4. Local return of tolerance at the site of infection

Because Lm was injected intraperitoneally, it was likely that the bulk of the infection, inflammation and anti-Lm immune responses were occurring in the abdomen. This raised the possibility that Lm infection may have abrogated transplantation tolerance permanently in the peritoneal cavity where the first heart was located, whereas distal tolerance may have persisted to permit acceptance of the second hearts placed in the cervical area. Intraperitoneal Lm infection indeed resulted in high bacterial counts in the spleen and liver of untransplanted, syngeneic and allogeneic graft recipients, and in the syngeneic and allogeneic abdominal grafts, while significantly lower counts were detected in the native heart, outside of the peritoneal cavity (Fig. 2.6a). The infection was associated with a strong anti-Lm response in the graft and spleen of allograft recipients, as determined using Lm engineered to express the model antigen Ovalbumin (OVA) and fluorescently-coupled OVA:$K^b$ pentamers to identify Lm-reactive T cells (Fig. 2.6b), and intra-graft Lm-specific T cells contributed to the overall increase in the total number of effector T cell subsets in the grafts (Fig. 2.6c). Furthermore similar Lm CFUs and anti-Lm T cells were detected in syngeneic cardiac grafts from infected mice (Fig. 2.6a,b), but only the allogeneic but not the syngeneic grafts stopped beating after Lm infection (Wang et al., 2010). These observations therefore confirm that a strong local inflammatory response to Lm was elicited within the abdominal graft, even though it was not sufficient to trigger graft loss in the absence of alloreactivity.

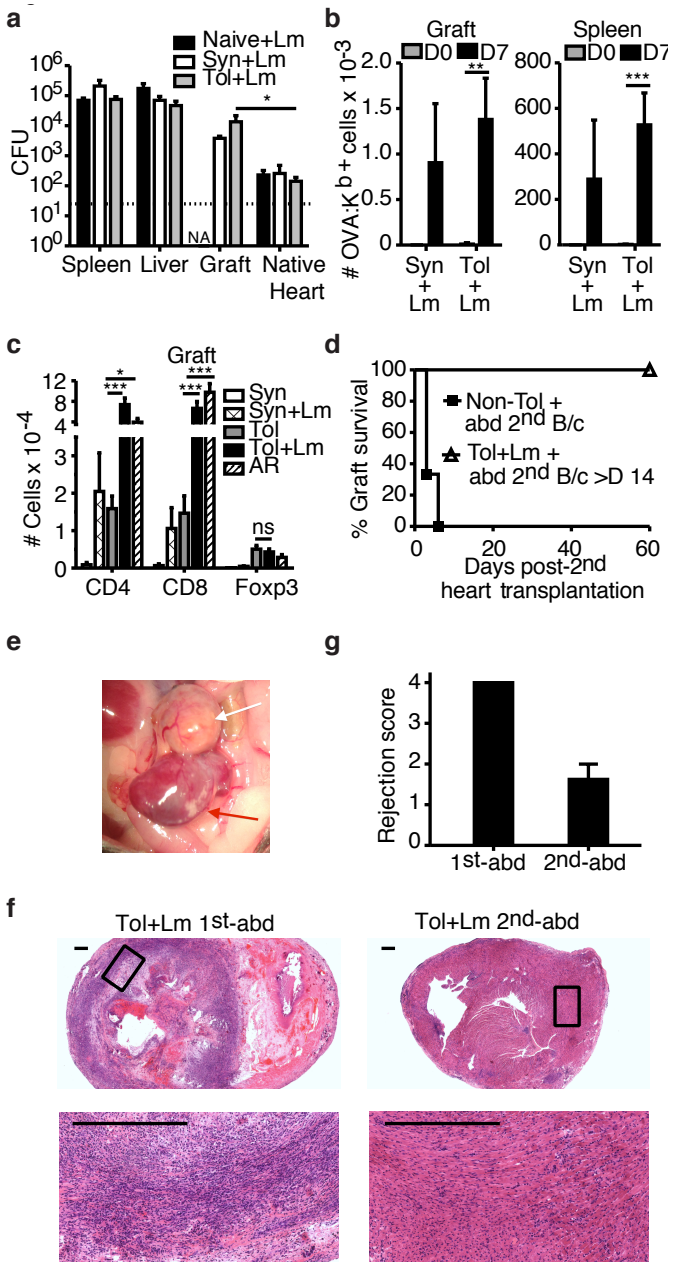


**Figure 2.6: Lm infection elicits a systemic alloresponse in tolerant mice**
(**a**) CFU numbers at 48 hours post-Lm infection in the spleen, liver, grafts, and native hearts of B6 mice that were either not transplanted (Naive), or were tolerant to an allogeneic B/c heart (Tol) or had received a syngeneic heart (Syn) at least 60 days prior to Lm infection. Results are pooled from two independent experiments: Naïve+Lm (n=7), Tol+Lm (n=10), Syn+Lm (n=3). NA: not applicable. *p<0.05 by Kruskal-Wallis with Dunn's post-test. (**b**) The total number of OVA:$K^b$-binding Lm-specific T cells in the graft and spleen of syngeneic (Syn) or tolerant (Tol) mice, on D 0 or D 7 post-infection, was determined by flow cytometry.

**Figure 2.6, continued:** Syn D 0 n=2, Syn+Lm D 7 n=4, Tol D 0 n=4, Tol+Lm D 7 n=5. Data are pooled from two independent experiments. **p<0.01 or ***p<0.001 by Two-way ANOVA with Bonferroni post-test. (**c**) Total numbers of $CD4^+$, $CD8^+$ and $CD4^+$ $Foxp3^+$ T cells within syngeneic (Syn) or tolerated (Tol) grafts, or 7-8 days post-Lm infection, and in grafts from untreated mice undergoing AR (data pooled from three independent experiments. Syn n=2, Syn+Lm n=4, Tol n=8, Tol+Lm n=8, AR n=4). *p<0.05 or ***p<0.001 by two-way ANOVA with Bonferroni post-test. (**d**) Tol+Lm mice that had rejected their allografts following Lm infection (3-5x$10^6$ CFU i.p. on day 0) were re-transplanted in the same location (abdominal area) on days 19-21 post-Lm with B/c allografts (abd n=5). AR mice (Non-Tol) were re-transplanted in the abdominal area on days 21-34, (n=3, p<0.01 by Log-rank test). (**e**) Example (from D) of a white fibrotic rejected first allograft in a Tol+Lm mouse (white arrow), and of the second well-vascularized beating allograft transplanted immediately below the rejected allograft 3 weeks after Lm infection, in the absence of immunosuppression (red arrow). The photograph was taken at sacrifice, D 60 after second transplantation. (**f**) Representative histology of $1^{st}$ abdominal B/c graft (Tol+Lm $1^{st}$ abd, left), and $2^{nd}$ B/c abdominal graft (Tol+Lm $2^{nd}$ abd, right), of the mice described in (D, E) taken at D 60 post-transplantation of the second allograft at lower (top) and higher (bottom) magnifications. Scale bar=200 μm. (**g**) Composite histological scores (Mean ± SEM of 4 hearts/group).

To test whether transplantation tolerance was permanently abrogated at the site of the Lm infection, mice post-Lm-mediated rejection of a primary allograft were re-transplanted 14-21 days post-first allograft rejection with a donor-matched cardiac allograft placed also in the abdominal cavity, immediately adjacent to the primary rejected heart (rather than in the cervical area as shown in Figure 2.2d). Remarkably, these second abdominal allografts were also spontaneously accepted (Fig. 2.6d,e,f). These data indicate that tolerance returned even at the site of maximal infection, thus underscoring the resilience of robust transplantation tolerance that could be transiently abrogated during an intense inflammatory response but returned to dominate over alloreactivity when the infection was cleared.

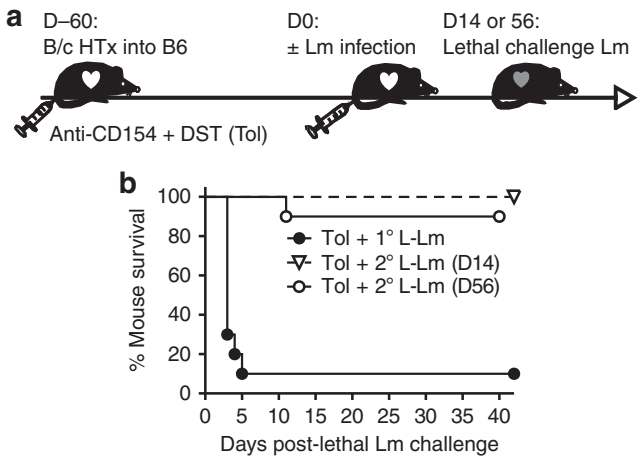


**Figure 2.7: Animals experiencing a return of transplantation tolerance can successfully develop protective immunity to a lethal challenge of Lm**
Tolerant mice received a primary lethal (L) challenge of Lm (1.2 x $10^7$ CFU) ($1^o$ L-Lm, n=10) or a sublethal Lm infection on day 0 followed by graft rejection and subsequent re-infection with a secondary lethal Lm challenge ($2^o$ L-Lm) on day 14 or 56 after the first infection (n=7-9/group). Data are pooled from two independent experiments. (**a**) Experimental design. (**b**) Data are presented as percent animal survival after lethal Lm challenge ($p<0.001$ for $2^o$ L-Lm on D 14 or D 56 compared to $1^o$ L-Lm by Log-rank test).

5. Development of memory to Lm in transplant-tolerant mice

The inability of tolerant mice to demonstrate a functional memory of the transplant rejection prompted us to test whether this defect extended to the development of protective immunity to Lm. Lm-infected tolerant animals were challenged with a dose of Lm that was lethal to tolerant recipients that had not been previously infected (Fig. 2.7a). Memory to Lm developed successfully as the Lm-infected tolerant animals were protected against a lethal challenge of Lm administered either 2 or 8 weeks after the first infection (Fig. 2.7b). Thus, Lm infection abrogated established transplantation tolerance without inducing a functional allograft-specific memory, whereas anti-Lm memory developed normally.

## E. Discussion

The spontaneous restoration of tolerance following infection-mediated rejection provides experimental evidence of a transplant outcome that does not follow the rules of allosensitization described for non-tolerant animals by Medawar and his colleagues (Gibson and Medawar, 1943; Medawar, 1944) as it demonstrates in mice with established transplantation tolerance that a memory of regulation can dominate over a memory of infection-triggered rejection. We previously reported that the loss of transplantation tolerance following Lm infection depended on the production of the inflammatory cytokines IL-6 and IFNβ (Wang et al., 2010). We and others have shown that IL-6 augments effector T cell responses, rendering them less susceptible to suppression by Tregs *in vitro* and preventing iTreg differentiation, whereas IFNβ promotes the acquisition by conventional T cells of IFNγ production and its consequent effector functions (Bettelli et al., 2006; Pasare and Medzhitov, 2003; Wang et al., 2010). Our current results suggest that the pro-inflammatory effects of the Lm-response are transient even at the site of infection, where the concentration of pro-inflammatory cytokines is presumed to be the highest.

The susceptibility to rejection following infection in recipients with established tolerance has implications for the notion that tolerance can achieve the goal of "one transplant for life" in humans (Kawai et al., 2014a). Indeed, viral as well as other types of bacterial infections have been described in immunosuppressed as well as tolerant transplant patients preceding episodes of acute rejection or allograft loss, especially when infections are in the vicinity of the graft (Ahmed et al., 2008; Brouard et al., 2012;

Kawai et al., 2014b; Leventhal et al., 2013). Even though Lm infections have been reported in transplanted patients (Fishman, 2007) thereby providing a clinical context for our experimental model, it is important to point out that we used this single infectious agent in two donor-recipient combinations, such that the generalizability of infections breaking established transplantation tolerance is not yet clear.

We speculate that infections close to or within the grafts may be more likely than distal infections to achieve local levels of inflammatory cytokines that are capable of uncoupling graft-infiltrating alloreactive T cells from the immunosuppressive pathways that keep them in check. We had previously demonstrated that the ability of Lm to break established transplantation tolerance is the result of synergy between the inflammatory axes of type I IFN and IL-6 signaling (Wang et al., 2010). While cytokine production is likely to be dampened in transplant patients maintained on pharmacological immunosuppression, it might become more relevant and have more potent immunological consequences when protocols to induce clinical tolerance or minimize immunosuppression become more widespread. It is therefore important to define the spectrum of microbes and pro-inflammatory signals that are capable of enhancing alloreactivity and triggering a loss of tolerance.

Our observation that only a subset of tolerant infected mice fully reject their hearts is also similar to the clinical observation that not all infected patients reject their allografts (Brouard et al., 2012). The underlying basis for this variable susceptibility to rejection following infection remains to be fully investigated, but we hypothesize that

stochastic differences in the strength of the immune response to infection and the robustness of tolerance in the recipients may be contributory factors.

The identification of a high percentage of Tregs in both primary tolerated and secondary 'return of tolerance' allografts and their expression of an activated phenotype, as well as the dependence on Tregs for the return of tolerance following infection is consistent with previous reports of regulatory memory (reviewed in Rosenblum et al., 2016). Rosenblum and colleagues described memory Tregs in transgenic mice that contain ovalbumin-specific DO11.10 T cells, and, upon doxycycline treatment, inducible ovalbumin expression in the skin (Rosenblum et al., 2011). In those mice, T cell-dependent dermatitis occurred following expression of ovalbumin, and Tregs with an activated phenotype accumulated in the skin and limited secondary responses more potently than primary ones, suggesting that a response to a self-antigen can imprint regulatory memory in tissues. Similarly, Rowe and colleagues reported regulatory memory in the context of immune responses to fetal antigens (Rowe et al., 2012). In that study, antigen-specific Tregs persisted after parturition and rapidly expanded during secondary pregnancy to more strongly suppress fetal resorption. Our results not only extend those observations to a transplantation setting but also demonstrate that regulatory memory can be therapeutically induced, and is migratory to a second allograft. More importantly, our data also establish a new paradigm for transplantation tolerance: that regulatory tolerance can be transiently overcome by acute infections to allow allospecific effector responses to emerge, but that the tolerance can be re-established when the inflammatory signals recede with the clearance of the

infection. That the spontaneous acceptance of second donor-matched allografts post-Lm was prevented by anti-CD25 treatment confirms the systemic presence of alloreactive T cells capable of driving rejection after Lm infection of tolerant mice, and implicates functional suppression by regulatory cells for tolerance to dominate over the memory of rejection.

This study left several remaining questions, which we set out to address. First, we had observed that the maintenance of established tolerance to heart allografts in uninfected recipients is not dependent on $CD25^+$ regulatory T cells. In contrast, in post-infected tolerant recipients, depletion of $CD25^+$ cells was sufficient to prevent the acceptance of secondary donor allografts. These results led us to hypothesize whether multiple redundant mechanisms of tolerance cooperate to maintain cardiac allograft survival after anti-CD154/DST therapy in the uninfected recipient and whether Lm impaired some other non-Treg tolerance mechanism more durably.

## III. RESULTS – TRACKING OF TCR-TRANSGENIC T CELLS REVEALS THAT MULTIPLE MECHANISMS MAINTAIN CARDIAC TRANSPLANT TOLERANCE IN MICE[1]

### A. Abstract

Solid organ transplantation tolerance can be achieved following select transient immunosuppressive regimens that result in long-lasting restraint of alloimmunity without affecting responses to other antigens. Transplantation tolerance has been observed in animal models following costimulation or coreceptor blockade therapies, and in a subset of patients through induction protocols that include donor bone marrow transplantation, or following withdrawal of immunosuppression. Previous data from our lab and others have shown that proinflammatory interventions that effectively prevent the induction of transplantation tolerance in mice often fail to break tolerance once it has been stably established. This suggests that established tolerance acquires resilience to proinflammatory insults, and prompted us to investigate the mechanisms that maintain a stable state of robust tolerance. Our results demonstrate that only a triple intervention of depleting $CD25^+$ Tregs, blocking PD-L1 signals, and transferring low numbers of alloreactive T cells was sufficient to break established tolerance. We infer from these observations that Tregs and PD-1/PD-L1 signals cooperate to preserve a low

[1] The following section titled "Tracking of TCR-transgenic T cells reveals that multiple mechanisms maintain cardiac transplant tolerance in mice" is reproduced with minor editing, and with figure renumbering, from reference (Miller et al., 2016a) as allowed by copyright by John Wiley & Sons. The article was accepted by the American Journal of Transplantation. Authors: Michelle L. Miller, Melvin D. Daniels, Tongmin Wang, Ying Wang, Jing Xu, Dengping Yin, Anita S. Chong and Maria-Luisa Alegre

Contributions:
- Performed the experiments and analysis of data in Figures 3.1, 3.3, and 3.4
- Assisted Melvin Daniels with the TCR75 cell transfers for the experiments in Figures 3.2 and 3.5
- Co-wrote manuscript with Anita Chong and Maria-Luisa Alegre

alloreactive T cell frequency to maintain tolerance. Thus, therapeutic protocols designed to induce multiple parallel mechanisms of peripheral tolerance may be necessary to achieve robust transplantation tolerance capable of maintaining one allograft for the life of a patient.

B. Introduction

Transplantation tolerance is defined as a state of allograft acceptance in the absence of immunosuppression. In the mouse, a short course of costimulation blockade combined with the infusion of donor splenocytes results in long-term cardiac allograft survival and donor-specific tolerance, as a second heart graft of donor origin can be accepted with no further immunosuppression, while maintaining immunocompetence to reject third party allografts. Other tolerizing protocols include treatment with non-depleting anti-CD4 and anti-CD8 antibodies (Waldmann et al., 2008). Our laboratories as well as others have investigated barriers that can challenge these tolerance induction protocols. Viral infections such as LCMV and Pichinde virus, bacterial infections such as *Staphylococcus aureus* and *Listeria monocytogenes,* parasitic infections such as *Leishmania major*, as well as toll like receptor (TLR) agonists can all prevent the induction of tolerance with anti-CD154 (Welsh et al., 2000; Ahmed et al., 2011a; Wang et al., 2008b; Pantenburg et al., 2002; Chen et al., 2006). In addition to infections, manipulations that increase the naïve or memory T cell precursor frequency, or those that directly target mechanisms of peripheral T cell tolerance i.e., preventing apoptosis, depleting/inhibiting regulatory cells or blocking signals through the negative regulatory

PD-1/PD-L1 pathway all result in the inability to induce tolerance (Ford et al., 2007; Haspot et al., 2008; Ito et al., 2005; Jiang et al., 2011; Lal et al., 2015; Lee et al., 2005b; Lu et al., 2006; Schroeder et al., 2004; Wells et al., 1999; Wu et al., 2004).

Interestingly, many of the manipulations that effectively prevent the induction of tolerance are unable to break tolerance once it has been stably established. Viral infections, including infections of graft tissue itself, did not break tolerance (Welsh et al., 2000). *S. aureus* infections and TLR agonists given during the maintenance phase did not precipitate allograft rejection [T. Wang and L. Chen, unpublished observations and (de Vries et al., 2009)]. Regulatory T cell (Treg) depletion in tolerant cardiac allograft recipients greater than 30 days post-transplantation also failed to break tolerance (Jiang et al., 2011; Miller et al., 2015). In our model, only infection with *Listeria monocytogenes* at ≥60 days post transplantation was capable of increasing alloreactivity and precipitating acute cardiac allograft rejection, in a manner dependent on expression of MyD88, type I interferon and interleukin-6 (Wang et al., 2010). And even in this unique case of breaking of tolerance, donor-specific tolerance was reestablished once the infection was cleared (Miller et al., 2015).

These data suggest that costimulation blockade-induced transplantation tolerance can be quite robust once established and that the requirements for tolerance maintenance are likely different than those for tolerance induction. This prompted us to investigate the mechanisms controlling its maintenance. Our results from using *Listeria monocytogenes* to break tolerance indicated that the loss of tolerance was associated with an increase in graft-infiltrating T cells concurrent with an inability of Tregs to

adequately suppress them (Miller et al., 2015; Wang et al., 2010). Thus, we hypothesized that one facet of tolerance maintenance was to keep alloreactive T cell numbers low. Furthermore, as Treg depletion alone was insufficient to break tolerance even in the presence of a new second donor-matched allograft, we hypothesized that multiple mechanisms must cooperate to keep residual alloreactive T cells in check. We used $CD4^+$ TCR75 cells that recognize a donor $K^d$ peptide presented on host $I\text{-}A^b$ as a tracer population seeded during the induction or maintenance phase of tolerance to fully MHC-mismatched allogeneic cardiac allografts. Our results demonstrate that abortive proliferation of alloreactive T cells occurs during the induction phase of tolerance, and cooperation between low numbers of alloreactive T cells, PD-L1 signals and presence of $CD25^+$ Tregs exists at the maintenance phase of tolerance. This supports the conclusion that multiple mechanisms of peripheral tolerance cooperate to maintain long-term cardiac allograft acceptance when tolerance is robust.

## C. Materials and Methods

### Mice

C57BL/6 and BALB/c mice were purchased from Envigo RMS, Inc. (Indianapolis, IN). CD45.1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TCR75 TCR-Tg mice were obtained from R. Pat Bucy (University of Alabama-Birmingham) and crossed to CD45.1 mice. Mice were housed under specific pathogen-free conditions and used in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the National Institutes of Health

guidelines for animal use.

Heart transplantation

Cardiac allograft transplantation was performed using a technique adapted from Corry et al. (Corry et al., 1973). Transplantation of cardiac allografts in the abdominal cavity was performed by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. The day of allograft rejection was defined as the first day of cessation of heartbeat as measured by palpation. In certain experiments, mice were treated with a single 0.4 mg dose of anti-CD25 (PC61) and/or 5 doses of anti-PD-L1 (0.5 mg, 0.25 mg, 0.25 mg, 0.25 mg, 0.25 mg) administered every other day.

Adoptive transfer of cells

Spleen and peripheral lymph node cells (from inguinal, brachial, axillary, cervical and mesenteric lymph nodes) were isolated from donor TCR75 mice. Cell counts were obtained with an Accuri C6 flow cytometer (BD Biosciences) and a subset of cells was stained for CD4, V$\beta$8.3, the congenic marker CD45.1 and CD44. The percentage of $CD44^{lo}$, congenically marked TCR-Tg T cells was used to calculate the total number of cells for the adoptive transfer. TCR75 cells were predominantly $CD44^{lo}$ (>97%) at the time of adoptive transfer. Cells were injected retro-orbitally in 200 $\mu$l of phosphate-buffered saline (PBS).

Isolation of graft-infiltrating cells

Cardiac allografts were rinsed *in situ* with Hanks balanced salt solution (HBSS) containing 1% heparin. Explanted hearts were dissected into small pieces and digested for 40 min at 37°C with 400 U/ml collagenase IV (Sigma), 10 mM N-2-hydroxyethylpiperazine-N′-2- ethanesulfonic acid (HEPES, Cellgro) and 0.01% DNase I (MP Biomedicals) in HBSS (Cellgro). Digested cell suspensions were washed with an equal volume of complete Dulbecco's Modified Eagle medium (DMEM) containing 5% fetal bovine serum, passed through a nylon mesh and centrifuged. Cells were either used directly for flow cytometry or first incubated for 4 h with phorbol myristate acetate (PMA, 50 ng/ml) and ionomycin (0.5 μg/ml) in the presence of brefeldin A (5 μg/ml).

Magnetic enrichment

CD45.1$^+$ TCR75 cells were magnetically enriched from spleen and peripheral lymph nodes of recipient mice following staining with anti-CD45.1-bio and incubation with streptavidin magnetic beads (Miltenyi). The cells were enriched using an AutoMACs machine (Miltenyi).

Intracellular staining and flow cytometry

Single cell suspensions of lymphocytes were prepared from isolated spleens and heart grafts. Cells were stained first with a fixable live/dead stain (Aqua, Invitrogen) and then with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), anti-Vβ8.3 (1B3.3), anti-PD-1 (J43), anti-CD44 (IM7). For interferon gamma (IFNγ) and tumor necrosis factor

(TNFα) intracellular staining, cells were stimulated with PMA (Sigma), ionomycin (Sigma) and brefeldin A (BD Pharmingen) for 4 h, prior to staining for viability and surface markers. Surface-stained cells were then fixed with the Foxp3 fixation permeablization buffer kit (eBioscience) for 15 min at room temperature, washed with 1 x permeabilization buffer, stained using anti-IFNγ (XMG1.2) and anti-TNFα (MP6-XT22) for 30 min at room temperature, washed again, and analyzed by flow cytometry. In certain experiments, unstimulated cells were fixed and stained with anti-Foxp3 (FJK-16s) using the Foxp3 fixation permeablization buffer kit (eBioscience). All mAbs were from BD Biosciences or eBioscience.

Data analysis

Flow cytometry data were analyzed using FlowJo (TreeStar). Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc).

## D. Results

### 1. Alloreactive T cells persist in an activated state in tolerant mice

Our previous results using tolerant mice infected with Listeria revealed a clear population of T cells responding to alloantigen following the infection (Miller et al., 2015; Wang et al., 2010), suggesting that if clonal deletion occurs during tolerance induction or maintenance, it must be incomplete. An alternative explanation is that clonal deletion was complete following costimulation blockade therapy, but that new thymic emigrants later restored a pool of alloreactive cells. To test the degree of deletion of alloreactive T

cells without the confounding factor of new thymic emigrants, a tracer population of $CD4^+$ $K^d$-reactive TCR75 cells was seeded into naïve C57BL/6 mice, either unimmunized, or prior to immunization with donor BALB/c splenocytes alone (DST), or prior to tolerization with anti-CD154, donor splenocytes and a BALB/c heart transplant (Figure 3.1A). At doses of $10^3$, $10^4$, and $10^5$ transferred TCR75 cells, tolerance was still successfully induced in the third group, and mice retained their allografts for the duration of the experiment (MST > 60 days). Following isolation of the TCR75 cells from the spleen and peripheral lymph nodes at day 60, a similar percentage and number of TCR75 cells was recovered from tolerant mice as from naïve mice, while ~10-fold more cells were recovered from mice immunized with donor splenocytes alone (Figure 3.1B). Thus, the quantity of alloreactive T cells in tolerant mice was not significantly reduced from that in naïve mice, where cell numbers may be decaying over time due to a lack of antigenic stimulation. The TCR75 cells isolated from tolerant mice were antigen-experienced, with similar percentages of $CD44^{hi}$ cells in the DST-immunized and tolerant groups (Figure 3.1C). These results indicate that while costimulation blockade-induced tolerance prevents accumulation of alloreactive T cells, it does not cause their complete deletion, nor does it depend on antigen ignorance, as the alloreactive T cells expressed high levels of CD44.

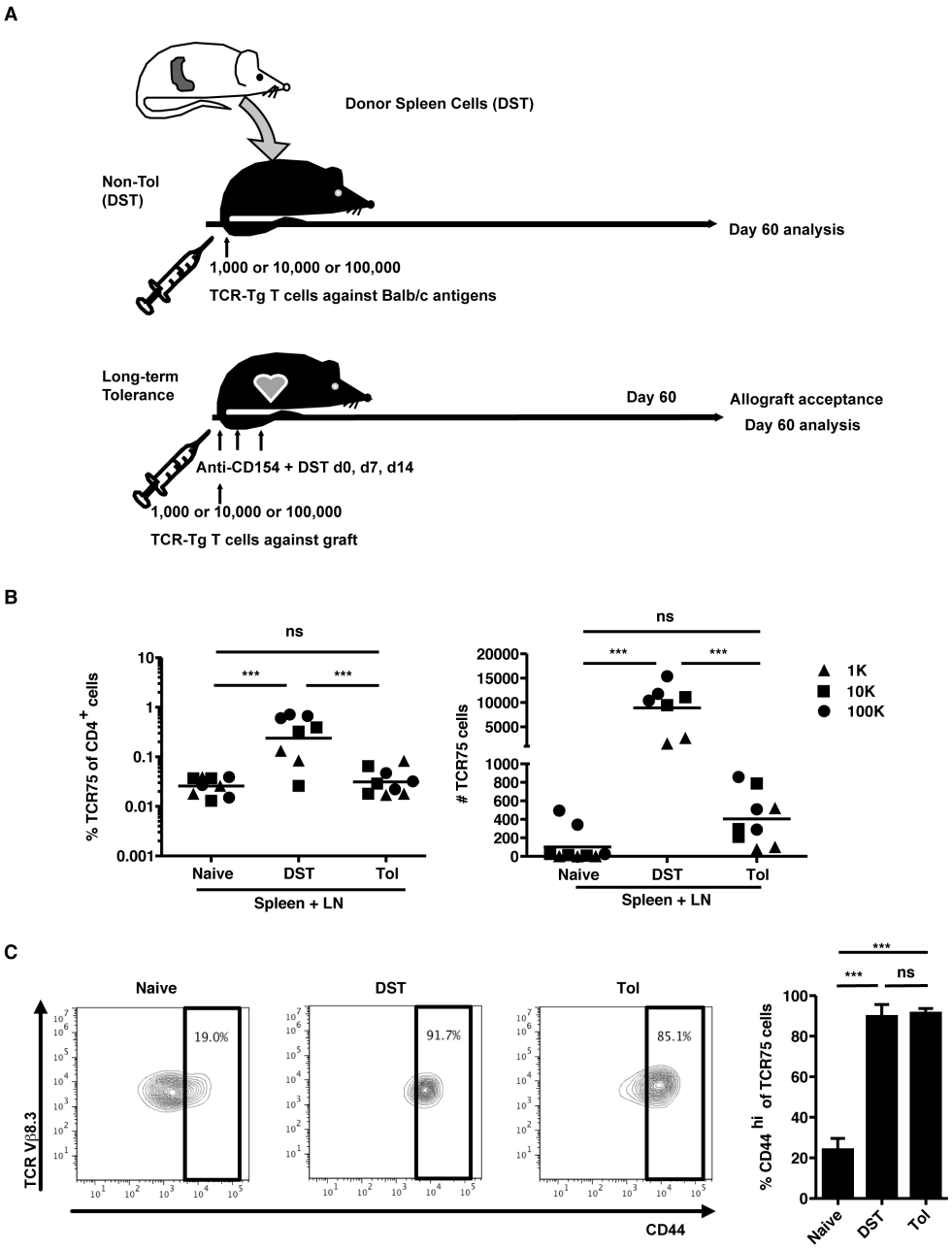


**Figure 3.1: Alloreactive T cells seeded in tolerant mice are not completely deleted and are not ignorant of the graft, but are maintained at low numbers**
**A.** Experimental design. **B.** The percentages and total numbers of $CD4^+$ TCR75 cells recovered from the spleen and peripheral lymph nodes 60 days post adoptive transfer into naïve untransplanted C57BL/6 mice, mice receiving BALB/c donor splenocytes (DST) and mice receiving BALB/c donor splenocytes, anti-CD154, and a heart transplant (Tol). n=3 mice per cell dose ($10^3$, $10^4$, $10^5$) per group. Different cell doses are represented by different symbols. **C.** Representative flow plots of percentages of $CD44^{hi}$ cells amongst TCR75 cells and their quantification. Different cell doses were pooled. Mean values were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons ns=not significant, ***p<0.001.

2. Adoptive transfer of high numbers of alloreactive T cells is sufficient to break tolerance

The persistence of alloreactive T cells at low levels, led us to hypothesize that one requirement for maintaining tolerance may be keeping the numbers of alloreactive T cells below a specific threshold. To test this hypothesis, we adoptively transferred a low number ($10^5$ cells) or a high number (2-4 x $10^6$ cells) of TCR75 cells into tolerant mice at ≥ 60 days post-transplantation (Figure 3.2A). The transfer of a low dose of TCR75 cells had no effect on graft acceptance, whereas the transfer of a high dose of TCR75 cells was sufficient to precipitate graft rejection (Figure 3.2B, low-dose MST > day 60 vs high-dose MST=12, p=0.0096). Thus, the maintenance of tolerance is dependent on keeping the numbers of alloreactive T cells low.

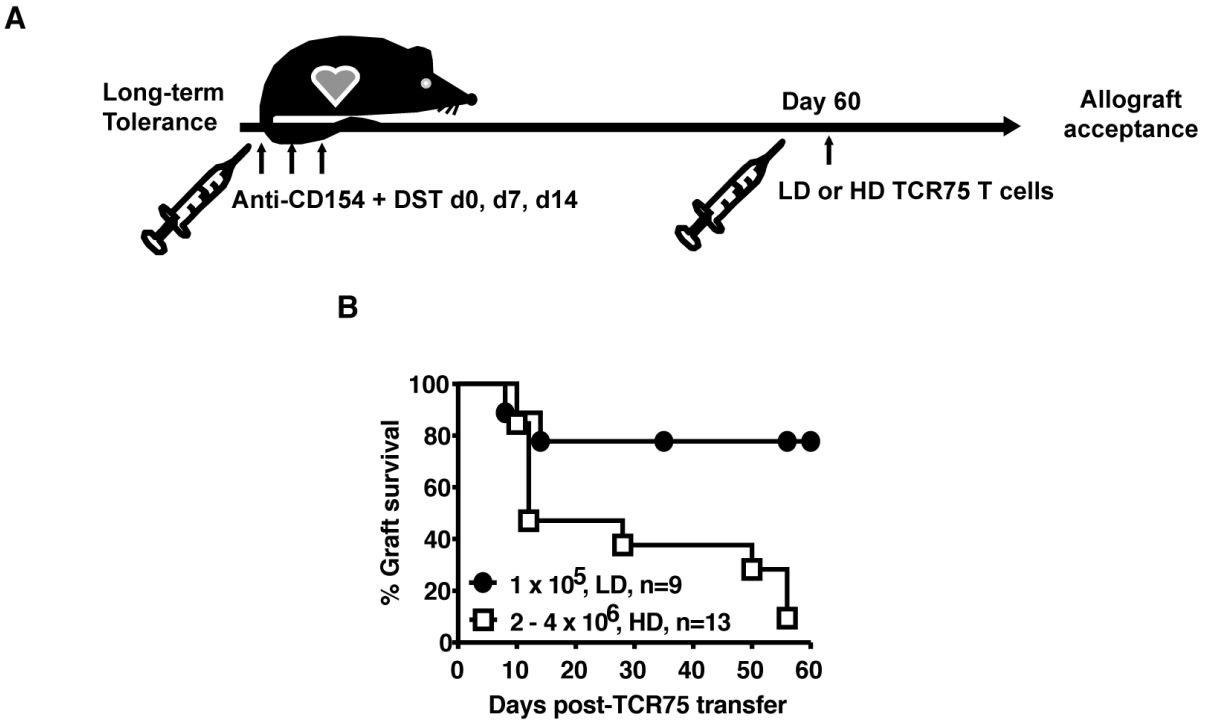


**Figure 3.2: Transfer of a high dose (HD) but not low dose (LD) of alloreactive T cells is sufficient to break established tolerance**
**A.** Experimental design. **B.** Graft survival in tolerant mice that were adoptively transferred at least 60 days post-transplantation with $10^5$ TCR75 cells (low dose, LD, n=9) or 2-4 x $10^6$ cells (high dose, HD, n=13), $p<0.05$ by log-rank test.

3. Low numbers of transferred TCR75 cells are controlled in a tolerant environment

The lack of graft rejection in tolerant mice receiving a low dose of alloreactive T cells on day 60 post-transplantation is reminiscent of previous descriptions of 'infectious tolerance' (Qin et al., 1993), whereby adoptively transferred naïve T cells are not only kept in check but can also acquire properties of tolerance. To understand how a low number of new alloreactive T cells could be prevented from destabilizing tolerance, we traced the fate of CFSE-labeled TCR75 cells transferred at low doses ($10^5$-$2.5x10^5$ cells) into tolerant recipients ≥ 60 days post-transplantation and compared them to those transferred into naïve untransplanted mice, or into naïve mice prior to BALB/c cardiac allograft transplantation and acute rejection (Figure 3.3A). TCR75 cells in tolerant recipients diluted CFSE (Figure 3.3B) and upregulated CD44 (Figure 3.3C) comparably to acutely rejecting mice, indicating that TCR75 cells in tolerant mice detected and responded to the cardiac allograft. However, the TCR75 cells failed to accumulate in tolerant mice, suggesting abortive proliferation (Chai et al., 2015; Ford et al., 2007; Quezada et al., 2005), as their percentages and total numbers remained similar to those observed in naïve mice, and were significantly lower than those in mice undergoing unmodified acute rejection (Figure 3.3D,E).

In addition to the lack of accumulation, lower percentages of TCR75 cells from tolerant hosts produced cytokines upon restimulation with PMA and ionomycin (Figure 3.3F), which suggested profound hyporesponsiveness or a reduced ability to fully differentiate into multi-functional effector cells, as restimulation with PMA and ionomycin bypasses proximal TCR signaling and would recover the function of anergic T cells.

Phenotypically, despite similar proliferation and acquisition of $CD44^{hi}$ expression (Figure 3.3B,C), a higher proportion of TCR75 cells expressed the negative costimulatory molecule PD-1 when transferred into tolerant recipients than into rejecting mice (Figure 3.3G). Of note, the TCR75 cells themselves do not upregulate Foxp3 in a tolerant environment (Chai et al., 2015) and therefore might be intrinsically controlled through PD-1/PD-L1 signals and/or extrinsically controlled through host regulatory cells. In sum, the tolerant environment prevented accumulation and ultimate cytokine production potential and also resulted in a greater proportion of cells expressing PD-1.

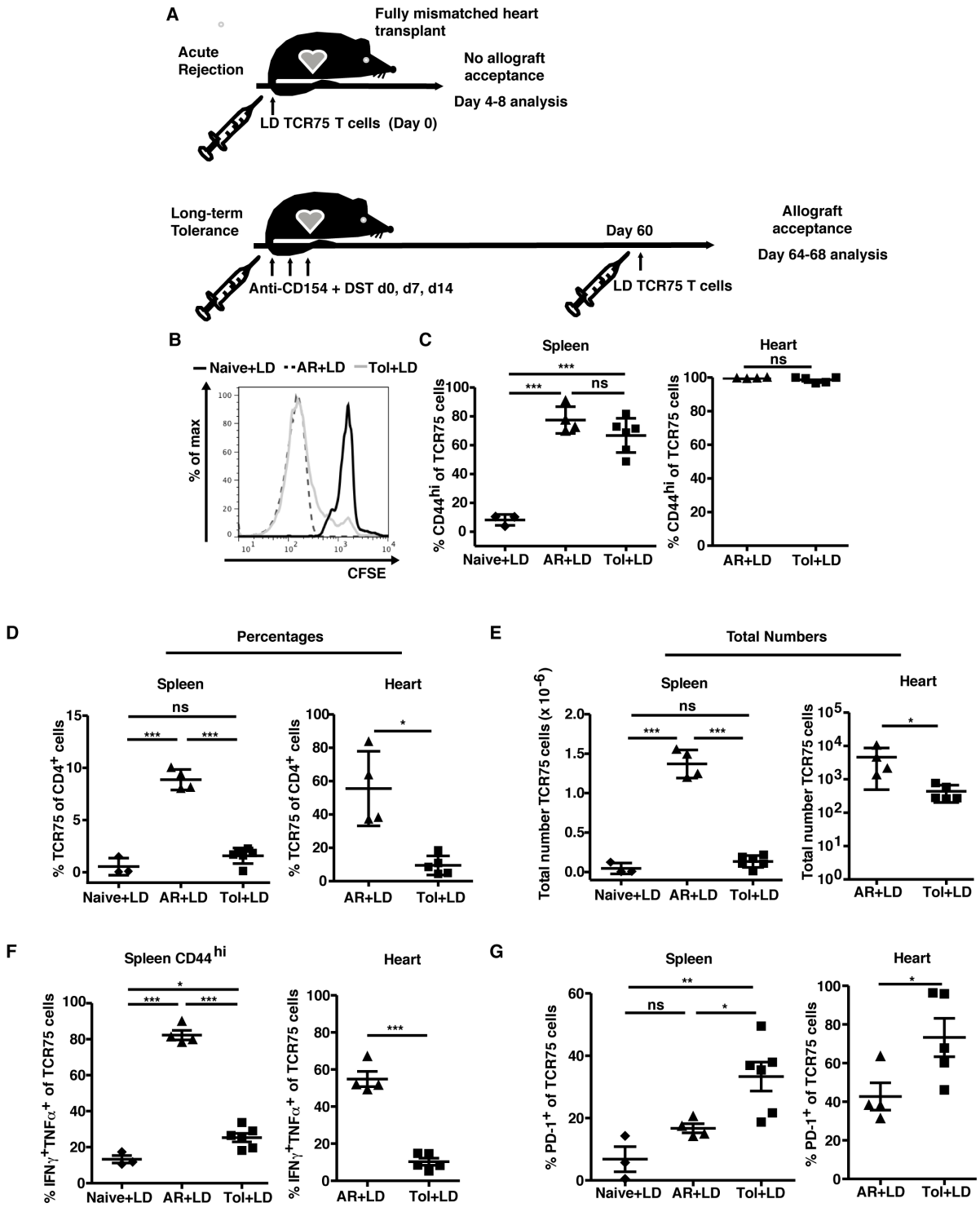


**Figure 3.3: Low dose (LD) alloreactive T cells seeded during the maintenance phase of tolerance are well controlled**

**A.** Experimental design. **B.** Representative plots of CFSE dilution of 2.5 x $10^5$ TCR75 cells adoptively transferred into naïve mice (Naïve+LD, n=3), or into mice undergoing acute rejection (AR+LD, n=3), or into tolerant mice (Tol+LD, n=3), at four days post-transfer in the spleen.

**Figure 3.3, continued: C.** Percentages of TCR75 cells expressing $CD44^{hi}$ in the spleen and heart graft 8 days post adoptive transfer. Percentages **(D)** and total numbers **(E)** of TCR75 cells recovered in the spleen and graft 8 days post adoptive transfer. Percentages of TCR75 cells expressing IFN$\gamma$ and TNF$\alpha$ upon PMA/ionomycin restimulation **(F)** and PD-1 without restimulation **(G)** 8 days after adoptive transfer. **C-G.** Results were pooled from two or more independent experiments. Mean values were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons or Student's t test where appropriate. ns=not significant, *p<0.05, **p<0.01, ***p<0.001.

4. Alloreactive T cells transferred in high numbers are controlled in the periphery but not in the graft

Because transfer of high numbers but not low numbers of TCR75 cells precipitated rejection in already tolerant recipients, we traced the fate of TCR75 cells when transferred at high numbers into tolerant or control animals. Surprisingly, numbers of TCR75 cells recovered from the spleens of tolerant mice 5 days after transfer were significantly lower than those from acutely rejecting recipients, and comparable to the numbers recovered from naïve mice (Figure 3.4A). Cytokine production by TCR75 cells from the spleens of tolerant mice was dramatically reduced compared to TCR75 cells from acutely rejecting mice (Figure 3.4B), similar to the observation with low numbers of TCR75 cells (Figure 3.3F), and was increased relative to cells from naïve mice. Finally, the proportion of TCR75 cells expressing PD-1 was high and persistent in tolerant mice (Figure 3.4C) compared to TCR75 cells from acutely rejecting or naïve mice. Extrapolation of the data from Figures 3 and 4 suggests that the phenotype and function of TCR75 cells recovered from the spleen was unexpectedly similar in tolerant mice injected with low versus high numbers, despite tolerance being maintained versus lost, respectively.

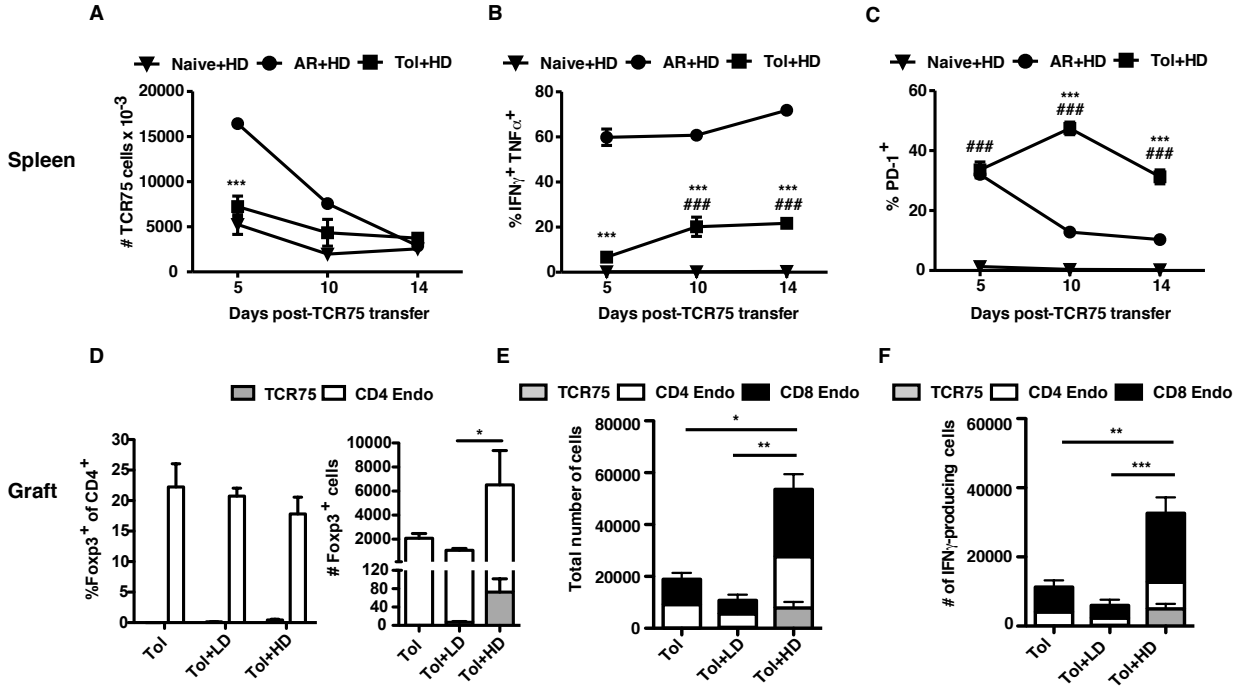


**Figure 3.4: High dose (HD) alloreactive T cells are controlled in the periphery of tolerant mice but not in the graft**

**A-C.** TCR75 cells injected at high doses (2-4 x $10^6$ cells, HD) into naïve mice or tolerant mice 60 days post transplantation and were recovered from spleens 5, 10, or 14 days later (n=2-4 mice per group per time point). **A.** Total numbers of TCR75 cells in the spleen. Percentages of splenic TCR75 cells expressing IFNγ and TNFα upon PMA/ionomycin restimulation **(B)** and PD-1 without restimulation **(C)** on the indicated days post adoptive transfer. Mean values for each time point were compared using two-way ANOVA with Bonferroni correction for pairwise comparisons $^{###}p<0.001$, between tolerant and naïve groups ***p<0.001, between tolerant and acute rejection groups. **D-F.** Graft-infiltrating cells were analyzed 7-14 days post adoptive transfer into tolerant mice (n=10-12 mice per group). **D.** Percentages and total numbers of $Foxp3^+$ cells within TCR75 and endogenous T cells within the graft. **E.** Total numbers of graft-infiltrating TCR75 cells and endogenous $CD4^+$ and $CD8^+$ T cells. **F.** Total numbers of IFNγ-producing graft-infiltrating TCR75 cells and endogenous $CD4^+$ and $CD8^+$ T cells. Results were pooled from three or more independent experiments. Mean values of total graft T cells were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons *p<0.05, **p<0.01, ***p<0.001.

Therefore, we evaluated whether the loss of tolerance with high dose TCR75 cell transfer was due to an increase in the numbers of functional alloreactive T cells in the

allograft, thereby overwhelming endogenous Tregs. We first investigated whether the percentages of Foxp3$^+$ cells were decreased within the grafts of high dose transfer mice. However, this was not the case as Treg percentages in the grafts were similar whether tolerant mice received a low or high dose of TCR75 cells (Figure 3.4D). In contrast, the numbers of T cells that accumulated in the allografts, and specifically the numbers of IFN$\gamma$-producing T cells, were significantly greater in the tolerant mice receiving high dose TCR75 cells when compared to the other groups (Figure 3.4E,F). This suggests that despite an increase in intragraft Tregs numbers (Figure 3.4D) to counter the increase in effector T cell numbers, resulting in equivalent Treg:Tconv ratios to the non-transfer group, intragraft effector T cells that accumulated following high dose TCR75 transfer were inadequately controlled.

5. Cell-intrinsic and cell-extrinsic mechanisms cooperate to control alloreactive T cells in a tolerant environment

Our data indicate that controlling the numbers of alloreactive T cells, to levels observed in naïve mice, is an essential component to the maintenance of tolerance, and this may be achieved through cell-intrinsic and/or cell-extrinsic mechanisms. To investigate the role of these mechanisms, tolerant mice were treated with either anti-CD25 to deplete Tregs, anti-PD-L1 to block signals from PD-1/PD-L1, alone or in combination with an infusion of low dose ($10^5$) TCR75 cells, and graft survival was assessed (Figure 3.5A). PD-L1 has been shown to be the dominant ligand for PD-1 in transplantation settings (Tanaka et al., 2007; Wang et al., 2007) and blocking anti-PD-L1 antibodies have been shown to have similar effects as anti-PD-1 antibodies (Koehn

et al., 2008). Each treatment alone or two treatments in combination were insufficient to induce the loss of tolerance, and only the triple combination was sufficient to break tolerance consistently (Figure 3.5B). We conclude that multiple mechanisms cooperate to maintain robust transplantation tolerance.

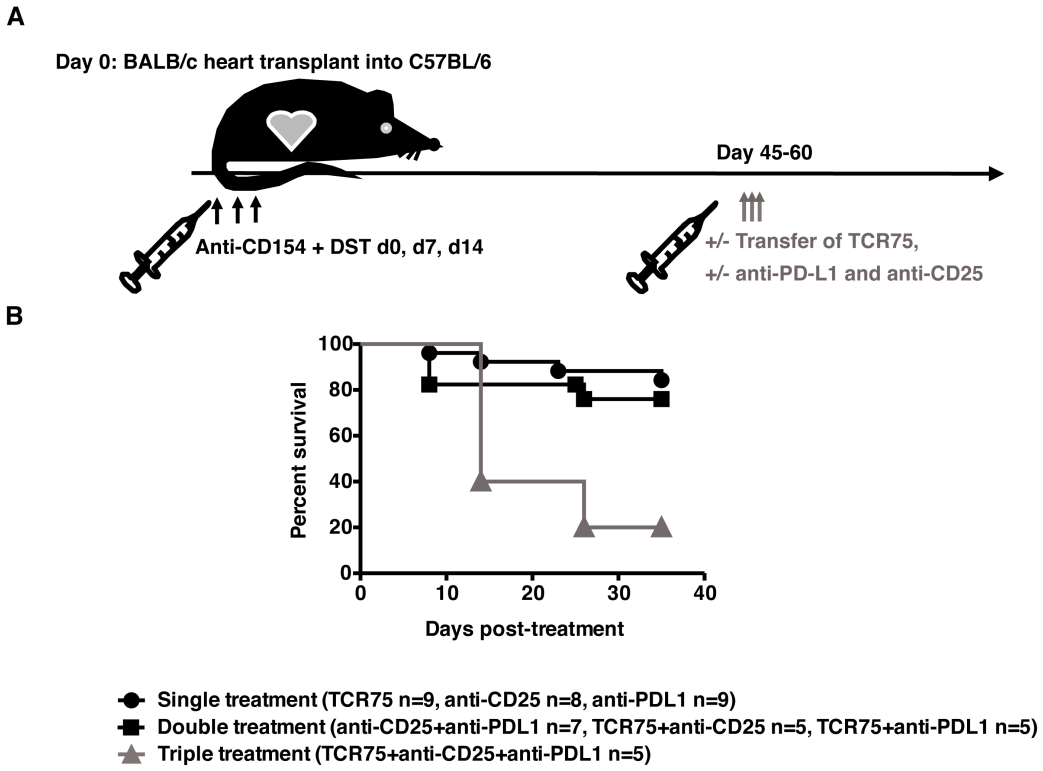


**Figure 3.5: Maintenance of tolerance of primary grafts is dependent on controlling the size of the alloreactive T cell pool through regulatory T cells and signals from PD-L1**

**A.** C57BL/6 mice were transplanted with BALB/c hearts and treated with anti-CD154+DST. On day >45 post-transplantation, tolerant recipients were treated with individual or combined treatments to block pathways of peripheral tolerance. **B.** Graft survival following transfer of naïve TCR75 T cell ($10^5$), blockade of PD-L1, or administration of anti-CD25 alone or in combination. Graft rejection was significantly induced only when all 3 therapies were combined (triangles, $p<0.05$).

## E. Discussion

Recent advances towards pursuing transplantation tolerance in the clinic are heightening the need to better identify the mechanisms required for maintaining tolerance in a robust and durable manner. Reports that some tolerant patients acutely reject their graft, often following an infectious episode (Brouard et al., 2012), suggest that tolerance in some individuals may not be sufficiently stable and robust to preserve life-long allograft acceptance. In mice, anti-CD154 and DST, without bone marrow cell transplantation, can achieve a robust state of peripheral allograft tolerance that is resistant to many inflammatory challenges (de Vries et al., 2009; Welsh et al., 2000). To understand why tolerance is so robust in this model, we investigated the fate of transferred TCR-transgenic alloreactive $CD4^+$ T cells in C57BL/6 mice treated with anti-CD154 and DST to induce tolerance to MHC- and non-MHC mismatched BALB/c cardiac grafts.

The seeding of low numbers of TCR75 T cells ($10^3$-$10^5$) at the time of transplantation permitted tolerance to develop normally, and revealed that clonal deletion of these cells was incomplete and that these cells persisted to day 60 post-transplantation at levels comparable to naïve, untransplanted mice. This partial deletion and persistence was similar to recent results by Chai et al. using TCR75 cells seeded in a model of single minor antigen-expressing skin transplantation (Chai et al., 2015) and to earlier results tracking TCR-Tg $CD8^+$ cells in synchimeric recipients of allogeneic skin grafts (Iwakoshi et al., 2000, 2001). Alloreactive T cell apoptosis has been shown to be an important mechanism for tolerance induction with costimulation blockade (Wells et

al., 1999); however, our data highlight that this process can be incomplete and tolerance of remaining T cells still needs to occur. Interestingly, whereas in the DST-immunized group there was a positive correlation between the number of TCR75 T cells seeded and the number of these cells recovered 60 days later, in the tolerant mice the same number of TCR75 cells was recovered at day 60 post-transplantation, irrespective of the dose of cells transferred suggesting strong constraints on the frequency of alloreactive T cells for the successful induction and maintenance of transplantation tolerance. These observations therefore complement and extend the well established notion that a high initial precursor frequency of naïve and memory alloreactive T cells presents a barrier to tolerance induction with costimulation blockade (Adams et al., 2003; Ford et al., 2007; Koehn et al., 2008).

The long-term persistence of low numbers of TCR75 T cells adoptively transferred during the initiation of tolerance induction could be due to cell-intrinsic changes turned on only during the tolerance induction phase. To control for this possibility, we transferred low numbers of TCR75 T cells into tolerant mice at 60 days after transplantation. Similar to adoptive transfer in the tolerance induction phase, alloreactive T cells transferred during the maintenance phase of tolerance did not undergo complete deletion but were maintained at levels comparable to naïve untransplanted mice. However, these TCR75 cells were only limited in their accumulation and function, but not in their proliferation or acquisition of the activation marker CD44. This observation is consistent with abortive proliferation that has been described during the tolerance induction phase, whereby T cells proliferate but then

undergo apoptosis leading to a lack of net accumulation of these cells (Chai et al., 2015; Ford et al., 2007; Quezada et al., 2005). In addition, this observation is also consistent with studies showing that $CD4^+$ T cells proliferate when encountering self-antigens and yet become tolerant (Adler et al., 2000), and that adoptively transferred naïve $CD8^+$ T cells into tolerant skin graft recipients have limited cytokine production and cytotoxicity without reduction in proliferation (Koehn et al., 2008; Lin et al., 2002). Thus, the net constraints on non-tolerant alloreactive T cells introduced into a tolerant environment are similar for both $CD4^+$ and $CD8^+$ T cells, and provide mechanistic insights into infectious tolerance of new thymic emigrants into a tolerant host.

In the tolerant environment, the transferred TCR75 cells acquired sustained PD-1 upregulation in both the spleen and the graft. We have shown elevated PD-1 expression on intra-graft polyclonal regulatory T cells in long-term tolerant mice, compared to peripheral Tregs in the same mice or to Tregs from naïve mice (Miller et al., 2015). We also find PD-1 expressed on polyclonal effector T cells in tolerant allografts (M. Daniels et al., in preparation). The expression of PD-1 by T cells infiltrating the graft is not surprising, because its expression is dependent on antigen encounter and TCR stimulation (Bennett et al., 2003); however the greater proportion of cells expressing this marker in tolerance than acute rejection in both the spleen and the graft may reveal a different quality or duration of T cell activation in these two environments. PD-1 expression on TCR75 cells in the spleen, albeit at lower levels than in the graft, suggests that there is persistent antigen presentation in the spleen or that these T cells are recirculating between the graft and the secondary lymphoid organs or both.

Because TCR75 cells recognize a peptide from donor H-2K$^{d}$, in an indirect manner, it is likely that there will be a continuous supply of cognate antigen for the lifespan of the graft. Whether T cells recognizing donor MHC II indirectly have fates different from TCR75 cells, because of different levels and duration of antigen expression, remains to be investigated (Ali et al., 2015). It is also unknown whether alloreactive T cells recognizing alloantigens directly would have the same fate in a tolerant environment as the TCR75 cells which use indirect recognition.

Precipitation of acute allograft rejection following adoptive transfer of high numbers of TCR75 cells into tolerant recipients correlated with increased intragraft accumulation of T cells and higher cytokine production when compared with transfer of low numbers of TCR75 cells. Interestingly, not only did greater numbers of TCR75 accumulate in these grafts, but endogenous T cell numbers were also elevated. Though the specificity of these endogenous T cells is unknown, this is reminiscent of recent reports indicating that TCR-mediated migration of alloreactive T cells into a transplant opens the door to chemokine-dependent migration of non-graft reactive effector T cells (Walch et al., 2013).

It was surprising to observe high doses of TCR75 cells in tolerant animals led to graft rejection despite as high a percentage of Tregs in those grafts as in those of fully tolerant hosts. Indeed, it is generally thought that the ratio of intragraft Tregs to Teffector cells determines tolerance versus rejection. Because the specificity of the intragraft Tregs cannot be assessed in this model, it is possible that not all Tregs were alloreactive and capable of suppressing the effector T cells in this setting. Moreover,

effector and memory T cells have been shown to be more resistant to the suppressive effect of Tregs (Yang et al., 2007) perhaps explaining their ability to produce IFNγ and reject the established allografts.

Depletion of Tregs at the maintenance phase of tolerance in skin-transplanted mice treated with combined coreceptor/costimulation blockade has been shown to be sufficient to precipitate rejection (Kendal et al., 2011) whereas it is not sufficient in the cardiac model of tolerance following costimulation blockade therapy (Jiang et al., 2011). In our experiments, even in the context of $10^5$ excess alloreactive T cells, Treg depletion was unable to precipitate graft rejection, showing at least in this model of allograft tolerance, that T cell-intrinsic mechanisms of tolerance, such as through PD-1/PD-L1 interactions, probably play a key role. Indeed, only when adding anti-PD-L1 to Treg depletion in the context of an increased frequency of alloreactive T cells did graft rejection ensue. While we believe this is most likely due to blocking PD-L1 on antigen presenting cells and preventing it from ligating to PD-1 on the TCR75 cells and endogenous T effector cells, we cannot exclude the possibility that it also blocks PD-L1 signals to PD-1 on any remaining endogenous regulatory cells that escaped depletion with the anti-CD25 treatment.

Blocking T cell-intrinsic PD-1/PD-L1 signaling alone was also insufficient to cause rejection in mice made tolerant to cardiac allografts with anti-CD154 and DST. However, this same intervention has been shown to be effective at inducing allograft rejection in mice made tolerant to cardiac allografts with CTLA4-Ig (Tanaka et al., 2007), in mice made tolerant to islet allografts with anti-CD3 (Baas et al., 2016) and in

synchimeric mice with long-term single-mismatch skin grafts following anti-CD154 and CTLA4-Ig treatment (Koehn et al., 2008). It is currently unknown to what extent each peripheral tolerance mechanism is induced and maintained following different costimulation-blockade tolerance induction strategies, but this may suggest that anti-CD154 + DST induces more robust tolerance than CTLA4-Ig, at least at the doses used. Investigating differences in efficacy of T cell control with these two regimens will be the subject of future research to identify additional pathways that can be targeted in patients receiving CTLA4-Ig to strengthen its ability to induce immunological hyporesponsiveness to an allograft.

It is also unknown to what extent each peripheral tolerance mechanism is engaged in other models of tolerance, such as in models of spontaneous transplantation tolerance. Kidney allografts can be spontaneously accepted between particular strain combinations of mice. One study found that Treg depletion alone was sufficient to break tolerance (Miyajima et al., 2011). This may suggest that the spontaneous tolerance in this model is less robust than that induced with anti-CD154+DST but future research should determine how many peripheral tolerance mechanisms are involved in this kidney transplant model.

Our data collectively show that a tolerant environment can constrain the numbers of alloreactive T cells, but this constraint can be overwhelmed and allograft rejection can occur with increased frequency of alloreactive T cells, or when multiple mechanisms of peripheral tolerance are compromised. To reveal the importance for preserving low numbers of alloreactive T cells for tolerance maintenance, our model relied on the

adoptive transfer of high numbers of TCR-Tg T cells. There are several limitations to using TCR-Tg T cells. The cells all have the same specificity and affinity, and are not able to reflect endogenous populations of differing affinities that would be able to compete with one another. While the specific affinity of TCR75 cells is not known, most TCR-Tg models are of high affinity T cells and may not be representative of endogenous polyclonal T cell responses. However, despite these known limitations, our results with this model may reveal an important underlying mechanism for tolerance maintenance. Indeed of the documented interventions that result in a loss of tolerance during the maintenance phase, almost all point to a loss of Tregs' ability to control an increased endogenous alloreactive effector T cell pool. These interventions include Listeria infection, high dose IL-2, lymphodepletion, mast cell degranulation, agonistic CD40 mAb, and peptide immunization in the absence of persistent donor antigen following graft removal have all been shown to break tolerance to donor antigens (Iida et al., 2013; Okumi et al., 2008; de Vries et al., 2009; Wang et al., 2010; Yamada et al., 2015). This interpretation is supported by our data showing that transfer of high numbers of alloreactive T cells alone is sufficient to precipitate rejection.

Further research into the kinetics and durability of each of the peripheral mechanisms of tolerance induced with costimulation blockade will help predict effective combinations of new or existing drugs in the clinic for tolerance protocols that simultaneously or sequentially induce multiple mechanisms of tolerance in patients. Having a better understanding of the mechanisms that are compromised when tolerance is lost in different settings may also lead to more targeted interventions that

can either reinforce particular arms of the tolerance repertoire that may have been compromised by infections, or bolster the existing unaffected pathways.

## IV. RESULTS – DISTINCT GRAFT-SPECIFIC TCR AVIDITY PROFILES DURING ACUTE REJECTION AND TOLERANCE

### A. Abstract

At the cellular level, mechanisms implicated in the development of robust transplantation tolerance include those that inhibit T cells intrinsically (clonal deletion, anergy, exhaustion), or extrinsically through regulatory cells. In this study, we investigated the possibility that additional mechanisms control T cells at the population level during therapeutically-induced peripheral tolerance. Because a given peptide/MHC complex is often recognized by a polyclonal population of T cells with distinct TCR sequences and unique peptide/MHC affinities, we investigated whether similarly to observations in central tolerance, peripheral transplantation tolerance selects for T cells with low avidity for graft antigens. Whereas high avidity T cells preferentially accumulated during acute rejection of allografts or allogeneic cells, the avidity profile of alloreactive cells in tolerant recipients was low on average and comparable to that in naive mice despite signs of T cell activation. These contrasting avidity profiles upon productive versus tolerogenic alloantigen stimulation were durable, and persisted upon alloantigen reencounter in the absence of immunosuppression. This was due, at least in part, to lack of expansion of high avidity T cell clones in tolerant hosts. Therapeutic approaches that achieve long-term elimination of T cells with high affinity for donor antigens may be desirable to achieve robust transplantation tolerance in the clinic.

B. Introduction

T cells express surface TCRs capable of recognizing antigens with a range of affinities/avidities. T cell affinity refers to the binding strength of a single TCR-pMHC interaction, while T cell avidity takes into account the sum of interactions between multiple TCR-pMHC molecules, as well as other factors such as co-receptors that contribute to the binding interaction (Van Den Berg and Rand, 2007). The intensity with which T cells bind to pMHC multimers as determined by flow cytometry has been used as a proxy to define the affinity/avidity of endogenous antigen-specific T cell populations because the amount of fluorescence of multimer staining has correlated with functional assays of T cell avidity (Crawford et al., 1998; Wang and Altman, 2003; Yee et al., 1999).

While having a broad range of affinities/avidities is advantageous to combat infections, high avidity T cells can be detrimental to maintaining tolerance to self. Therefore, the immune system has evolved mechanisms to mitigate autoimmunity while preserving a diverse repertoire for encountering pathogens. One such mechanism is the establishment of central tolerance to self-antigens within the thymus (Anderson et al., 2002; Watanabe-Fukunaga et al., 1992). In central tolerance, T cells with high avidity to self-antigens are deleted through negative selection, while $CD4^+$ T cells of intermediate affinity to self are diverted to become regulatory cells (Malhotra et al., 2016). This has been demonstrated in mouse models using monoclonal TCR-Tg T cells reviewed in (Ohashi et al., 1989), as well as through examining oligoclonal endogenous T cell populations with pMHC multimers (Malhotra et al., 2016; Moon et al., 2011). Two

recent studies extending this question of humans have also revealed that residual self-reactive T cells in healthy individuals are low avidity, as MelanA-specific T cells from healthy controls were lower avidity than in patients with active psoriatic autoimmunity (Maeda et al., 2014), and H-Y-specific T cells from male individuals were fewer in number and of lower avidity than those from females (Yu et al., 2015). Thus, central tolerance shapes the T cell repertoire to be of low avidity for self-antigens. Whether peripheral mechanisms of tolerance are also able to shape T cell avidity profiles for a given antigen is unknown.

Several animal models have demonstrated that peripheral T cells increase in avidity following foreign antigen encounter (Busch and Pamer, 1999; Moon et al., 2007; Savage et al., 1999). This is thought to occur through the selective expansion of high affinity T cells based on their ability to better compete for antigen and to get a stronger signal to proliferate and survive, as well as via preferential inhibition of low-avidity T cells by regulatory T cells (Pace et al., 2012). Furthermore, the clustering of TCRs on the cell surface as well as their degree of co-localization with their co-receptor, CD4 or CD8, upon T cell activation can also impact T cell avidity (Demotte et al., 2008; Fahmy et al., 2001). This change in pMHC binding dependent on accumulation of high affinity/avidity T cells has been termed ‘T cell affinity maturation’ at the population level (Busch and Pamer, 1999).

How T cells behave at the population level in response to alloantigens is not clear, and such investigations have been hampered by the lack of tools to study endogenous donor-specific T cells. Using a novel mouse model for transplantation that

affords the ability to track endogenous graft-reactive T cells with pMHC multimers (Moon et al., 2011), we found that, during productive alloimmunity, endogenous CD4 and CD8 T cells increased in avidity at the population level. In contrast, following therapeutically-induced peripheral tolerance, the avidity profile of tolerant alloreactive T cells remained of low avidity, comparable to naive T cells, despite upregulation of CD44 by tolerant T cells. This low avidity profile in tolerant mice was stable, even upon re-encounter with donor antigen, and was due, at least in part, to a costimulation blockade-dependent inhibition of expansion of high avidity clones. Our study reveals that the phenomenon of affinity maturation at the T cell population level occurs not only following infections, but also during unmodified alloreactivity. Moreover, our data reveal that therapeutically-induced peripheral tolerance, much like central tolerance, can shape T cell repertoires at the population level, and may point to a novel approach to inducing stable transplantation tolerance in the clinic through reducing the abundance of high avidity donor-specific clones.

## C. Materials and Methods

### Mice

C57BL/6 and BALB/c mice were purchased from Envigo RMS, Inc. (Indianapolis, IN). CD45.1$^+$ mice and OT-I/RAG-KO mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in our facility. 2W1S-mOVA mice on a C57BL/6 background, previously described (Moon et al., 2011), were crossed to BALB/c mice to generate F1 progeny. TEa mice were obtained from Alexander Rudensky (when at the University of

Washington). BALB/c-OVA mice were obtained from Dr. Elizabeth Ingulli (when at the University of Minnesota). Mice were housed under specific pathogen-free conditions and used in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Heart transplantation

Cardiac allografts were transplanted, adapting a technique from Corry et al. (Corry et al., 1973). Cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. Tolerant mice were treated with three 0.6 mg doses of anti-CD154 (MR1) on days 0, 7 and 14 post-transplantation as well as one-quarter spleen for donor splenocyte transfusion (DST) injected i.p. on day 0. In certain experiments, mice were treated with a 100 μg dose of agonistic anti-CD40 (FGK4.5) on days 0 and 7 post-transplantation.

Magnetic enrichment

T cells were magnetically enriched by negative selection from spleens of recipient mice following staining with anti-CD19-bio, anti-Ter119-bio and anti-CD11b-bio and incubation with streptavidin magnetic beads (Pierce).

Flow cytometry

Five million T-enriched cells were stained first with a fixable live/dead stain

(Aqua, Invitrogen) followed by PE-coupled-2W:I-$A^b$ tetramers for 1 hour in a room temperature water bath, washed and then stained with PE-coupled-OVA:$K^b$ pentamers (ProImmune) for 20 minutes in a room temperature water bath. Cells were then stained with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD44 (IM7) and anti-B220 (RA3-6B2). Surface-stained cells were then fixed with the Foxp3 fixation permeablization buffer kit (eBioscience) for 15 min at room temperature, washed with 1 x permeabilization buffer, and analyzed by flow cytometry. All mAbs were from BD Biosciences or eBioscience.

Adoptive cell transfer

Spleen and lymph node cells were isolated into single-cell suspensions and counted with an Accuri C6 flow cytometer (BD Biosciences). The percentage of $CD8a^+$ $Va2^+$ $CD45.2^+$ $CD44^{lo}$ cells was determined by flow cytometry and used to calculate the total number of OT-I cells for the adoptive transfer. $10^5$ cells were injected retro-orbitally in 200 μl of phosphate-buffered saline (PBS) on the day before or the day of transplantation.

TCRβ spectratyping

Multimer-positive and -negative T cells were sorted from T cell-enriched spleens of naïve mice, mice receiving an immunization with DST, or of DST with a single dose of anti-CD154 (0.6 mg) one week prior. The cells were sorted into Qiagen's RLT Plus lysis buffer, and frozen prior to isolating RNA using RNEasy mini and micro kits according to

the manufacturer's instructions (Qiagen). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. CDR3β regions were amplified by PCR with 21 different Vβ 5' primers (Table 4.1) paired with a FAM (Fluorescein amidite)-Cβ1.1 primer. The Vβ11 PCR reactions did not reach significant amplification for analysis and were removed from the analysis. CDR3 peaks were measured on an Applied Biosystems 3130XL or 3730XL machine and aligned using the Liz500 ladder.

Data analysis

Flow cytometry data were analyzed using FlowJo (TreeStar). For TCR spectratyping data, the areas under each peak were analyzed using PeakStudio software (McCafferty et al., 2012). Hamming distances were calculated as previously described (Currier and Robinson, 2001). Briefly, the relative area under each binned curve was determined for sets of base pair lengths and the sum of the absolute values of the differences at each position was calculated and divided by two. Therefore 0= 100% overlap of peak areas, with no skewness, and 1= 0% overlap of peak areas, representing high skewness. A minimum area of 2000 units was used as a threshold before including samples in the Hamming distance calculations. Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc).

| | Primer Sequences | | |
|---|---|---|---|
| | **Wilson** | **IMGT** | <-Nomenclatures |
| 0 | Cβ1.1 | TRBC1 | CTCAAACAAGGAGACCTTGGGTGG |
| 1 | Vβ1 | TRVB5 | CAGACAGCTCCAAGCTACTTTTAC |
| 2 | Vβ2 | TRVB1 | ATGAGCCAGGGCAGAACCTTGTAC |
| 3 | Vβ3 | TRVB26 | GAAATTCAGTCCTCTGAGGCAGGA |
| 4 | Vβ4 | TRVB2 | CTAAAGCCTGATGACTCGGCCACA |
| 5 | Vβ5.1 | TRVB12-2 | CTTTGGAGCTAGAGGACTCTGCCG |
| 6 | Vβ5.2 | TRVB12-1 | CCTTGGAACTGGAGGACTCTGCTA |
| 7 | Vβ6 | TRVB19 | GCCCAGAAGAACGAGATGGCCGTT |
| 8 | Vβ7 | TRVB29 | GGATTCTGCTAAAACAAACCAGACATCTGT |
| 9 | Vβ8.1 | TRVB13-3 | GCTTCCCTTTCTCAGACAGCTGTA |
| 10 | Vβ8.2 | TRVB13-2 | GCTACCCCCTCTCAGACATCAGTG |
| 11 | Vβ8.3 | TRVB13-3 | GGCTTCTCCCTCTCAGACATCTT |
| 12 | Vβ9 | TRVB17 | CTCTCTCTACATTGGCTCTGCAGG |
| 13 | Vβ10 | TRVB4 | CTTCGAATCAAGTCTGTAGAGCCG |
| 14 | Vβ11 | TRVB16 | TGAAGATCCAGAGCAGCGGGCCCC |
| 15 | Vβ12 | TRVB15 | CCACTCTGAAGATTCAACCTACAGAACCC |
| 16 | Vβ13 | TRVB14 | CAAGATCCAGTCTGCAAAGCAGGG |
| 17 | Vβ14 | TRVB31 | GCACGGAGAAGCTGCTTCTCAGCC |
| 18 | Vβ15 | TRVB20 | GCATATCTTGAAGACAGAGGC |
| 19 | Vβ16 | TRVB3 | CTCTGAAAATCCAACCCACAGCACTGG |
| 20 | Vβ17 | TRVB24 | TCTGAAGAAGACGACTCAGCACTG |
| 21 | Vβ18 | TRVB30 | GCAAGGCCTGGAGACAGCAGTATC |

**Table 4.1: Primer sequences used for amplifying TCR CDR3 regions for spectratyping**
This table lists the primers that were used to PCR-amplify TCR CDR3 regions for spectratyping analysis.

D. Results

1. Endogenous allospecific tolerant T cells do not accumulate but become antigen-experienced

To understand the nature of the endogenous T-cell mediated alloimmune response during rejection and tolerance, we utilized donor mice ubiquitously expressing a transgene for the model antigens 2W1S presented on recipient I-$A^b$, and ovalbumin presented on recipient $K^b$ to enable the use of peptide-MHC multimers to track endogenous allospecific $CD4^+$ and $CD8^+$ T cells respectively. These populations are

rare in naïve, antigen-inexperienced mice (Moon et al., 2011), such that in all experiments T cell enrichment was first performed to increase the frequency of multimer-binding T cells during flow cytometric analysis. T cell enrichment by negative selection, as opposed to multimer enrichment, afforded the ability to increase the frequency of multimer-binding events without the potential loss of low-multimer-binding T cells.

2W1S-mOVA transgenic mice on the C57BL/6 background were crossed once to BALB/c mice to generate F1 progeny as donors. This allows for the study of rejection (as minor antigen-mismatched hearts are not acutely rejected (Ehst et al., 2003; Honjo et al., 2004)) as well as of tolerance in a haploidentical physiological setting. The numbers of 2W:I-$A^{b+}$ and OVA:$K^{b+}$ T cells in untransplanted naïve C57BL/6 mice as well as in mice transplanted and left untreated (Rejected, Rej) and in mice transplanted and tolerized with anti-CD154 + donor splenocytes (DST) were enumerated at early time points (day 8-9) as well as at late time points (day 60-120). As expected, at both early and late time points, the numbers of graft-reactive T cells recovered from spleens of mice that had rejected their grafts was greater than in tolerant mice. In contrast, the numbers of tolerant 2W:I-$A^{b+}$ and OVA:$K^{b+}$ T cells were not different from those in naïve mice (Figure 4.1A,B). However, tolerant T cells were significantly more activated than naïve T cells as measured by the percentage of cells that were $CD44^{hi}$, albeit not as uniformly activated as cells from mice having undergone acute rejection (Figure 4.1C). Thus, despite having encountered antigen, graft-specific T cells failed to accumulate in tolerant mice.

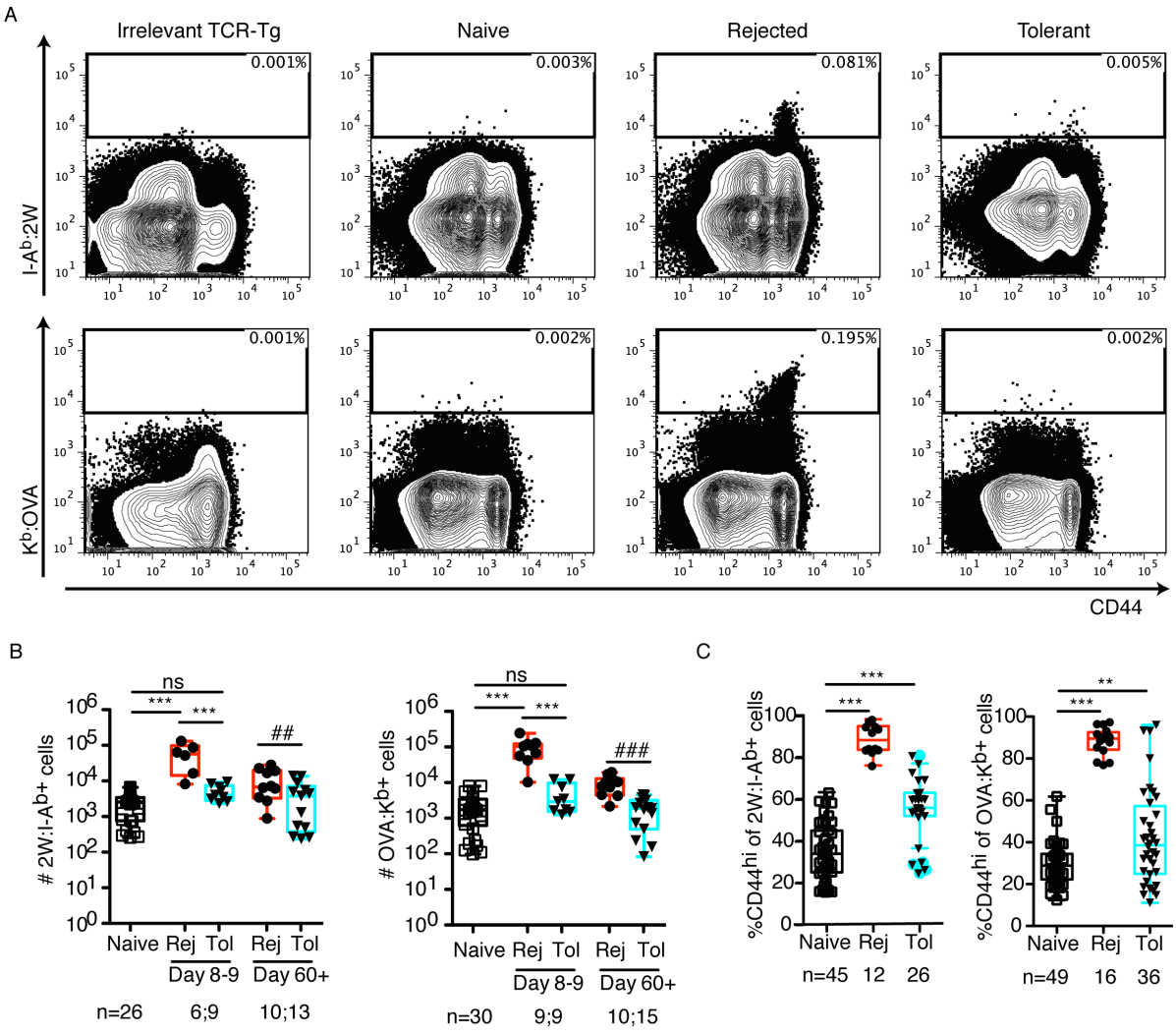


**Figure 4.1: Endogenous graft-specific T cells do not accumulate but are antigen-experienced in tolerant mice**

**A.** Representative flow cytometry plots of 2W:I-A$^b$- and OVA:K$^b$-binding populations in the spleens of irrelevant TCR-Tg (TEa) mice, naïve untransplanted C57BL/6 mice, mice transplanted four months prior with a BALB/c x C57BL/6 F1 2W1S-mOVA or BALB/c-OVA heart and left untreated to undergo acute rejection (Rej) or transplanted and tolerized with anti-CD154 and DST (Tol). **B.** Numbers of 2W:I-A$^b$- and OVA:K$^b$-binding cells isolated from the spleens of naïve, rejected and tolerant mice. **C.** Percentages of 2W:I-A$^b$- and OVA:K$^b$-binding cells that were CD44$^{hi}$, data pooled from early and late time points. The numbers of mice in each group are listed under each figure. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01, ***p<0.001 (**B** Naïve, Day 8-9 Rej and Tol; **C**). $^{\#\#}$0.01, $^{\#\#\#}$p<0.001 (**B** Naïve, Day 60 Rej and Tol).

2. Endogenous allospecific T cells have higher avidity profiles in rejection compared to tolerance

As self-tolerant T cell populations have been shown to become of lower avidity following central tolerance, we tested whether peripherally tolerant T cell populations might be constrained in a similar manner. To this end, we analyzed the mean fluorescence intensities of pMHC multimer-binding on endogenous graft-specific T cells in the spleen of naïve, rejecting and tolerant mice and found much higher multimer-binding in recipients that rejected their allografts than in tolerant or naïve mice (Figure 4.2 A,B). The differences in multimer binding between rejecting and tolerant T cells were not just due to the greater proportion of antigen-inexperienced T cells in tolerant than rejecting hosts, as pMHC multimer-binding differences were still present at most time points when analyzing only $CD44^{hi}$ 2W:I-$A^{b+}$ and OVA:$K^{b+}$ cells (Figure 4.3). Moreover, they were not due to differential TCR expression, as staining with the anti-TCR$\beta$ clone H57-597 that does not interfere with TCR-pMHC binding (Wang et al., 1998) revealed similar TCR$\beta$ levels in naïve, rejecting and tolerant mice at any time point (Figure 4.2 A,B). CD8$\alpha$ has been shown to contribute to OVA:$K^b$ multimer-binding (Daniels and Jameson, 2000), but, if anything, it was downregulated in rejecting mice during the early time point (Figure 4.4), indicating the rejecting T cells may be even higher avidity than multimer-binding alone would suggest. Thus, these data suggest that non-tolerant T cell populations undergo avidity maturation in response to alloantigen encounter, similarly to T cells responding to a pathogen (Busch and Pamer, 1999), and that this avidity maturation is much reduced in tolerant mice.

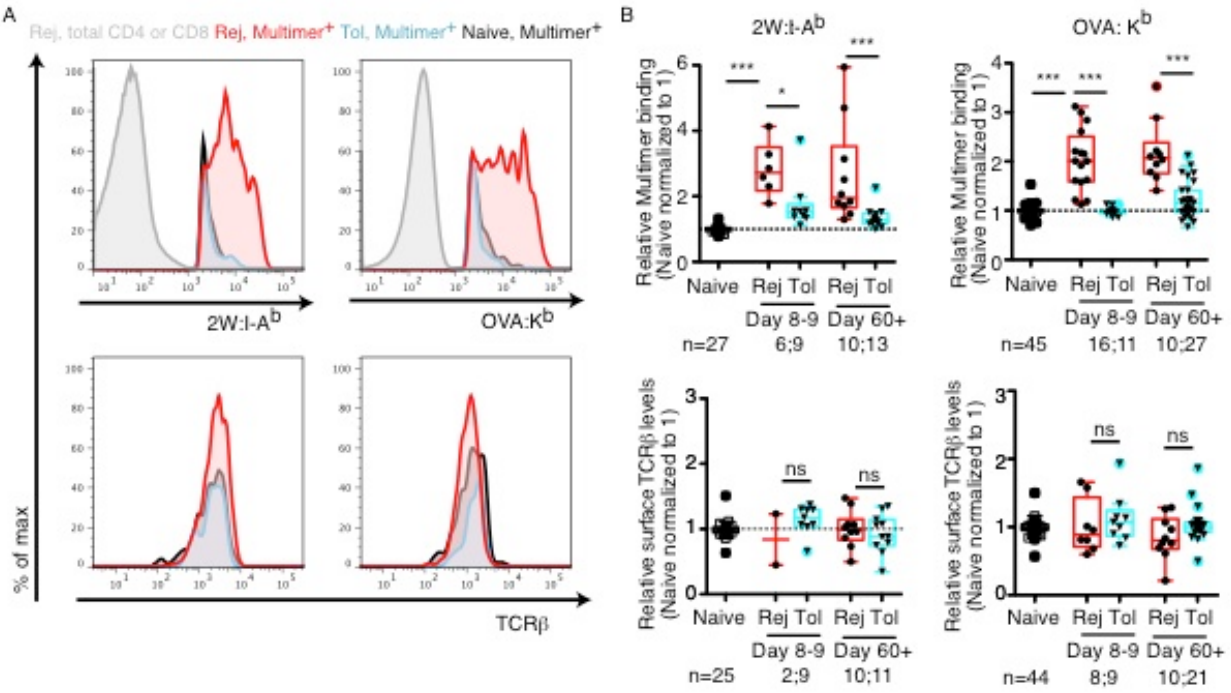


**Figure 4.2: Graft-specific T cells from tolerant mice bind less pMHC multimer than from non-tolerant mice**

T cells reactive to 2W:I-A$^b$ and OVA:K$^b$ were stained with peptide MHC multimers in naïve untransplanted mice, and mice having undergone acute rejection (Rej) or tolerance induction (Tol). **A.** Histograms of 2W:I-A$^b$ and OVA:K$^b$ multimer staining and TCRβ levels. **B.** Relative 2W:I-A$^b$ and OVA:K$^b$ multimer mean fluorescence intensity (MFI) and surface TCRβ levels (assessed by MFI) of endogenous alloreactive T cells over time in naïve, acute rejection and tolerant recipients. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments. Numbers of mice per group are listed below each panel. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, ***p<0.001, ns=not significant.

A

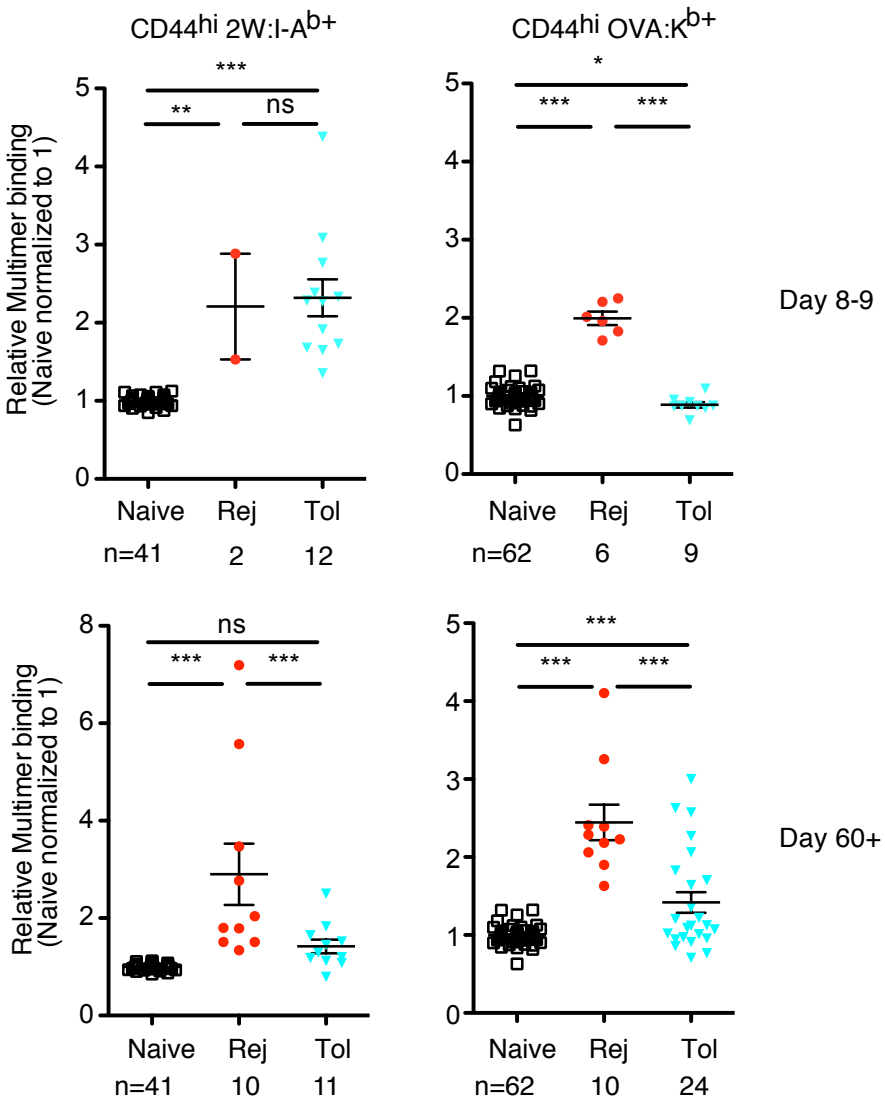


**Figure 4.3: Multimer-binding in CD44$^{hi}$ 2W:I-A$^{b}$- and OVA:K$^{b}$-specific T cells in rejecting and tolerant mice over time**

Relative 2W:I-A$^{b}$ and OVA:K$^{b}$ multimer MFI of CD44$^{hi}$ alloreactive T cells over time in naïve, acute rejection and tolerant recipients. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments and displayed as Mean +/- SEM; numbers of mice per group are listed below each panel. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, ***p<0.001, ns=not significant.

3. Costimulation blockade prevents avidity maturation of allospecific T cells exposed to alloantigen in the absence of a graft

To determine if the presence of an allograft was required for the differences in T cell avidity observed between rejecting and tolerant mice, we exposed mice to donor antigen only in the form of donor splenocyte transfusion in the absence (DST) or presence of costimulation blockade (DST+αCD154). As observed following cardiac transplantation, pMHC multimer-binding was increased during productive alloimmunity in response to DST, and this avidity maturation was blunted in the absence of costimulation (Figure 4.5 A-C). These differences persisted at late time points (Figure 4.5C) and were retained when gating only on antigen-experienced $CD44^{hi}$ T cells (Figure 4.6). Thus, administration of anti-CD154 was sufficient to block avidity maturation following exposure to donor antigen in the presence of absence of a persistent allograft.

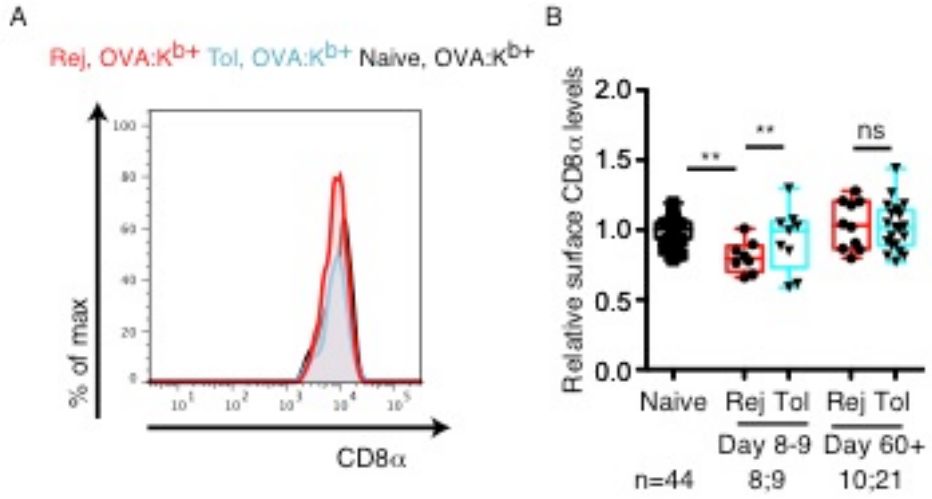


**Figure 4.4: Surface CD8α levels do not account for differences in OVA:$K^b$ multimer-binding on alloreactive $CD8^+$ T cells**
Relative CD8α MFI of endogenous alloreactive $CD8^+$ T cells over time in naïve, acute rejection and tolerant recipients.

**Figure 4.4, continued:** Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments; numbers of mice per group are listed below each panel. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. **p<0.01, ns=not significant.

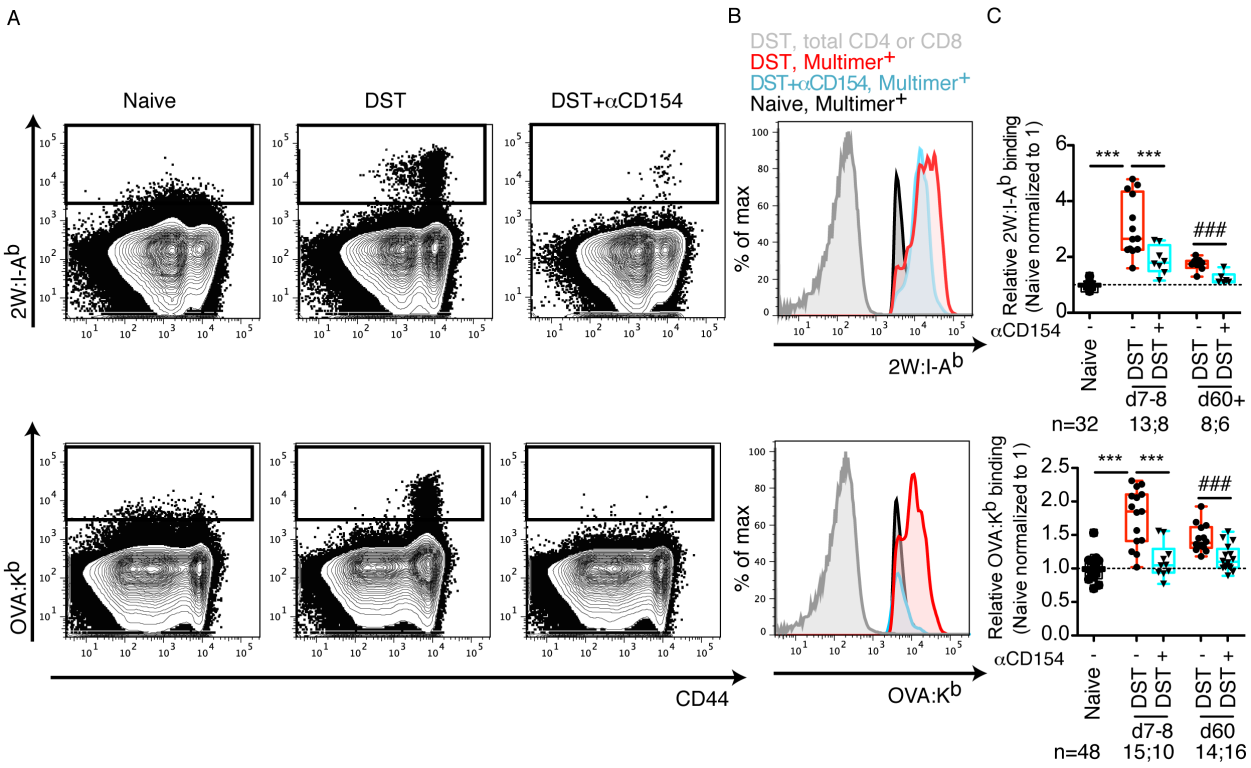


**Figure 4.5: Exposure to donor antigen alone without an allograft promotes avidity maturation of alloreactive T cell populations, which is blunted in the absence of costimulation**

**A.** Representative flow cytometry plots of 2W:I-A$^b$- and OVA:K$^b$-binding populations in the spleens of naïve mice, and mice immunized one week prior with BALB/c x C57BL/6 F1 2W1S-mOVA donor splenocytes in the absence (DST) or in the presence of anti-CD154 (DST+αCD154). **B.** Histograms showing the levels of 2W:I-A$^b$ and OVA:K$^b$ multimer-binding. **C.** 2W:I-A$^b$ and OVA:K$^b$ multimer mean fluorescence intensity (MFI) of endogenous alloreactive T cells isolated from spleens on the days indicated. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments; numbers of mice per group are listed below each panel. Data were analyzed by one-way ANOVA with Bonferroni post-tests for multiple comparisons. ***p<0.001 (Naïve, Day 7-8 DST, DST+αCD154). $^{###}$p<0.001 (Naïve, Day 60+ DST, DST+αCD154).

A

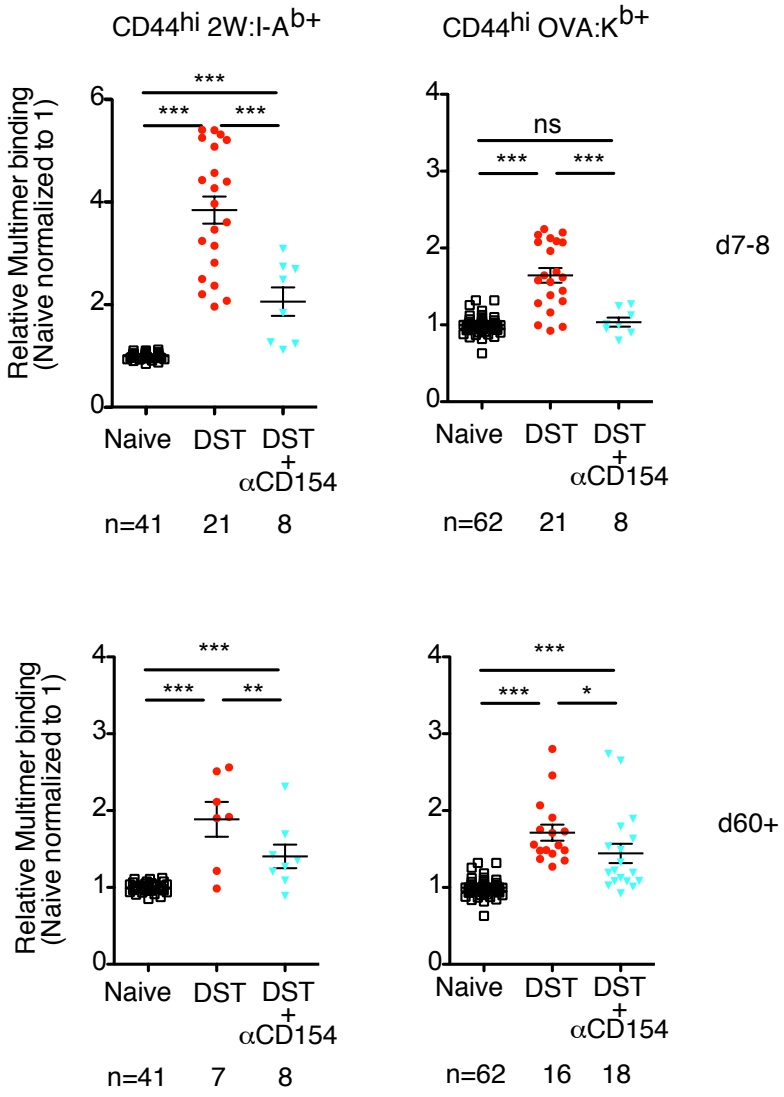


**Figure 4.6: Multimer-binding differences in CD44$^{hi}$ 2W:I-A$^{b}$- and OVA:K$^{b}$-specific T cells in mice treated with and without costimulation blockade**

Relative 2W:I-A$^{b}$ and OVA:K$^{b}$ multimer MFI of CD44$^{hi}$ alloreactive T cells over time in naïve, DST- and DST+anti-CD154-treated recipients. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments and displayed as Mean +/- SEM; numbers of mice per group are listed below each panel. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, *** p<0.001, ns=not significant.

4. The modified avidity profile of allospecific T cells in tolerance resists alloantigen rechallenge

To determine the long-term impact of costimulation-blockade-mediated low avidity skewing in alloreactive T cell populations, we rechallenged mice with donor antigen in the absence of any immunosuppression (Figure 4.7A). Similarly to the increased population avidity reported upon secondary response to infections (Busch and Pamer, 1999; Savage et al., 1999), rechallenge of DST-immunized mice with donor splenocytes resulted in a further increase in pMHC multimer-binding (Figure 4.7B). In contrast, mice initially exposed to DST+anti-CD154 and rechallenged with DST alone 60 days later did not increase their alloreactive T cell avidity profile (Figure 4.7B). Therefore, even though the avidity of alloreactive T cell populations is similar in naïve and tolerant mice 60 days post costimulation-blockade treatment, these cell populations exhibited distinct behaviors when responding to the day 60 DST. These results suggest the T cell avidity profile is permanently altered upon antigen exposure in the presence of costimulation blockade.

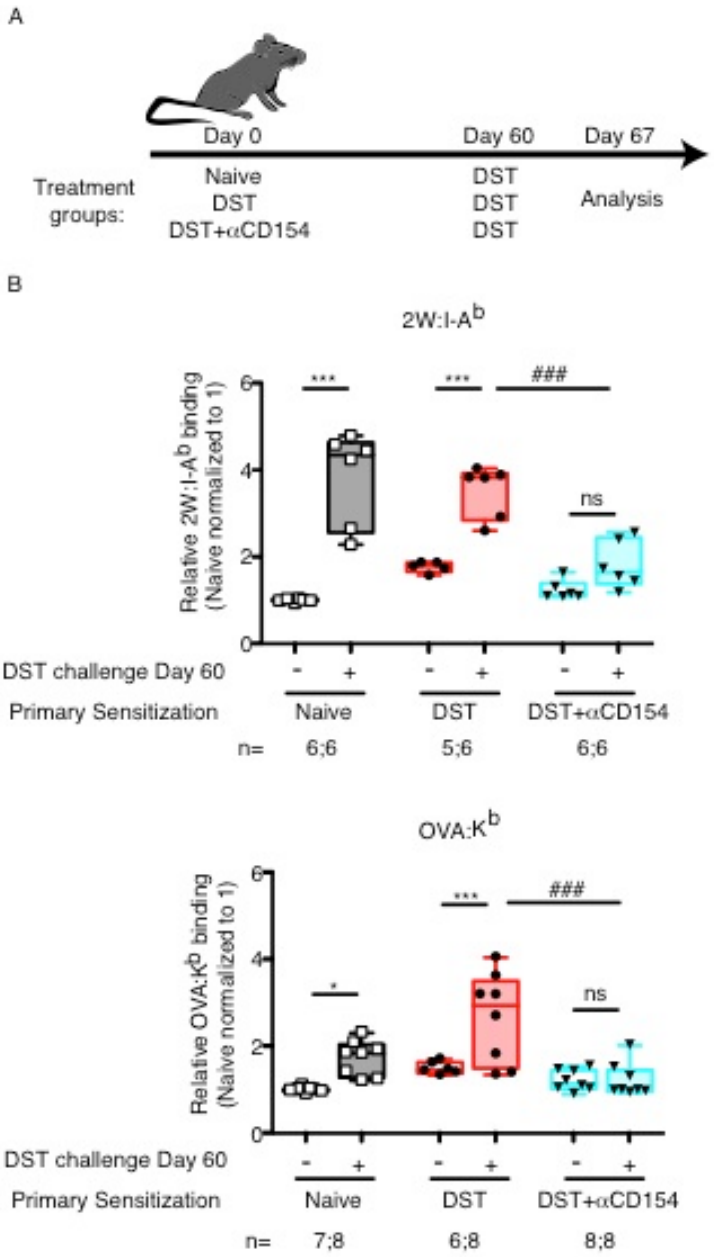


**Figure 4.7: The avidity profile of T cells primed in the presence of costimulation blockade persists upon antigen rechallenge**

**A.** Experiment design. **B.** Normalized MFI of 2W:I-A$^b$- and OVA:K$^b$- binding endogenous alloreactive T cells isolated from spleens of mice one week post-challenge with DST. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-3 independent experiments; numbers of mice per group are listed below each panel. Data from non-rechallenged groups are the same as those displayed in Figure 3C. Data were analyzed by two-way ANOVA with Bonferroni post-tests for multiple pairwise comparisons. *p<0.05; ***p<0.001, and by one-way ANOVA, $^{###}$p,0.001.

5. Avidity maturation occurs at the population level

Changes to T cell avidity can occur on a cell-intrinsic basis through post-translational modifications such as glycosylation or nitration of the TCR (Daniels et al., 2001; Kuball et al., 2009; Nagaraj et al., 2007). To determine if costimulation blockade treatment led to a low avidity profile through post-translational modification of tolerant TCRs, we adoptively transferred congenically marked CD45.2$^+$ OT-I T cells on a Rag-KO background to track avidity of T cells of a monoclonal specificity with OVA:K$^b$ multimers in rejecting and tolerant CD45.1$^+$ recipients. Surprisingly, in contrast to the increased avidity maturation of endogenous T cells in rejecting mice, OT-I T cells in the same animals bound less pMHC multimer in rejecting than in tolerant or naïve mice (Figure 4.8A). This was most likely due to activation-induced downregulation of surface TCR$\beta$ and CD8$\alpha$ (Figure 4.8B,C). These results indicate that avidity maturation in rejecting mice is occurring through population-levels changes to the T cell repertoire, rather than through post-translational modifications to the TCRs at the cell-intrinsic level. While we cannot exclude that post-translational modifications may also be occurring, the dominant effect appears to be through population level changes leading to avidity maturation or lack thereof in rejection versus tolerance, respectively.

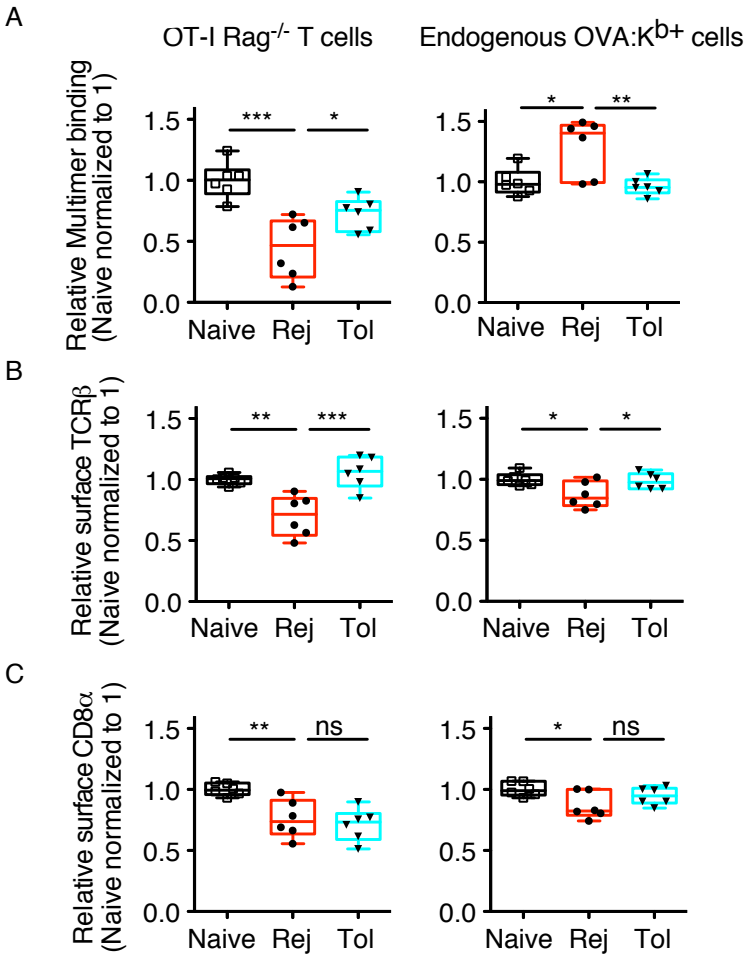


**Figure 4.8: Differences in T cell avidity between rejecting and tolerant mice result from population-level changes post-alloantigen encounter**

1 x $10^5$ OT-I $Rag^{-/-}$ T cells were adoptively transferred into naïve mice, mice transplanted with a BALB/c-OVA heart and left untreated (Rej) or tolerized with anti-CD154 (Tol). Normalized values of the geometric mean fluorescence intensity of multimer-binding **(A),** surface TCRβ **(B),** and CD8α **(C)** are shown. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2 independent experiments, with n=6 mice total per group. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, ***p<0.001, ns=not significant.

6. Lack of T cell avidity maturation in tolerant mice is not rescued by overexpression of Bcl-$x_L$ or Bcl-2

One hypothesis for the lack of T cell avidity maturation in tolerant mice is that high affinity clones may be deleted. Deletion plays an important role in central tolerance, and it has been shown to play a role in costimulation-blockade induced tolerance to alloantigens (Wells et al., 1999). T cells in tolerant mice have been shown to undergo abortive proliferation (Chai et al., 2015; Ford et al., 2007; Iwakoshi et al., 2001), in which T cells proliferate but fail to accumulate, but whether abortive proliferation leads to selective loss of high avidity T cells is not known. High avidity T cells though are thought to be the most susceptible to activation-induced cell death (Dalla Santa et al., 2014). To test whether deletion of high affinity clones was underlying the low avidity profile in tolerant mice, Bcl-$x_L$-Tg and Bcl-2-Tg mice were transplanted and tolerized with DST and anti-CD154. Despite the fact that Bcl-$x_L$ overexpression in T cells prevents tolerance induction (Wells et al., 1999), overexpression of either of these anti-apoptotic factors failed to rescue high multimer-binding in costimulation-blockade-treated mice (Figure 4.9A, B). Thus high avidity T cells are not deleted in a manner rescuable by forced expression of the anti-apoptotic factors Bcl-$x_L$ or Bcl-2.

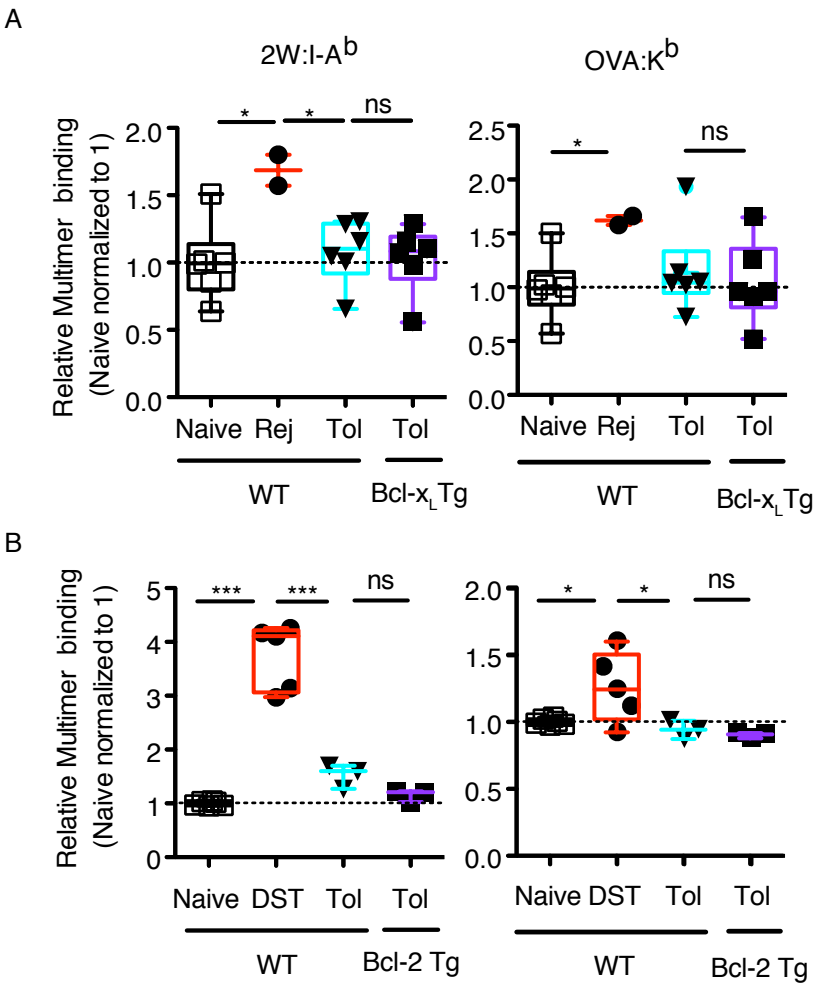


**Figure 4.9: Overexpression of Bcl-$x_L$ or Bcl-2 does not increase avidity of graft-specific T cell populations in tolerant mice**

Endogenous 2W:I-$A^b$-reactive and OVA:$K^b$-reactive T cells were stained with 2W:I-$A^b$ and OVA:$K^b$ multimers in wild-type (WT) naïve, rejecting, and tolerant mice as well as Bcl-$x_L$-Tg **(A)** or Bcl-2-Tg **(B)** tolerant mice, and the relative multimer staining of each was quantified by normalizing the MFI of the naïve group to 1. Data are pooled from 2 independent experiments each and were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, *** p<0.001, ns=not significant.

7. Avidity maturation is dependent on positive costimulation downstream of CD40 signaling

An alternative possibility is that reduced avidity maturation of alloreactive T cells in tolerant hosts is due to lack of expansion of high avidity clones because of their activation in the absence of costimulation, rather than due to their deletion. To test this possibility, we treated mice concurrently with agonistic anti-CD40 to restore signaling to antigen presenting cells while still having blocking anti-CD154 on board. The agonistic anti-CD40 treatment rescued avidity maturation in transplanted mice treated with DST + anti-CD154 (Figure 4.10A), suggesting positive signaling to CD40 is required for alloreactive T cell populations to increase their avidity upon antigen exposure. The observed increase in population avidity coincided with a significant increase in allospecific T cell numbers (Figure 4.10B). These data suggest that selective costimulation-dependent expansion of high avidity T cells may be required for allospecific T cell avidity maturation.

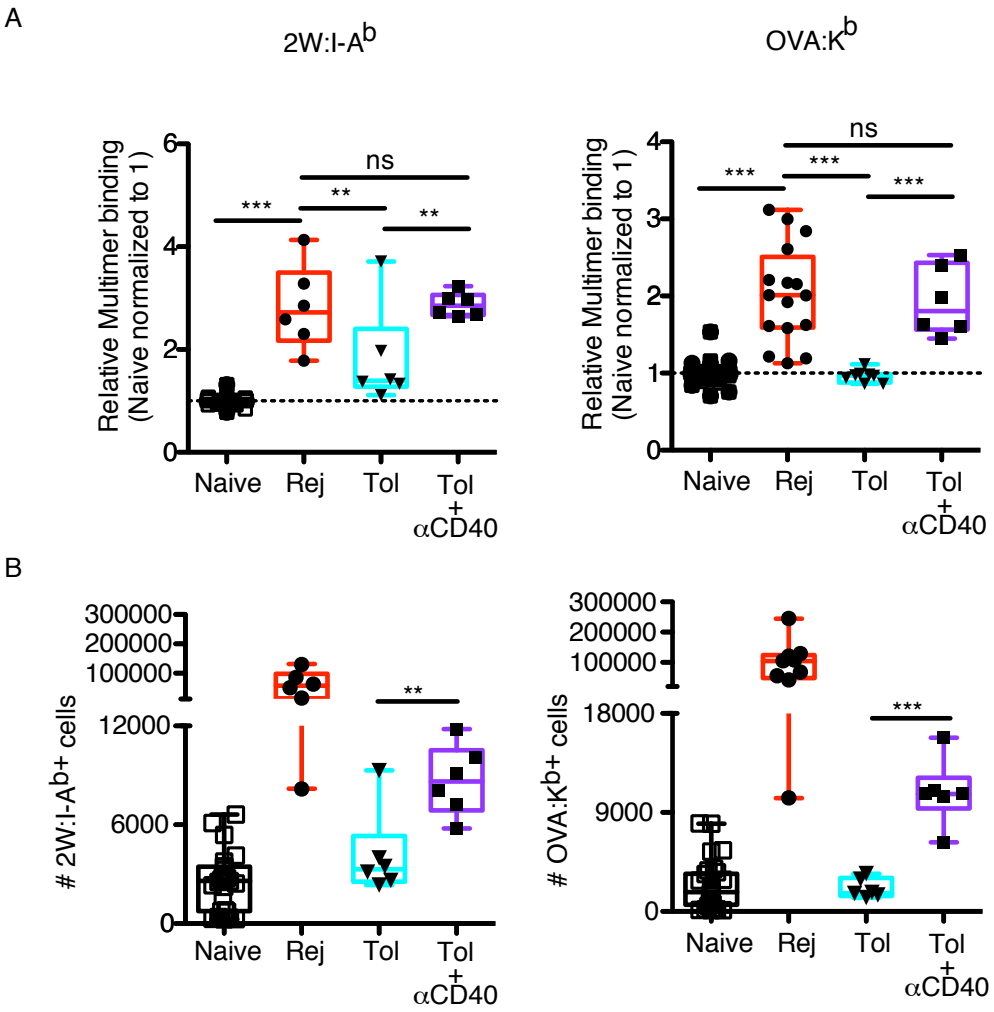


**Figure 4.10: Concurrent agonistic anti-CD40 treatment allows costimulation blockade-treated alloreactive T cell populations to undergo avidity maturation and expansion**

**A.** Normalized MFI of multimer-binding on 2W:I-A$^b$- and OVA:K$^b$-reactive splenic T cells from naïve, rejected, and tolerant mice. In addition, a group of transplanted mice received a single concurrent dose of agonistic anti-CD40 on day 0 at the same time as the DST and anti-CD154 used for tolerance induction. **B.** Numbers of 2W:I-A$^b$- and OVA:K$^b$-binding cells in the spleen. All transplanted mice were analyzed 8-9 days post-transplantation. Data points in Naïve, Rejected, and Tolerant groups were also presented in Figures 4.1B and 4.2B. Tol + anti-CD40 data are pooled from two independent experiments, with n=6 mice total per group. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing (A) or student's T test (B). **p<0.01, ***p<0.001, ns=not significant.

8. Allosensitized mice have a skewed T cell repertoire that is not observed in mice treated with costimulation blockade

To determine if lack of avidity maturation in transplantation tolerance correlated with a distinct TCR repertoire following alloantigen exposure from that in non-tolerant hosts, we sorted 2W:I-$A^{b-}$and OVA:$K^{b}$-binding cells from DST-treated and DST+αCD154–treated mice and analyzed their respective repertoires through TCRβ spectratyping. Examples of spectratype traces of a highly represented 2W:I-$A^{b}$-specific Vβ family member (Moon et al., 2007), Vβ8.2, are shown for 2W:I-$A^{b-}$ and 2W:I-$A^{b+}$ T cells in Figure 7A. 2W:I-$A^{b-}$ cells represent all polyclonal $CD4^{+}$ T cells in the respective groups, and for the most part followed a Gaussian distribution of CDR3 lengths. The DST-treated mice are exposed to numerous BALB/c antigens in addition to 2W1S and OVA, such that the 2W:I-$A^{b-}$ spectratypes sometimes revealed slight skewing. 2W:I-$A^{b}$-reactive T cells in DST-treated mice had a more restricted Vβ8.2 repertoire than their DST+αCD154 counterparts, suggesting lack of expansion of specific clones in the tolerant animals (Figure 4.11A).

Immunization with ovalbumin has been shown to expand T cells that express Vβ5.2, and Vβ5.2 transgenic mice have a high proportion of OVA-reactive T cells (Dillon et al., 1994). Similarly to the alloreactive $CD4^{+}$ T cells, OVA:$K^{b+}$ $CD8^{+}$ T cells from DST-treated mice had a more skewed Vβ5.2 repertoire than T cells from DST+αCD154 treated mice (Figure 4.11B). To measure the degree of skewness of each population of pMHC multimer-binding T cells, Hamming distances were calculated for each TCR Vβ family for each individual mouse using the relative areas under each peak in the spectratypes compared between pMHC multimer-binding T cells in treated mice relative

to pMHC multimer-binding T cells in naïve mice (Figure 4.11C). A value of zero for the Hamming distance indicates there was no difference in the two populations compared, while a value of one signifies the two populations were completely distinct. TCR lengths across all Vβ families were more skewed in DST- than in the DST+αCD154-treated groups both relative to multimer non-binding T cells in the same mice, as well as relative to multimer-binding T cells in naïve mice. In combination with the individual spectratype traces showing more restricted repertoires, we interpret this to mean that productive alloimmunity results in an expansion of T cell clones of which some are likely high avidity, and this leads to the overall avidity maturation of the allospecific T cell populations observed following cardiac allograft rejection or DST-immunization. In contrast, the less skewed repertoire and the more Gaussian-looking spectratypes in the costimulation-blockade-treated mice reveals a lack of selective expansion of particular T cell clones, correlating with the failure of this population to undergo avidity maturation in the absence of positive costimulation.

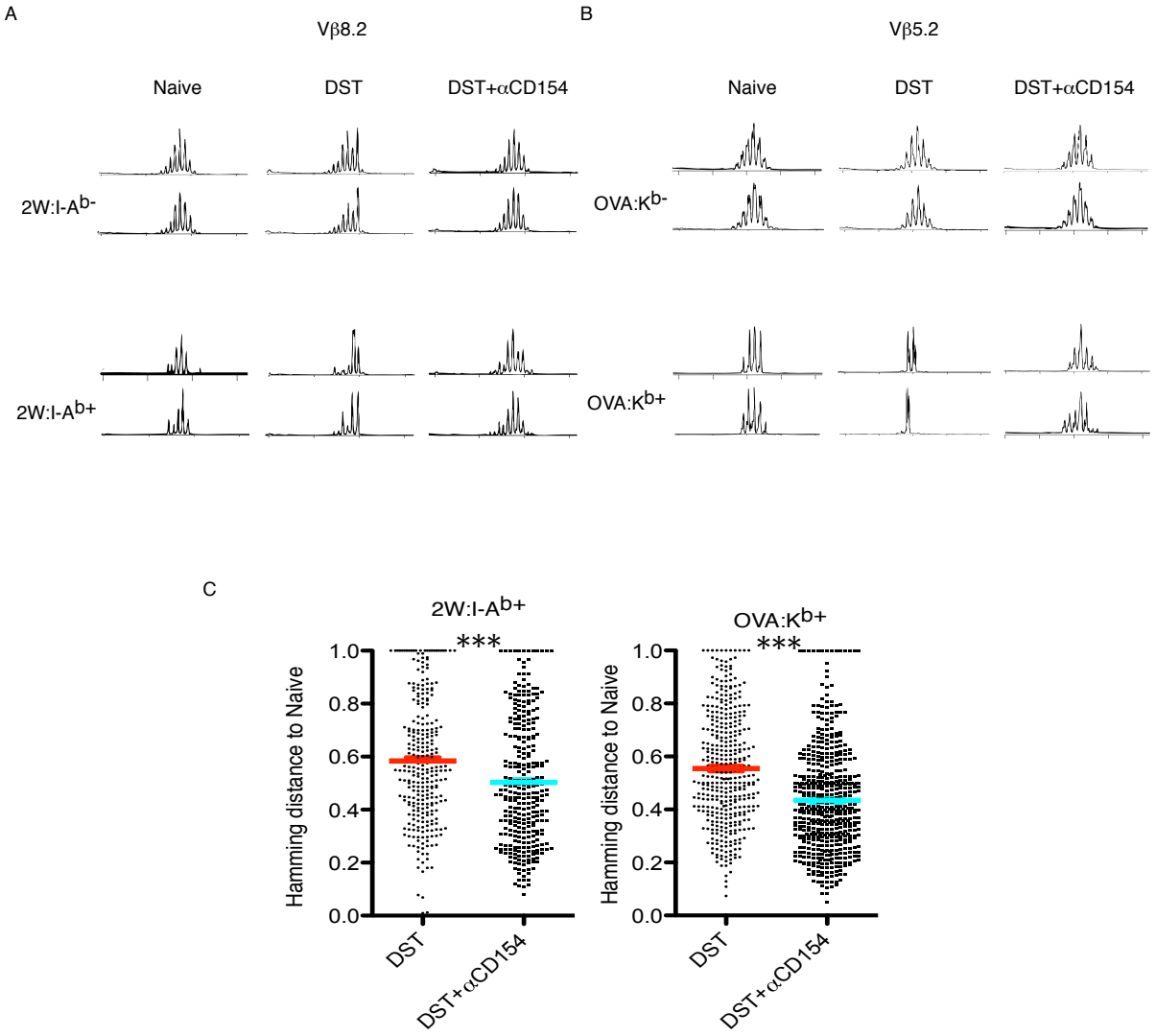


**Figure 4.11: Costimulation blockade dampens skewing of the T cell repertoire that normally occurs following alloantigen encounter**

**A.** Representative TCR Vβ 8.2 spectratypes for $CD4^+$ T cells that were non-multimer-binding (2W:I-$A^{b-}$) or that bound multimer (2W:I-$A^{b+}$), from splenic T cells isolated from naïve mice or one week post DST or DST+anti-CD154. **B.** Representative TCR Vβ 5.2 spectratypes for $CD8^+$ T cells that were non-multimer-binding (OVA:$K^{b-}$) or that bound multimer (OVA:$K^{b+}$), from splenic T cells isolated from naïve mice or one week post DST or DST+anti-CD154. Traces were normalized to the height of the tallest peak. **C.** Hamming distances comparing the degree of similarity (=0) or skewness (=1) of populations of TCR Vβ lengths across all 18 Vβ families sampled between the multimer-positive samples in the DST and DST+anti-CD154 groups compared to the multimer positive samples in the naïve group. **A.** and **B.** Two traces are shown out of 4-8 mice per group, pooled from two independent experiments. **** $p<0.0001$ by Student's t test.

E. Discussion

In transplantation, tolerance to donor antigens can be induced both centrally and peripherally. Prospective transplantation tolerance in patients has been induced successfully with concurrent donor bone marrow and solid organ transplantation (Sachs et al., 2014), and deletion of donor specific cells has been implicated as a major mechanism of this tolerance (Morris et al., 2015). In animal models, costimulation blockade therapy induces robust donor-specific tolerance through peripheral T cell mechanisms (Markees et al., 1998), including at a cell-intrinsic level, clonal deletion and anergy/exhaustion, and at a cell-extrinsic level through regulatory cells (Pinelli et al., 2013; Quezada et al., 2005). Whether additional tolerance mechanisms, including population-level changes to the allospecific T cell repertoire, similar to what occurs in central tolerance, could follow from a peripheral tolerance induction regimen, was an open question. We hypothesized peripheral tolerance could also select for T cells with low avidity for alloantigens. Alloreactive T cells in tolerant mice were not only lower avidity than in non-tolerant hosts, but differences were stable and resistant to TCR rechallenge, as their avidity profile did not change upon subsequent encounter of alloantigen. This low avidity profile was dependent upon lack of costimulation to prevent selective expansion of particular high avidity T cell clones. These findings highlight a previously unknown element of peripheral tolerance, shaping alloreactive T cell repertoires during transplantation.

Graft-specific T cells can persist in a tolerant environment, but they fail to appreciably accumulate over levels found in naïve hosts. Persistence of alloreactive T

cells in tolerant settings has been demonstrated using TCR-Tg transfer studies, as well as experiments using synchimeric mice, and by revealing remaining cells functionally through breaking the maintenance phase of tolerance (Chai et al., 2015; Ford et al., 2007; Iida et al., 2013; Iwakoshi et al., 2001; Kendal et al., 2011; Miller et al., 2015). Using a robust model of transplantation tolerance and simultaneous tracking of endogenous alloreactive T cells, we confirm that endogenous graft-specific T cells persist but fail to increase in numbers following transplantation.

Peripheral tolerance to self-antigens and the induction of oral tolerance can result in abortive proliferation of antigen specific T cells. Studies using CFSE-labeled TCR-Tg T cells transferred into tolerant recipients have shown that tolerance induction does not inhibit proliferation of T cells ((Chai et al., 2015) and our unpublished results), but prevents their accumulation. This suggests that preventing cells from undergoing apoptosis should lead to increased accumulation of T cells. Indeed, this was argued by Wells et al., as Bcl-$x_L$ Tg mice failed to develop long-term graft acceptance following costimulation blockade (Wells et al., 1999). However, that study did not analyze alloreactive T cell numbers under these conditions (Wells et al., 1999). Our data show that Bcl-$x_L$ or Bcl-2 overexpression does not restore high avidity T cells, as there is likely to be an impact of circulating anti-CD154 antibody actively limiting accumulation of T cells at this time. Corroborating these data, Lehnert and colleagues (Lehnert et al., 2007) did not find any prevention of tolerance to islet allografts with costimulation blockade in Bcl-2-Tg mice or in Bim-KO mice, concluding that inhibition of passive cell death pathways in T cells does not block tolerance induction. Although we cannot rule

out that other cell death pathways may be preventing T cell population-avidity maturation in tolerant animals, our data point instead to the lack of expansion of high avidity T cell clones. While TCR-Tg studies imply there is no defect in proliferation in a tolerant environment, these studies are limited in that they only compare proliferation of T cells of a single specificity, and thus these experiments are unable to recapitulate the endogenous response to alloantigens, where interclonal competition occurs as revealed by selective expansion and accumulation of certain T cell clones.

Indeed, our results with OT-I Rag-KO T cells emphasize that using a single TCR sequence to study a polyclonal response could lead to misleading conclusions. Using pMHC multimer-binding of these cells alone one would conclude that T cells in tolerant mice are of higher avidity than those in acutely rejecting animals, whereas the opposite is revealed by tracking the endogenous polyclonal population specific for the same model antigen. This is due to population-level changes occurring in the polyclonal setting. The monoclonal OT-I cells have less OVA:$K^b$ binding in the rejecting group primarily because T cell activation results in a reduction of TCR and CD8$\alpha$ levels on the cell surface. This highlights the importance of including these parameters when measuring T cell avidity by pMHC multimers-binding studies. The result of this experiment points to population-level changes enabling differences in multimer-binding of alloreactive populations in rejecting and tolerant mice, rather than modifications that occur at the individual T cell level. Any cellular-level changes such as TCR/co-receptor post-translational modifications or changes in clustering of these molecules on the cell surface may still be occurring but are not the dominant changes leading to avidity

maturation or lack thereof, rather the dominant effect is due to population-level alterations.

The population-level changes in endogenous T cell avidity between rejection and tolerance were directly observed through analyzing the repertoires of individual TCR Vβ families using TCRβ spectratyping. That $CD8^+$ $V\beta5.2^+$ cells were clonally restricted upon immunization with DST expressing ovalbumin recapitulates in our model what has been known for immunization with ovalbumin in other settings, supporting the conclusion that this Vβ family harbors T cells containing greater affinity for recognizing OVA:$K^b$ (Dillon et al., 1994). In contrast, mice treated with DST+anti-CD154 exhibited a much less restricted Vβ5.2 repertoire. These results were extended across all TCR Vβ families examined, and both $CD4^+$ and $CD8^+$ T cells in DST+anti-CD154-treated mice had less skewed T cell repertoires than those from DST-treated mice. Thus, costimulation blockade reduces population-level avidity maturation through limiting expansion of high avidity T cells. This conclusion is supported by a study from Kwun et al., who showed that blocking LFA-1 prevented T cell expansion in a minor mismatch transplantation model, and, although not discussed by the authors, this correlated with a concurrent loss of high avidity T cells observed from the lower pMHC multimer-binding of T cells in the anti-LFA-1-treated group (Kwun et al., 2015).

The rescue of T cells with higher pMHC multimer-binding in mice treated with agonistic anti-CD40 occurred with a concurrent increase in T cell numbers. This is consistent with expansion of high avidity T cells being required to raise the overall avidity profile of T cells from naive levels. Indeed, our results support other studies in

models of cancer, vaccines or tolerance to self-antigens showing that costimulation has a positive impact on T cell avidity maturation (Black et al., 2014; Hodge et al., 2005; Yang et al., 2005). Providing enhanced $CD4^+$ T cell help also appears to increase the avidity of $CD8^+$ T cells (Figure AI.3). How APCs program populations of T cells to become high avidity or to stay low avidity remains an open question. It is interesting to note this programming is remarkably stable in tolerant mice, as avidity remains unchanged upon antigen rechallenge. This is reminiscent of T cell-intrinsic hyporesponsiveness, which is also thought to be programmed, and makes cells resistant to secondary encounter of antigen (Schietinger et al., 2012). How much of an anergic/hyporesponsive phenotype may be contributed to a cell's avidity for antigen is unknown. Studies of T cells in tumor microenvironments that are deemed dysfunctional, and of self-specific cells, including regulatory CD8 T cells to self and donor antigens (Black et al., 2014; Cai et al., 2004; Kuball et al., 2009; Malhotra et al., 2016; Moon et al., 2011) have also revealed that many of these cells are low avidity at the population level, supporting the idea that tolerance may be simultaneously enforced through multiple mechanisms, one of which being to have low avidity for the relevant antigen.

There are strong correlations between high avidity T cells and clearance of infections (Busch and Pamer, 1999), elimination of tumors (Black et al., 2014; Kuball et al., 2009; Soong et al., 2014), better memory responses (Turner et al., 2008; Zehn et al., 2009) and autoimmunity (Maeda et al., 2014). In contrast, low avidity T cells are found in many settings of tolerance to self-antigens (Malhotra et al., 2016; Moon et al., 2011; Yu et al., 2015), to tumors (Black et al., 2014; Soong et al., 2014; Souders et al.,

2007; Wong et al., 2008), and to non-inherited maternal antigens (van Halteren et al., 2009). Our study demonstrates that costimulation-blockade can maintain alloreactive T cell populations of lower avidity, which correlates with robust transplantation tolerance, while unmodified rejection correlates with population-avidity maturation. Thus, therapeutic approaches that achieve long-term elimination of T cells with high affinity for donor antigens may be desirable to achieve robust transplantation tolerance in the clinic.

# V. RESULTS – PERSISTENT DONOR ANTIGEN CONTRIBUTES TO AN ALLOREACTIVE CD4$^+$ T CELL EXHAUSTION-LIKE PHENOTYPE IN TRANSPLANTATION

## A. Abstract

Tolerance can be quite resilient. Tumor patients can relapse following immunotherapy-mediated regression. Autoimmune patients can have long periods of remission after a relapse. Transplantation tolerance can spontaneously re-emerge after rejection of a donor allograft in mice. What underlies the resilience of tolerance is not well understood. Our previous work with mice that spontaneously restored their tolerance following infection-mediated rejection of a primary allograft illustrated a functional lack of memory of the rejection event (Miller et al., 2015). This was unexpected because rejection events are thought to result in sensitization and generate immunologic memory. These data led us to question whether T cell memory can develop from a tolerant state. We found tolerant T cells did not develop into conventional memory cells; instead they resembled exhausted cells and were intrinsically impaired in their ability to proliferate upon reencountering antigen. This hyporesponsiveness of tolerant T cells was unchanged upon infection with Listeria or by other pro-inflammatory challenges tested even if the intervention resulted in graft rejection. Persistent antigen was the only parameter that predicted cell-intrinsic hyporesponsiveness during secondary challenge. Thus, similar to chronic viral infections, persistent antigen from the tolerant allograft led to a phenotype resembling T cell exhaustion. This phenomenon of duration of antigen exposure leading to T cell

exhaustion may help to explain why the most predictive factor for patients to spontaneously develop tolerance is the time from transplantation before weaning immunosuppression (de la Garza et al., 2013). These results may also have important implications for other settings of chronic antigen exposure such as cancer and autoimmunity.

B. Introduction

Adaptive immune cell memory develops in a host from a naïve, antigen inexperienced state in order to allow for more rapid clearance of repeat encounter of antigen to protect the host from infections, and is the basis of vaccination (Jaigirdar and MacLeod, 2015). Memory T cells have enhanced ability to respond to repeat antigen through existing at a higher frequency than their naïve counterparts as well as being transcriptionally poised to produce cytokines and proliferate faster than naïve cells (Youngblood et al., 2013). Resident memory T cells also have an advantage of being already in the right location to respond (Mueller and Mackay, 2015). T cell memory in transplantation, however, is a known barrier to tolerance induction (Ford and Larsen, 2010; Valujskikh and Li, 2007). Memory T cells that can react to a transplant are thought to be generated prior to transplantation through heterologous immunity from T cells that can cross react with microbial antigens and the allograft, as well as through prior exposure to alloantigens during pregnancy, blood transfusions, or previous transplantation. Memory T cells are notoriously more difficult to immunosuppress with

conventional agents and can be resistant to costimulation blockade therapies (Ford and Larsen, 2010; Valujskikh and Li, 2007).

Many studies have looked at the role of pre-existing memory T cells in transplantation (Brook et al., 2006; Golshayan et al., 2010; Krummey and Ford, 2012; Macedo et al., 2009; Nadazdin et al., 2011; Traitanon et al., 2014); however, whether memory is productively generated from a tolerant state is currently unknown. This question has importance for patients that undergo episodes of acute rejection that are successfully treated to determine if these patients would progressively be sensitizing themselves and generating memory cells that would in turn be resistant to immunosuppression, requiring escalating dosing. Empirical data suggests otherwise. Patients can often return to baseline immunosuppression following an acute rejection episode and episodes of rejection do not prevent a patient from successfully becoming tolerant (Brouard et al., 2012; Lerut and Sanchez-Fueyo, 2006). We have recently generated a mouse model in which tolerant recipients infected with *Listeria monocytogenes* acutely reject their allografts, yet despite this rejection event the mice are not sensitized to the transplant and are able to spontaneously accept secondary allografts of donor origin once the inflammation from the infection has subsided (Miller et al., 2015). As the second transplant was accepted, this reflects a lack of functional memory of the previous rejection event.

In this study we tested whether T cell memory was generated but controlled following an infection-mediated rejection event, or whether a tolerant T cell was incapable of differentiating into a memory cell. Tolerant T cells, rather than taking on

characteristics of memory T cells, instead resembled exhausted T cells, expressing high levels of PD-1, low levels of IL-7R, and were cell-intrinsically hypoproliferative when rechallenged with donor antigen. This exhaustion phenotype was not specific to our tolerizing protocol using antigen exposure in the absence of costimulation blockade, but instead required an allograft as a source of persistent antigen. This is reminiscent of chronic viral infections, where persistent antigen leads to T cell exhaustion, as for a period of time post-rejection though the heart stops beating, there is still tissue present as a continuous source of antigen until the organ is completely resorbed. These data support the conclusion that persistent antigen from a tolerant allograft leads to cell-intrinsic T cell exhaustion-like hyporesponsiveness.

## C. Materials and Methods

### Mice

BALB/c and C57BL/6 mice were purchased from Envigo RMS, Inc. (Indianapolis, IN). TCR75 $CD4^+$ TCR-transgenic mice were obtained from R. Pat Bucy (University of Alabama-Birmingham) and crossed to CD45.1 mice, which were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed under specific pathogen-free conditions and used in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Heart transplantation

Cardiac transplantation was performed as previously described (Miller et al., 2015) using a technique adapted from Corry et al. (Corry et al., 1973). Cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. Allograft rejection was measured by palpation and is defined as a complete cessation of heartbeat. Mice were treated with 600 μg of anti-CD154 (BioXCell) on days 0, 7, 14 post-transplantation. In some experiments, mice were treated with 400 μg anti-PC61 on day -1, or 100 μg anti-CD40 on day 0, day 7 or CpG on days 0, 7 post-transplantation.

Listeria infection

Mice were infected with Listeria as previously described (Miller et al., 2015). Doses of 1.5 x $10^6$ CFU (Lm-GFP) were administered i.p. All Lm-infected mice were kept in biosafety facilities.

Adoptive transfer of cells

Cells were isolated from donor mice from the spleen and inguinal, brachial, axillary, cervical and mesenteric lymph nodes. An Accuri C6 flow cytometer (BD Biosciences) was used to obtain cell counts and a subset of cells were stained for CD4 or CD8, Vβ or Vα, the congenic marker CD45.1 and CD44. The percentage of $CD44^{lo}$, congenically marked TCR-Tg T cells was used to calculate the total number of cells for the adoptive transfer. In certain experiments, cells were first labeled with 2 μM CFSE

prior to adoptive transfer. Cells were resuspended in 200 μl of phosphate-buffered saline (PBS) and injected retro-orbitally.

Donor splenocyte transfusion

Mice received one-quarter spleen homogenized in PBS, washed and twice-filtered as a donor splenocyte transfusion. Cells were resuspended in 200 μl PBS and injected retro-orbitally or intraperitoneally.

Isolation of graft-infiltrating cells

Graft-infiltrating cells were isolated as previously described (Miller et al., 2015). Briefly, cardiac allografts were rinsed in situ with Hanks balanced salt solution (HBSS) containing 1% heparin. Explanted hearts were dissected into small pieces and digested for 40 min at 37°C with 400 U/ml collagenase IV (Sigma), 10 mM HEPES (Cellgro) and 0.01% DNase I (MP Biomedicals) in HBSS (Cellgro). Digested cell suspensions were washed and filtered through 40 μm nylon mesh.

Magnetic enrichment

CD45.1$^+$ TCR75 cells from spleen and peripheral lymph nodes of recipient mice were stained with anti-CD45.1-bio (eBioscience) and incubated with streptavidin magnetic beads (Miltenyi) for magnetic enrichment with an AutoMACs machine (Miltenyi).

FACS

Magnetically enriched CD45.1$^+$ cells were stained with fluorescently coupled antibodies against CD45.1, CD4 and Vβ8.3, as well as fluorescently labeled streptavidin and were further sorted for CD45.1$^+$ CD4$^+$ Vβ8.3$^+$ cells (FACSAria, BD Biosciences).

Intracellular staining and flow cytometry

Single cell suspensions of lymphocytes were prepared from isolated spleens and heart grafts. First, cells were stained with a fixable live/dead stain (Aqua, Invitrogen) for 30 min at room temperature and then with anti-CD4 (L3T4), anti-CD45.1 (A20), anti-Vβ8-3 (1B3.3), anti-PD-1 (J43), anti-CD127 (A7R34), and anti-CD44 (IM7). Surface-stained cells were then fixed with the Foxp3 fixation permeablization buffer kit (eBioscience) for 15 min at room temperature, washed with 1 x permeabilization buffer, stained using anti-Ki-67 (SolA15) for 30 min at room temperature, washed again, and analyzed by flow cytometry. All mAbs were from BD Biosciences or eBioscience.

Data analysis

Flow cytometry data were analyzed using FlowJo (TreeStar). Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc).

D. Results

1. Tolerant T cells survive following an infection-mediated rejection

Our previous results with infection mediated-rejection of cardiac allografts from a tolerant state revealed that the loss of tolerance was only transient, and it was spontaneously restored following resolution of the infection (Miller et al., 2015). This rejection event was dependent on the bystander activation of T cells through the cytokines IL-6 and IFNβ and coincided with a transient increase in alloreactive T cells in the spleen (Miller et al., 2015; Wang et al., 2010). One possibility for the lack of memory of the rejection event was that the alloreactive T cells did not survive following the infection. To investigate this possibility, we seeded $CD4^+$ TCR75 TCR-Tg T cells into tolerant C57BL/6 recipients just prior to transplantation with a BALB/c heart so that we would have a fixed traceable population of alloreactive T cells that would not be confounded by new thymic emigrants. TCR75 cells recognize a BALB/c $K^d$ peptide presented on host I-$A^b$. These seeded TCR75 cells did not prevent tolerance induction with anti-CD154 and donor splenocyte transfusion (DST, MST > 60 days) and were present at naïve levels in tolerant mice 60 days post-transplantation (Figure 5.1A). Tolerant mice infected with Listeria at day 30 post-transplantation retained significantly more TCR75 cells 30 days post the infection than in uninfected tolerant recipients (Figure 5.1A). In both tolerant mice and tolerant mice post-Lm, the TCR75 cells were antigen experienced as a high proportion expressed the activation marker CD44 (Figure 5.1B). Thus alloreactive $CD4^+$ T cells did survive following the infection.

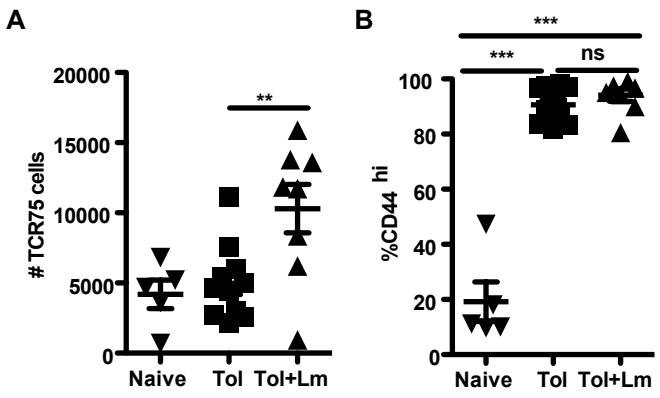


**Figure 5.1: Allospecific T cells persist following infection-mediated rejection**
$10^5$ TCR75 cells were seeded 1-3 days prior to transplantation and tolerance induction with anti-CD154+DST. A subset of mice were infected with Lm-GFP. **A.** The numbers of TCR75 cells and the percentages that were $CD44^{hi}$ (**B**) recovered from the spleen and peripheral lymph nodes 30 days post-Lm infection and 60 days post-transplantation/adoptive transfer in naïve (n=5), Tol (n=11) and Tol+Lm (n=8). Data are pooled from 2-3 independent experiments. Data were analyzed with a one-way ANOVA test and Bonferroni correction, ** $p<0.01$, ***$p<0.001$, ns=not significant.

2. Tolerant T cells have an exhausted phenotype

We next wanted to determine whether these tolerant cells were differentiating into memory T cells but were being controlled extrinsically by host regulatory cells following the infection-mediated rejection event, or if these cells were incapable of differentiating into memory cells from a tolerant state. We phenotyped the cells for IL-7R (CD127) expression, a cytokine receptor on the surface of naïve and memory T cells, important for cell survival. IL-7R is more highly expressed in memory T cells compared to naïve cells (Figure 5.2A,C). TCR75 cells from mice that undergone an acute rejection event 30-80 days prior and from mice that had been immunized with donor splenocytes (DST) 30-60 days prior both expressed high levels of IL-7R, as would be expected for conventional memory cells. On the other hand, TCR75 cells from

tolerant mice and tolerant mice infected with Listeria, while positive for IL-7R as in naïve mice, did not express high levels of CD127 like in the control rejection and DST-immunized groups (Figure 5.2A,C). With respect to the canonical memory cell marker IL-7R, tolerant T cells did not express high levels, irrespective of whether the allografts had undergone infection-mediated rejection.

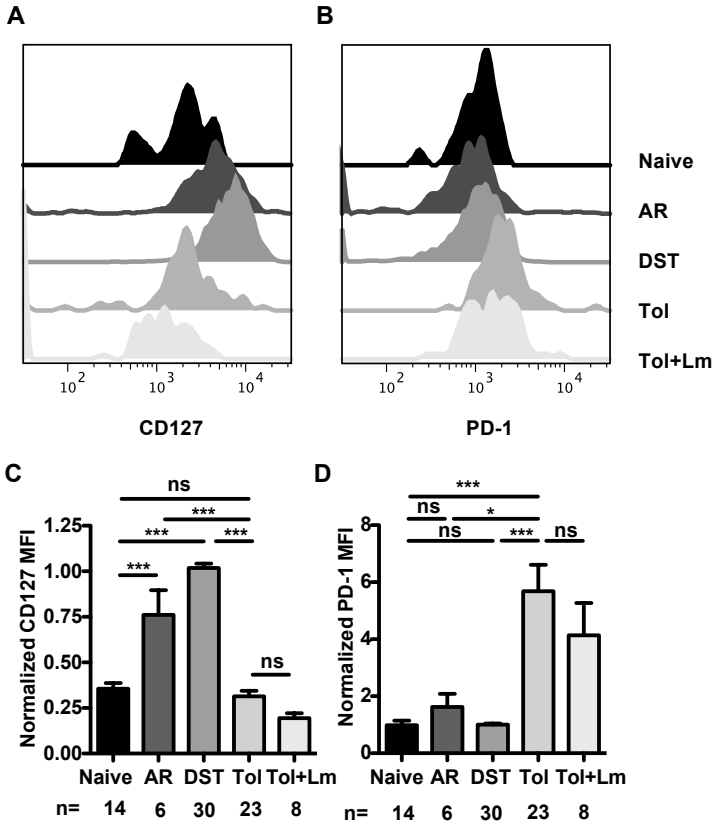


**Figure 5.2: Tolerant T cells have an exhausted phenotype**
**A.** and **B.** Representative flow plots showing CD127 (**A**) and PD-1 (**B**) staining on TCR75 cells 60 days post-transfer in naïve mice, mice that underwent acute rejection (AR), mice immunized with donor splenocytes (DST), mice tolerant to their allograft (Tol) and tolerant mice that were infected with Listeria 30 days post-transplantation. **C** and **D**. Relative mean fluorescence intensity (MFI) of CD127 (**C**) and PD-1 (**D**). Data were normalized, with the values in the DST group set to 1. Data are pooled from 2-6 independent experiments. Data were analyzed with a one-way ANOVA test and Bonferroni correction, * $p<0.05$, ***$p<0.001$, ns=not significant.

IL-7R expression is downregulated downstream of TCR-signaling in naïve T cells as they transition into antigen-experienced effector T cells (Cekic et al., 2013). As tolerant hearts are alive and able to provide a constant source of $K^d$ antigen, we asked if another marker of chronic antigen stimulation, PD-1 (Barber et al., 2006), was expressed on tolerant T cells. Indeed, TCR75 cells from tolerant and tolerant mice infected with Lm both expressed high levels of PD-1 while naïve mice, mice with acutely rejected allografts, and DST-immunized mice all expressed low levels of this marker (Figure 5.2B,D). Together, the tolerant T cell profile of $CD127^{lo}PD\text{-}1^{hi}$ is an exhausted phenotype.

3. Tolerant T cells have impaired recall proliferation

Memory and exhausted cells are not only characterized phenotypically but also functionally. Memory T cells have high proliferative capacity upon repeat stimulation but exhausted cells are impaired in their recall proliferation. To test whether tolerant T cells behaved functionally as memory or exhausted cells, we sorted the TCR75 cells from their non-tolerant and tolerant hosts and adoptively transferred similar numbers into new naïve hosts (Figure 5.3A). This allowed us to assess the cells' proliferative capacity on a per cell basis, eliminating bias from certain populations existing at higher frequencies before rechallenge. Adoptively transferring the cells into new naïve hosts also allowed us to assess proliferation in the absence of extrinsic regulation and removed the cells from the transplant antigens. One day after transferring the TCR75 cells to new hosts,

we rechallenged a subset of those mice with DST and assessed recall proliferation 5 days post rechallenge (Figure 5.3A).

As expected, TCR75 cells transferred from mice that had undergone acute rejection (AR) or had been immunized with DST had high proliferative capacity and we recovered 300-400x as many cells from rechallenged mice as from non-rechallenged mice (Figure 5.3B). In contrast, TCR75 cells from tolerant mice or Lm-infected tolerant mice were impaired in their recall proliferation, and only about 30x as many cells were recovered from rechallenged mice as from non-rechallenged mice, a 10-fold reduction compared to the AR and DST groups (Figure 5.3B). We obtained similar numbers of cells from mice in all of the non-rechallenged groups, suggesting the reduced recovery of cells from tolerant mice is due to reduced proliferation or accumulation of the cells. By using Ki-67 staining to mark actively proliferating cells, nearly all transferred cells in mice rechallenged with DST were actively proliferating 5 days post-DST, whereas there was much less proliferation in cells transferred into mice not given DST (Figure 5.3C). Thus despite some proliferation, T cells from tolerant mice were impaired in their accumulation post-rechallenge with donor antigen, irrespective of whether the mice had undergone infection-mediated rejection of their allograft.

4. T cells from tolerant mice retain a CD127$^{lo}$ phenotype when removed from antigen

In chronic viral infection it has been shown that T cells that are exposed to chronic antigen for at least two weeks are able to retain their exhausted phenotype even when transferred to new hosts (Angelosanto et al., 2012). In the mice in which the

TCR75 cells were parked for 6 days and did not receive DST for rechallenge, the TCR75 cells from the AR and DST-immunized groups remained $CD127^{hi}$, while those from tolerant mice and tolerant mice infected with Lm remained $CD127^{lo}$ (Figure 5.3D). All of the TCR75 cells in mice receiving DST for rechallenge had low levels of CD127 (Figure 5.3D), which is to be expected as all of these cells would have recently seen antigen. Thus phenotypically and functionally, these tolerant T cells behave less like memory cells and more like exhausted T cells.

5. Persistent donor antigen impairs memory generation in tolerant recipients

Since it appeared T cell memory development was impaired from a tolerant state, we next asked what aspects of the tolerant environment were restraining memory generation (Figure5.4A). We first tested whether Tregs were restraining memory development by depleting these cells at the time of tolerance induction. Treg depletion at the time of tolerance induction prevents long-term graft acceptance and median survival time is around day 55 (Jiang et al., 2011). At 6 weeks post-transplantation, TCR75 cells from Treg-depleted mice were still as phenotypically exhausted and hypoproliferative upon antigen rechallenge as tolerant mice (Figure 5.4B-C). Therefore it did not appear Tregs were constraining alloreactive T cell memory development from a tolerant environment.

We next wondered if providing additional inflammation at the time of tolerance induction would restore memory development. We have previously shown administration of toll-like receptor (TLR) ligands at the time of tolerance induction prevents

graft acceptance (Chen et al., 2006). The recall proliferative capacity of T cells from tolerant mice given the TLR ligand CpG 5 weeks post-transplantation was as impaired as from tolerant mice not given CpG (Figure 5.4C), despite CpG causing heart graft rejection in all of the initial recipients (MST <14 days) and lower PD-1 MFI (Figure 5.4B). Thus, providing CpG as adjuvant did not restore recall proliferation to tolerant T cells.

We next wanted to test if providing costimulation back to T cells would restore memory formation. To test this hypothesis, we provided agonistic anti-CD40 in the presence of anti-CD154+DST. This treatment again caused rejection in all of the initial mice (MST <14 days); however, these T cells were still phenotypically exhausted and were impaired in their accumulation upon antigen rechallenge (Figure 5.4B-C). Despite the addition of positive costimulation to T cells, providing agonistic anti-CD40 did not restore memory function to tolerant T cells.

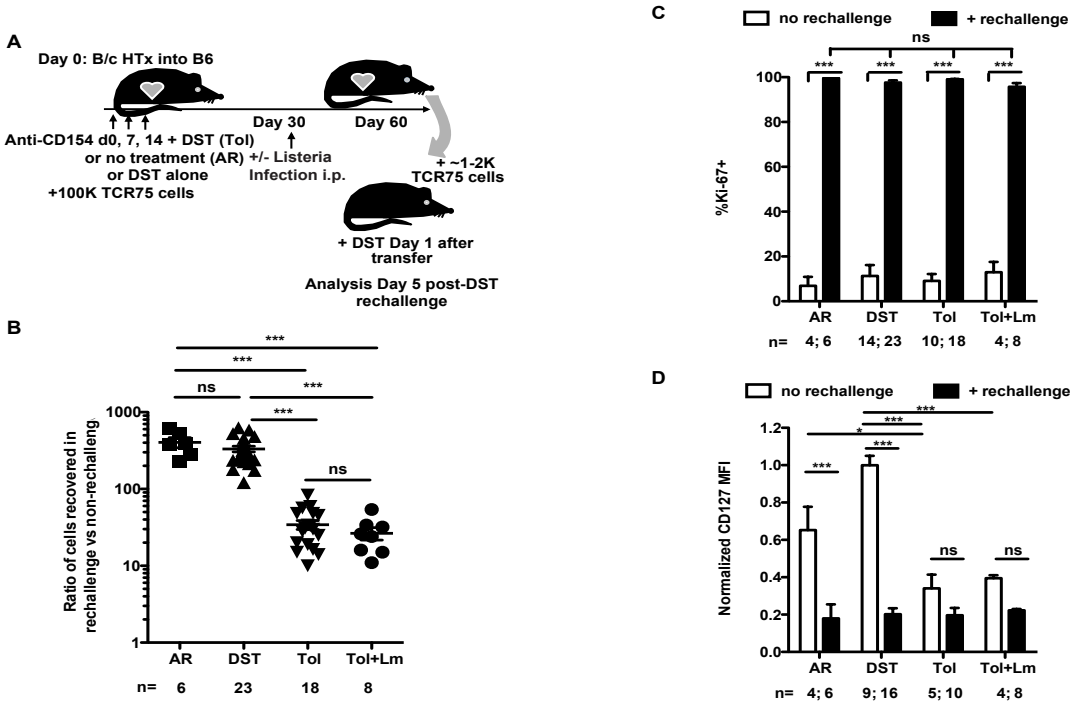


**Figure 5.3: Tolerant T cells are functionally impaired following antigen rechallenge**

**Figure 5.3, continued: A.** Experimental design. **B.** Ratio of TCR75 cells recovered in mice 5 days after rechallenge with DST compared to mice that were not given DST. Groups depict the treatments the original mice received before adoptive transfer of the TCR75 cells into new hosts prior to rechallenge. **C.** Percentages of TCR75 cells that were Ki-67$^{+}$ in non-rechallenged and rechallenged mice. **D.** Relative CD127 MFI on TCR75 cells in non-rechallenged and rechallenged mice. Data were normalized, with the values in the DST group set to 1. The numbers below each group represent the number of mice in each group. Data were analyzed with one-way ANOVA (B) or two-way ANOVA (C,D) and Bonferroni correction for multiple pairwise comparisons, ***p<0.001, ns=not significant.

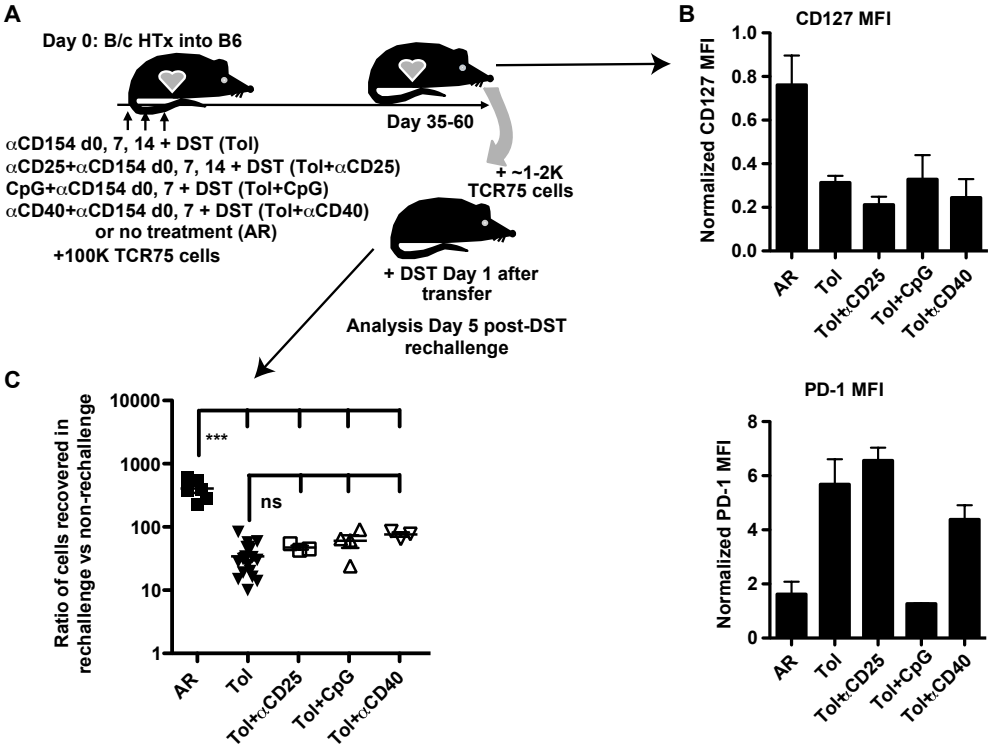


**Figure 5.4: Tolerant T cell hypoproliferation upon antigen recall is not restored by interventions that prevent tolerance induction**

**A.** Experiment design. **B.** Relative mean fluorescent intensity (MFI) of CD127 and PD-1 on T cells in the intact mice before adoptive transfer. **C**. The relative recovery of TCR75 cells recovered from the spleens and peripheral lymph nodes of AR, Tol, Tol+αCD25, Tol+CpG and Tol+αCD40 treated groups 6 days post-adoptive transfer into new hosts without rechallenge or hosts 5 days post-rechallenge with DST. Data from the AR and Tol groups is also depicted in Figures 5.2 and 5.3. Data were analyzed with one-way ANOVA and Bonferroni correction for multiple pairwise comparisons, ***p<0.001, ns=not significant.

In all of the mice that had rejected their transplants following tolerance induction, we noted that the hearts grafts, though not beating, remained palpable and similar in size to non-rejecting hearts. We wondered if, despite acute rejection, these hearts could provide a lingering supply of persistent antigen. To test whether persistent antigen alone or tolerance induction with costimulation blockade was required for impaired T cell memory development, we compared the phenotype and function of T cells tolerized with DST+anti-CD154 alone with no heart transplant to those with anti-CD154+DST with an allograft (Tol), and to their respective controls without anti-CD154 (AR and DST alone). Mice treated with anti-CD154+DST alone have been previously shown to be capable of clearing >90% of these cells in less than 24 hours (Brehm et al., 2007). As expected, treatment with anti-CD154 led to impaired accumulation of allospecific T cells in the intact recipients (Figure 5.5A). However, the TCR75 cells in the DST+anti-CD154 group phenotypically resembled the cells in the AR and DST groups, in that they were $CD127^{hi}$ $PD\text{-}1^{lo}$, in contrast to cells from tolerant mice, which were $CD127^{lo}$ $PD\text{-}1^{hi}$ (Figure 5.5B,C). Thus, phenotypic markers of T cell memory correlated with an absence of persistent antigen rather than treatment with anti-CD154. Functionally, when controlling for equal cell numbers, these DST+anti-CD154-treated cells responded as well to repeat antigen stimulation as those from mice that had undergone acute rejection or had been immunized with DST alone, unlike T cells from tolerant mice, which were impaired in their accumulation post-antigen rechallenge (Figure 5.5D). Consistent with their functional behavior, T cells from DST+anti-CD154-treated mice also retained high CD127 expression in non-rechallenged hosts (Figure 5.5E). Therefore, these data

support the conclusion that persistent antigen rather than costimulation blockade leads to a lack of memory formation in tolerant mice.

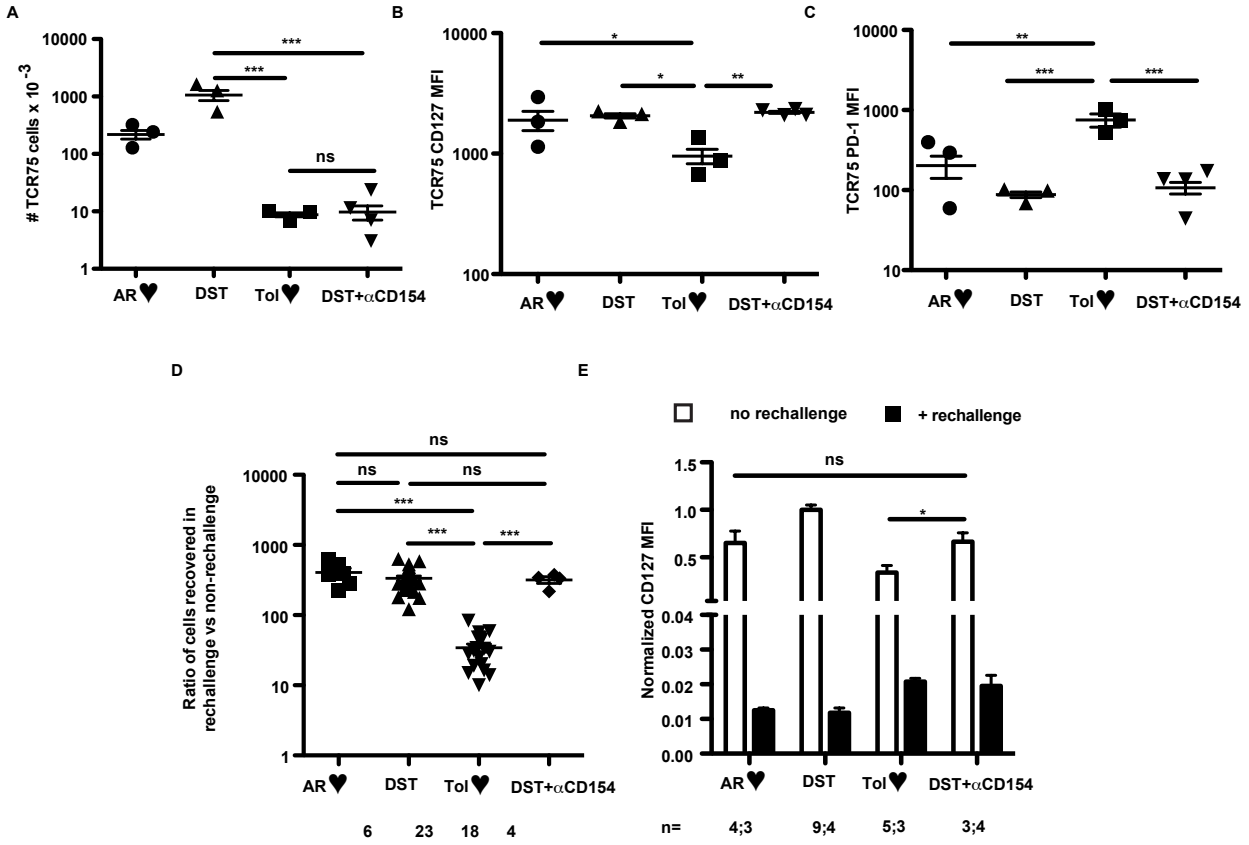


**Figure 5.5: Persistent antigen, not treatment with costimulation blockade, predicts T cell exhaustion**

**A.** The numbers of TCR75 cells recovered from the spleens and peripheral lymph nodes of AR, DST, Tol, and anti-CD154+DST (MR1 DST) treated groups 30 days post-treatment and adoptive transfer. **B.** CD127 MFI of TCR75 cells. **C.** PD-1 MFI of TCR75 cells. **D** and **E.** The relative recovery of TCR75 cells (**D**) and CD127 MFI (**E**) 6 days post-adoptive transfer into new hosts without rechallenge or hosts 5 days post-rechallenge with DST. Data were analyzed with one-way ANOVA (A-C) or two-way ANOVA (D,E) and Bonferroni correction for multiple pairwise comparisons, *p<0.05, ** p<0.01, ***p<0.001, ns=not significant.

## E. Discussion

A limited number of transplanted patients have become spontaneously tolerant to their allografts after ceasing immunosuppression. Studies to identify factors predictive

of a patient's ability to develop spontaneous tolerance have revealed that prior acute rejection events do not preclude a patient from later becoming tolerant (Benítez et al., 2013; Brouard et al., 2012). These data seem incongruent with the current paradigm that rejection leads to allosensitization. Yet consistent with these clinical data, we have shown that infection-triggered rejection of a tolerant allograft also did not lead to sensitization and did not preclude tolerance from being spontaneously restored (Miller et al., 2015). As discussed above, restoration of tolerance was dependent on the presence of Tregs as their depletion prevented the spontaneous acceptance of donor-matched secondary allografts transplanted shortly after resolution of the infection. These data suggested that regulatory T cells could suppress these Lm infection-reinvigorated alloreactive T cells at the quiescence of their effector stage. However, it remained unclear what the long-term phenotype and function of such reinvigorated T cells was. It is well established that naïve T cells can develop into memory T cells following successful antigen elimination. We asked whether T cell memory could develop from a state of T cell tolerance. Our results indicate that tolerant T cells did not differentiate into memory T cells following their transient reinvigoration by infection-mediated bystander activation. They instead resembled tolerant T cells that had not been exposed to an infection and displayed phenotypic signs of exhaustion and impaired recall proliferation. This state of hyporesponsiveness was programmed during the induction of tolerance if the grafts persisted for greater than 2 weeks under the cover of costimulation blockade and irrespective of whether the transplant was accepted or rejected thereafter. These data support the conclusion that T cell exhaustion from

chronic antigen exposure contributes to T cell hyporesponsiveness during transplantation tolerance and can drive T cell dysfunction even if tolerance fails and the graft is rejected.

Alloreactive T cells can persist in a tolerant environment, as they can be detected following an insult that results in tolerance being broken. Many manipulations that result in a loss of established tolerance are due to reactivation/expansion of these residual alloreactive T cells. These include Lm infection, high dose IL-2, lymphodepletion, mast cell degranulation, agonistic CD40 mAb in tolerant recipients, and peptide immunization after a graft has been removed (Iida et al., 2013; Okumi et al., 2008; de Vries et al., 2009; Wang et al., 2010; Yamada et al., 2015). Our data show, at least with respect to Lm infection, that these cells may only be transiently rescued in effector function, and despite their persistence do not appear to become *bona fide* memory T cells.

These tolerant cells instead resemble exhausted T cells. T cell exhaustion has been best characterized for CD8 T cells in chronic viral infections, though investigations into CD4 exhaustion are also beginning (Crawford et al., 2014). Exhausted viral specific CD8 T cells, like the CD4 T cells we observe in tolerant mice, express high levels of the negative regulator PD-1 and low levels of the IL-7R (Barber et al., 2006; Lang et al., 2005; Wherry et al., 2004). These exhausted cells are also hypoproliferative compared to their memory cell counterparts (Wherry et al., 2004). Exhaustion has been identified to play a role in transplantation in one study of chronic rejection in fucosyltransferase VII (Fut7)-deficient mice (Sarraj et al., 2014). CD4 T cells from mice deficient in Fut7, an enzyme important for selectin biosynthesis, were impaired in their migration and

proliferation following transplantation into untreated hosts and expressed an exhausted CD127$^{lo}$ PD-1$^{hi}$ phenotype (Sarraj et al., 2014). It is currently unknown if persistent antigen can affect Fut7 levels or T cell migration directly or if these pathways to T cell exhaustion following transplantation (costimulation blockade-treated wildtype mice versus untreated Fut7-deficient mice) are entirely independent.

Progression through the cell cycle may transiently rescue exhausted T cells, such as in settings where exhausted T cells are allowed to homeostatically proliferate (Brown et al., 2006; Schietinger et al., 2012). Reversing T cell exhaustion may be one mechanism by which lymphodepletion results in a loss of transplantation tolerance (Iida et al., 2013). Treatment with anti-PD-1 has also been shown to reinvigorate exhausted cells (Barber et al., 2006), but whether these cells return to an exhausted state or become *bona fide* memory cells following the anti-PD-1 treatment is unknown. Combined treatment with anti-PD-1 and administration of IL-2 did increase IL-7R expression on exhausted T cells (West et al., 2013). Whether IL-2 used to expand Tregs for tolerance induction would also prevent T cell exhaustion from occurring needs to be investigated.

Interestingly, the only factor predictive of T cell exhaustion versus T cell memory formation was persistence of antigen, whereas the tolerogenic regimen in the absence of persistent antigen was insufficient to program dysfunction. A recent paper by Chai et al. used TCR75 cells seeded at the time of tolerance induction in the absence of an allograft and concluded that tolerant mice maintain fully functional conventional T cells (Chai et al., 2015). Our data using anti-CD154+DST alone are consistent with this

study, but the addition of an allograft to this treatment did result in durably impaired T cell function.

Many studies utilizing presensitization protocols to generate memory prior to transplantation have used DST or skin transplants as the source of donor antigen, while others have used acute infections that elicit cross-reactive T cells, and have shown that this makes recipients resistant to tolerance induction (Adams et al., 2003; Hancock et al., 2002; Zhai et al., 2002). DST can be cleared effectively in >24 hours (Brehm et al., 2007) and skin transplants in mice are smaller organs than hearts or kidneys, providing a more limited source of antigen, and visibly disappear as they scab and detach. We propose that these sources of donor antigen may in fact be cleared efficiently enough to produce a good memory response, whereas slower rejection of a larger organ with a higher antigen load may not, as a high antigen load has also been shown to induce exhaustion (Mueller and Ahmed, 2009). Indeed, He et al. showed differential rejection kinetics for organs with differing amounts of antigen—one skin transplant was rejected faster than two skin transplants and than a heart transplant (He et al., 2004).

The duration of antigen exposure has been shown to be a critical determinant of T cell memory versus exhaustion (Angelosanto et al., 2012; Han et al., 2010). Virus-specific CD8 T cells that were exposed to a chronic infection for just two weeks were unable to become memory cells, even if they were then removed from antigen (Angelosanto et al., 2012). However, if the cells were removed from the chronically infected host after only one week they could become memory cells (Angelosanto et al., 2012). This suggests there is a limited window whereby T cells can be overexposed to

antigen and become durably exhausted. It is likely that the transplants that underwent rejection with Treg depletion, CpG or anti-CD40 treatment were viable long enough to retain alloantigen and allow T cell exhaustion to form.

Kidney transplants that become dysfunctional in the clinic are sometimes left in place, even upon subsequent re-transplantation. If some level of alloantigen is still present following organ failure, one could imagine that this may help tolerize T cells to any shared antigens between the primary and secondary graft, and that it may be beneficial to have a rejected organ remain in the patient if there is no clinical reason to remove it.

Our results would predict that CTLA4-Ig and other therapies that prolong graft acceptance would also promote exhaustion of alloreactive T cells in transplant recipients. However, there may be some exceptions to this as Sarraj et al. noted that different immunosuppressive agents used clinically can have varying effects on T cell expression of PD-1 (Sarraj et al., 2014). Specifically they mentioned that cyclosporine can inhibit induction of PD-1 (Oestreich et al., 2008), while mycophenolate can induce PD-1 expression (He et al., 2011). They speculated the decreased incidence of cardiac allograft vasculopathy observed in human heart transplant patients taking mycophenolate compared to calcineurin inhibitor-based regimens (Kaczmarek et al., 2006) may be due to mycophenolate's ability to promote PD-1 expression in T cells and perhaps T-cell exhaustion. Moreover, recent data show that NFAT, the transcription factor whose activation is blocked by calcineurin inhibitors, induces T cell exhaustion when activated without its partners (Wherry and Kurachi, 2015), further suggesting that

regimens that include calcineurin inhibitors may be detrimental if inducing this mechanism of dysfunction is important for robust tolerance. Further investigations into the role of T cell exhaustion in transplantation tolerance will aid in the development of targeted therapies to manipulate the balance between rejection and tolerance.

# VI. DISCUSSION

## A. Introduction

The work presented here demonstrates that transplantation tolerance can be quite robust and resilient, with its ability to spontaneously return after an acute rejection episode. Robust transplantation tolerance is induced and maintained through the cooperation of *multiple* mechanisms of tolerance, which explains its resistance to being broken by the disruption of a single tolerance mechanism. We have identified additional contributions to tolerance not previously described: the persistence of a low avidity T cell repertoire and T cell dysfunction/exhaustion due to chronic antigen exposure.

## B. Working Model

Based on data presented in this thesis and put in the broader context of literature on transplantation tolerance, we propose a working model explaining outcomes of transplantation tolerance versus rejection based on a gradient rather than a binary state (Figure 6.1). Tolerance is a delicate balance with multiple fail-safes to prevent rejection from occurring. Only when several mechanisms are simultaneously disrupted does rejection ensue. Furthermore, our data supports loss of tolerance as reversible, with tolerance able to be spontaneously restored after a sufficient number of tolerance mechanisms are re-engaged to rise above the threshold of rejection.

That multiple mechanisms need to be simultaneously disrupted to allow for rejection to occur from a tolerant setting is illustrated in Table 6.1. For example, our observation that the maintenance of established tolerance to heart allografts in

uninfected recipients is not dependent on $CD25^+$ regulatory T cells is consistent with a previous report showing that anti-CD25 treatment prevented the induction of tolerance to cardiac allografts by anti-CD154, but did not abrogate tolerance after it had been established (Jiang et al., 2011) (Table 6.1). Because anti-CD154/DST treatment has been shown to also drive partial deletion and anergy of alloreactive T cells (Iwakoshi et al., 2001; Quezada et al., 2005), we hypothesized that multiple redundant mechanisms of tolerance cooperate to maintain cardiac allograft survival after anti-CD154/DST therapy in the uninfected recipient, and that the disruption of a single pathway is not sufficient to precipitate rejection. In contrast, in post-infected tolerant recipients, depletion of $CD25^+$ cells was sufficient to prevent the acceptance of secondary donor allografts. If not all mechanisms are fully restored following the rejection event this may lead to an erosion of robustness of tolerance during the restoration phase. This may explain why Treg depletion, in the presence of the added inflammation provided by a second heart transplant in a recipient post-infection harboring increased numbers of alloreactive T cells, is sufficient to prevent acceptance of the second graft, as there are three disruptions in this setting (Table 6.1). We reason that Lm infection-mediated expansion of alloreactive T cells may be more durable than its effect on Tregs, thereby allowing Tregs to play a dominant role in the return of tolerance. Thus, depending on the mechanisms of maintenance of tolerance elicited by specific therapeutic regimens, the types of inflammatory pathways triggered by particular infections, and the duration of their impact, one could conceive that certain infections might have no significant impact on tolerance while others might precipitate an irreversible loss of tolerance. The latter

may be achieved by simultaneously permitting the expansion of effector T cells and permanently impairing regulatory T cell maintenance or function. Indeed, chronic inflammation including inflammatory cytokines such as IL-6 and TNF, have been shown to promote the instability of the Treg lineage and impair the function of regulatory cells (Barbi et al., 2014). Whether the intrinsic responsiveness of alloreactive T cells can be reversed or whether dysfunctional T cells can trigger rejection if in sufficient numbers and in the presence of poor Treg suppression remains to be determined and will be discussed in the Future Directions section, as will the participation of a low avidity TCR repertoire in rejection.

To validate the model of multiple mechanisms of tolerance simultaneously needing to be disrupted in order for rejection to occur, we examined the literature to determine whether this was also the case in other models of transplantation tolerance, when documented interventions were described to either be sufficient or insufficient to break established tolerance. A non-exhaustive summary of many experiments described in the literature can be found in Table 6.2. These data are consistent with our own results showing, in each case of breaking of transplantation tolerance, multiple significant disruptions in different tolerance pathways are necessary before an individual is able to cross the threshold of rejection and a loss of tolerance occurs.

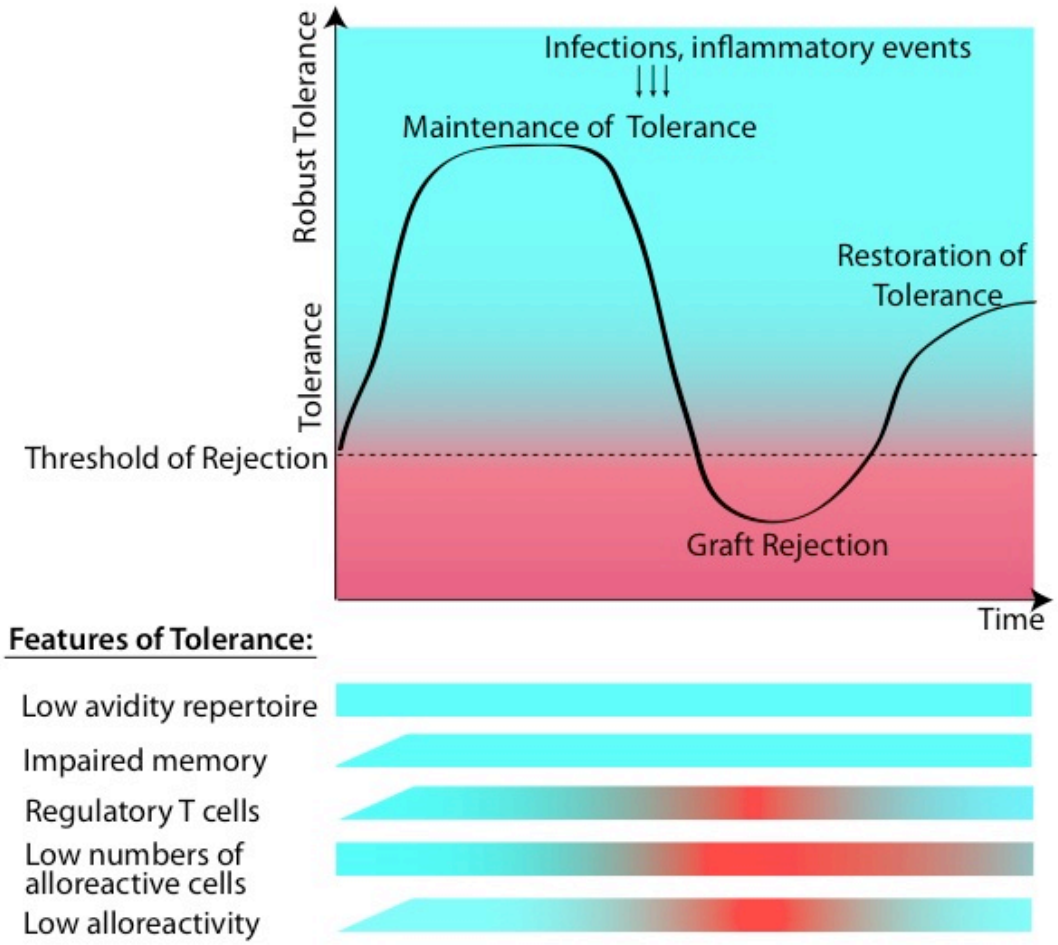


**Figure 6.1: Threshold model of tolerance and mechanisms involved**
Tolerance is not an all or none state but rather exists as a gradient of robust tolerance resulting from the number of combined T cell mechanisms intact. If multiple mechanisms are simultaneously compromised this may result in dipping below the threshold of rejection. Tolerance mechanisms may later recover resulting in a restoration of tolerance. Whether all tolerance mechanisms are fully restored or not will determine whether the restored tolerance state is as robust as the initial tolerance, or is potentially eroded. The example shown above lists different tolerance mechanisms examined in this study and how their strength changes during a Listeria-triggered loss of tolerance and return of tolerance. A low avidity T cell repertoire is present from a naïve state and persists throughout rejection crisis and recovery (shown in Figure 6.5). T cell hyporesponsiveness that most closely resembles exhaustion is programmed from a naïve state in the tolerant setting and persists despite rejection following Listeria infection and during recovery of tolerance. Graft-specific regulatory T cells are expanded or induced following tolerance induction but these cells can be overwhelmed transiently (red gradient) by alloreactive effector T cells and Listeria-specific T cells in the graft. Tolerant mice maintain low numbers of alloreactive T cells, similar to naïve mice but these cells expand and stay elevated post-Listeria infection (red-grey gradient).

**Figure 6.1, continued:** Naïve T cells have the potential for high alloreactivity without modification through tolerance induction, which can inhibit cytokine production from these cells either extrinsically or intrinsically. Alloreactivity transiently increases (red gradient) during infection-mediated rejection but returns to baseline levels during the restoration of tolerance.

| Intervention during maintenance phase of tolerance | ↑Cell # [a] | Treg brake removed[b] | PD-1 brake removed[c] | IFNγ[d] | ↑Cell # +IFNγ[e] | Rejection? [f] | Score [g] |
|---|---|---|---|---|---|---|---|
| Lm | + | + | - | + | + | ✔ | 4 |
| 100K TCR75 | + | - | - | - | - | - | 1 |
| HD TCR75 | + | - | - | + | + | ✔ | 3 |
| αCD25 | - | +/- | - | - | - | - | 0.5 |
| αPD-L1 | - | - | + | - | - | - | 1 |
| αCD25+100K | + | +/- | - | - | - | - | 1.5 |
| αPD-L1+100K | + | - | + | - | - | - | 2 |
| αCD25+αPD-L1 | - | +/- | + | - | - | - | 1.5 |
| αCD25+αPD-L1+ 100K TCR75 | + | +/- | + | +[h] | +[h] | ✔ | 4.5 |
| αCD25 post-Lm 2nd🖤 | + | + | - | + | + | ✔ | 4 |
| αCD25 no Lm 2nd🖤 | - | + | - | +/- | - | - | 1.5 |

**Table 6.1: Multiple mechanisms of tolerance need to be compromised to result in graft rejection**

[a] Increased cell number "+" given for known increase in alloreactive T cells that occurs post-Lm and for increasing the numbers of cells with adoptive transfer. "-" no known increase in cells, but not experimentally determined (grey)

[b] Treg brake removed "+" given for known reduction in Treg percentages in the graft or for αCD25 given prior to a new graft. "+/-" given for αCD25 treatment with a pre-existing graft because depletion of Tregs is less efficient in tissues "–" given for no known change in Treg percentages, but some values not experimentally determined (grey)

[c] PD-1 brake removed "+" given for αPD-L1 "–" given for no αPD-L1 given and high PD-1 expression intact, but some values not experimentally determined (grey)

[d] IFNγ production "+" given for Lm and HD TCR75 transfer based on experimental data or "+/-" given for post-Lm day 14 (having recently expressed IFNγ and perhaps being poised to do so again provided inflammation) and a new transplant as it is a source of inflammation (combined two (+/-) values (each 0.5) = 1 = +) "-" given for no known change in IFNγ; grey values not experimentally determined

**Table 6.1, continued:** [e] If a given treatment resulted in an increase in allospecific T cells and IFNγ, the same value was given in this column as what was in the IFNγ column to give additional weight to what is likely causing rejection (an increased number of IFNγ-producing T cells)

[f] "✔"=rejection in the majority of recipients, "-"= no rejection

[g] The sum of each value across a row was used to determine the score with each "+" = 1, "+/-" = 0.5 and "-" = 0. The rejection column was not used in the score.

[h] "+" values were given based on the hypothesis that naïve T cells with two brakes removed would produce IFNγ because this group did undergo rejection. It would be surprising to have rejection in the absence of any IFNγ production. This needs to be experimentally verified.

**Table 6.2: Previously published interventions resulting in loss of tolerance reveal that multiple disruptions of tolerance mechanisms are required for rejection**

| | Graft | Treatment | HP[a] | No Treg brake[b] | Inc naïve cells[c] | Iflm new graft[d] | Major vs Minor[e] | Treg Depl +Major (Incr Freq/#Fxl | Rej?[g] | Score[h] | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | ■ | ATx Tol w/non-depl αCD4 αCD8 then later αCD4 αCD8 depl | + | + | - | - | Minor - | - | - | 2 | (Qin et al,. 1993) |
| 2 | ■ | ATx Tol w/non-depl αCD4 αCD8 + later hCD2 depl | + | + | - | - | Minor - | - | - | 2 | (Qin et al,. 1993) |
| 3 | ■ | ATx Tol w/non-depl αCD4,αCD8 + 50 mil N spl + graft | - | - | + | + | Minor - | - | - | 2 | (Qin et al,. 1993) |
| 4 | ■ | ATx Tol w/non-depl αCD4 αCD8 + hCD2 depl + 50 mil N spl + graft | +/- | + | + | + | Minor - | - | ✔ | 3.5 | (Qin et al,. 1993) |
| 5 | ■ | ATx Tol w/non-depl αCD4 αCD8 + hCD2 depl + 50 mil N spl + 50 mil Tol CD4-depl cells + graft | +/- | + | + | + | Minor - | - | ✔ | 3.5 | (Qin et al,. 1993) |
| 6 | ■ | ATx Tol w/non-depl αCD4 αCD8 + hCD2 depl + 50 mil N spl + 50 mil Tol CD8-depl cells + graft | +/- | - | + | + | Minor - | - | - | 2.5 | (Qin et al,. 1993) |
| 7 | ■ | ATx Tol w/non-depl αCD4 αCD8 + 50 mil N spl + graft + 2 wks later hCD2 depl of Tol host cells only-- not transferred cells | - | - b/c new Treg | + | + | Minor - | - | - | 2 | (Qin et al,. 1993) |
| 8 | ■ | ATx Tol w/non-depl αCD4 αCD8 + hCD52 depl + 20 mil naïve T cells + graft | + | + | +/- | + | Minor - | - | ✔ | 3.5 | (Graca et al., 2002) |
| 9 | ■ | ATx Tol w/non-depl αCD4 αCD8 + hCD52 depl + 20 mil N T cells + 20 mil Tol T cells treated w/ αCTLA-4 + αCD25 + graft | - also depl activ cells that would HP | + | +/- | + | Minor - | - | - | 2.5 | (Graca et al., 2002) |
| 10 | ■ | αCD4, αCD8 αCD154 + 2nd graft | - | - | - | + | Minor - | - | - | 1 | (Kendal et al., 2012) |
| 11 | ■ | 5 mil N+20 mil Tol (depl of Foxp3 cells) into Rag-KO + graft | + | + | + | + | Minor - | - | ✔ | 4 | (Kendal et al., 2012) |

| | Graft | Treatment | HP[a] | No Treg brake[b] | Inc naïve cells[c] | Iflm new graft[d] | Major vs Minor | Treg Depl +Major (Incr Freq/#Fxl | Rej?[g] | Score[h] | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 12 | ▢ | 1 mil Prim+20 mil Tol (depl Foxp3 cells) into Rag-KO + graft | + | + | + | + | Minor - | - | ✔ | 4 | (Kendal et al., 2012) |
| 13 | ▢ | 20 mil Tol into Rag-KO + graft | + | - | - | + | Minor - | - | - | 2 | (Kendal et al., 2012) |
| 14 | ▢ | 20 mil Tol into Rag-KO +graft +Treg depl | + | + | - | + | Minor - | - | ✔ | 3 | (Kendal et al., 2012) |
| 15 | ▢ | αCD4, αCD8 αCD154 + 2nd graft | - | - | - | + | Major + | - | - | 2 | (Kendal et al., 2012) |
| 16 | ▢ | αCD4, αCD8 αCD154 + Treg depl + graft | - | + | - | + | Major + | + | ✔ | 4 | (Kendal et al., 2012) |
| 17 | ▢ | αCD4, αCD8 αCD154 + Treg depl | - | + | - | - | Major + | + | ✔ | 3 | (Kendal et al., 2012) |
| 18 | ▢ | Marilyn iTreg into Rag-KO + graft | + | - | - | + | Minor - | - | - | 2 | (Kendal et al., 2012) |
| 19 | ▢ | Marilyn iTreg into Rag-KO + 100K N Marilyn + graft | + | - | +/- (100K) | + | Minor - | - | - | 2.5 | (Kendal et al., 2012) |
| 20 | ▢ | 100K N Marilyn into Rag-KO + graft | + | + | +/- (100K) | + | Minor - | - | ✔ | 3.5 | (Kendal et al., 2012) |
| 21 | ▢ | Move Tol graft from Marilyn to Rag-KO | + | - | - | + | Minor - | - | - | 2 | (Kendal et al., 2012) |
| 22 | ▢ | Move Tol graft from Marilyn to Rag-KO + Treg depl | + | + | - | + | Minor - | - | ✔ | 3 | (Kendal et al., 2012) |
| 23 | ▢ | 500K iTreg Marilyn | + | - | - | + | Minor - | - | - | 2 | (Kendal et al., 2012) |
| 24 | ▢ | 500K iTreg+100K N Marilyn | + | - | +/- (100K) | + | Minor - | - | ✔/- | 2.5 | (Kendal et al., 2012) |
| 25 | ♥ | αCD154/CTLA-4-Ig + d60 irradiation | + | + | - | - | Major + | + | ✔ | 4 | (Iida et al., 2013) |
| 26 | ♥ | αCD154/CTLA-4-Ig + d60 αCD4 αCD8 depl | + | + | - | - | Major + | + | ✔ | 4 | (Iida et al., 2013) |
| 27 | ☽ | BMTx + late high dose IL-2 | +[i] | + | - | - | Major + | + | ✔ | 4 | Yamada et al., 2015) |
| 28 | ▢ | αCD154/DST + late agonistic αCD40 | +[i] | - | - | + infl | Major + | - | ✔ | 3 | (deVries et al., 2009) |
| 29 | ▢ | αCD154/DST + late mast cell degranulation | - | + | - | + infl | Major + | - | ✔ | 3 | (deVries et al., 2009) |

**Table 6.2, continued:** ATx=Thymectomized recipient; BMTx=bone marrow transplant; Depl=depleting/depletion; HP=homeostatic proliferation; Freq=frequency; Fxl=functional; Inc=increased; Infl=inflammation; mil=million; N=naïve; Prim=primed; Rej=rejected; spl=splenocytes; 100K=100,000; ▢ =skin, ♥ =heart, ☽=kidney

**Table 6.2, continued:** [a] If cells were allowed to strongly homeostatically proliferate a "+" was given, whereas if many cells were adoptively transferred following depletion there would be less space for the cells to homeostatically proliferate and a +/- was given. "-" no homeostatic proliferation
[b] If regulatory T cells were depleted or functionally inactivated, a "+" was given; a "–" given for no known change in Tregs
[c] Increased cell number "+" given for known increase in alloreactive T cells that occurs increasing the numbers of naïve or in one case of primed cells with adoptive transfer. "+" given for slightly increasing the numbers with only 100K cell transfer; "-" no increase in cells through adoptive transfer
[d] If a new transplant was added at the time of intervention or the intervention resulted in strong inflammation, a "+" was given, otherwise a "–" was assigned
[e] Major mismatch vs minor mismatch transplant. A "+" was given for a major mismatch as this likely reflects a higher overall precursor frequency of allospecific T cells.
[f] If a given treatment resulted in Treg depletion in the context of a major mismatch a "+" was given to give additional weight to what is likely causing rejection (an increased number of non-suppressed allospecific T cells)
[g] "✔"=rejection in the majority of recipients, "-"= no rejection "✔/-" = 50% of grafts rejected
[h] The sum of each value across a row was used to determine the score with each "+" = 1, "+/-" = 0.5 and "-" = 0. The rejection column was not used in the score.
[I] High dose IL-2 and agonist anti-CD40, while not allowing the cells to homeostatically proliferate, do allow the cells to proliferate

The concept that tolerance can be overridden transiently during inflammatory events, but then resurfaces when the inflammation resolves may have wide clinical implications. It may help explain why certain transplant patients can be successfully weaned of immunosuppression revealing a state of operational tolerance despite having experienced prior acute rejection events (Benítez et al., 2013; Brouard et al., 2012). Moreover, several autoimmune diseases are known to undergo phases of relapse and stages of remission. It is possible that disease relapse is triggered by pro-inflammatory events that overwhelm an already suboptimal self-tolerant state in individuals genetically predisposed to autoimmunity. With the quiescence of inflammation, regulation may dominate again to explain disease remission. Similarly, initial immune-dependent

regression of tumors can be followed by tumor recurrence. Tumor elimination by anti-tumor T cells may be aided by bystander inflammation, while tumor recurrence may be facilitated by activated Tregs (Goding et al., 2013). In fact, Lm is currently being used in clinical trials to improve anti-tumor immunity (Wood and Paterson, 2014) suggesting that similar mechanisms of tolerance in the context of cancer as in our transplant model may be overcome by Lm infection. Therefore, a better understanding of the loss and spontaneous restoration of antigen-specific tolerance may have wide clinical applicability for therapeutic approaches to transplantation, autoimmunity and cancer.

## C. Future Directions

### 1. The mechanisms of tolerance disrupted during rejection crises

Patients almost exclusively have rejection episodes that are crises, diagnosed by biopsy and treated, rather than the complete rejection events used in mouse models. Only a subset of mice infected with Lm undergo complete rejection, whereas another subset undergoes a rejection crisis with a slowed heartbeat and can recover. Do T cells in these mice that undergo rejection crises have the same reduction in regulatory T cells and recovery as in mice with complete rejection? Do they also have increases in allospecific T cell numbers and transient alloreactivity? What underlies the difference between a rejection crisis and a complete rejection event? We anticipate that T cells in mice with rejection crises will have similar perturbations in tolerance mechanisms following Lm but of lesser magnitude than those with complete rejection episodes.

Determining the different mechanisms or levels of their disruption affected by a rejection crisis versus a complete rejection episode may inform which mechanisms need to be bolstered to shift the balance back to tolerance to prevent graft loss.

2. Specificity of T cells mediating loss and return of tolerance

Most of the observations made with the loss of tolerance and restoration of tolerance model were made with bulk T cells. As we identified a significant proportion of graft-infiltrating T cells being Lm-specific, another important area of future research is to determine if the same increases in graft-infiltrating T cells and changes in Treg percentages in these experiments observed for bulk T cell populations occur within endogenous allospecific T cell populations. Our preliminary data with a model of rejection crisis in which mice did not reject their transplants following Lm infection do show that the alloreactive T cells themselves are expanded likely through increased proliferation, and that consequently there is a reduction in the percentage of graft-specific Tregs at the time of the rejection crisis (Figure 6.2). We still have to determine if Treg percentages increase again later on, and if endogenous allospecific T cell numbers remain persistently elevated following the rejection episode.

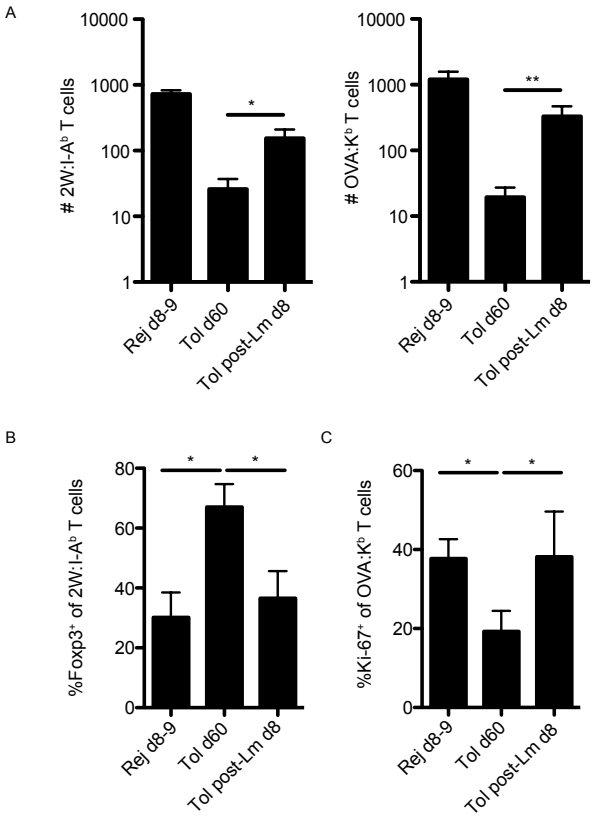


**Figure 6.2: Allospecific effector T cells expand during a rejection crisis**
**A.** The numbers of 2W:I-$A^b$-specific and OVA:$K^b$-specific T cells in the grafts of mice undergoing acute rejection (AR), tolerant mice (Tol), or tolerant mice infected with Lm 8 days prior. **B.** The percentages of $Foxp3^+$ 2W:I-$A^b$-specific T cells in the graft. **C.** The percentages of Ki-$67^+$ OVA:$K^b$-specific T cells in the graft. Data were analyzed by one-way ANOVA with Bonferroni correction, *$p<0.05$, **$p<0.01$.

Treg depletion before secondary transplantation restores alloreactive T cell infiltration into the second graft. In contrast, in the return of tolerance a high proportion of Tregs populate the second allograft. One other outstanding question is whether these Tregs originate from the first graft. TCR$\alpha$ sequencing of endogenous allospecific regulatory T cells in TCR$\beta$-Tg mice receiving a primary or secondary allograft can help address the degree of overlap in these two Treg populations. The result of this experiment would address whether preserving the initial pool of Tregs is essential to the restoration of tolerance or whether tolerance is restored simply through the reversion to

an exhausted/hyporesponsive state long enough to allow new Tregs (either from new thymic emigrants or differentiation of new iTregs) to infiltrate the second grafts. While iTreg differentiation has been shown to occur in an infectious manner to new naïve T cells, it is unknown whether these cells can also be differentiated from a tolerant effector state.

3. Duration of high dose T cell transfer-mediated loss of tolerance

High dose transfer of alloreactive $CD4^+$ T cells is sufficient to break established transplantation tolerance, resulting in increased IFN$\gamma$-production by transferred and endogenous T cells within the graft. When tolerance is broken with high dose transfer of TCR75 cells, is tolerance lost also in the endogenous T cell population? Is the loss of tolerance that follows injection of a high number of TCR75 cells permanent or only transient as that with Lm-triggered rejection model? To test this, we can transplant mice with Balb/c hearts, use high doses of TCR75 cells that express the congenic marker Thy1.1 to break tolerance and then rechallenge these mice with second heart grafts with and without Thy1.1-depletion of TCR75 cells to determine whether tolerance is also broken in the endogenous T cell population. We anticipate that this loss of tolerance may also be transient because transferred TCR75 cells, despite mediating acute rejection, still did not proliferate well upon antigen rechallenge with DST (Figure 6.3). Another future area of investigation is to determine if mice receiving high-dose allospecific $CD4^+$ T cells would generate effective T follicular helper T cells and be able to elicit an alloantibody response. In Appendix I, we show TCR75 cells are able to drive

a strong alloantibody response in a non-tolerant environment (Figure AI.4). If $CD4^+$ T cells are not able to promote alloantibodies, this may point to a lack of differentiation into Tfh cells or an inability to stimulate tolerant allospecific B cells. Allospecific B cells do persist in a tolerant environment albeit at low numbers (Chen et al., 2013), similar to tolerant T cells not undergoing complete deletion.

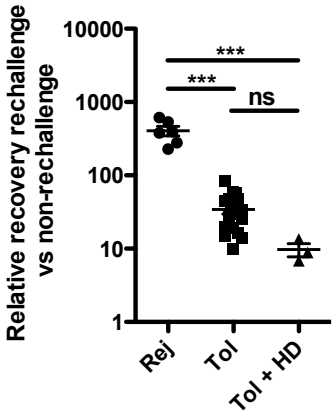


**Figure 6.3: TCR75 cells mediating rejection in tolerant mice are still impaired in their accumulation post-antigen rechallenge**
$10^5$ TCR75 cells were seeded at the time of transplantation into untreated mice (Rej) or mice transplanted and treated with anti-CD154 and DST (Tol). Other mice were transplanted and tolerant for 60 days and then were given 4 x $10^6$ TCR75 cells to break tolerance. >30 days later in all groups these cells were sorted and the same number of cells were adoptively transferred into new naïve mice and one day later half of these mice were challenged with DST. The ratio of the recovery of cells 5 days post-DST rechallenge versus non-rechallenge is plotted. Data were analyzed by one-way ANOVA with Bonferroni correction, ***$p<0.001$, ns=not significant

4. Stability and programming of low avidity repertoire in tolerant mice

Alloreactive T cells in tolerant mice have low avidity T cells. The avidity profile of T cells in tolerant mice resembles that of naïve mice, yet T cells in these 2 contexts are fundamentally different because the cells from tolerant mice are unable to undergo avidity maturation upon antigen challenge whereas the cells from naïve mice do. What

programs the avidity profile in the peripheral TCR repertoire? How does lack of costimulation lead to lack of selective expansion of high avidity T cells? Is this low avidity repertoire in tolerant mice permanently fixed? Whereas TCR75 cells removed from a host sensitized with anti-CD154+DST exhibited good recall proliferation when rechallenged with DST in a new host, we have shown that endogenous alloreactive T cells displayed a low TCR avidity profile in mice sensitized with anti-CD154+DST and that this profile was resistent, in these same primary animals, to antigen-rechallenge with DST. Moreover, our additional data show that these tolerant endogenous alloreactive T cells did also not expand upon rechallenge (Figure 6.4A).

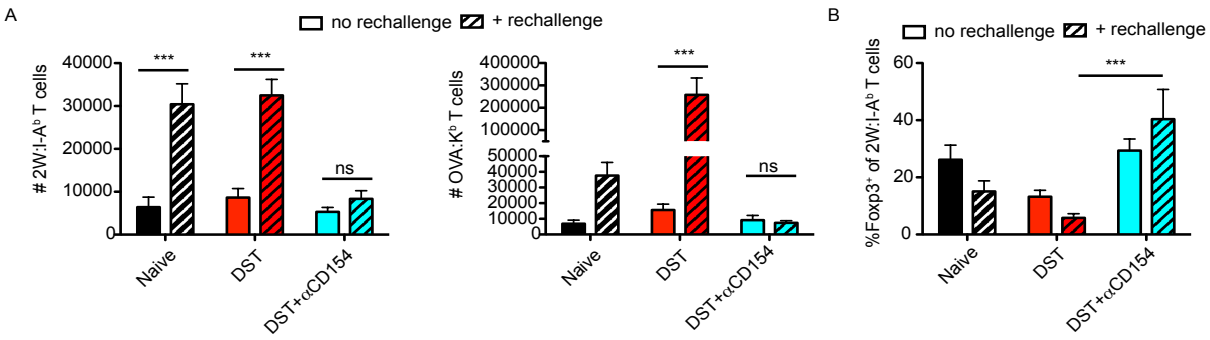


**Figure 6.4: Donor-specific T cells in mice treated with DST+anti-CD154 do not expand in the intact hosts following donor antigen rechallenge**
Naïve mice, mice sensitized with DST, and mice treated with DST+αCD154 were challenged >60 days later with DST. **A.** The numbers of donor-specific 2W:I-$A^b$-specific and OVA:$K^b$-specific T cells in the spleen 7 days post the DST rechallenge. **B.** The percentages of $Foxp3^+$ 2W:I-$A^b$-specific T cells in the spleen 7 days post DST rechallenge. Results pooled from three independent experiments, n=5-8 mice per group. Data were analyzed by two-way ANOVA (A) or one-way ANOVA (B) with Bonferroni correction, ***p<0.001, ns=not significant.

As there are increased percentages of Tregs in the intact mice treated with DST+anti-CD154 (Figure 6.4B), it is possible Tregs are constraining expansion of the endogenous alloreactive T cells. To test this hypothesis, we would deplete Tregs before rechallenging the mice with DST and then assess the avidity profile of the T cells post-rechallenge. It has been shown that Tregs can alter avidity profiles of effector cell populations. Pace et al. described that Tregs can promote avidity maturation and memory development during a productive immune response (Pace et al., 2012), perhaps by preferentially suppressing low avidity clones. In contrast, in settings of lymphopenia-induced proliferation, Tregs have been shown to help maintain diversity of the repertoire, presumably by preventing the skewing of the population to high avidity clones (Winstead et al., 2010). It is also possible that other manipulations that promote enhanced T cell proliferation, such as with the agonistic anti-CD40 (see Figure 4.10), could potentially also rescue a high avidity profile of endogenous alloreactive T cell populations. One bit of evidence to suggest Tregs and lack of T cell proliferation may not be the only factors programming T cells to be low avidity in tolerant mice: during bystander inflammation in an Lm infection that results in a rejection crisis, there is no increase in T cell avidity of graft-reactive T cells at early or late time points following the infection (Figure 6.5). As donor antigen alone and bystander inflammation alone seem to be insufficient to change the avidity profile of tolerant T cells, would rechallenge with donor antigen and inflammation (such as with Lm infection expressing 2W and OVA) to mimic an infection with cross-reactive antigens rather than bystander inflammation

provided in the Lm experiments used thus far be sufficient to rescue expansion of high avidity T cells?

Though changes to the avidity profile occur dominantly through population level changes that result from selective expansion of particular T cell clones, additional changes to a T cell's individual avidity at the clonal level also likely occur (Fahmy et al., 2001). To determine if the type of clustering of TCR and co-receptor on the cell surface contributes to the programming of low avidity populations in tolerant mice, T cells from rejecting and tolerant mice can be stained with pMHC multimers, antibodies to the TCRβ and the co-receptors CD4 and CD8, and analyzed by confocal microscopy. We hypothesize this may be another level of restriction on intrinsic T cell avidity in tolerant mice.

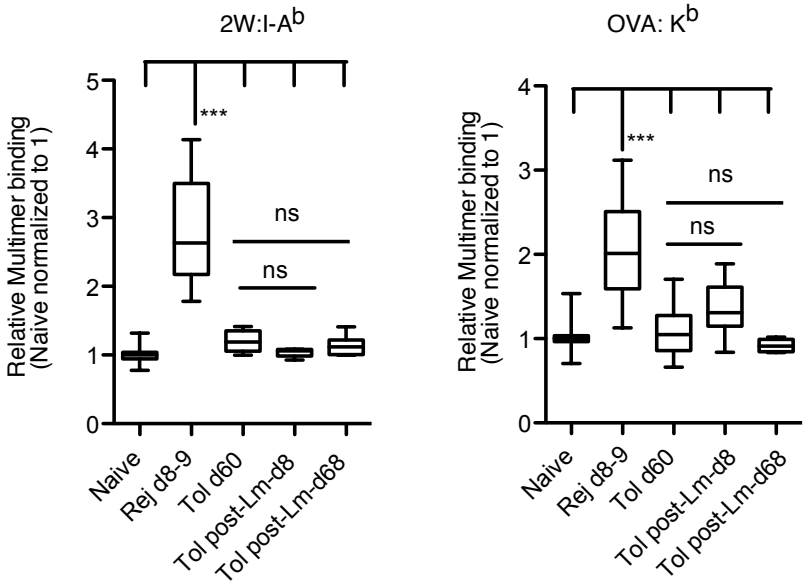


**Figure 6.5: TCR avidity does not increase following bystander activation from an Lm infection**

T cells reactive to 2W:I-Ab and OVA:Kb were stained with pMHC multimers in naïve untransplanted mice, and mice having undergone acute rejection (Rej) or tolerance induction (Tol).

**Figure 6.5, continued:** Tolerant mice were infected with Lm 60 days post-transplantation and analyzed 8 days or 68 days post-infection. **A.** Relative 2W:I-$A^b$ and OVA:$K^b$ multimer mean fluorescence intensity (MFI) of endogenous alloreactive T cells in naïve, acute rejection and tolerant recipients before and after Lm infection. Values were normalized, with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2-20 independent experiments. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *** $p<0.001$, ns=not significant.

Populations of alloreactive T cells with a low avidity profile correlated with tolerance while those with a high avidity profile correlated with rejection. However, whether a low avidity profile is required for the induction and/or maintenance of transplantation tolerance remains to be investigated. In other words, it is not clear if high avidity clones have a higher capacity to reject a transplant than lower avidity clones. To address this question, high avidity and low avidity endogenous alloreactive T cells can be sorted from rejecting mice and their TCRs sequenced. Recurrent TCR sequences in each population can be cloned and retrogenic mice can be generated (Bettini et al., 2012). The generation of these mice would allow one to obtain large enough numbers of low avidity and high avidity T cells (albeit of a monoclonal specificity) to test the rejection kinetics upon transfer of these cells into transplanted hosts. In addition, it will allow us to address whether tolerance can be induced more easily in mice transferred with low avidity than high T cells. We anticipate based on the efficacy of high avidity T cells to mediate tumor rejection (Kuball et al., 2009) that restricting T cells to being low avidity may be important for the induction and maintenance of robust tolerance.

The generation of retrogenic mice expressing T cells of high and low avidity will also facilitate the analysis of the interaction of multiple tolerance mechanisms. Are low avidity T cells more susceptible to T cell regulation, or to becoming intrinsically exhausted? Low frequency T cells were more susceptible to exhaustion than high frequency T cells in a tumor model (Malandro et al., 2016) and low affinity T cells are less able to become memory T cells as has been shown in infection/vaccination models (Baumgartner et al., 2012; Day et al., 2007). Do low avidity T cells persist in a tolerant environment at lower frequencies than high avidity T cells? It is possible that the alternative is true and that high avidity T cells are more susceptible to activation-induced cell-death, as has been shown previously for viral-specific T cells (Dalla Santa et al., 2014). Determining the ability of individual T cell mechanisms of tolerance to reinforce others will be informative for choosing which other mechanisms to bolster to increase the robustness of tolerance if a particular mechanism is compromised.

5. Programming and stability of T cell dysfunction in tolerant mice

Our studies point to a programmed cell-intrinsic exhaustion in T cells in tolerant mice that doesn't change with inflammatory events that promote rejection. This is reminiscent of the model that Schietinger and colleagues have described in which cell-intrinsic T cell hyporesponsiveness was epigenetically imprinted such that it could spontaneously return even after transient recovery of effector functions (Schietinger et al., 2012). In the Schietinger et al., study, $CD8^+$ TCR transgenic T cells tolerant to a Friend murine leukemia virus (FMuLV) GAG epitope expressed in hepatocytes as a self

antigen, transiently recovered effector function when these cells were adoptively transferred into lymphodeficient hosts lacking the GAG epitope but then returned to an unresponsive state that was found to be due to epigenetic programming in the tolerant cells (Schietinger et al., 2012). Whether similar cell-intrinsic epigenetic changes program unresponsiveness of alloantigen-specific T cells will be of interest to pursue in future studies. T cells from tolerant mice could be treated with demethylating agents to ask whether transiently changing epigenetic marks in these cells would restore T cell accumulation during antigen rechallenge. Measuring gene expression differences in T cells in tolerant versus rejecting mice in comparison with ATAC-Seq (Assay for Transposase-Accessible Chromatin with highthroughput Sequencing) data, which provides a map of chromatin accessibility and can be performed on as few as 5000 cells, can also address whether epigenetic changes are programming the tolerant T cells to be dysfunctional. This experiment could also begin to address how widespread the T cell dysfunction is and whether the defects we see in accumulation of T cells post-antigen rechallenge extend to impaired cytokine production or cytotoxicity as well. It will also help to define molecular characteristics of these cells further on the spectrum of T cell dysfunction/anergy/exhaustion/senescence. Technology affording the ability to assess gene expression in single cells is beginning to emerge. One study using this method identified an “anergic” genetic profile of antigen-specific T cells that was predictive of a successful response to therapeutic desensitization to peanut allergens (Ryan et al., 2016).

In the Schietinger et al. study, T cells were transiently rescued from their hyporesponsiveness after undergoing homeostatic proliferation in a lymphopenic environment. In fact, many manipulations that rescue effector functions of hyporesponsive T cells involve T cell proliferation with productive accumulation. To test whether homeostatic proliferation could restore T cell function of the tolerant T cells, these cells can be adoptively transferred into congenic Thy1.1-expressing recipients to permit depletion of only host T cells so that these cells can homeostatically proliferate prior to sorting these T cells and transferring them into new hosts for rechallenge with donor antigen. Other manipulations that "reinvigorate" exhausted T cells that have been tested in LCMV models, such as treating the mice with anti-PD-1/PD-L1 (Barber et al., 2006) or high doses of IL-7 (Nanjappa et al., 2011), could also be tried in our model to see if these manipulations are able to reverse the T cell hyporesponsiveness to donor antigens we see by allowing these cells to proliferate. High dose IL-7 has been shown to prevent the induction of tolerance to cardiac allografts induced with costimulation blockade (Wang et al., 2006), while blocking the receptor has been shown to promote tolerance induction in a setting of lymphopenia (Mai et al., 2014). Other treatments that promote T cell proliferation and are being explored clinically to restore dysfunctional T cells in tumor models include use of agonistic anti-41BB (Fisher et al., 2012) or the combination of blocking anti-PD-1 and anti-CTLA-4 (Spranger et al., 2014) antibodies. These could also be explored to see if they reverse the function of tolerant T cells in our model. Understanding how robust the individual mechanisms of tolerance are and how each can be impaired is important for knowing which pathways are susceptible to

disruption to contribute to a loss of tolerance and which pathways to target to reinforce robust tolerance.

Another important question to address is whether long-term exposure of antigen is necessary and sufficient for the programming or maintenance of T cell dysfunction/exhaustion in tolerant mice. Our results comparing recall proliferation of T cells from tolerant mice with a heart transplant to tolerant mice only exposed to DST+anti-CD154 suggest that persistent antigen was required for the T cell exhaustion to be programmed; however, we did not formally show that antigen was cleared in DST+anti-CD154-treated mice. This experiment does however indicate that costimulation blockade is not sufficient to program exhaustion. To address whether there is persistence of antigen in DST+anti-CD154-treated mice, we can use PCR to detect residual expression of donor antigens. This method is very sensitive and not influenced by Tregs that could suppress CFSE-dilution of new naïve T cells transferred as an alternative method to detect persistent antigen. PCR has been used to detect rare microchimeric cells in a pregnancy model (Kinder et al., 2015). We can additionally test the duration of antigen exposure that is required to program the exhaustion by removing heart transplants from tolerant mice one week or three weeks after transplantation. In LCMV infections, two weeks is a sufficient time period to program $CD8^+$ T cells to become exhausted (Angelosanto et al., 2012). If the T cells are still functional following only one week in the presence of persistent antigen from the allograft, then this also helps to categorize these T cells are exhausted because they will have then undergone a “progressive loss of function.”

A related question is to determine whether costimulation blockade is necessary to program the exhausted state in transplanted mice. If a particular duration of antigen exposure is found above, this can be used to test whether prolongation of graft survival and antigen persistence in the absence of costimulation blockade can program T cell exhaustion. As mentioned above, cyclosporine can inhibit induction of PD-1 (Oestreich et al., 2008), while mycophenolate can induce PD-1 expression (He et al., 2011). Experiments with both types of immunosuppression will be performed in case one of these directly inhibits exhaustion from being programmed. Preliminary data with untreated BALB/c hearts show that antigen can persist for >60 days in up to 20% of mice (Figure 6.6), and data with F1 hearts that cease beating but are not resorbed for >120 days have endogenous T cells that express high levels of PD-1 and low levels of CD127 relative to T cells that have responded to a Lm-model antigen infection that was acutely cleared (Figure 6.7). If T cells can be programmed to be exhausted without costimulation blockade, this may be a mechanism that also occurs in transplanted patients on conventional immunosuppression and may explain why prior acute rejection events do not preclude some of these patients from becoming spontaneously tolerant after immunosuppression withdrawal (Benítez et al., 2013; Brouard et al., 2012).

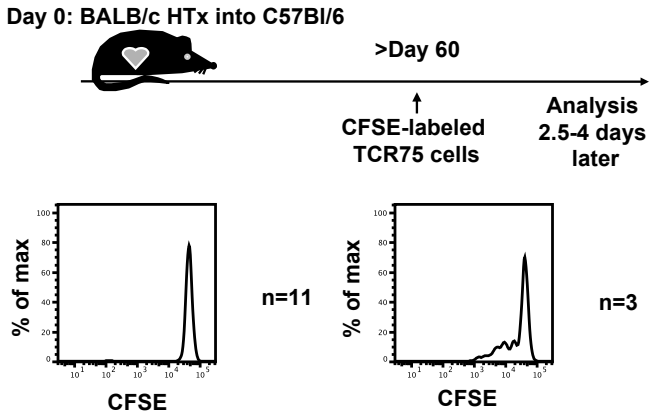


**Figure 6.6: Donor antigen can persist and is actively presented for >60 days post cessation of heartbeat**

Mice were transplanted with fully allogeneic hearts and left untreated. These grafts were rejected acutely. Greater than 60 days post-rejection 250,000 CFSE-labeled TCR75 cells were adoptively transferred and proliferation was assessed 2.5-4 days later. Data are representative from three independent experiments.

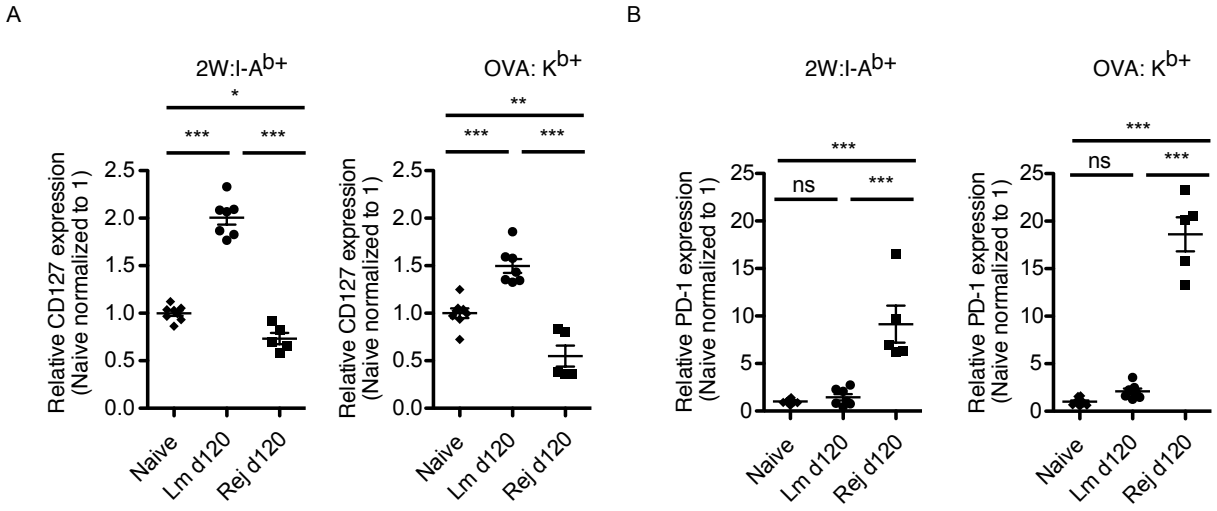


**Figure 6.7: T cells in mice with non-resorbed acutely rejected hearts can exhibit an exhausted CD127$^{lo}$ PD-1$^{hi}$ phenotype**

T cells reactive to 2W:I-A$^b$ and OVA:K$^b$ were stained with pMHC multimers in naïve untransplanted, uninfected mice, in mice having acutely cleared a Lm-2W-OVA infection and in mice having undergone acute rejection (Rej) to an F1/2W-OVA-expressing heart 120 days prior. **A.** Relative CD127 expression and (**B**) PD-1 expression. Normalized values calculated with the average of the MFI for the naïve mice in each experiment set to 1. Data are pooled from 2 independent experiments. Data were analyzed by one-way ANOVA with Bonferroni correction for multiple testing. *p<0.05, **p<0.01, *** p<0.001, ns=not significant.

## D. Conclusion

In summary, work described in this thesis has identified transplantation tolerance to be a graded state rather than an all-or-none state. Two mechanisms of T cell tolerance have been newly identified to also occur in therapeutically-induced robust transplantation tolerance. Each represents new and exciting avenues of research with the potential to be developed into novel biomarkers for transplantation tolerance in the clinic and may lead to new therapeutic targets to induce and sustain tolerance. Finally, as discussed, future investigations will be necessary for a more complete understanding of how various distinct tolerance mechanisms individually and cooperatively contribute to robust transplantation tolerance so that tolerance can be stably achieved and maintained in patients.

## APPENDIX I – ADOPTIVE TRANSFER OF TRACER ALLOREACTIVE CD4$^+$ TCR-TRANSGENIC T CELLS ALTERS THE ENDOGENOUS IMMUNE RESPONSE TO AN ALLOGRAFT[1]

### A. Abstract

T cell receptor transgenic (TCR-Tg) T cells are often used as tracer populations of antigen-specific responses, to extrapolate findings to endogenous T cells. The extent to which TCR-Tg T cells behave purely as tracer cells or modify the endogenous immune response is not clear. To test the impact of TCR-Tg T cell transfer on endogenous alloimmunity, recipient mice were seeded with CD4$^+$ or CD8$^+$ TCR-Tg or polyclonal T cells at the time of cardiac allograft transplantation. Only CD4$^+$ TCR-Tg T cells accelerated rejection, and unexpectedly led to a dose-dependent decrease in both transferred and endogenous T cells infiltrating the graft. In contrast, recipients of CD4$^+$ TCR-Tg cell exhibited enhanced endogenous donor-specific CD8$^+$ T-cell activation in the spleen and accelerated alloantibody production. Introduction of CD4$^+$ TCR-Tg T cells also perturbed the intra-graft accumulation of innate cell populations. In sum, CD4$^+$ TCR-Tg T cells alter many aspects of endogenous alloimmunity, suggesting that caution should be used when interpreting experiments utilizing these adoptively-transferred cells, as the overall nature of allograft rejection may be altered. These

[1] The following section titled "Adoptive transfer of tracer alloreactive CD4$^+$ TCR-transgenic T cells alters the endogenous immune response to an allograft" is reproduced with minor editing, and with figure renumbering, from reference (Miller et al., 2016b) as allowed by copyright by John Wiley & Sons. The article was accepted by the American Journal of Transplantation. Authors: Michelle L. Miller, Jianjun Chen, Melvin D. Daniels, Matthew G. McKeague, Ying Wang, Dengping Yin, Vinh Vu, Anita S. Chong and Maria-Luisa Alegre

Contributions:

- Performed the experiments and analysis of data in all figures including assisting Jianjun Chen with experiments in Figures AI.8B-D
- Co-wrote manuscript with Anita Chong and Maria-Luisa Alegre

results may also have implications for CD4 adoptive T cell immunotherapy in tumor and infectious clinical settings as cell infusion may have additional effects on natural immune responses.

B. Introduction

In animal studies, T cell receptor transgenic (TCR-Tg) T cells, which are monoclonal T cells specific for a known antigen, are often used as a convenient tool for the study of antigen-specific T cell responses. It is easy to obtain sufficient numbers of antigen-specific T cells from TCR-Tg mice and these cells, once transferred, can be identified by their particular TCR V$\alpha$ or V$\beta$ subunit, a clonotypic antibody, or a congenic marker. This approach has been used to track the fate and function of antigen-specific T cells including their deletion, proliferation, differentiation, or cytokine production. Furthermore, this approach with its ability to transfer a set number of cells has allowed estimation of the number of antigen-specific cells required to clear an infection, reject an organ or tumor, or to overcome a therapeutic intervention (Ford et al., 2007; Honjo et al., 2004; Jensen et al., 2012; Wirth et al., 2009). The fate and numbers of the TCR-Tg cells are then often extrapolated to endogenous T cell responses. Importantly, it has been demonstrated in models of infection that TCR-Tg T cells can compete with endogenous T cells specific for the same antigen (Badovinac et al., 2007). These observations suggest that the introduced TCR-Tg T cells are not simply recruited into the immune response but they may also in fact alter the immune responses on which they are supposed to report.

In this study, we transferred TCR-Tg and polyclonal T cells into mice receiving fully allogeneic cardiac allografts to investigate their fate and effects on the endogenous alloimmune response. The transfer of $10^4$-$10^5$ $CD4^+$ TCR-Tg T cells/mouse, a similar dose to what has been frequently used for mechanistic studies of allograft rejection and tolerance, significantly modified the endogenous immune response to the allograft, increasing both CD8 and alloantibody responses, while paradoxically limiting the accumulation of intra-graft T cells and altering the profile of graft-infiltrating innate immune cells. These pleiotropic alterations in the endogenous immune response as a result of $CD4^+$ TCR-Tg T cell transfer urge caution for the use of these cells in animal models and patient therapies.

## C. Materials and Methods

### Mice

C57BL/6 and BALB/c mice were purchased from Envigo RMS, Inc. (Indianapolis, IN). OT-I Rag-KO, MD4, $\mu MT^{-/-}$ and CD45.1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Ovalbumin-transgenic (OVA-Tg) mice on a BALB/c background, that express membrane OVA under the control of the β-actin promoter, were a generous gift by Elizabeth Ingulli (when at the University of Minnesota). 2W1S-OVA mice were provided by James Moon (Harvard) and crossed once to BALB/c mice to generate F1 mice. TCR75 TCR-Tg mice were obtained from R. Pat Bucy (University of Alabama-Birmingham) and crossed to CD45.1 mice. TEa TCR-Tg mice were obtained from Alexander Rudensky (when at the University of Washington) and crossed

to CD45.1 mice. Mice were housed under specific pathogen-free conditions and used in agreement with the University of Chicago's Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Heart transplantation

Transplantation of cardiac allografts was performed using a technique adapted from that originally described by Corry et al. (Corry et al., 1973). Cardiac allografts were transplanted in the abdominal cavity by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava, respectively. The day of rejection was defined as the first day of cessation of heartbeat in the graft. Graft rejection was verified in selected cases by necropsy.

Adoptive transfer of cells

Spleen and lymph node cells were isolated from donor mice. Cells were enumerated with an Accuri C6 flow cytometer (BD Biosciences) and a subset of cells were stained for CD4 or CD8, V$\beta$ or V$\alpha$, the congenic marker CD45.1 or CD45.2 and CD44. The percentage of $CD44^{lo}$, congenically marked TCR-Tg T cells was used to calculate the total number of cells for the adoptive transfer. Cells were injected retro-orbitally in 200 $\mu$l of phosphate-buffered saline (PBS) on the day of transplantation unless otherwise indicated.

Isolation of graft-infiltrating cells

Cardiac grafts were rinsed in situ with Hanks balanced salt solution (HBSS) containing 1% heparin. Explanted hearts were cut into small pieces and digested for 40 min at 37°C with 400 U/ml collagenase IV (Sigma), 10 mM N-2-hydroxyethylpiperazine-N′-2- ethanesulfonic acid (HEPES, Cellgro) and 0.01% DNase I (MP Biomedicals) in HBSS (Cellgro). Digested suspensions were washed with an equal portion of complete Dulbecco's Modified Eagle medium (DMEM) containing 5% fetal bovine serum, passed through a nylon mesh and centrifuged. Cells were either used for flow cytometry or incubated for 4 h with phorbol myristate acetate (PMA) and ionomycin in the presence of brefeldin A.

Intracellular staining and flow cytometry

Lymphocytes were isolated from spleens and heart grafts and processed into single cell suspensions. Cells were stained first with a fixable live/dead stain (Aqua, Invitrogen) and then with anti-CD4 (L3T4), anti-CD8 (Ly2), anti-CD45.1 (A20), anti-Vβ8.3 (1B3.3) or anti-Vα2 (B20.1) and anti-CD45.2 (104), anti-CD11b (M1/70), anti-Gr-1 (RB6-8C5), anti-TCRβ (H57-597), anti-CD19 (eBio1D3), anti-CD45 (30-F11) mAbs. For interferon gamma (IFNγ) intracellular staining, cells were stimulated with PMA (Sigma), ionomycin (Sigma) and brefeldin A (BD Pharmingen) for 4 h, prior to staining for viability and surface markers. Surface-stained cells were then fixed with the Foxp3 fixation permeablization buffer kit (eBioscience) for 15 min at room temperature, washed with 1x permeabilization buffer, stained using anti-IFNγ (XMG1.2) for 30 min at room

temperature, washed again, and analyzed by flow cytometry. For T follicular helper (Tfh) analysis, cells were stained with surface antibodies and CXCR5-bio for 2 hours at 4ºC, followed by two washes with FACS buffer and 30 minute staining with streptavidin-Percp-e710 at 4ºC. For staining B cells, anti-B220 (RA3-6B2), anti-Fas (15A7) and anti-GL-7 (GL7) were used as well as a dump gate including anti-CD3 (145-2C11), anti-Ter119 (TER119), anti-F4/80 (BM8), anti-CD49b (DX5), and anti-Gr-1 (RB6-8C5). All mAbs were from BD Biosciences or eBioscience.

Multimers and monomers

OVA:$K^b$ pentamers were used to stain OVA-specific $CD8^+$ T cells (ProImmune) following T-cell magnetic enrichment by negative selection to remove $CD19^+$ and $CD11b^+$ cells. One test was used to stain 5 x $10^6$ cells resuspended at 50 x $10^6$ cells/ml.

H2-$K^d$- biotin monomers, H2-I-$E^d$ -biotin monomers, H2-$K^d$ or H2-I-$E^d$ tetramers were obtained from the NIH Tetramer Core Facility (Atlanta, GA). The peptides bound to H2-$K^d$ and H2-I-$E^d$ were SYIPSAEKI (Plasmodium berghei circumsporozoite peptide 252-260) (Kessler et al., 1999) and SSIEFARL (herpes simplex virus glycoprotein B peptide 498 to 505) (Blaney et al., 1998), respectively. In some experiments, magnetic enrichment of tetramer positive B cells was performed before cell surface staining as previously described (Chen et al., 2015). For staining 5 × $10^6$ cells, saturating (0.1 µg) concentrations of each tetramer were used.

ELISpot

For detection of total IgG secreting B cells, plates (Millipore, Cat: MAIPSWU10) were coated with anti-mouse IgG F(ab)' specific antibody (Jackson Immunoresearch, Cat: 115-005-072) overnight at 4 °C , then washed with PBS, and blocked with DMEM (10% FBS) 2 hours at 37°C. Cells were plated and cultured overnight, then washed and alkaline phosphatase conjugated anti-mouse IgG Fc specific antibody was added (Jackson Immunoresearch, Cat: 115-055-071). After 1 hour at room temperature, plates were washed and developed with substrate BCIP/NPT (Sigma, Cat: B5655-25tab). For detecting H2-$K^d$ specific IgG secreting plasma cells, plates were coated with anti-mouse IgG Fc-gamma specific antibody overnight at 4°C, washed and blocked. Cells ($10^3$, $10^4$, and $10^5$) were then plated and cultured overnight. Plates were washed and H2-$K^d$-biotin monomers were added. After one hour at room temperature, plates were washed and streptavidin-ALP (Mabtech, Cat:3310-10) was added. After one additional hour incubation at room temperature, plates were then washed and developed. The numbers of spots per well were enumerated using the ImmunoSpot Analyzer (CTL Analyzers LLC).

Donor-specific antibody (DSA) assay

For donor-specific antibody detection, serum was collected before and after transplantation and was diluted (1:50) and incubated with 1 x $10^6$ BALB/c splenocytes resuspended at 20 x $10^6$ cells/ml for 1 hour at 4°C. After washing twice, the cells were incubated with anti-IgG (Southern Biotech, Cat. # 1030-02) or anti-IgM (eBioscience,

Cat. # 125790-82) and anti-B220 (BD Biosciences, Cat. # 561226) for 30 minutes. Mean fluorescence intensity (MFI) was measured by flow cytometry gating on $B220^{lo}$ cells.

D. Results

1. $CD4^+$ TCR-Tg T cell transfer leads to reduced intra-graft T cells

Adoptive transfer of antigen-specific TCR-Tg T cells is a tool most often used to follow monoclonal T cell responses and extrapolate conclusions to endogenous responses. In order to determine whether the adoptive transfer of TCR-Tg cells alters endogenous immune responses, a model of fully MHC-mismatched cardiac allograft transplantation with and without TCR-Tg cell transfer was examined. 1,000-250,000 (1K-250K) naïve $CD4^+$ TCR75 T cells that recognize a BALB/c $K^d$ peptide presented on $I\text{-}A^b$ were transferred into C57BL/6 mice on the day of transplantation with a BALB/c heart (Figure AI.1A). The time of rejection was slightly accelerated in mice receiving 100K TCR75 cells (MST Day 6 vs Day 8 with no transfer, Figure AI.1B) and the majority of $CD4^+$ cells present in the graft were TCR75 (79.4%±13.4%, Figure AI.1C). Unexpectedly, the TCR75 cell transfer led to a dose-dependent decrease in the total number of T cells within the rejected allografts, with the most striking reduction being in the number of endogenous $CD8^+$ T cells (Figure AI.1D). Whereas graft-infiltrating $CD8^+$ T cells vastly outnumbered $CD4^+$ T cells in unmodified acute rejection (AR), the addition of 100K or more TCR75 cells not only reduced the numbers of T cells in the graft but also shifted the ratio of CD8:CD4 T cells closer to 1:1 (Figure AI.1D).

We also examined the effects of an adoptive transfer of a second $CD4^+$ TCR-Tg T cell, TEa, which recognizes an I-$E^d$ peptide presented on I-$A^b$ (Figure AI.1D). In contrast to the TCR75 cells, TEa cells comprised only a small proportion of the total $CD4^+$ T cells within the allografts (4.4% ± 5.1%, Figure AI.1C). Despite this difference, TEa cells also mediated accelerated graft rejection that was characterized by reduced total numbers of graft-infiltrating T cells compared to rejection in the absence of TCR-Tg $CD4^+$ T cells.

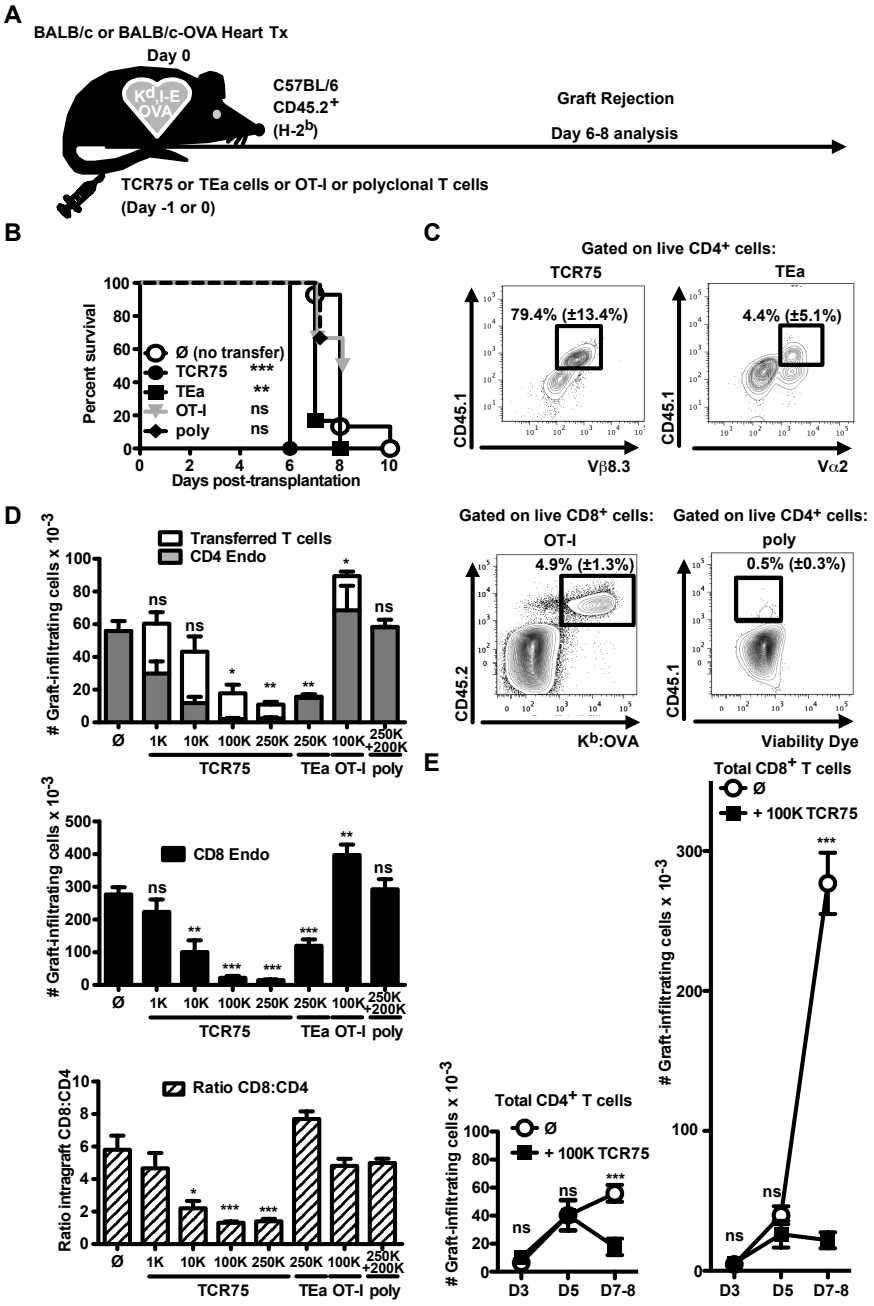


**Figure AI.1: Adoptive transfer of CD4$^+$ TCR-Tg T cells prevents accumulation of transferred and endogenous CD4$^+$ and CD8$^+$ T cells**

**A.** Experiment design. **B.** Graft survival following transplantation without T cell transfer (⦸, n=14) or with $10^5$ TCR75 (100K, n=7), 2.5 x $10^5$ TEa (250K, n=6), $10^5$ OT-I (100K, n=6), or 2.5 x $10^5$ CD4$^+$ plus 2 x $10^5$ CD8$^+$ polyclonal T cells (poly, n=6). **C.** Representative flow cytometry plots of intra-graft T cells of mice receiving TCR75 (10K-250K cells transferred, n=16), TEa (250K cells transferred, n=6), OT-I (100K cells transferred, n=6) and polyclonal T cells ((250K CD4$^+$ + 200K CD8$^+$ T cells transferred, n=6).

**Figure AI.1, continued:** Numbers are the mean percentage of transferred cells out of $CD4^+$ or $CD8^+$ graft-infiltrating T cells ± SD. **D.** Numbers of endogenous $CD4^+$, TCR-Tg, and $CD8^+$ cells infiltrating the grafts 6-8 days post-transplantation, and ratio of CD8:CD4 T cells; ⦸ (no transfer, n=14), TCR75: 1K (n=6) 10K (n=3) 100K (n=6) 250K (n=7), TEa: 250K (n=6), OT-I: 100K (n=6), polyclonal (250K $CD4^+$ + 200K $CD8^+$ cells transferred, n=6). **E.** Numbers of total $CD4^+$ and $CD8^+$ T cells infiltrating the grafts on days 3, 5, or 7-8 post-transplantation; ⦸ (no transfer, n=3,3,14), +100K TCR75: (n=3,3,6). Data from Day 7-8 is the same as in AI.1D. Results are pooled from 2-10 independent experiments. Survival data was analyzed by log-rank test, and mean values were compared using one-way analysis of variance (ANOVA) (D) or two-way ANOVA (E) with Bonferroni correction for pairwise comparisons, ns=not significant, *p<0.05, **<p<0.01, ***p<0.001

Accelerated graft rejection and reduction in total intra-graft T cells was specific to adoptive transfer of antigen-specific $CD4^+$ TCR-Tg T cells, as transfer of similar numbers of naïve $CD8^+$ TCR-Tg OT-I T cells into recipients of OVA-expressing BALB/c allografts or transfer of wild-type polyclonal $CD4^+$ $CD45.1^+$ T cells, which contain lower percentages of alloreactive T cells and a majority of T cells of irrelevant specificities, did not have these effects (Figure AI.1B,D). In fact, adoptive transfer of OT-I cells resulted in an increase in overall numbers of intra-graft T cells (Figure AI.1D).

One possibility for observing reduced numbers of T cells in Day 7-grafts from mice with $CD4^+$ TCR-Tg T cell transfer was that we may have missed an earlier peak of T cell infiltration and T cells may have died or exited the graft by the time the mice were analyzed on Day 7. However, a time course analysis revealed that mice transferred with 100K TCR75 cells had few intra-graft T cells on days 3 and 5 post-transplantation, similarly to non-transferred animals (Figure AI.1E). Thus the addition of modest numbers of graft-specific $CD4^+$ but not $CD8^+$ TCR-Tg T cells profoundly limited the total accumulation of intra-graft $CD4^+$ and $CD8^+$ T cells.

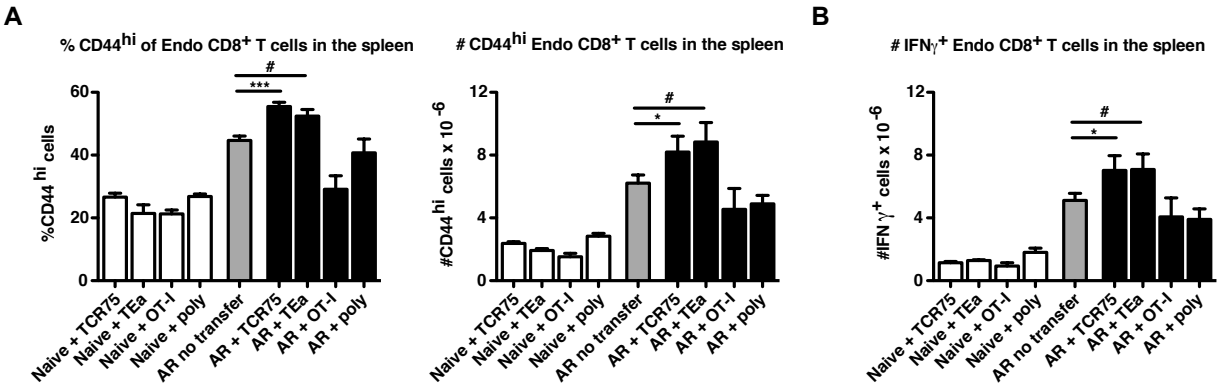


**Figure AI.2: Adoptive transfer of CD4$^+$ TCR-Tg T cells augments endogenous CD8$^+$ T cell responses in the spleen**

Percentages and numbers of CD44$^{hi}$ (**A**) or IFN$\gamma^+$ (**B**) transferred and endogenous CD4$^+$ or CD8$^+$ T cells in the spleens of mice receiving no transfer of cells (n=22), adoptive transfer of TCR75 (10K-250K cells, n=12-13), TEa (250K cells, n=5-6), OT-I (100K cells, n=6) or polyclonal (poly, 250K CD4$^+$ + 200K CD8$^+$ cells, n=6), and did (acute rejection, AR, with or without transfer) or did not (naïve) receive a heart transplant analyzed on day 7-8 post-transfer or post-transplantation. Mean values were compared using one-way analysis of variance (ANOVA) with Bonferroni correction for pairwise comparisons, ns=not significant, *p<0.05, ***p<0.001 for TCR75 groups $^{\#}$p<0.05 for TEa groups.

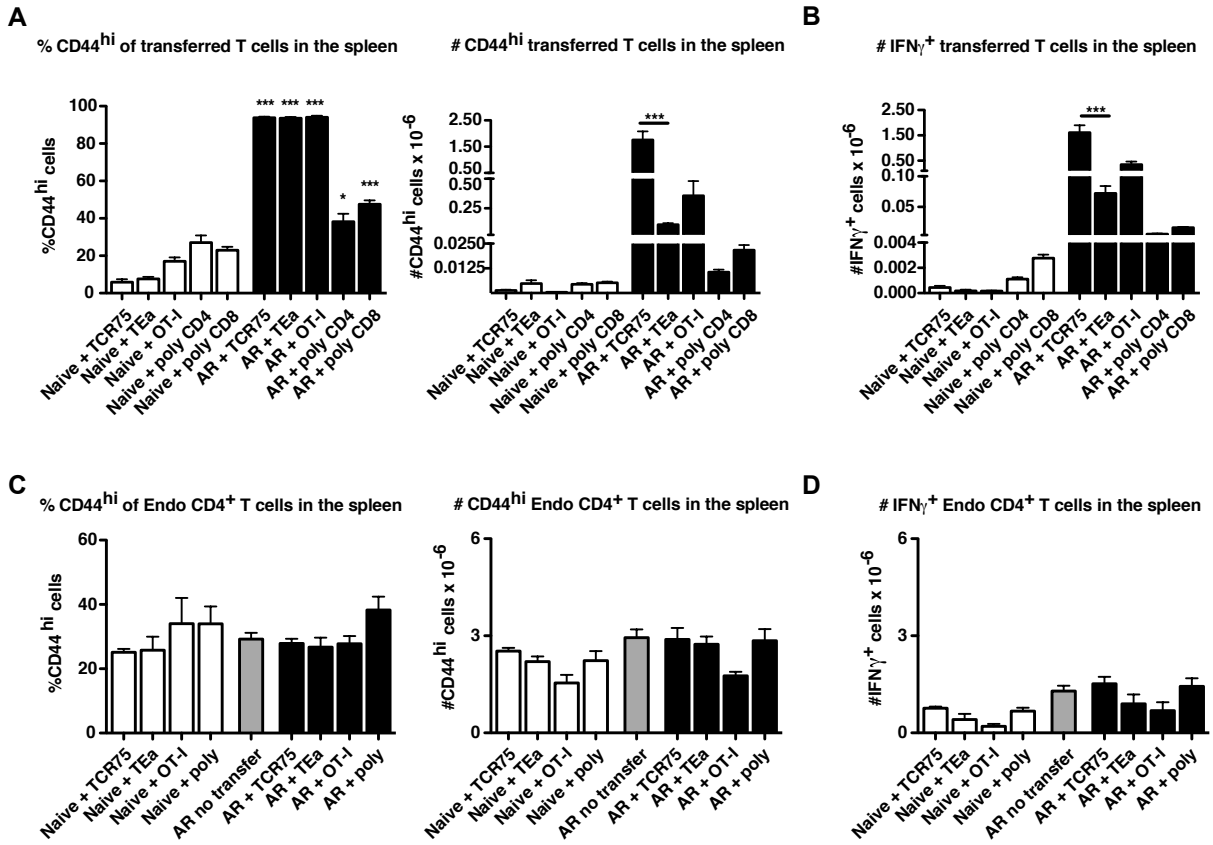


**Figure AI.3: Transferred T cells are activated during acute rejection but do not perturb endogenous CD4$^+$ T cells**

Percentages and numbers of CD44$^{hi}$ (**A**) or IFNg$^+$ (**B**) transferred and endogenous CD4$^+$ T cells in the spleens of mice receiving no transfer of cells (n=22), adoptive transfer of TCR75 (10K-250K cells, n=12-13), TEa (250K cells, n=5-6), OT-I (100K cells, n=6) or polyclonal (250K CD4$^+$ + 200K CD8$^+$, "poly CD4" and "poly CD8", n=6) T cells, and did (acute rejection, AR, with or without transfer) or did not (naïve) receive a heart transplant analyzed on Day 7-8 post-transfer. Mean values were compared using one-way and two-way ANOVA with Bonferroni correction for pairwise comparisons, ns=not significant, *p<0.05, ***p<0.001.

2. CD4$^+$ TCR-Tg T cells enhance priming of graft-specific CD8$^+$ T cells in the spleen

One possibility for the observed reduction in intra-graft T cell numbers following CD4$^+$ TCR-Tg T cell transfer was that the TCR-Tg cells were interfering with the priming of endogenous T cells in the spleen. To investigate this possibility, the numbers of

activated CD44$^{hi}$ TCR-Tg and endogenous T cells were enumerated in the spleen 7-8 days following adoptive transfer of TCR75, TEa, OT-I or polyclonal T cells (Figure AI.2 and AI.3). Rather than inhibiting T cell priming, transfer of CD4$^{+}$ TCR-Tg T cells, but not OT-I or polyclonal cells, provided additional help to endogenous CD8$^{+}$ T cells, with overall increased percentages and numbers of CD44$^{hi}$ and interferon gamma (IFN$\gamma$)-producing CD8$^{+}$ T cells in the spleens of those mice receiving TCR75 or TEa cells (Figure AI.2). There was no significant change in the percentages or numbers of regulatory T cells in the spleens of mice receiving CD4$^{+}$ TCR-Tg T cells (Figure AI.4), suggesting that the CD4$^{+}$ TCR-Tg T cells are not promoting stronger CD8$^{+}$ T cell responses by affecting total numbers of Tregs or ratios of Tregs to T effector cells.

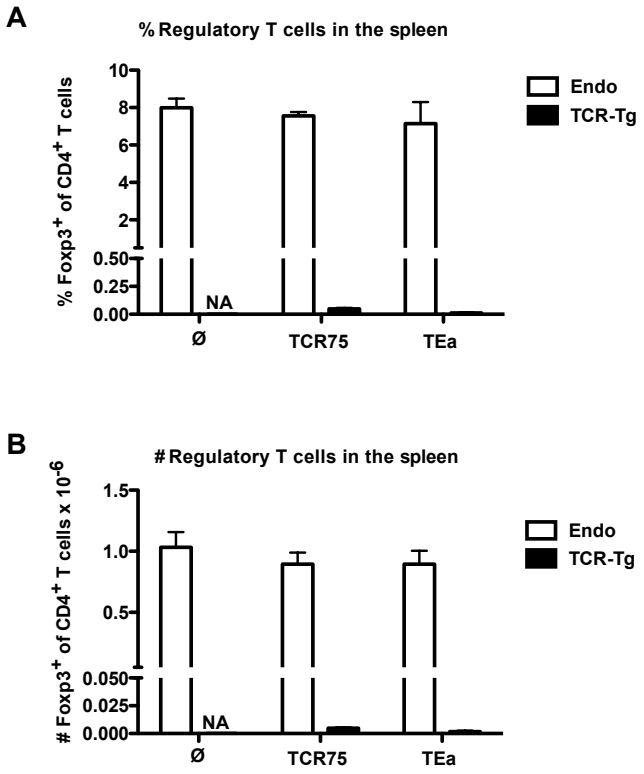


**Figure AI.4: Regulatory T cell percentages and numbers in the spleen are not changed by adoptive transfer of CD4$^{+}$ TCR-Tg T cells**

**Figure AI.4, continued:** The percentages (**A**) and total numbers (**B**) of endogenous and TCR-Tg Foxp3$^+$ Tregs amongst CD4$^+$ T cells in the spleen 7-8 days post-transplantation in mice receiving no transfer (⃠, n=14), 10K-250K TCR75 cells (n=13) or 250K TEa cells (n=6).

CD4$^+$ helper T cells are so named for their ability to help other immune cells, including CD8 responses. To determine if TCR75 cell transfer could impact antigen-specific endogenous CD8$^+$ T cells that recognize a different alloantigen than the K$^d$ antigen recognized by the TCR75 cells, B6 mice were transplanted with BALB/c hearts expressing OVA so that endogenous CD8$^+$ T cell responses could be tracked using OVA:K$^b$ multimers (Figure AI.5, AI.6). Adoptive transfer of 100K TCR75 cells resulted in a 14-fold increase in the number of OVA-specific CD8$^+$ T cells within the spleen (Figure AI.6B and AI.6C). These results indicate that TCR75 transfer can alter endogenous CD8$^+$ T cell responses that do not require the recognition of the same alloantigen. However, despite the increased accumulation of splenic OVA:K$^b$-reactive cells in the spleen, the TCR75 transfer resulted in a log-decrease in the numbers of intra-graft OVA:K$^b$-reactive CD8$^+$ T cells (Figure AI.6C). Thus TCR75 transfer enhanced non-cognate endogenous allospecific CD8$^+$ T cell accumulation in the spleen but inhibited their accumulation in the graft in a similar manner to what was observed for polyclonal CD8$^+$ T cells.

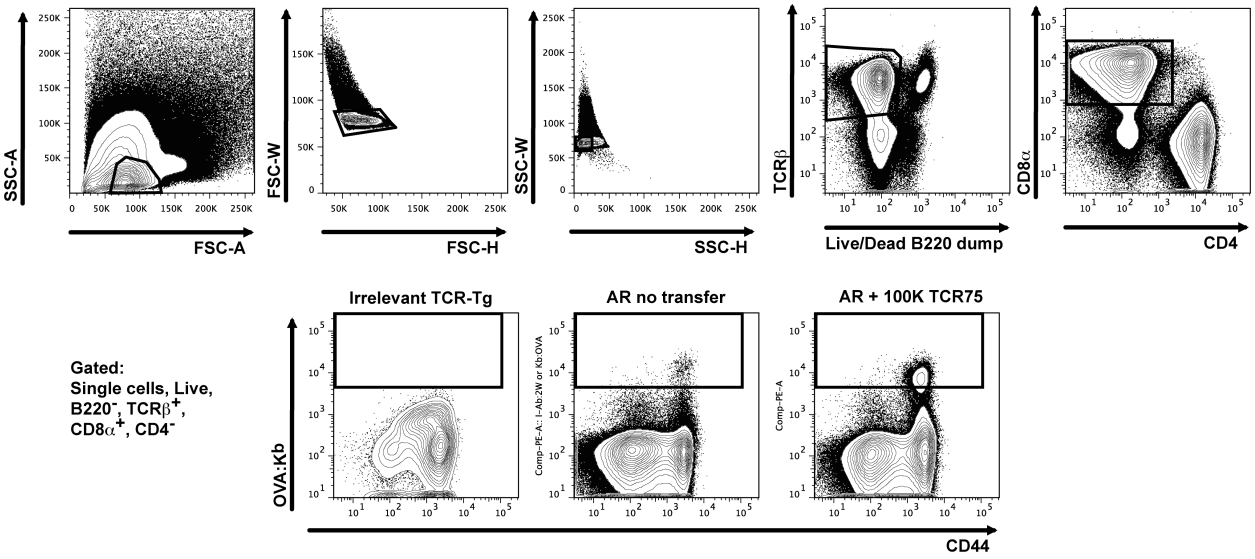


**Figure AI.5: Gating strategy of OVA:$K^{b+}$ cells**
Spleen cells from mice undergoing acute rejection of OVA-expressing BALB/c x B6 F1 hearts without transfer or with 100K TCR75 cells were magnetically enriched for T cells and stained with OVA:$K^b$ multimers. As a negative control, $CD8^+$ T cells from an irrelevant TCR-Tg mouse were used (TEa on a RAG-sufficient background). Representative of n=5-6 per group from two independent experiments.

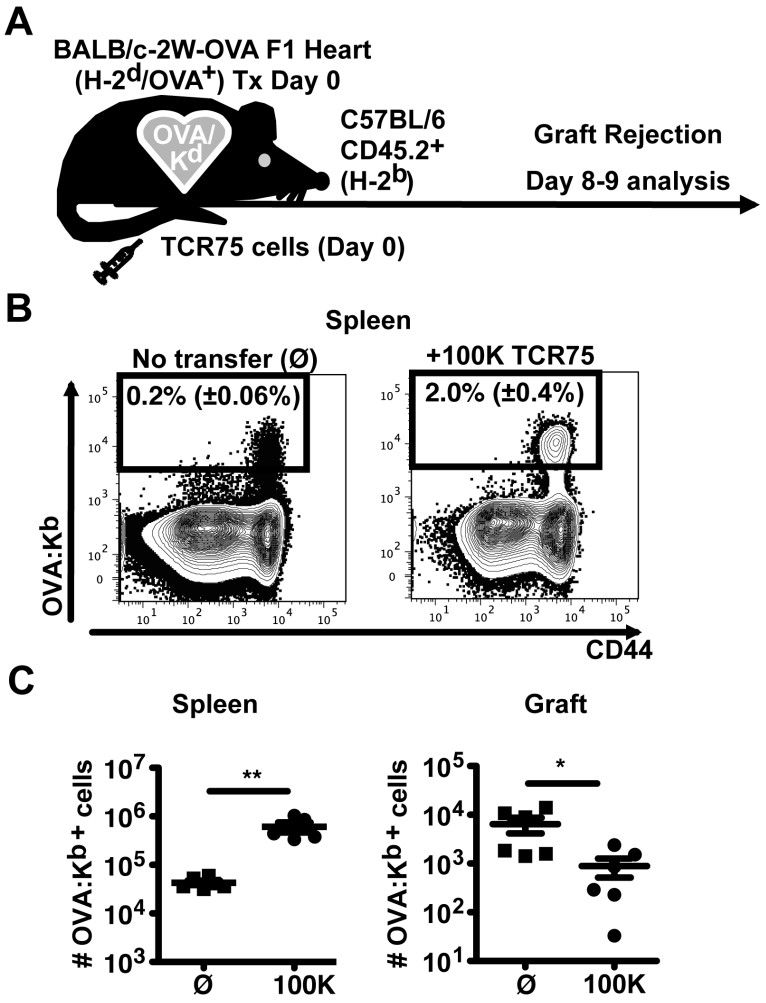


**Figure AI.6: TCR75 cell transfer affects endogenous CD8$^+$ T cell responses even when they recognize distinct antigens**

**A.** C57BL/6 mice were transplanted with OVA-expressing BALB/c x B6 F1 hearts and half of the mice were adoptively transferred with 100K TCR75 cells. **B.** Percentages of OVA:K$^{b+}$ cells in the spleen ± SD (n=5-6 per group). **C.** Numbers of endogenous OVA:K$^{b+}$ cells identified in the spleen (n=5-6 mice per group) and graft (n=6 per group) 8-9 days post transplantation. Mean values were compared using Student's t test, *p<0.05, **p<0.01.

3. CD4$^+$ TCR-Tg T cells enhance allograft-specific B cell responses

In addition to helping CD8$^+$ T cell responses, CD4$^+$ T cells are also known to provide B cell help. To determine whether the transferred CD4$^+$ TCR75 cells were similarly providing help to alloreactive B cells, splenocytes were first stained with

CXCR5 and PD-1, which are markers of T follicular helper cells (Tfh cells). A high proportion of transferred TCR75 cells differentiated into Tfh cells while the number of endogenous Tfh cells remained similar in transferred versus non-transferred mice (Figure AI.7, AI.8A); thus, in mice receiving TCR75 cells, the total numbers of Tfh cells were effectively doubled (Figure AI.8A). This finding correlated with an expansion of allospecific B cells that recognized $K^d$, which reflects their ability to engage in cognate interaction with the TCR75 cells (Figure AI.8B). The $K^d$–specific B cells were not enriched in cells expressing germinal center markers ($GL\text{-}7^+$, $Fas^+$). Instead, they had already begun to differentiate into plasmablasts that downregulate B220 (Figure AI.8C) and CD19 (Figure AI.9) by the time of acute rejection. Indeed, as measured by ELISpot, TCR75 transfer increased antibody-producing splenocytes at day 7 post-transplantation (Figure AI.8D) and serum IgM and IgG alloantibodies as early as 6 days post-transplantation (Figure AI.8E).

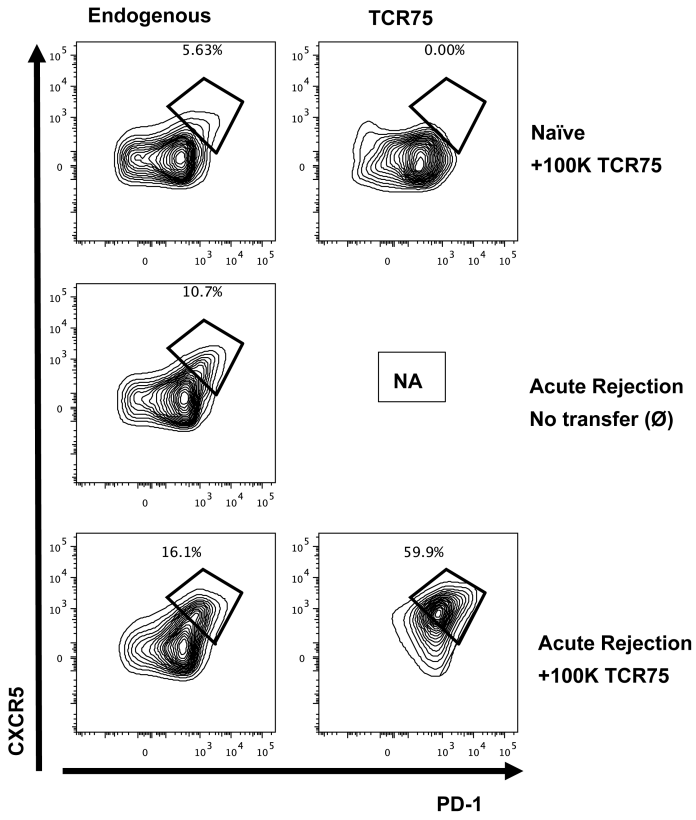


**Figure AI.7: Gating of T follicular helper cells**
Representative flow cytometry plots of T follicular helper cells (PD-1$^{hi}$, CXCR5$^{hi}$) from endogenous CD4$^{+}$ T cells and TCR75 cells in naïve untransplanted mice, or in mice undergoing acute rejection without or with 100K TCR75 cells. Representative of n=4-6 per group from two independent experiments.

We also quantified the numbers of Class II (I-E$^{d}$)-specific B cells, which are not able to engage in cognate interactions with TCR75 cells. While the total numbers of I-E$^{d}$-specific B cells were not significantly increased, the proportion of these B cells that downregulated B220 was significantly augmented (Figure AI.4C). In addition, the ELISpot revealed many more total antibodies produced than those specific for K$^{d}$ (Figure AI.4D), illustrating that TCR75 transfer enhances the differentiation of alloreactive B cells that can engage in both cognate and non-cognate interactions with TCR75 cells.

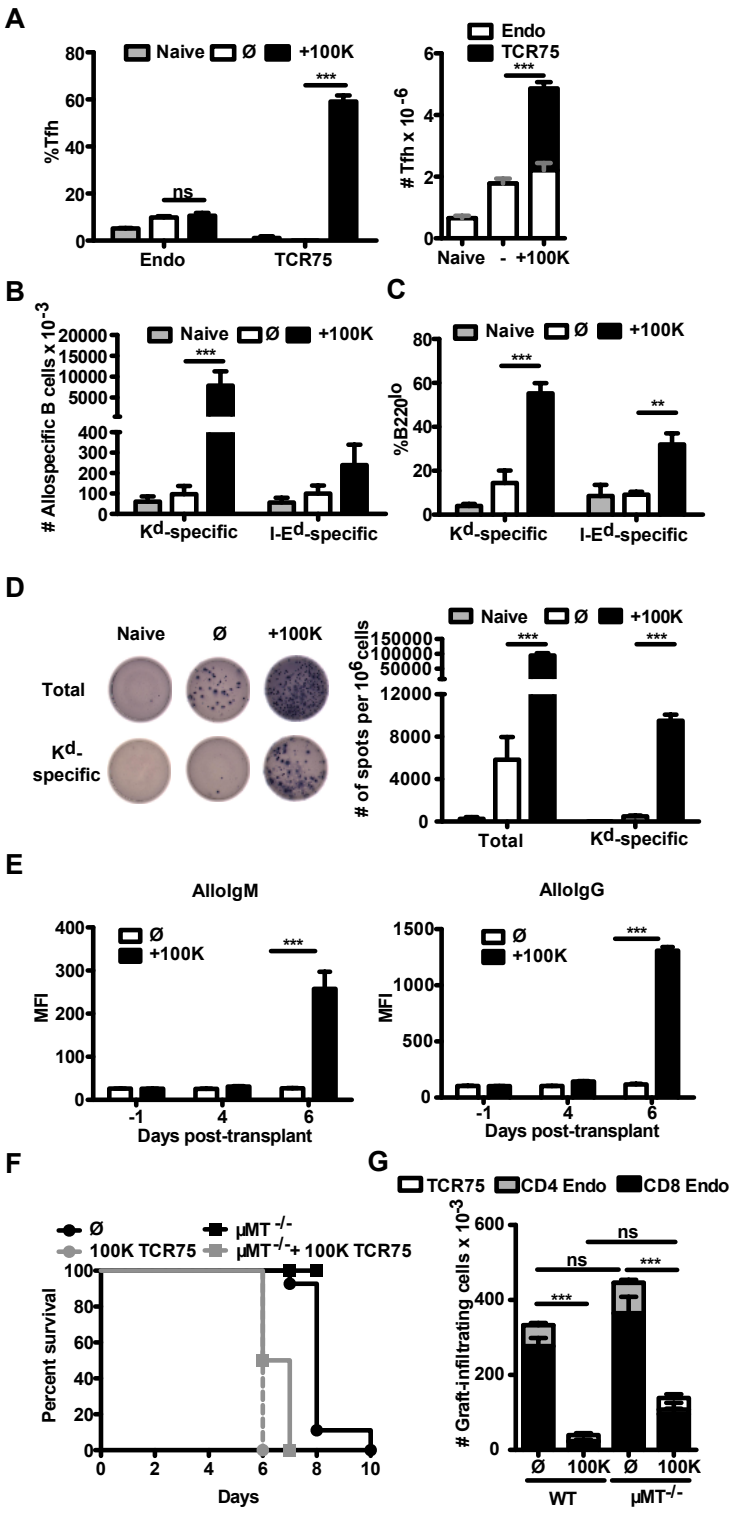


**Figure AI.8: CD4$^{+}$ TCR-Tg T cell transfer leads to increased cognate and non-cognate alloantibody responses**

**A.** Percentages and total numbers of Tfh cells (PD-1$^{hi}$, CXCR5$^{hi}$) **B.** Numbers of K$^{d}$-specific (MHC I) and I-E$^{d}$ specific (MHC II) B cells in the spleens of naïve mice or mice undergoing acute rejection with or without TCR75 transfer.

**Figure AI.8, continued: C.** Percentages of $B220^{lo}$ cells amongst MHC I- or MHC II-specific B cells. For A-C, n=4-6 per group pooled from two independent experiments. **D.** ELISpot of antibody-producing cells that made alloantibodies (total IgG) or to specifically to $K^d$. $10^4$ cells were plated per well. Data are representative of two experiments of triplicate wells for each of 2-3 mice per group. **E.** Donor-specific antibodies were measured in the serum one day prior to transplantation and on days 4 and 6 post-transplantation. n=3 mice per group, representative of 2 independent experiments. **F and G.** Survival of hearts post-transplantation in wild-type (WT) or $\mu MT^{-/-}$ mice with or without $10^5$ TCR75 cells (+100K) (**F**) and numbers of endogenous CD4, CD8, and TCR-Tg cells infiltrating the grafts 6-8 days post transplantation (**G**), ⦸ (no transfer, n=14), 100K TCR75 (n=6) $\mu MT^{-/-}$ (n=6), $\mu MT^{-/-}$ + 100K TCR75 (n=6). For F & G, data from WT and WT+100K TCR75 are the same as those shown in Figure AI.1, and data are pooled from two experiments. Mean values were compared using Student's t-test or one-way ANOVA with Bonferroni correction for pairwise comparisons where appropriate **p<0.01, ***p<0.001.

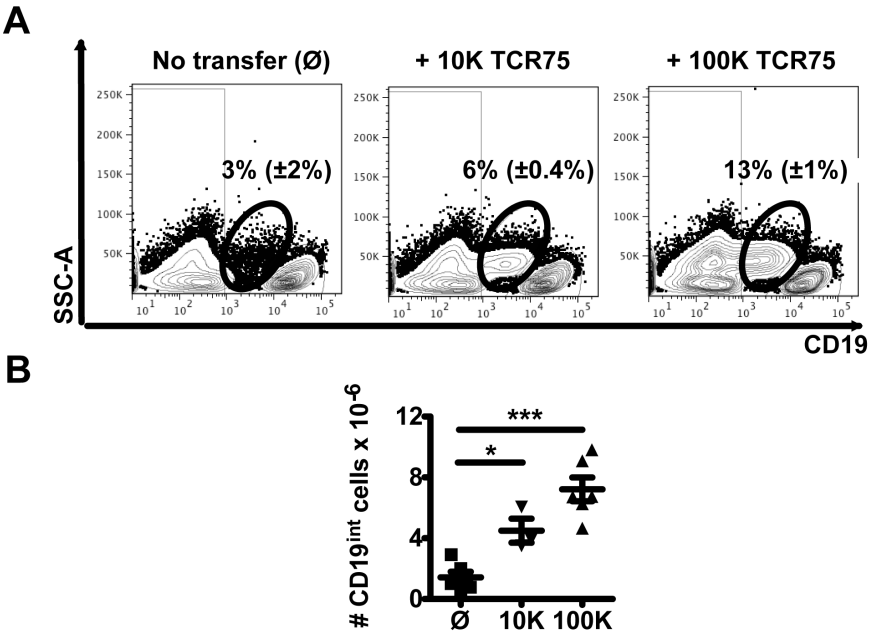


**Figure AI.9: Transfer of TCR75 cells increases the numbers of $CD19^{int}$ cells in the spleen**

**A**. The mean percentages ± SD (**A**) and total numbers (**B**) of $CD19^{int}$ cells in the spleen day 7-8 post-transplantation. No transfer: ⦸ n=6, TCR75: 10K n=3, 100K n=6 pooled from 3-4 independent experiments. Mean values were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons, *p<0.05, ***p<0.001.

To further illustrate the potency of the help provided by transferred $CD4^+$ TCR-Tg T cells, we transplanted the quasi-monoclonal MD4 BCR-Tg mice with or without transferred TCR75 cells. As 90% of B cells in MD4 mice are specific for Hen Egg Lysozyme (HEL) (Chen et al., 2013), these animals provide a model in which most B cells are not capable of producing alloantibodies. Remarkably, transfer of 100K TCR75 cells was able to drive allospecific IgG production by day 6 in these mice (Figure AI.10A). These observations underscore the potency of transferred TCR75 cells at providing B cell help, even in the face of a dramatically reduced alloreactive B cell repertoire. Notably, TCR75 transfer into MD4 hosts still resulted in reduced intra-graft T cell infiltration (Figure AI.10B).

Biopsies of patients undergoing acute antibody-mediated rejection often contain minimal cellular infiltrates (Berry et al., 2013). Because of the reduced intra-graft cellular infiltrate upon $CD4^+$ TCR-Tg T cell transfer, we tested the possibility that the allografts in the presence of TCR75 underwent antibody-mediated instead of T cell-mediated rejection. To test whether alloantibodies and their downstream effects were inhibiting intra-graft T cell accumulation in animals that had received $CD4^+$ TCR-Tg cell transfer we used $\mu MT^{-/-}$ mice, which lack membrane-bound IgM and mature B cells. $\mu MT^{-/-}$ mice did not make allo-IgM or allo-IgG upon transplantation and transfer of 100K TCR75 cells (Figure AI.10C). However, accelerated graft rejection (Figure AI.8F) and a reduction of T cells within the allografts were still observed (Figure AI.8G). Collectively these observations support the conclusion that early production of alloantibodies was

not required for the reduction of intra-graft T cell accumulation following $CD4^+$ TCR-Tg T cell transfer.

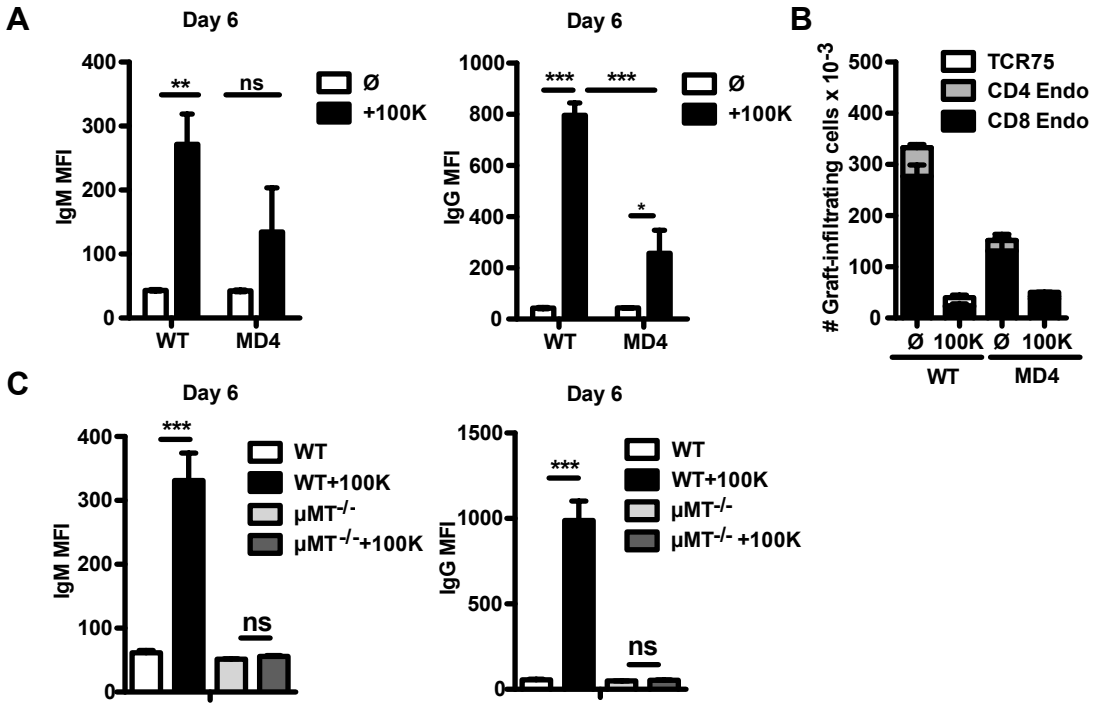


**Figure AI.10: Alloantibody production in MD4 and $\mu MT^{-/-}$ mice**
**A**. Allospecific IgM and IgG production in the serum of indicated mice on day 6 post-transplantation. **B.** Intra-graft T cells from day 6-8 post-transplantation in indicated mice. Data from WT and WT+100K TCR75 are the same as those shown in Figure AI.1D. **C.** Allospecific IgM and IgG production in the serum of indicated mice on day 6 post-transplantation. Mean values were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons, **p<0.01, ***p<0.001.

4. The transfer of $CD4^+$ TCR-Tg T cells alters intra-graft accumulation of innate immune cells

To determine if $CD4^+$ TCR-Tg T cell transfer had additional consequences on the endogenous alloresponse and gain insight into the mechanism of graft rejection, we analyzed the phenotype of other intra-graft leukocytes. TCR75 cell transfer resulted in a dose-dependent decrease in B cells and $CD11c^+$ dendritic cells in the grafts (Figure

AI.11A), and a striking increase in neutrophils (Figure AI.11B). This reduction in graft-infiltrating antigen-presenting cells was unique to the $CD4^+$ TCR-Tg T cell transfer, as OT-I transfer had no effect on B cell accumulation, and increased $CD11c^+$ cells within the allografts (Figure AI.12A), although similarly to the $CD4^+$ TCR-Tg T cell transfer, OT-I transfer also resulted in increased neutrophils in the graft (Figure AI.12A). Antigen specificity was required for T cell transfer to have an effect on accumulation of endogenous immune cell populations, as transfer of 250K polyclonal CD4s and 200K polyclonal CD8s had no effect on B cell, $CD11c^+$ cell or neutrophil accumulation compared to mice without transfer (Figure AI.12B).

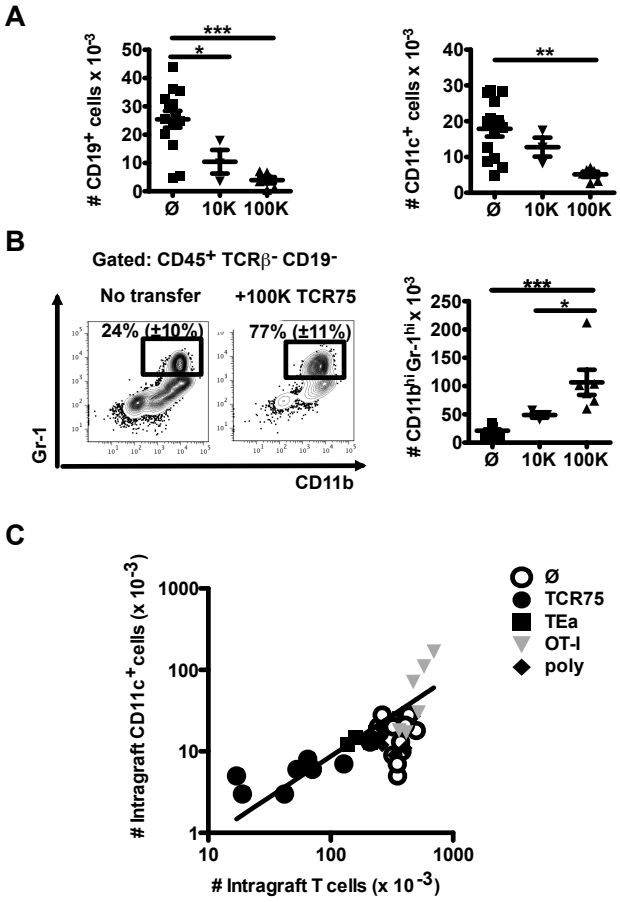


**Figure AI.11: Transfer of TCR75 cells alters the numbers of non-T cell populations in the graft**

**Figure AI.11, continued:** C57BL/6 mice were transplanted with BALB/c heart grafts. Six to eight days post-transplantation intra-graft antigen-presenting cells and neutrophils were enumerated by flow cytometry. **A.** The numbers of intra-graft B cells and $CD11c^+$ cells in mice with no transfer (⃠, n=14), 10K TCR75 cells (n=3) or 100K TCR75 cells (n=6). **B.** Representative flow cytometry plots of mean percentages of graft-infiltrating neutrophils ($Gr\text{-}1^{hi}$, $CD11b^{hi}$) ± SD after gating on $CD45^+TCRb^-CD19^-$ cells and their total numbers in mice with no transfer (n= 17), 10K TCR75 cells (n=3) or 100K TCR75 cells (n=6). For A and B, mean values were compared using one-way ANOVA with Bonferroni correction for pairwise comparisons *p<0.05, **p<0.01, ***p<0.001. **C.** The numbers of intra-graft $CD11c^+$ cells versus total T cells are positively correlated, Pearson r=0.66, p<0.0001. Results are pooled from 2-10 experiments.

As the total numbers of graft-infiltrating T cells was lowest in mice with TCR75 transfer and highest in mice with OT-I transfer, and this trend was recapitulated with intra-graft $CD11c^+$ cells, we tested whether there was a correlation between the two factors. To this end, data from 38 animals were combined from each of the groups of non-transfer, TCR75, TEa, OT-I and polyclonal T cell transfer. There was a robust positive correlation between the numbers of $CD11c^+$ cells and the total number of intra-graft T cells (Figure AI.11C, Pearson r=0.66, p<0.0001), suggesting that transfer of CD4 or CD8 TCR-Tg cells may affect the numbers of intra-graft $CD11c^+$ cells, and these intra-graft $CD11c^+$ cells may in turn affect the total numbers of intra-graft T cells, as has been described in tumor settings (Spranger et al., 2015).

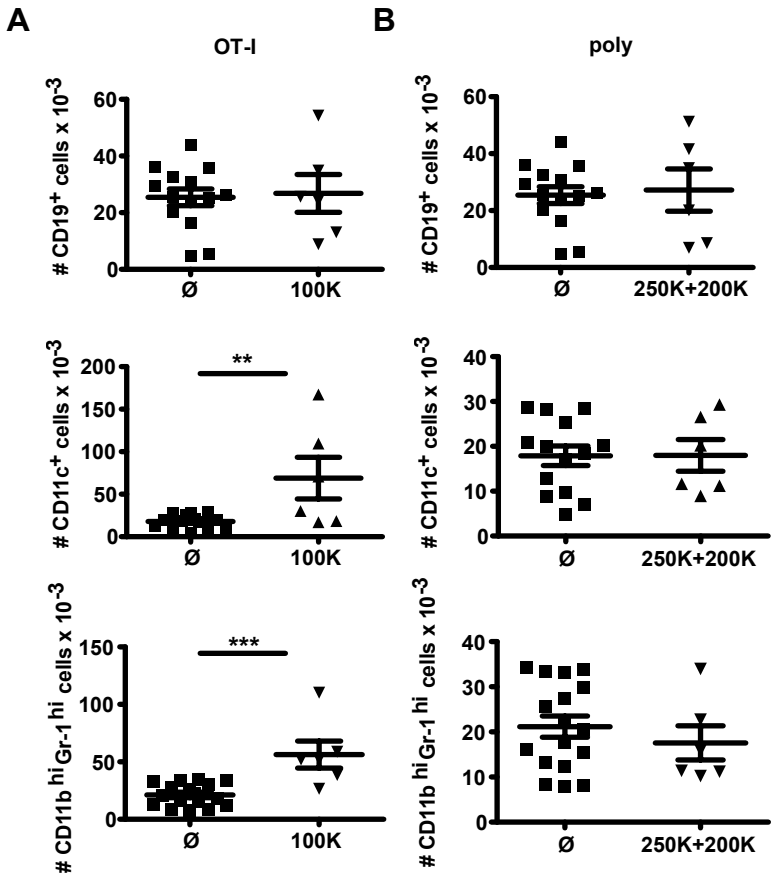


**Figure AI.12: Transfer of CD8$^+$ OT-I TCR-Tg cells but not polyclonal T cells changes non-T cell accumulation in the allograft**
The total numbers of endogenous CD19$^+$ B cells, CD11c$^+$ APCs and Gr-1$^{hi}$, CD11b$^{hi}$ neutrophils in mice with no transfer, (⦸, n=14-17) and mice with 100K OT-I transfer (n=6, **A**) or 250K polyclonal CD45.1$^+$ CD4$^+$ T cell transfer (n=6, **B**). Means were compared with Student's t test and results are pooled from at least two independent experiments. No transfer group is the same in panels A and B. OT-I cells were transferred either 1 day prior to or on the day of transplantation. CD45.1$^+$ T cells were transferred on the day of transplantation.

E. Discussion

TCR-Tg T cells are useful tools for following antigen-specific immune responses *in vivo* but they must be used cautiously. Using infectious disease models, it has been previously shown that OVA-specific OT-I cells can effectively compete for antigen with

endogenous OVA-reactive $CD8^+$ T cells, such that only transfer of less than 500 TCR-Tg OT-I cells can preserve the endogenous T cell response to the same antigen (Badovinac et al., 2007). Our data is consistent with this conclusion as few endogenous OVA-specific T cells were detectable in the graft following OT-I transfer ($OVA:K^{b+}CD45.1^+$, Figure AI.1C). Badovinac et al as well as others reported that CD8 memory T cell differentiation kinetics depended on initial precursor frequency (Badovinac et al., 2007; Hataye et al., 2006; Marzo et al., 2005). Ford and colleagues have demonstrated different levels of resistance of TCR-Tg T cells to costimulation blockade based on their initial seeded frequency in a skin transplant model (Ford et al., 2007). For $CD4^+$ TCR-Tg cells, Marshall et al compared the magnitude of the response of $CD4^+$ SMARTA T cells with that of endogenous T cells reactive to the same LCMV antigen, but in separate mice (Marshall et al., 2011). This study concluded that SMARTA T cells were representative of the endogenous response but no analysis of endogenous responses in the mice that received SMARTA T cells, compared to those that did not receive SMARTA cells, was shown (Marshall et al., 2011).

Our study shows, in contrast, that the addition of naïve $CD4^+$ TCR-Tg T cells not only likely competes with T cells recognizing the same antigen but also alters the response of $CD4^+$ and $CD8^+$ T cells recognizing other donor-specific antigens following allograft transplantation. The numbers and types of non-T cell subsets able to accumulate within the allografts were also altered by the addition of modest numbers of $CD4^+$ TCR-Tg T cells, further demonstrating a significant reshaping of the endogenous alloresponse by the transferred graft-reactive T cells. Of the hematopoietic cell

populations that were altered in the graft, the reduction in intra-graft T cells correlated most strongly with a reduction in the number of intra-graft $CD11c^+$ cells. This result is interesting in the context of recent data from Spranger et al who showed in a melanoma model that the presence of $CD11c^+$ cells positively correlated with intra-tumoral T cells, and that the addition of dendritic cells intra-tumorally allowed for increased T cell accumulation (Spranger et al., 2015). Antigen-specific $CD4^+$ T cells have been shown to have the ability to kill antigen-presenting cells (APCs) (Grogg et al., 1992; Umeshappa et al., 2009), and APCs have been shown to be particularly sensitive to CD4-mediated killing in the presence of high numbers of $CD4^+$ TCR-Tg T cells (Ingulli et al., 1997). Therefore, a possible explanation for the reduction in $CD11c^+$ cells observed in allografts with TCR75 transfer is that the TCR-Tg cells may have killed some of the APCs. The reduced number of APCs could then lead to a reduction in the chemokines produced by APCs that attract T cells or retain them within the allograft.

The reshaped endogenous immune response observed with TCR75 cell transfer was also observed with transfer of T cells from a second $CD4^+$ TCR-Tg mouse, TEa, although the TEa cells infiltrated the grafts at a lower frequency (Figure AI.1) and accumulated in the spleen at lower numbers (Figure AI.3). Differences in intra-graft accumulation of these two types of TCR-Tg T cells may be due to the respective antigens recognized by each TCR ($K^d$ for the TCR75 cells which is expressed by all cells in the allograft versus I-E for TEa cells which is only expressed on professional antigen-presenting cells and activated endothelial cells within the graft), or to a difference in the TCR affinity of each monoclonal population. Despite these differences,

our data suggest that transfer of TEa cells is also associated with a decrease in $CD11c^+$ cells, correlating with a reduction in the accumulation of endogenous allograft-specific T cells in the allograft.

Many groups utilizing TCR-Tg T cells in transplantation studies have transferred much higher numbers of T cells than those used here, up to $2 \times 10^7$ T cells (Burrell and Bromberg, 2012; Conlon et al., 2012a; Diamond and Gill, 2000; Ford et al., 2007). In this study, as few as $10^4$ TCR75 cells had profound effects on the endogenous immune response, with elevated alloantibody responses detected with as few as $10^3$ cells, and with greater perturbations with increasing numbers of transferred TCR-Tg T cells. In light of these observations, some past studies should be revisited and perhaps re-interpreted. For example, if a new immunosuppressive agent were to be tested in the mice used in this study it would not only have to overcome the effects of the adoptively transferred $CD4^+$ T cells specific for the graft, but also would have to counter an enhanced (in magnitude and kinetics) endogenous CD8 and alloantibody response. Thus, the efficacy of the immunosuppressive agent may be significantly underestimated. Future investigations using these cells should limit the numbers of TCR-Tg cells used for transfer so studies may more accurately reflect endogenous precursor frequencies.

Interestingly the naïve $CD4^+$ TCR-Tg T cell transfer was able to promote both cognate and non-cognate B cell responses. This result supports published data showing that B cells are directly able to receive help from non-cognate $CD4^+$ T cells provided these B cells express MHC-II to present the antigen, and they have acquired the non-cognate antigen from the same cell that expresses the cognate antigen (Conlon

et al., 2012b). Our data also show that transfer of $CD4^+$ TCR-Tg T cells promotes a non-cognate alloreactive $CD8^+$ T cell response, which invokes an indirect and/or semi-direct model of help. This could occur when the $CD4^+$ T cell activates APCs that present both alloantigens, such that the APCs, in turn, can stimulate the $CD8^+$ T cells recognizing a distinct alloantigen from that recognized by the $CD4^+$ TCR-Tg T cells.

Overall, the data give insight into the "cooperativity" of rejection first described by Bucy and colleagues, who when adoptively transferring TCR75 cells into C57BL/6 recipients of B6.$K^d$ hearts noted a non-linear relationship between the number of cells transferred and the kinetics of rejection (Honjo et al., 2004). This is likely due to reaching a threshold number of $CD4^+$ T cells that not only facilitate rejection themselves but also coordinate the rejection event by promoting more $CD8^+$ T cell responses in the spleen and a strong alloantibody response. Indeed, in B cell-competent mice, $CD4^+$ but not $CD8^+$ T cells have been shown to be essential for cardiac allograft rejection (Krieger et al., 1996). However, in B cell-deficient mice depleted of $CD8^+$ T cells, the remaining naïve $CD4^+$ T cells were incapable of rejecting a cardiac allograft (Nozaki et al., 2008), suggesting that CD4 help to either $CD8^+$ T cells or B cells is necessary to elicit graft rejection. In contrast, memory $CD4^+$ T cells might lead to allograft rejection by other mechanisms, as they could still trigger rejection in B cell-deficient mice depleted of $CD8^+$ T cells (Nozaki et al., 2008). Our data reveal increasing the numbers of naïve alloreactive $CD4^+$ T cells provides enhanced help to $CD8^+$ T cells and allospecific B cells but it also leads to an overall reduction in intragraft T cells as well as $CD11c^+$ cells. The mechanism of rejection is most likely altered by the presence of these increased

numbers of $CD4^+$ T cells, as graft rejection ensues without ever achieving high numbers of T cells within the graft and can still be accelerated without alloantibodies as the results of the $\mu MT^{-/-}$ experiments showed. It is possible that the transferred $CD4^+$ TCR-Tg T cells provide help to allospecific $CD8^+$ T cells, which in turn may destroy donor endothelial cells, causing graft rejection before many T cells infiltrate the graft.

Our data showing that transfer of naïve TCR75 cells which results in reduced accumulation of T cells, B cells and dendritic cells in the allograft contrast with data obtained by Martin-Orozco and colleagues in a tumor model. In this B16-OVA melanoma model, transfer of activated, Th17-skewed $CD4^+$ TCR-Tg OT-II T cells enhanced endogenous CD4, CD8, antigen-presenting cell and neutrophil accumulation into the tumor (Martin-Orozco et al., 2009). Whether the differences are due to the tumor microenvironment or to the TCR-Tg T cells being pre-activated rather than naïve remains to be established.

$CD8^+$ T cell transfer also perturbed endogenous alloimmunity, albeit in different ways than the $CD4^+$ T cell transfer. $CD8^+$ T cell transfer led to an overall increase in the numbers of graft-infiltrating T cells, $CD11c^+$ and neutrophils, though it did not accelerate graft rejection. Taken with $CD4^+$ T cell transfer data, the alterations in endogenous alloimmunity following transfer of $CD8^+$ T cells provides evidence that there are unique effects of distinct adoptively transferred populations on endogenous alloimmune responses.

Assessing the effects of adoptive transfer of antigen-specific T cells in a clinical setting should be the subject of future investigations as recently completed and ongoing

clinical trials are evaluating the efficacy of transferring very large numbers of expanded antigen-specific T cells in settings of tumor and of viral infection (for example NCT00393029, NCT02210104, NCT00880789, NCT00110578). To date, endogenous immune responses have not been monitored in these patients and it is not known if the adoptive cell therapy would have unintended long-term consequences on the endogenous immune response against the tumor or virus being targeted, or against a concurrent infection. Clinical trials are also ongoing in transplantation, transferring expanded antigen-specific Tregs to prolong allograft survival, anti-tumor T cells to combat post-transplant lymphoproliferative disorder (PTLD) or viral-specific T cells to control infection (NCT02371434, NCT00063648, NCT00880789). Supporting our results, one recent preclinical study using adoptive Treg therapy in a non-human primate model of heart transplantation revealed that transfer of Tregs into a lymphopenic host can result in enhanced alloantibody responses and T cell memory, as the transferred cells provided help to endogenous alloreactive cells (Ezzelarab et al., 2015). Whereas our study assessed the consequences of transferring naïve T cells, many clinical trials use transfer of activated T cells, which may alter the endogenous immune response differently. In addition, humans have a higher proportion of endogenous memory T cells than laboratory mice, which may react differently that naïve T cells to the adoptive cell therapy. The consequences of T cell transfers on endogenous immune responses should be investigated in human clinical trials.

## APPENDIX II – BASAL NF-κB CONTROLS IL-7 RESPONSIVENESS OF QUIESCENT NAÏVE T CELLS[1]

### A. Abstract

T cells are essential for immune defenses against pathogens, such that viability of naïve T cells prior to antigen encounter is critical to preserve a polyclonal repertoire and prevent immunodeficiencies. The viability of naïve T cells before antigen recognition is ensured by IL-7, which drives expression of the pro-survival factor Bcl-2. Quiescent naïve T cells have low basal activity of the transcription factor NF-κB, which was assumed to have no functional consequences. In contrast to this postulate, our data demonstrate that basal nuclear NF-κB activity plays an important role in the transcription of IL-7Rα (CD127), enabling responsiveness of naïve T cells to the pro-survival effects of IL-7, and allowing T cell persistence *in vivo*. Moreover, we show this property of basal NF-κB activity is shared by mouse and human naïve T cells. Thus, NF-κB drives a distinct transcriptional program in T cells prior to antigen encounter, by controlling susceptibility to IL-7. Our results reveal an evolutionarily conserved role of

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Contributions:
- Performed experiments and analyzed data in Figures AII.1B, AII.2A, AII.4B-D, AII.5, AII.7B-8 (with Luciana Molinero), AII.9-10, AII.11B,F,G, AII.12
- Generated the luciferase constructs (with Luciana Molinero)
- Design of experiments and statistical analyses
- Co-wrote manuscript with Maria-Luisa Alegre and Luciana Molinero

NF-κB in T cells prior to antigenic stimulation and identify a novel molecular pathway that controls T cell homeostasis.

B. Introduction

Survival of naïve quiescent T cells is essential to maintain a pool of polyclonal T cells ready for activation by their cognate antigen. Upon egress from the thymus, survival of peripheral naïve T cells ($CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$) depends on intermittent tonic engagement of the T cell receptor (TCR) and signaling by the cytokine IL-7 (Kimura et al., 2013; Mackall et al., 2011). Tonic TCR engagement is generated by the interaction of the TCR with weakly reactive self-peptides (Surh and Sprent, 2008). Survival of quiescent CD8 T cells requires MHC class I-TCR engagement, as indicated by dwindling numbers of naïve CD8 T cells following transfer into MHC class I-deficient mice (Markiewicz et al., 2003; Murali-Krishna et al., 1999). In addition, long (but not short) term survival of CD4 T cells requires the presence of MHC class II (Martin et al., 2003).

Interleukin-7 is important for survival and homeostatic proliferation of naïve T cells, as demonstrated by reduced recovery of naïve T cells transferred into $IL\text{-}7^{-/-}$ mice (Schluns et al., 2000; Tan et al., 2001) and impaired survival and homeostatic proliferation of T cells from IL-7 receptor α subunit (IL-7Rα)-deficient mice (Maraskovsky et al., 1996; Tani-ichi et al., 2013). The receptor for IL-7 is a heterodimer consisting of the IL-7Rα (CD127) and common gamma-chain receptor ($\gamma_c$, CD132) subunits. Triggering of IL-7R activates Stat5 via Jak1/Jak3 and the PI3K/Akt/mTOR axis

(Henriques et al., 2010; Pallard et al., 1999). Interleukin-7-mediated survival involves upregulation of the pro-survival factors Bcl-2 and Mcl-1 as well as reduction of pro-apoptotic molecules Bax, Bad and Bim (Jiang et al., 2005). Interestingly, IL-7 negatively regulates the expression of its receptor, promoting endocytosis, degradation and the transcriptional inhibition of *Il7r* expression (Henriques et al., 2010; Park et al., 2004). This enables a pool of T cells that have not yet encountered IL-7 to be preferentially responsive to limiting concentrations of this cytokine. Several transcription factors are involved in the control of *Il7r* expression in T cells, including positive regulation by GA-binding protein, glucocorticoid receptor (GR), Ets1, Runx1, Runx3 and Foxo1, and repression by Foxp1 and Gfi1, the latter exclusively in CD8 T cells (Carrette and Surh, 2012).

The transcription factor NF-κB is critical for T cell activation, proliferation and survival following TCR engagement. NF-κB exists mostly as heterodimers between the transactivating proteins RelA, RelB, and c-Rel and their DNA-binding partners p50 (p105, NF-κB1) and p52 (p100, NF-κB2) (Bonizzi and Karin, 2004). Upon TCR engagement, the kinase IKKβ, part of the IKK complex, phosphorylates IκBα, the inhibitor of NF-κB, targeting it for degradation and allowing NF-κB to translocate into the nucleus. In activated T cells, NF-κB induces upregulation of the pro-survival molecules Bcl-$x_L$, A1, A20, and cellular inhibitors of apoptosis (c-IAPs) (Harlin et al., 2002a; Hayden and Ghosh, 2004; Wu et al., 1996). IκBαΔN mice bear transgenic expression of a non-degradable form of IκBα in early thymocyte development resulting in NF-κB-impaired T cells (Boothby et al., 1997). These mice have diminished survival of

activated mature T cells and reduced numbers of peripheral naïve T cells (Boothby et al., 1997; Mora et al., 2003). The mechanism for decreasing survival of IκBαΔN naïve T cells is not clear. Using this and other genetic mouse models of NF-κB impairment in T cells, as well as pharmacological inhibition of NF-κB, our results show that basal NF-κB activity controls survival of naïve quiescent T cells at least in part by enhancing *Il7r* transcription, a mechanism conserved in both mice and humans. Our findings demonstrate an essential role of NF-κB in the control of naive T cell homeostasis.

## C. Materials and Methods

### Mice

C57BL/6 were obtained from Harlan Laboratories (Indianapolis, IN) while $Lck^{Cre}$, Rosa26-$Stop^{FL}IKK\beta^{CA}$-GFP and $CD45.1^{+}$ mice (all C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME). IκBαΔN mice (C57BL/6 background), whose T cells express a dominant negative form of IκBα (driven by the proximal Lck promoter and the CD2 enhancer) and DO11.10 x IκBαΔN mice generated by breeding IκBαΔN (Balb/c background) to DO11.10 mice, which express an ovalbumin-specific TCR transgene were a generous gift from Mark Boothby (Vanderbilt University, TN). NF-κB1/p50-deficient and NF-κB2/p52-deficient mice (C57BL/6 background) were provided by Yang-Xin Fu (University of Chicago). Bcl-$2^{Tg}$ mice, obtained from Marcus Clark (University of Chicago), express human Bcl-2 under control of a Vav promoter (Ogilvy et al., 1999), and OVA-specific OT-II TCR transgenic mice (Taconic) were crossed to IκBαΔN mice at the University of Chicago. $IKK\beta^{fl/fl}$, $CD4^{Cre}$

and IL-7R$^{Cre+/-}$ [a Cre knock-in that disrupts normal *Il7r* mRNA (Schlenner et al., 2010)] mice were generously provided by Michael Karin (La Jolla Institute, CA), Fotini Gounari and Barbara Kee (both University of Chicago, IL), respectively. All mice were bred at the University of Chicago SPF facility in agreement with our Institutional Animal Care and Use Committee (IACUC) and according to the National Institutes of Health guidelines for animal use.

Reagents

Recombinant mouse and human IL-7 were obtained from Peprotech (Rocky Hill, NJ). The NF-κB Activation Inhibitor 6-amino-4-4-phenoxyphenylethylamino-quinazoline was purchased from EMD Millipore (Darmstadt, Germany).

Splenocytes and lymph node cells isolation

Spleens were homogenized in hypotonic ammonium chloride potassium (ACK) lysis buffer for 1 minute to lyse red blood cells. Lymph nodes were homogenized with glass slides. Cell suspensions were diluted in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS (HyClone, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (50 μM), 2-mercaptoethanol (50 μM), and non-essential amino acids (1% final volume) (complete DMEM). Cells were filtered through nylon gauze, centrifuged and resuspended in complete DMEM. Live cells were counted in a light microscope or with the Countess Automated Cell Counter, (Invitrogen) using the trypan blue exclusion method. To measure IFNγ production, T cells were stimulated

in the presence of phorbol myristate acetate (PMA, 150 ng/ml) and ionomycin (500 ng/ml), in the presence of brefeldin A (10 μg/ml, Biolegend).

T cell purification

T cells, $CD4^+$ or $CD8^+$ T cells were purified by negative selection using magnetic beads according to the instructions of the manufacturer (Stem Cell, Vancouver, Canada). In some experiments, $CD4^+$ $CD44^{lo}$ and $CD8^+$ $CD44^{lo}$ naïve T cells were further purified by FACS cell sorting with BD FACSAria (San Jose, CA). In other experiments $CD44^{hi}$ cells were depleted from CD4 and CD8 T cells through negative magnetic selection. Purity of naïve T cells was verified in each experiment to be equal or superior to 95%.

Flow cytometry

Flow cytometric analyses were performed on single-cell suspensions, washed, stained in FACS buffer (PBS, 1% BSA and 0.01% $NaN_3$), washed again and resuspended in FACS buffer. Alternatively, cells were fixed and permeabilized with either eBioscience Foxp3 staining buffer (for immunodetection of Bcl-2 or IFNγ) or 1% formaldehyde and 90% methanol (for immunodetection of phospho-STAT5). Cells were labeled with PE-, FITC-, APC-, PerCP-, PE-Cy-7- or biotin-conjugated antibodies coupled to streptavidin (SA)-PE or SA-allophycocyanin (APC). The antibodies used targeted murine CD4 (GK1.5), CD8α (53-6.7), CD44 (IM7), IL-7Rα (CD127; B12-1), CD45.1 (A20), CD45.2 (104), Thy1.1 (HIS51), Thy1.2 (30-H12), CD132 (TUGh4), CD5

(53-7.3), CD3ε (145-2C11), Bcl-2 (BCL/10C4), IFNγ (XMG1.2) and phospho-STAT5 (47/Stat5[pY694]). All of these antibodies were obtained from BD Biosciences (San Jose, CA), eBioscience (San Diego, CA) or Biolegend (San Diego, CA). In certain experiments cells were stained with propidium iodide (PI) or 7-AAD obtained from Invitrogen (Carlsbad, CA). Samples were acquired in either LSR Fortessa or LSRII (BD Biosciences) flow cytometers. Data were analyzed by FlowJo software (TreeStar).

Electrophoretic mobility shift assays (EMSA)

Nuclear proteins were extracted and quantified as previously described (Molinero et al., 2011). Double stranded oligonucleotides coding for NF-κB consensus sequence (5'-CAACGGCAGGGGAATTCCCCTCTCCTT-3')(Boothby et al., 1997), IL2R-NF-κB (5'-CAAGGCAGGGGAATTCCCCTCTCCTT-3') and NF-κB/IL-7Rα (5'-ACCAGTGGAAATCCCCTGAGCA-3') were either purchased biotinylated or labeled with [γ-$^{32}$P] ATP (MP Biomedicals, Irvine, CA) using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Equal amounts of nuclear extracts were added to 8 μl of gel shift binding buffer (2 μl 10X HGE buffer containing 200 mM HEPES, pH 7.9, 50% glycerol, 10 mM EDTA, 50 mM NaCl, 0.5 mM EDTA, 1μl 1mg/ml BSA, 1μl DTT 0.1M, 2 μl 10% NP-40, 2 μl poly-dIdC). The mixture was incubated for 30 min at 37 °C with 20-64 fmole $^{32}$P- or biotin-labeled oligonucleotide probes. Five microliters of loading buffer was added and the sample was electrophoresed in a 6 % polyacrylamide gel. Radioactive EMSA: the dried gel was exposed to X-ray film (Pierce, Rockford, IL) at –80°C. The intensity of the NF-κB complex was quantified by densitometry. Non-radioactive EMSA:

gel was transferred onto positively charged nylon membranes, and DNA was cross-linked to the membrane with a UV Stratalinker (Stratagene, Agilent Technologies, Santa Clara, CA). Membranes were developed using LightShift Chemiluminescent EMSA kit (Thermo Scientific) according to manufacturer's instructions. For supershift assays, the protein extracts were pre-incubated with 1 μg of rabbit anti-p50 or anti-RelA antibodies (Santa Cruz Biotechnology, Santa Cruz CA) for 30 minutes.

*In vivo* T cell survival

6-8 week-old DO11.10/IκBαΔN mice and littermate controls were thymectomized. Approximately 45-50 μl of mouse peripheral blood was obtained weekly through the retro-orbital plexus using heparin-treated calibrated capillary tubes. Red blood cells were lysed for 5 minutes in 1 ml of ACK lysis buffer. Peripheral mononuclear cells were analyzed by flow cytometry.

Adoptive transfer

WT (Thy1.1$^+$ CD45.2$^+$ and Thy1.2$^+$CD45.1$^+$) and IκBαΔN (Thy1.2$^+$CD45.1$^+$) CD4$^+$CD44$^{lo}$ and CD8$^+$CD44$^{lo}$ naïve T cells were purified by FACS cell sorting and equal numbers were injected i.v. into Thy1.2$^+$ CD45.2$^+$ C57BL/6 recipients. Seven days later spleen and peripheral lymph nodes were isolated and cells in each organ were analyzed by flow cytometry.

*In silico* analysis

Assessment of putative NF-κB-binding sites upstream and downstream of the *Il7r* gene (Ensemble Gene ID ensmusg00000003882) was performed using the ECR Browser (http://ecrbrowser.dcode.org/) comparing mouse to rat, rabbit, human, dog, shrew, elephant and opossum. The results were further confirmed by Multiz alignment as viewed in the UCSC Genome Browser for the *Mus musculus* Dec. 2011 (GRCm38/mm10) genome assembly (http://genome.ucsc.edu/cgi-bin/hgTracks).

Quantitative RT-PCR

Total RNA was prepared from $CD4^{+}CD44^{lo}$ and $CD8^{+}CD44^{lo}$ T cells with the use of RNEasy Plus Mini Kit (QIAGEN). cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad) and the samples diluted in water (1:10). A total volume of 20 μl containing 5 μl cDNA template, 0.3 μM of each primer and SYBR Green PCRMaster Mix (Applied Biosystems) was loaded in triplicates. Gene expression was analyzed with an ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.9.1 (Applied Biosystems). Results were normalized to *Actb* levels. The primers used in the reactions were: IL-7Rα-Forward 5'-TCCGATCCATTCCCCATAACGA-3', IL-7Rα-Reverse 5'-TGGCAAGACAGG ATCCCATCCT-3'; Actin-F 5'-TGGAATCCTGTGGCATCCATGAAAG-3' and Actin-R 5'-TAAAACGCAGCCTCAGTAACAGTCCG-3'. Samples were then analyzed using the standard curve method.

Cloning of pGL4.23-ECR2 and pGL4.23-ECR3

The plasmid pGL4.23 (Promega) containing a minimal promoter upstream of firefly luciferase was used to test enhancer activity of the NF-κB-containing sequences present in the evolutionary conserved regions (ECR) 2 and 3 upstream of mouse *Il7r*. C57BL/6 mouse genomic DNA was used to amplify the sequences of ECR2 and ECR3, using oligonucleotides containing NheI and XhoI restriction enzyme sites (ECR2-NheI 5'-AAGCTAGCAATTGTATCATGTCTCTTTAAATTCT-3', ECR2-XhoI 5'-ACTCGAGCTTCCCGCACTCTATTTAGA-3'; ECR3-NheI 5'-AAGCTAGCATGTACTTCAACTCCAACTGAAC-3' and ECR3-XhoI 5'-AACTCGAGAGAAAGAAGAGAAAGAAAAAGAAG-3'). The purified PCR products and pGL4.23 were digested with NheI and XhoI, ligated with T4 DNA ligase and transformed into *E. coli* competent cells. The fidelity of the construct was verified by DNA sequencing.

Luciferase reporter assays

293T cells were seeded into 24-well plates at $7x10^4$ cells per well and cultured for 12 hours in DMEM-10% FCS before transfection. 0.35 μg of pGL4.23, pGL4.23.ECR2 or pGL4.23.ECR3 firefly-luciferase reporter plasmid DNA was cotransfected with expression plasmids encoding, GFP, $IKKb^{CA}$ or RelA (0.35, 0.48 or 0.45 μg of DNA) using a calcium chloride method (Invitrogen). Cells were also cotransfected with 2 ng pRL-TK-Renilla luciferase reporter plasmids to permit normalization for differences in transfection efficiency occurring in the individual cultures. 48h after transfection firefly

and *Renilla* luciferase activity were assayed in triplicates using the dual luciferase assay system according to manufacturer's instructions (Promega).

Chromatin immunoprecipitation assay (ChIP)

ChIP was carried out using a commercial kit (Upstate Biotechnologies, Lake Placid, NY) according to the manufacturer's instructions. Briefly, $10^6$ wild-type and IκBαΔN $CD4^+$ T cells were enriched by negative selection. DNA-binding proteins were cross-linked to DNA by adding formaldehyde (1% final concentration) and incubated for 10 minutes at 37°C and then quenched with 125 mM Glycine for 5 min. Cells were lysed and DNA in the supernatant was sheared by a Branson Digital Sonifer (Branson Ultrasonics, Danbury, CT). After removing an aliquot of sonicated samples as input material, the remainder was used for IP. Five μg of anti-RelA (Santa Cruz Biotechnologies, sc-372) or normal rabbit IgG (Santa Cruz Biotechnologies) antibodies and protein A+G magnetic-beads (Millipore) were added to the supernatant fraction and incubated overnight at 4°C with rotation. To reverse protein-DNA crosslinks, 20 μL of 5M NaCl and 1 μl of 20 mg/ml proteinase K were added to each sample before heating at 65°C for 2 hours. DNA was purified using the QIAquick PCR Purification Kit (Qiagen) and qPCR was performed using SYBR Green pre-mixed reagents (Applied Biosystems) in ABI PRISM 7300 Sequence Detector and ABI Prism Sequence Detection Software version 1.9.1 (Applied Biosystems). Reactions were prepared in triplicates and results were normalized with respect to IgG and input. The primers used were: IL7r.ECR2-F 5'-GGGCAGGAGATGCTGAAAGG-3', IL7r.ECR2-R 5'-CCTGCTTTCGGCTTCAAAGG-3';

Il7r.ECR3-F 5'-TGGGCACCTGCTTTCTTCTG-3' and Il7r.ECR3-R 5'-AACCACCCTCGCCTACCTGA-3'.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from healthy human volunteers as previously described (Molinero et al., 2002).

Statistical analyses

Comparisons of means were performed with GraphPad Prism (GraphPad Software) using the Student's t test, one-way ANOVA or two-way ANOVA where appropriate with Bonferroni's correction for multiple comparisons (one-way ANOVA). Normality was assessed by Kolmogorov-Smirnov tests, and non-parametric tests such as Mann-Whitney and Kruskal-Wallis (with Dunn's) were used where appropriate. Differences were considered significant for p values $<0.05$.

D. Results

1. Basal NF-κB contributes to the survival of quiescent naïve T cells

Activation of NF-κB upon TCR engagement is essential for survival of activated T cells (Molinero and Alegre, 2012). Basal NF-κB activity has been noted in unstimulated T cells, though at much lower levels than in TCR-stimulated T cells, but its functional significance was unknown (Harlin et al., 2002b). To investigate the NF-κB subunits at play in naïve T cells, electrophoretic mobility assays (EMSA) were performed using nuclear extracts from FACS-cell sorted purified $CD4^{+}CD44^{lo}$ and $CD8^{+}CD44^{lo}$ naïve wild-type (WT) and NF-κB-impaired IκBαΔN T cells. NF-κB activity in IκBαΔN naïve T cells was greatly reduced compared to naïve WT T cells (Fig. AII.1A). Supershift assays revealed that the predominant NF-κB complexes present in quiescent naïve WT T cells were RelA/p50 and p50/p50 dimers (Fig. AII.1B).

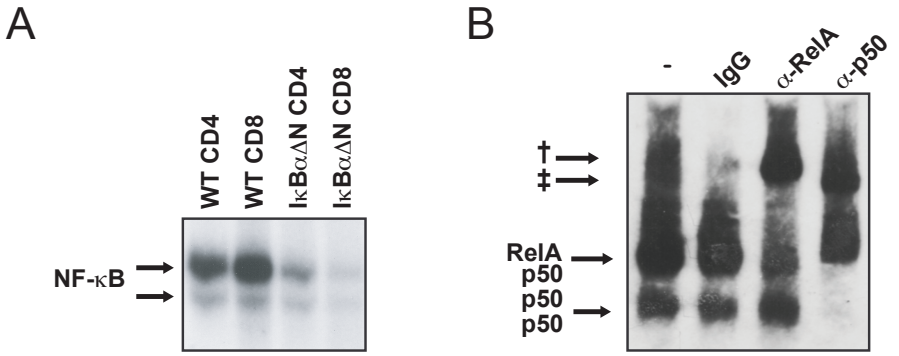


**Figure AII.1: Naïve T cells display basal NF-κB activity**
**A**. Electrophoretic mobility shift assay (EMSA) for NF-κB using nuclear extracts of WT and IκBαΔN FACS-cell sorted $CD4^{+}CD44^{lo}$ and $CD8^{+}CD44^{lo}$ T cells. **B.** EMSA supershift for NF-κB subunits RelA and p50 performed with nuclear extracts from WT naïve T cells. †: supershifted bands for RelA; ‡: supershifted band for p50. Results are representative of at least three independent experiments.

Because NF-κB signaling is required for lymphopenia- and activation-induced proliferation of T cells (Mora et al., 2003), we tested whether NF-κB drives quiescent naïve T cell survival *in vivo*. To this end, equal numbers of WT (CD45.1/2) and IκBαΔN (CD45. 2) $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ cells were co-adoptively transferred into congenic WT recipients (CD45.1). One week later, analysis of spleen and peripheral lymph nodes (Fig. AII.2A) revealed that the ratio of recovered IκBαΔN to WT $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells was significantly reduced. To rule out a causal role of TCR repertoire differences in developing NF-κB-impaired thymocytes, survival of T cells with a fixed TCR specificity was analyzed. Ovalbumin-specific TCR transgenic DO11.10 WT and DO11.10 IκBαΔN mice were thymectomized and the half-life of DO11.10 CD4 T cells was assessed over time in peripheral blood cells. The decay of DO11.10 IκBαΔN T cells was faster and more pronounced than that of WT controls (Fig. AII.2B), further indicating that basal NF-κB activity is necessary for survival of quiescent naïve T cells *in vivo.*

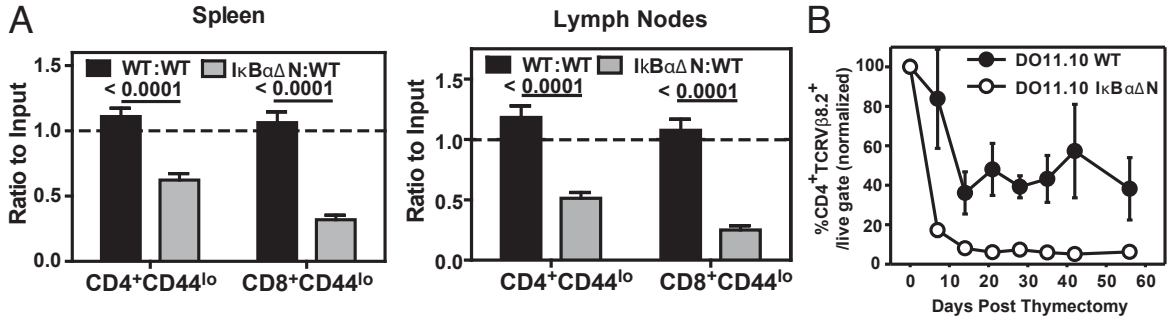


**Figure AII.2: Basal NF-κB is required for T cell survival *in vivo***
**A.** Equal numbers of CD45.1/2 WT and CD45.2 WT or IκBαΔN $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ cells were co-adoptively transferred into CD45.1 recipients. Seven days later, ratios of IκBαΔN:WT and WT:WT spleen and lymph node $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells were assessed as follows: $(\%CD45.2_{final} / \%CD45.1/2_{final})/ (\%CD45.2_{initial} / \%CD45.1/2_{initial})$. Graph represents recipients receiving WT:WT (n = 13) and IκBαΔN:WT (n = 14) cells.

**Figure AII.2, continued:** Data is pooled from four-five independent experiments and analyzed by Kruskal-Wallis test with Dunn's post-test. **B.** DO11.10/WT and DO11.10/IκBαΔN mice were thymectomized and presence of $CD4^+TCRV\beta8.2^+$ T cells in peripheral blood was assessed weekly by flow cytometry (WT, n=6; IκBαΔN, n=8). Values displayed are percentage of $CD4^+TCRV\beta8.2^+$ T cells with respect to the live gate and relative to the value obtained at the time of thymectomy (day 0). Data is representative of two independent experiments.

2. Basal NF-κB does not control tonic TCR signaling

The reduced lifespan of NF-κB-impaired naïve T cells suggests that NF-κB regulates the expression of one or more genes important for naïve T cell survival. As tonic TCR signaling is required for survival of naïve T cells, we hypothesized that basal NF-κB activity may control tonic TCR signaling in naïve T cells. At steady state, expression of CD5 in naïve T cells reflects proximal TCR signal strength in response to self-peptide/MHC (Mandl et al., 2013). To test if basal NF-κB controls tonic TCR signals, expression of CD5 was analyzed in peripheral naïve CD4 and CD8 T cells from WT and IκBαΔN mice. Remarkably, CD5 expression in IκBαΔN CD4 and CD8 naïve T cells was comparable to WT T cells (Fig. AII.3A). To rule out any compensatory effect due to a potentially disparate TCR repertoire in NF-κB-impaired T cells, expression of CD5 was analyzed in ovalbumin-specific TCR-transgenic WT and IκBαΔN OT-II $CD4^+$ naïve T cells. Again, no differences were observed in levels of CD5 expression between the two strains (Fig. AII.3B), suggesting that basal NF-κB does not control tonic TCR signaling.

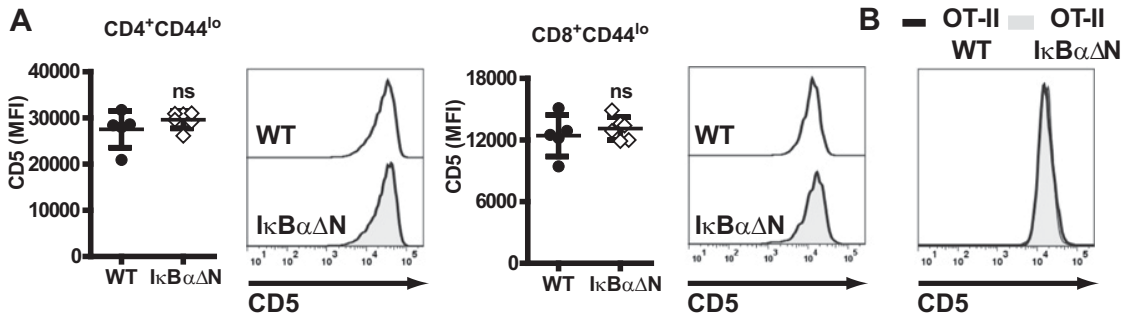


**Figure AII.3: Tonic TCR signaling in naïve T cells is independent of basal NF-κB activity**

**A.** Levels of CD5 were assessed in peripheral blood $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells from WT (n = 5) and IκBαΔN (n = 5) mice by flow cytometry. Data is pooled from two independent experiments. ns = not significant. **B.** CD5 expression in peripheral blood $CD4^+TCRV\beta\ 5.1^+CD44^{lo}$ T cells representative from OT-II WT (n = 6) and IκBαΔN (n = 5) mice.

3. Basal NF-κB activity controls IL-7-dependent T cell survival

Homeostasis of naïve T cells requires intermittent interaction of IL-7 with its receptor, IL-7R, which in turn activates the Jak3/Stat5 signaling. This pathway promotes the expression of the pro-survival factor Bcl-2 (Mackall et al., 2011). To test the susceptibility of IκBαΔN T cells to IL-7-mediated survival, WT and IκBαΔN splenocytes were cultured *in vitro* in the presence of exogenous IL-7 and viability of naïve CD4 and CD8 T cells was analyzed. Surprisingly, addition of IL-7 enhanced survival of WT but not IκBαΔN naïve CD4 and CD8 T cells (Fig. AII.4A). Consistent with this observation, IL-7-mediated STAT5 phosphorylation (Fig. AII.4B) and Bcl-2 upregulation (Fig. AII.4C) were severely compromised in the majority of IL-7-stimulated IκBαΔN T cells, suggesting that IL-7 signaling in T cells depends on basal NF-κB activity.

To test if diminished IL-7-mediated Bcl-2 upregulation was responsible for defective survival of naïve IκBαΔN T cells, IκBαΔN mice over-expressing human Bcl-2

(Ogilvy et al., 1999) were generated. Indeed, Bcl-2$^{Tg}$ rescued the percentage of CD4 and CD8 naïve T cells in IκBαΔN mice (Fig. AII.5A) and, *in vitro*, Bcl-2$^{Tg}$ significantly improved viability of IκBαΔN naïve T cells after 3d of culture with and without IL-7 (Fig. AII.5B-C). Finally, co-adoptive transfer of CD45.1/2 WT and CD45.2 WT, IκBαΔN or IκBαΔNxBcl-2$^{Tg}$ CD4 and CD8 naïve T cells into congenic recipients showed that Bcl-2$^{Tg}$ partially restored survival of IκBαΔN naïve T cells *in vivo* (Fig. AII.4D). Our data suggest that the defective survival of NF-κB-impaired naïve T cells in response to IL-7 is at least in part due to reduced IL-7-dependent Bcl-2 upregulation.

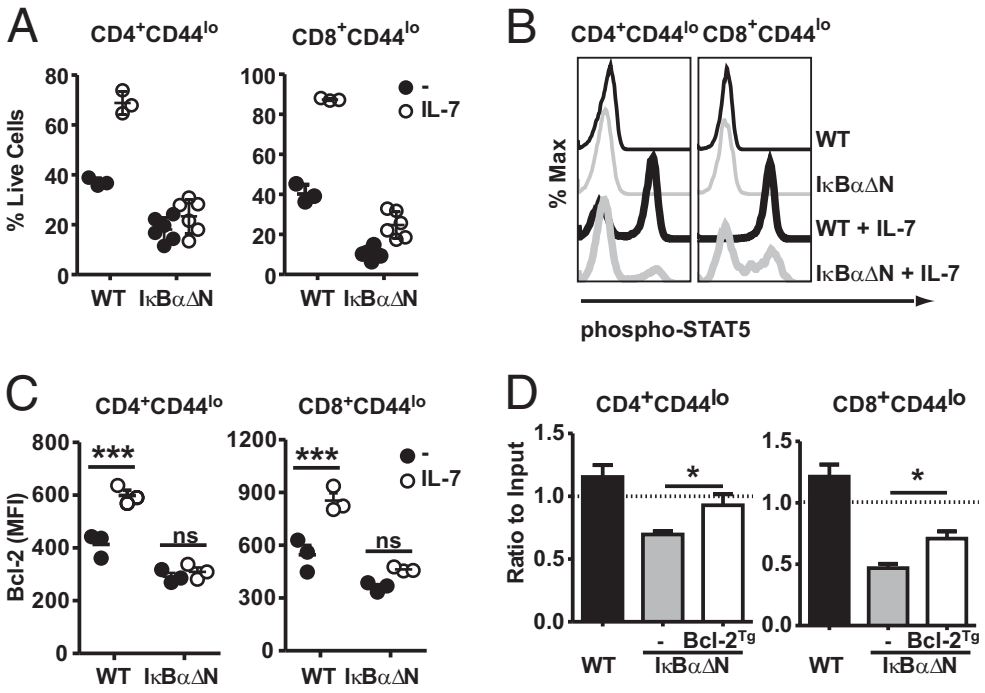


**Figure AII.4: Basal NF-κB activity is required for IL-7-mediated survival**
**A.** Splenocytes from WT (n = 3) and IκBαΔN (n = 6) mice were cultured for 3 days in the presence (open symbols) or absence (filled symbols) of 1 ng/ml IL-7. Percentages of CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ live (7AAD negative) cells were assessed by flow cytometry. **B.** WT and IκBαΔN splenocytes were cultured *in vitro* for 30 min in the presence or absence of 1 ng/ml IL-7 and (Y694) STAT5 phosphorylation in CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ was assessed by intracellular flow cytometry. Data are representative of n=6 mice each.

**Figure AII.4, continued: C.** Splenocytes from WT (n = 3) and IκBαΔN (n = 3) mice were cultured in the presence (open symbols) or absence (filled symbols) of IL-7 and 24h later expression of Bcl-2 was assessed by intracellular flow cytometry in $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ cells. Data was analyzed by two-way ANOVA with Bonferroni post-tests. **D.** Equal numbers of CD45.1/2 WT and CD45.2 WT, IκBαΔN or IκBαΔNxBcl-$2^{Tg}$ $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ cells were co-adoptively transferred into CD45.1 recipients. Results were analyzed as in Fig. 2A. Graph represents recipient mice for WT:WT (n = 8), IκBαΔN:WT (n = 9) and IκBαΔNxBcl-$2^{Tg}$:WT (n = 9). Data is pooled from three independent experiments and analyzed by Kruskal-Wallis test with Dunn's post-test. All experiments were performed at least three times.

4. NF-κB is required for IL-7Rα expression

The requirement of NF-kB activity for IL-7R signaling implied that NF-κB might control the expression or activation of components of the IL-7R pathway. To investigate this hypothesis, levels of IL-7Rα (CD127) and the common $\gamma_c$ chain (CD132) subunits of the IL-7R were assessed in WT and IκBαΔN CD4 and CD8 naïve T cells by flow cytometry. Interestingly, levels of IL-7Rα were reduced on IκBαΔN $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells (25% and 20% of WT, respectively), whereas levels of CD132 or CD3ε were not (Fig. AII.6A and AII.7), indicating that NF-κB selectively regulates the IL-7Rα chain in T cells. Due to impaired positive and negative selection of CD8SP but not CD4SP IκBαΔN (Voll et al., 2000), levels of IL-7Rα were also assessed in OT-II IκBαΔN CD4 naïve T cells (Fig. AII.6B). Similar to results in polyclonal T cells, IL-7Rα levels were diminished in OT-II IκBαΔN cells suggesting that the impact of NF-κB on IL-7Rα expression is independent of effects on thymic selection.

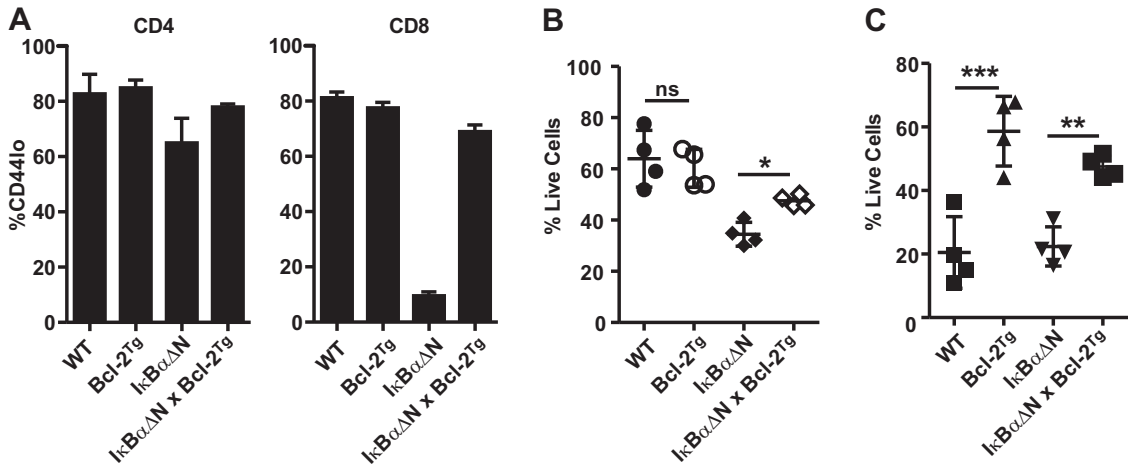


**Figure AII.5: Bcl-2$^{Tg}$ restores percentages and survival of IκBαΔN naïve T cells**
**A.** Percentages of CD44$^{lo}$ cells were assessed by flow cytometry in CD4$^{+}$ and CD8$^{+}$ T cells from WT (n = 3), Bcl-2Tg (n = 3), IκBαΔN (n = 6) and IκBαΔN x Bcl- 2$^{Tg}$ (n = 2) mice. **B and C.** *In vitro* survival of WT (n = 4), Bcl-2$^{Tg}$ (n = 4), IκBαΔN (n = 4) and IκBαΔN x Bcl-2$^{Tg}$ (n = 4) CD4$^{+}$CD44$^{lo}$ T cells cultured in the presence of 1 ng/ml IL-7 for 48h (**B**) or in medium only (**C**). Each point represents the average of technical triplicates and data are pooled from 4 independent experiments. ns = not significant; *, $p < 0.05$; ***, $p<0.001$. Data was analyzed by one-way ANOVA with Bonferroni post-test.

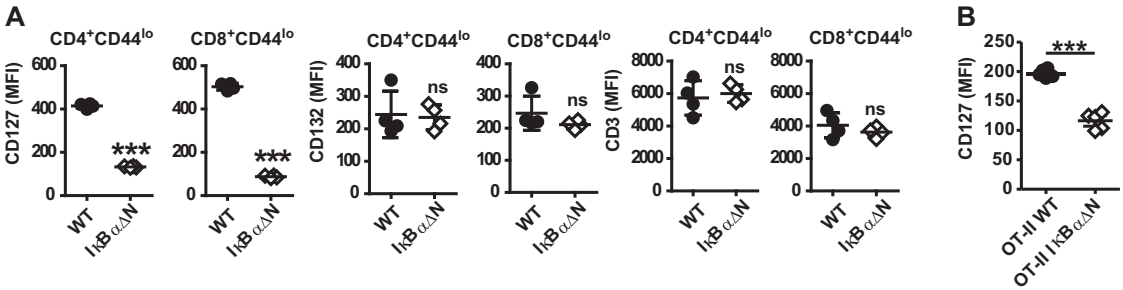


**Figure AII.6: Basal NF-κB controls IL-7Rα expression**
**A.** Mean fluorescence intensity of CD127, CD132 and CD3ε for WT (n = 3) and IκBαΔN (n = 3) CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ T cells. **B.** CD127 was analyzed in peripheral blood CD4$^{+}$TCRVβ5.1$^{+}$ CD44$^{lo}$ T cells from OT-II WT (n = 6) and IκBαΔN mice. ***, $p<0.001$; ns = not significant. Results are representative of at least three independent experiments.

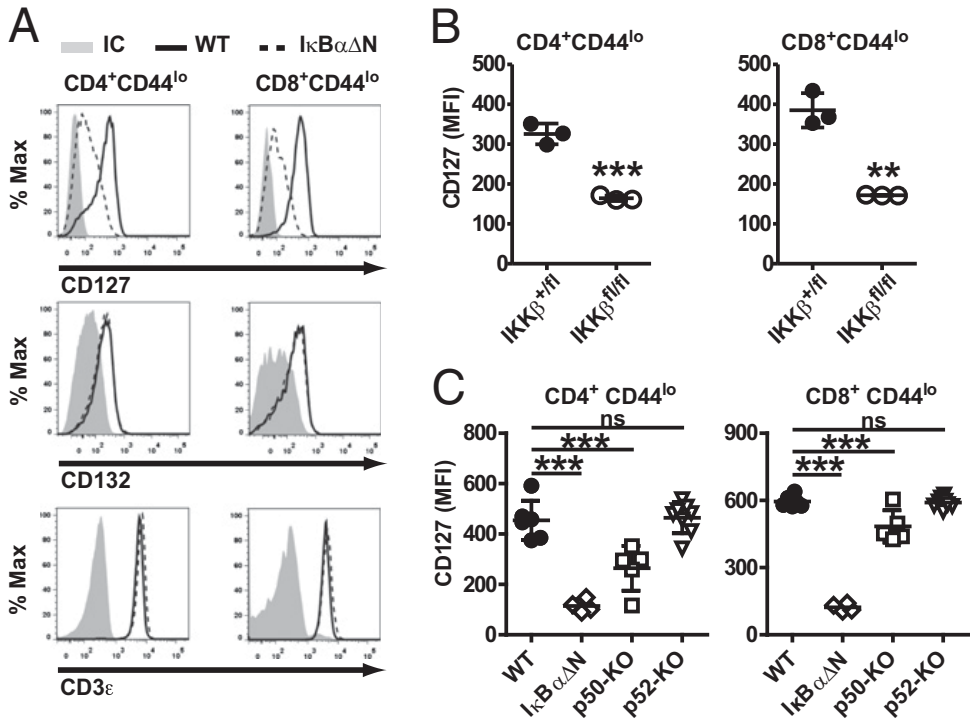


**Figure AII.7: Basal NF-κB is required for IL-7Rα expression**
**A.** Histograms displaying IL-7Rα/CD127 (top), $\gamma_c$/CD132 (middle) and CD3ε (bottom) as analyzed by flow cytometry in WT (solid line) and IκBαΔN (broken line) $CD4^+CD44^{lo}$ (left) and $CD8^+CD44^{lo}$ (right) T cells. **B**. Expression of CD127 in $CD4^+CD44^{lo}$ (left) and $CD8^+CD44^{lo}$ (right) splenocytes from $CD4^{Cre}IKK\beta^{+/fl}$ or $CD4^{Cre}IKK\beta^{fl/fl}$ mice. Data was analyzed by Student's t-test. **C**. Expression of CD127 in WT (n=4), IκBαΔN (n=5), $p50^{-/-}$ (n=5) and $p52^{-/-}$ (n=8) mice, as assessed by flow cytometry and one-way ANOVA with Bonferroni post-tests for pairwise comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Results are representative of at least two independent experiments.

The IκBαΔN transgene is driven by the proximal Lck promoter, therefore its expression is turned on early during thymic development (Boothby et al., 1997), which results in reduced IL-7Rα expression in IκBαΔN CD4SP and CD8SP thymocytes (Fig. AII.8A). $CD4^{Cre}$ $IKK\beta^{fl/fl}$ mice, which delete IKKβ in double positive thymocytes and have overall normal thymic development (Fig. AII.8B) (Schmidt-Supprian et al., 2003), were used to confirm the requirement of NF-κB for IL-7Rα expression. Indeed, IL-7Rα levels on peripheral $CD4^{Cre}$ $IKK\beta^{fl/fl}$ CD4 and CD8 naïve T cells were half those in WT controls

(Fig. AII.7B) and below those in IL-7Rα$^{+/-}$ T cells (Fig. AII.9A). The impact of reduced IL-7Rα expression on CD4$^{Cre}$IKKβ$^{fl/fl}$ and IL-7R-heterozygous T cells on IL-7-mediated survival was determined *in vitro*. Survival of IL-7R$^{Cre+/-}$ and WT T cells was equivalent, while that of CD4$^{Cre}$IKKβ$^{fl/fl}$ naïve CD8 T cells was slightly impaired but significantly higher than that of IκBαΔN T cells (Fig. AII.9B). Thus, these data suggest a threshold of IL-7Rα is required to confer full survival in the presence of IL-7, and that NF-κB is a limiting factor to attain this threshold.

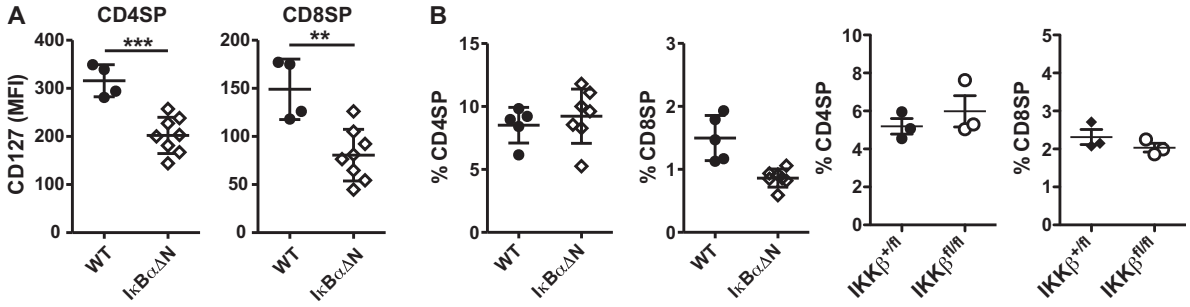


**Figure AII.8: Thymic development in CD4$^{Cre}$IKKβ$^{fl/fl}$ and IκBαΔN mice**
**A.** *Ex vivo* CD127 expression was assessed by flow cytometry in CD4$^{+}$SP and CD8$^{+}$SP WT (n = 4) and IκBαΔN (n = 8) thymocytes. Results are pooled from two independent experiments and analyzed by Student's t test. **, p<0.01; ***, p<0.001. **B.** Percentage of CD4SP and CD8SP thymocytes in WT (n = 5) vs IκBαΔN (n = 7) and CD4$^{Cre}$IKKβ $^{+/fl}$ (n = 3) vs CD4$^{Cre}$IKKβ$^{fl/fl}$ (n = 3) mice.

Two pathways of NF-κB activation have been described: RelA/p50 and cRel/p50 dimers are activated in the canonical (IKKβ and IκBα) pathway whereas the alternative pathway is dominated by RelB/p52 dimers (Hayden and Ghosh, 2004). IL-7Rα expression was reduced in p50- but not p52- deficient CD4 and CD8 naïve T cells (Fig. AII.7C), but to a lesser extent than in IκBαΔN or CD4$^{Cre}$IKKβ$^{fl/fl}$ T cells. Our results indicate that the canonical, but not the alternative NF-κB pathway regulates IL-7Rα expression.

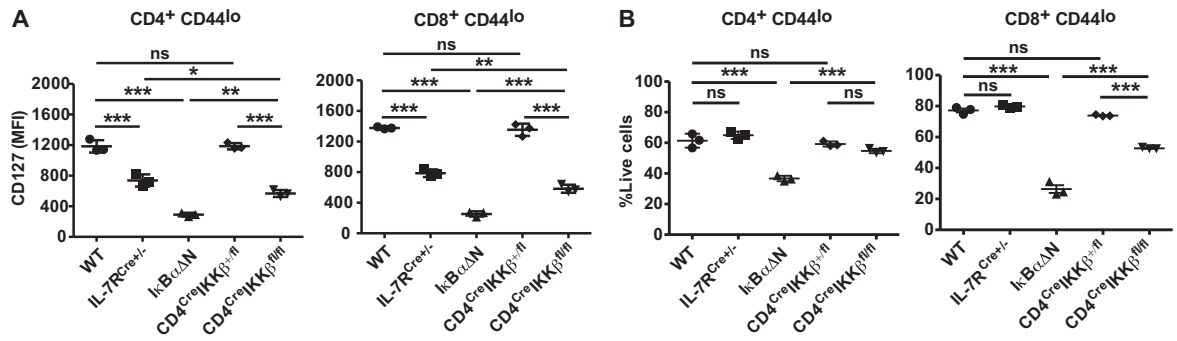


**Figure AII.9: IL-7Rα expression and *in vitro* survival of IL-7R$^{+/-}$ and CD4$^{Cre}$IKKβ$^{fl/fl}$ naïve T cells**

*Ex vivo* CD127 expression (**A**) and viability upon 48h culture in 1 ng/ml IL-7 (**B**) of splenic CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ T cells from WT (n= 3), IL-7R$^{Cre+/-}$ (n = 3), IκBαΔN (n = 3), CD4$^{Cre}$IKKβ$^{+/fl}$ (n = 3) and CD4$^{Cre}$ IKKβ$^{fl/fl}$ (n = 3) mice. ns = not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Results were analyzed by one-way ANOVA with Bonferroni post-tests.

IL-7 availability is increased in lymphopenic mice and humans (Mackall et al., 2011). To address whether reduced IL-7Rα in IκBαΔN T cells was due to increased IL-7 availability and consequent receptor downregulation *in vivo*, IκBαΔN T cells were harvested from their *in vivo* environment and cultured *in vitro* in the absence of IL-7 (18). In contrast to WT T cells that increased their IL-7Rα over a 48h culture, IκBαΔN T cells were unable to do so (Fig. AII.10A). Additionally, to avoid potential *in vitro* artifacts, WT and IκBαΔN T cells were co-adoptively transferred into lymphoreplete mice. Similar to the results *in vitro*, IL-7Rα was not restored in IκBαΔN T cells (Fig. AII.10B), suggesting that NF-κB is intrinsically required for IL-7Rα expression.

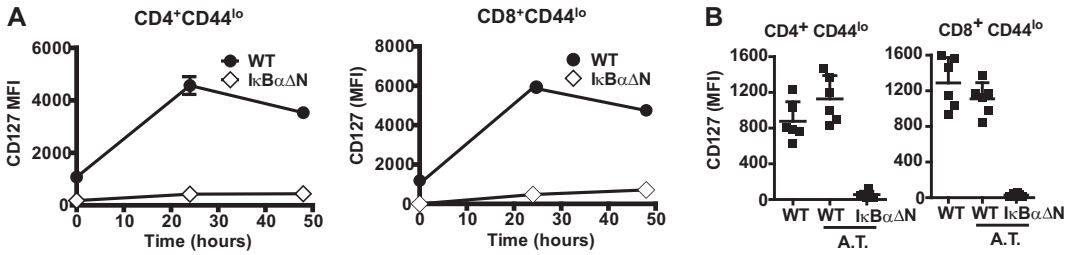


**Figure AII.10: Reduced IL-7Rα expression in IκBαΔN T cells is not due to lymphopenic environment**

**A.** WT and IκBαΔN splenocytes were cultured in medium and expression of CD127 was analyzed by flow cytometry over time in $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}7AAD^-$ live cells. Data is representative of three different experiments. **B**. WT (n = 6) and IκBαΔN (n = 6) $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells were adoptively transferred (A.T.) into WT CD45.1 congenic recipients and CD127 expression was assessed one week later expression by flow cytometry. Data is pooled from two different experiments.

5. Transcriptional control of IL-7Rα by NF-κB

To address whether NF-κB governs IL-7Rα transcription, *Il7r* mRNA was analyzed by RT-qPCR in WT and IκBαΔN CD4 and CD8 naïve T cells. Levels of *Il7r* mRNA were reduced in IκBαΔN CD4 and CD8 T cells (Fig. AII.11A), suggesting that NF-κB is required for *Il7r* transcription. To test the requirement of NF-κB for *de novo* IL-7Rα expression in mature T cells, the IKKβ pharmacological inhibitor 6-amino-4-4-phenoxyphenylethylamino-quinazoline (NF-κBi) was used (Tobe et al., 2003). IL-7 inhibits transcription of *Il7r*, and re-expression of IL-7Rα requires *de novo* transcription. To assess whether NF-κB controls *de novo* gene expression of IL-7Rα, WT T cells were incubated for 24h with IL-7, washed and cultured alone or in the presence of NF-κBi. $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells treated with NF-κBi had a substantial reduction in *de novo* expression of IL-7Rα mRNA (Fig. AII.11B) and protein (Fig. AII.12). These data prompted us to investigate whether NF-κB might directly regulate *Il7r* transcription.

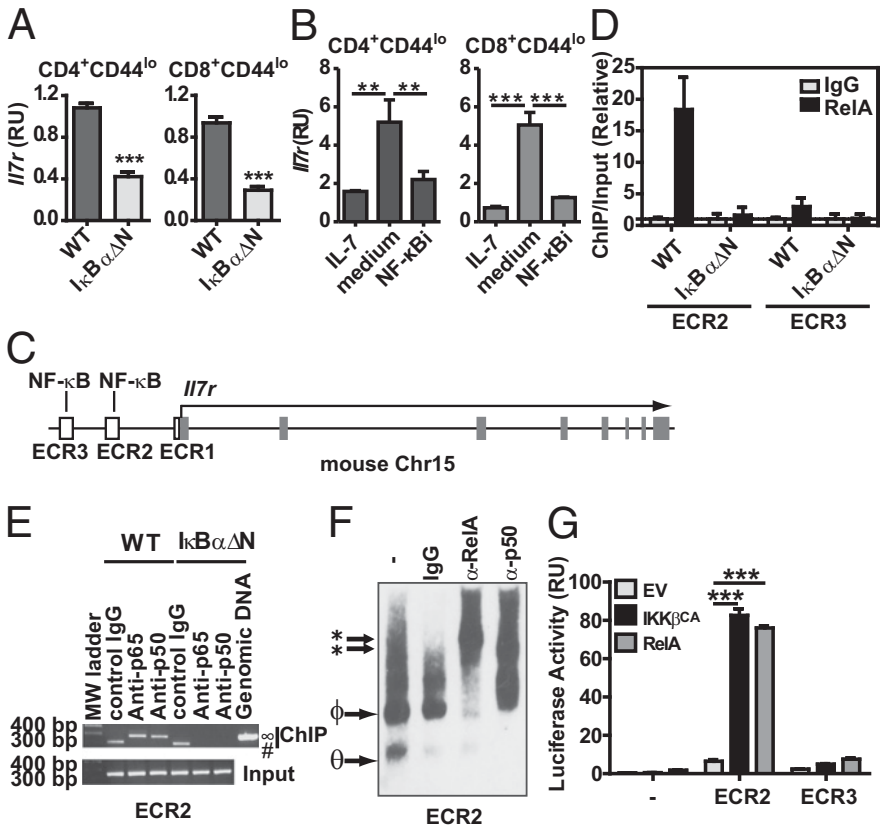


**Figure AII.11: Basal NF-κB controls transcription of *Il7r* in naïve T cells**
**A.** *Il7r* mRNA from WT and IκBαΔN CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ cells was assessed by RT-qPCR, and triplicates were normalized to *Actb*. Results are shown as mean ± SD of triplicates, analyzed by Student's t test, representative of three independent experiments. **B.** WT splenocytes were cultured for 24h with 1 ng/ml IL-7, washed and incubated 24h further with 1 ng/ml IL-7 (IL-7), no IL-7 (medium), or 5 nM amino-4-4-phenoxyphenylethylamino quinazoline (NF-κBi). CD127 expression was analyzed by RT-qPCR in FACS-cell sorted CD4$^{+}$CD44$^{lo}$ live cells with one-way ANOVA and Bonferroni post-tests. **C**. *In silico* analysis of murine *Il7r* locus highlighting evolutionary conserved regions (ECRs) upstream of the 5' transcription start site. ECR2 (-3.6 kb) and ECR3 (-5.6 kb) contain sequences with putative NF-κB binding sites. **D.** Chromatin immunoprecipitation (ChIP) for ECR2 and ECR3 using anti-RelA antibody or isotype control (IgG) in WT and IκBαΔN naïve T cells, as assessed by qPCR. **E.** Semiquantitative PCR following ChIP for ECR2 using anti-RelA, anti-p50 antibodies or isotype control (IgG) in WT and IκBαΔN naïve T cells. ∞: 282 bp expected PCR product; #: non-specific band. **F**. Supershift EMSA for NF-κB-binding site contained in ECR2 using antibodies against RelA, p50 or isotype control. Φ: RelA/p50 and θ: p50/p50 dimers; *: supershifted bands. **G.** Dual-luciferase assay of lysates of 293T cells transfected for 48 h with the luciferase reporter plasmid pGL4.23 alone, or containing ECR2 and ECR3 sequences from *Il7r* gene, plus pRL-TK and control plasmid (EV) or plasmids encoding IKKβ-CA and RelA.

**Figure AII.11, continued:** Results were analyzed by two-way ANOVA with Bonferroni post-tests for pair-wise comparisons. ***: $p < 0.001$. Results are representative of at least two independent experiments.

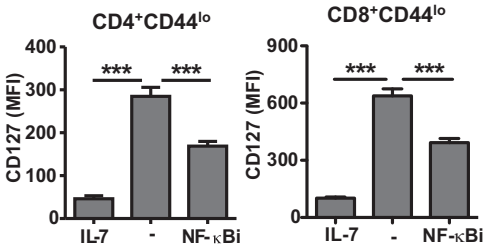


**Figure AII.12: Pharmacological inhibition of NF-κB prevents re-expression of IL-7Rα**
CD127 expression was assessed by flow cytometry in WT and IκBαΔN $CD4^{+}CD44^{lo}$ and $CD8^{+}CD44^{lo}$ T cells that had been treated for 24 hours with 1 ng/ml IL-7, washed and further cultured for 24 hours in 1 ng/ml IL-7 (IL-7), medium (-), or 5 nM of NF-κBi. Graphs depict mean fluorescence intensity ± SD of triplicates. Results are representative of at least three independent experiments. Results were analyzed by Student's t-test, ***: $p < 0.001$.

*In silico* analysis of *Il7r* genomic DNA in eight mammalian species revealed three evolutionary conserved regions (ECR) upstream of the *Il7r* transcription start site (TSS): ECR1 in the proximal *Il7r* promoter, ECR2 at -3.6 kb, and ECR3 at -5.6 kb from the *Il7r* TSS. ECR2 and ECR3 each contain a potential NF-κB-binding site (Fig. AII.11C). Chromatin immunoprecipitation (ChIP) assays revealed that NF-κB/RelA present in WT T cells bound to ECR2 but marginally to ECR3 (Fig. AII.11D). NF-κB1/p50 was also recruited to ECR2 (Fig. AII.11E). In contrast neither subunit was recruited to *Il7r* ECR2 or ECR3 in IκBαΔN T cells (Fig. AII.11D-E). Additionally, EMSA supershift assays confirmed that RelA and p50 bound to this region (Fig. AII.11F). Finally, to test the capacity of NF-κB to enhance *Il7r* expression, reporter assays in 293T cells were performed using ECR2 and ECR3 as enhancers of a minimal promoter driving the

luciferase gene. Co-transfection with plasmids coding for constitutively active IKKβ (IKKβ$^{CA}$) or RelA demonstrated that only ECR2 possessed a functional NF-κB enhancer (Fig. AII.11G). Taken together, these data imply that RelA/p50 controls transcription of *Il7r* through the -3.6 kb ECR2 enhancer.

6. Forced NF-κB activity enhances *Il7r* expression

Given the requirement of canonical NF-κB activation for IL-7Rα expression, we reasoned that manipulations enhancing canonical NF-κB signaling may further augment IL-7Rα levels. To test this possibility, we generated mice expressing constitutively active IKKβ (IKKβ$^{CA}$) specifically in T cells by crossing the Rosa26-Stop$^{FL}$IKKβ$^{CA}$-GFP mice (Sasaki et al., 2006) with Lck$^{Cre}$ transgenic mice (Hennet et al., 1995). The resulting IKKβ$^{CA}$-expressing T cells (Lck$^{Cre}$IKKβ$^{CA}$) co-express the green fluorescent protein (Fig. AII.13A) and display enhanced NF-κB activity (Molinero et al., 2011). The penetrance of the Lck$^{Cre}$ transgene is variable (Schmidt-Supprian et al., 2003), allowing for discrimination of IKKβ$^{CA}$ transgenic (GFP$^{+}$) from non-transgenic cells (GFP$^{-}$) in the same mouse (Fig. AII.13A). IKKβ$^{CA}$-GFP$^{+}$ CD4$^{+}$CD44$^{lo}$ and CD8$^{+}$CD44$^{lo}$ T cells had double the amount of IL-7Rα compared to their WT or GFP$^{-}$ counterparts, both at the protein (Fig. AII.13B) and mRNA (Fig. AII.14) levels. These data further support the conclusion that basal NF-κB activity intrinsically enhances *Il7r* expression in naïve T cells.

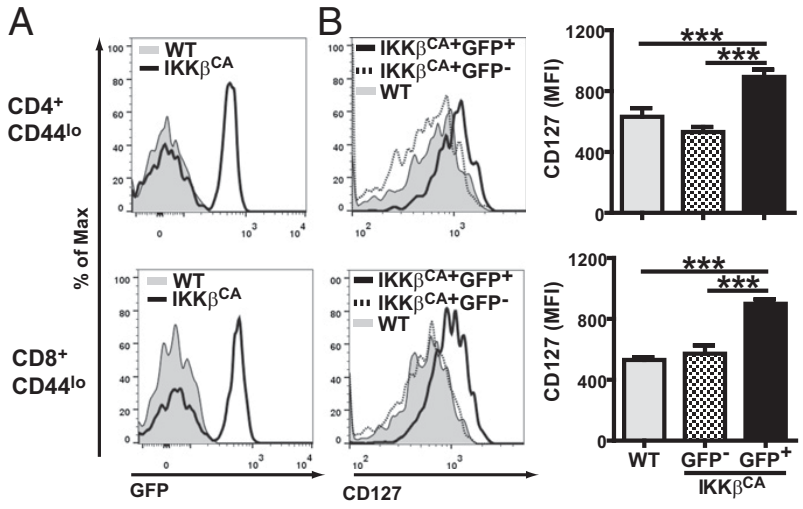


**Figure AII.13: Constitutive active IKKβ enhances IL-7Rα expression**
**A.** Expression of GFP was assessed by flow cytometry in $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells from WT and $Lck^{Cre}$ $IKK\beta^{CA}$ mice. **B.** CD127 expression was assessed by flow cytometry in $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ T cells from WT (n=4) and $Lck^{Cre}$ $IKK\beta^{CA}$ (n=6, gated on $GFP^-$ and $GFP^+$ events) mice. Results were analyzed with one-way ANOVA and Bonferroni post-tests. ***: $p < 0.001$.

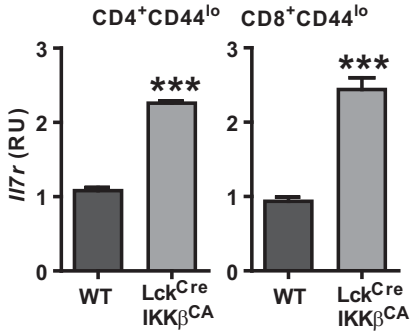


**Figure AII.14: Constitutive active IKKβ promotes transcription of *Il7r***
**A.** *Il7r* mRNA from WT and $Lck^{Cre}IKK\beta^{CA}$ $CD4^+CD44^{lo}$ and $CD8^+CD44^{lo}$ cells was assessed by RT-qPCR, and triplicates were normalized to *Actb*. Results are shown as mean ± SD of triplicates, representative of three independent experiments. Results were analyzed by Student's t-test, ***: $p < 0.001$.

7. NF-κB controls IL-7Rα expression in human T cells

To test whether basal NF-κB activity is important for IL-7Rα expression in human T cells, the IKKβ pharmacological inhibitor (NF-κBi) was used. Human peripheral blood

mononuclear cells (PBMCs) from healthy volunteers were subjected to IL-7-mediated IL-7Rα downregulation and re-expression as described for mouse splenocytes (Fig. AII.11B). Doses of NF-κBi of 5 nM or greater, while not affecting cell viability (Fig. AII.16), limited *de novo* IL-7Rα expression by 35-47% in $CD4^+CD45RO^-$ and $CD8^+CD45RO^-$ naïve T cells, as assessed by flow cytometry in live-gated cells (Fig. AII.15). These data suggest that NF-κB controls the expression of IL-7Rα in both human and mouse T cells, therefore therapies targeting NF-κB activity could potentially impact naïve T cell survival in human patients.

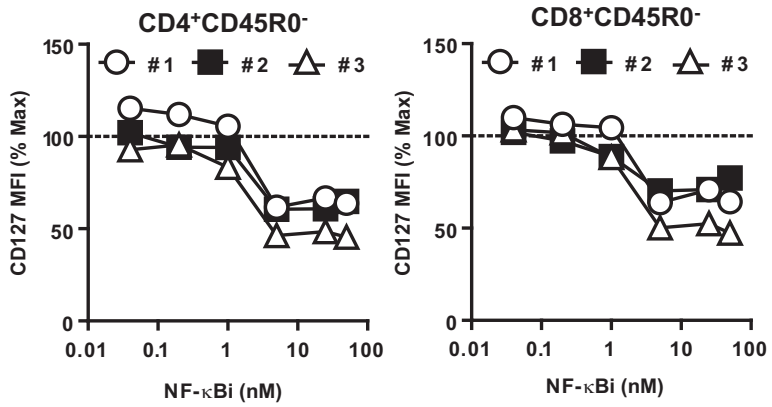


**Figure AII.15: Pharmacological inhibition of NF-κB reduces expression of IL-7Rα in human peripheral T cells**
PBMCs from healthy individuals were cultured for 24h with 10 ng/ml rhIL-7, washed and incubated for 24h with increasing doses of amino-4-4-phenoxyphenylethylamino quinazoline (NF-κBi)**.** Expression of CD127 was assessed in $CD3^+CD4^+CD45R0^-$ and $CD3^+CD8^+CD45R0^-$ live-gated cells and represented as compared to maximum (NF-κBi = 0 nM). Results are representative of two independent experiments.

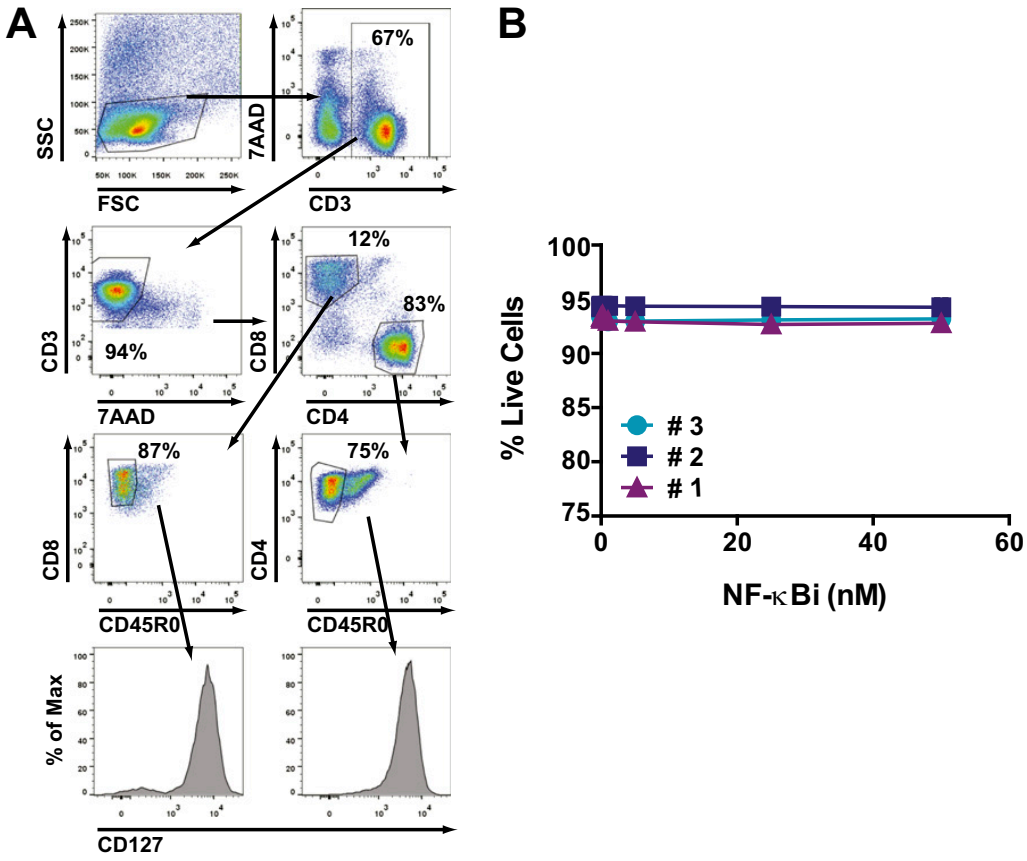


**Figure AII.16: Flow cytometry gating strategy and viability of cultured human PBMCs**

**A.** Gating strategy **B**. Viability of PBMCs from three healthy donors that had been stimulated for 24h in the presence of 10 ng/ml recombinant human IL-7 (Peprotech), washed and further cultured for 24h in complete RPMI alone, 10 ng/ml IL-7, or 0.04, 0.2, 1, 5, 25 and 50 nM of NF-κBi (6-Amino-4-(4-phenoxyphenylethylamino)-quinazoline). Viability was analyzed as percentage of 7AAD- cells.

E. Discussion

Survival of naïve T cells is essential to maintain T cell homeostasis and prevent lymphopenia and immunodeficiency. Persistence of naïve T cells depends on intermittent signaling through IL-7Rα and tonic TCR stimulation (Boyman et al., 2012). In the present study we show that basal NF-κB activity is essential for the survival of naïve T cells. Rather than controlling homeostatic signals induced by tonic TCR

stimulation, basal NF-κB activity in naïve T cells is a limiting factor to enhance transcription of *Il7r*. In antigen-experienced T cells NF-κB activity directly induces expression of pro-survival factors such as c-FLIP and Bcl-$x_L$ (Mora et al., 2003). In contrast, our findings suggest that NF-κB-dependent survival of naïve T cells relies, at least in part, on Bcl-2 upregulation upon IL-7 exposure. Supporting our results, Silva *et al*. recently reported that IKKβ is required for IL-7Rα expression and homeostatic proliferation (Silva et al., 2014). Together with our findings, both studies define a role for NF-κB in the control of naïve T cells homeostasis both in lymphoreplete and lymphopenic hosts.

The signals driving basal NF-κB signaling in quiescent naïve T cells are not known. Several receptors of the TNFR superfamily present in naïve T cells may deliver basal NF-κB signaling. TNFR and CD27 signaling have been shown to promote IL-7Rα expression in an NF-κB dependent manner (Park et al., 2004; Silva et al., 2014). Therefore, it is possible that these receptors are part of the physiological network driving basal NF-κB activity in naïve T cells.

The models of NF-κB-deficient T cells used in the present study display varying degrees of impairment in IL-7Rα expression, with the lowest in IκBαΔN naïve T cells, followed by $CD4^{Cre}IKK\beta^{fl/fl}$ and finally p50-deficient cells. Differences in the quantity and/or quality of basal nuclear NF-κB in the different NF-κB-impaired T cells may account for their different degrees in reduction of IL-7Rα expression. While RelA nuclear translocation is mostly impeded in IκBαΔN T cells by virtue of the IκBα superrepressor (Boothby et al., 1997), IKKα partially compensates the canonical NF-κB pathway in

IKKβ-deficient T cells (Schmidt-Supprian et al., 2003) and RelA/p52 complexes [generated only in particular circumstances (Hayden and Ghosh, 2004)] might suboptimally compensate for the canonical RelA/p50 complexes.

The impact of canonical NF-κB activity in the development and survival of naïve T cells is stronger in CD8 than CD4 T cells, as observed in several mouse models with defective canonical NF-κB signaling, either by overexpression of dominant-negative IκBα proteins in T cells, or by deletion of either IKKβ or NEMO (Boothby et al., 1997; Jimi et al., 2008; Schmidt-Supprian et al., 2003). Given the importance of IL-7Rα in CD8 thymic development (Park et al., 2007; Tan et al., 2001), our results suggest that the reduced number of CD8SP thymocytes in IκBαΔN mice may be due to their defective IL-7Rα expression (Fig. AII.8). Deficiencies of other transcription factors that control IL-7Rα expression, such as Foxo1 and Ets1, also lead to a more profound reduction of peripheral naïve CD8 than CD4 T cells (Grenningloh et al., 2010; Kerdiles et al., 2009; Ouyang et al., 2009), strengthening reports that IL-7Rα controls development and survival of naïve CD8 T cells more stringently than of CD4 naïve T cells (Park et al., 2007; Tani-ichi et al., 2013). Our findings that NF-κB regulates IL-7Rα in both T cell compartments, but has a stronger impact in CD8 T cell survival, further support this theory.

In addition to Foxo1 and Ets1, several other transcription factors have been implicated in the genetic control of *Il7r*, mostly through the binding to two evolutionary conserved regions upstream of the *Il7r*. ECR1 is located in the proximal *Il7r* promoter, spanning 200 bp, and it contains the binding sites for Runx1, PU.1 (active only in B

cells), Ets1 and GABPα (DeKoter et al., 2007; Grenningloh et al., 2010; Lee et al., 2005a). ECR2 is located 3.6 kb upstream of *Il7r*, and this region was previously described to contain binding sites for NF-κB, GATA3, Foxo1/Foxp1 (Kerdiles et al., 2009; Ouyang et al., 2009) and glucocorticoid receptor (Lee et al., 2005a). Although the NF-κB binding site in the ECR2 had been previously predicted, we demonstrate for the first time that RelA/p50 dimers bind to this sequence and confer enhancer characteristics. Interestingly, the NF-κB-binding site located in ECR3 (-5.7 kb from *Il7r* TSS) had marginal RelA-binding and enhancer activity, suggesting that NF-κB controls *Il7r* transcription mainly through ECR2.

Our *in vitro* data assessing the enhancer capacity of ECR2 correctly predicted that constitutively active IKKβ would potentiate IL-7Rα expression *in vivo*. Despite higher levels of IL-7Rα, IKKβ$^{CA}$ T cells have increased cell death *ex vivo* (Jimi et al., 2008; Krishna et al., 2012). Kimura *et al.* elegantly showed that continuous IL-7 stimulation in T cells with suboptimal TCR triggering promoted cell death in an IFNγ-dependent manner (Kimura et al., 2013). Interestingly, IKKβ$^{CA}$ T cells produce large amounts of IFNγ, and express half the amount of TCRβ chain compared to WT counterparts (Fig. AII.17), thus raising the possibility that IKKβ$^{CA}$ T cells might die through this mechanism.

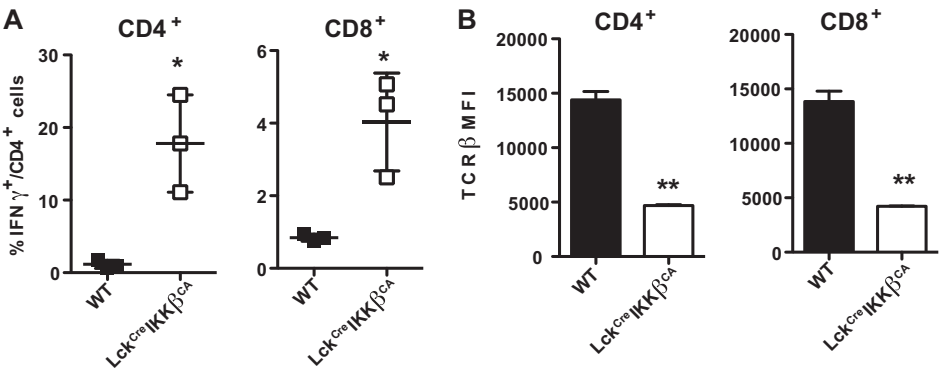


**Figure AII.17: Constitutive IKKβ increases IFNγ production and reduces TCRβ expression**

**A.** IFNγ production in $CD4^+$ and $CD8^+$ T cells from WT (n=3) and $Lck^{Cre}IKK\beta^{CA}$ (n=3, gated on $GFP^+$ events) mice was assessed by flow cytometry following 4h restimulation in the presence of PMA, ionomycin and Brefeldin A. **B.** The mean fluorescence intensities of TCRβ in $CD4^+$ and $CD8^+$ T cells from WT (n=2) and $Lck^{Cre}IKK\beta^{CA}$ (n=2, gated on $GFP^+$ events) mice were assessed by flow cytometry, and are presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$.

Antigen-mediated TCR stimulation, which potently activates NF-κB, also ablates IL- 7Rα expression in T cells. Cekic and colleagues reported that PI3K activation is partially responsible for IL-7Rα reduction in TCR-stimulated cells (Cekic et al., 2013), possibly by phosphorylation and inhibition of Foxo1 (Kerdiles et al., 2009). Transcriptional control of the *Il7r* ECR2 enhancer likely requires a minimal occupancy by multiple transcription factors. As such, TCR/PI3K-mediated Foxo1 inhibition may deprive ECR2 of a key component for its enhancer. We speculate that without Foxo1, the *Il7r* enhancer is not functional despite abundant active NF-κB in TCR-stimulated T cells. In contrast, in naïve T cells, both Foxo1 and NF-κB are available and required for IL-7Rα expression.

The NF-κB binding site present in the ECR2 is conserved between mice and humans. As in mice, pharmacological inhibition of NF-κB following exposure to IL-7

impaired *de novo* IL-7Rα re-expression in human T cells. By contrast, deletion of IKKβ in mouse post-thymic T cells did not affect constitutive levels of IL-7Rα (Silva et al., 2014), suggesting that in mature T cells NF-κB may be required for *de novo* IL-7Rα re-expression but not for physiological maintenance of IL-7Rα. TNF stimulation has been reported to upregulate *IL7R* mRNA in human HeLa cells in an NF-κB-dependent manner (Tian et al., 2005) and ChIP-Seq data from the ENCODE project revealed RelA binding to the homologous *IL7R* ECR2 sequence in human lymphoblastoid cell lines (ENCODE Project Consortium, 2011). Because this evidence supports a role for NF-κB in IL-7Rα regulation in human cells, caution should be exercised in therapies designed to inhibit the NF-κB pathway in immunoinflammatory diseases (Yamamoto and Gaynor, 2001), as these regimens may have deleterious effects on the naïve T cell pool, with potential consequences on immune responses to pathogenic threats.

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