Rapamycin-Induced Allograft Tolerance:
Elucidating Mechanisms and Biomarker Discovery

by

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A thesis submitted in conformity with the requirements

for the degree of Master of Science

Graduate Department of Immunology

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Abstract

The long-term success of transplantation is limited by the need for immunosuppression; thus, tolerance induction is an important therapeutic goal. A 16-day treatment with rapamycin in mice led to indefinite graft survival of fully mismatched cardiac allografts, whereas untreated hearts were rejected after 8-10 days. Specific tolerance was confirmed through subsequent skin grafts and *in vitro* lymphocyte assays that showed recipient mice remained immunocompetent towards 3rd party antigens but were impaired in responding to donor antigens. Mechanisms that account for this tolerant state were then investigated. Splenic $CD8^+CD44^+$ memory T-cells were reduced in tolerant mice but had increased frequencies of the $CD62L^{LO}$ population. $CD4^+CD25^+Foxp3^+$ regulatory T-cells were increased in tolerant mice. Through multiplex PCR, 4 regulatory T-cell related genes were found up-regulated and 2 proinflammatory genes were down-regulated in accepted hearts. This expression pattern may serve as a putative biomarker of tolerance in patients undergoing transplantation.

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List of Abbreviations

$A_{2A}R$	Adenosine receptor 2A
ACR	Acute cellular rejection
Ag(s)	Antigen(s)
AICD	Activation-induced cell death
AIRE	Autoimmune regulator
alloAg(s)	Allogeneic Antigen(s)
alloMHC	Allogeneic major histocompatibility complex
ANOVA	Analysis of variance
APC	Antigen presenting cell
APC*	Allophycocyanins
ARP	Actin-related protein
AVR	Acute vascular rejection
Bcl-x_L	B-cell lymphoma-extra large
BCR	B cell receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
Ci	Curie (includes micro [μ])
CMHD	Centre for Modeling of Human Diseases
CMV	Cytomegalovirus
CNI	Calcineurin inhibitor
cpm	counts per minute
Cr	Chromium
Cs	Cesium
CsA	Cyclosporine A
CSR	Class switch recombination
CTL	Cytotoxic T-lymphocyte
CTLA4	Cytotoxic T lymphocyte antigen 4
Da	Dalton
DAB	3,3'-diaminobenzidine
DAMPs	Danger-associated molecular patterns
DC(s)	Dendritic cell(s)
DN	Double negative
DNA	Deoxyribonucleic acid
dT	Deoxythymidine
DTH	Delayed-type hypersensitivity
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
Fc	Constant region fragment
FcR	Fc receptor

FGL2	Fibrinogen-like protein 2
FITC	Fluorescein isothiocyanate
FKBP12	FK506-binding protein 1A, 12kDa
Foxp3	Forkhead box P3
FRB domain	FKBP12-rapamycin-binding domain
g	Gram (includes nano [n], micro[μ], and milli [m])
GzmB	Granzyme B
H&E	Hematoxylin & eosin
HLA	Human leukocyte antigen
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horse Radish Peroxidase
HSD	Honestly Significant Difference
ICOS	Inducible costimulator
IDO	Indoleamine 2, 3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iNKT cells	Invariant natural killer T cells
i.p.	Intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
ITAM	Immunoreceptor tyrosine-based activation motif
iTregs	Inducible regulatory T cells
IVIg	Intravenous immunoglobulin
KAN^r	Kanamycin resistance gene
KLRF1	Killer cell lectin-like receptor subfamily F
L	Liter (includes micro[μ] and milli [m])
LAG3	Lymphocyte activating gene 3
LCMV	Lymphocytic choriomeningitis virus
LN	Lymph Nodes
LPS	Lipopolysaccharide
m	Meter (includes milli [m] and centi [c])
M	Molar (includes nano [n], micro[μ], and milli [m])
MEM	Modified Eagles Media
mH	minor histocompatibility antigens
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
ND	Not detected
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κ-light-chain enhancer of activated B cells
NK cells	Natural killer cells
NKT cells	Natural killer T cells

nTregs	Natural regulatory T cells
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinases
PMP	Per million population
PRA	Panel reactive antibody
PTEN	Phosphatase and tensin homologue
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAG	Recombination-activating gene
RAPTOR	Regulatory associated protein of mTOR
RNA	Ribonucleic acid
RNA_i	RNA interference
RPM	Rapamycin-only group (C3H/HeJ mice without transplant, but with induction protocol)
SD	Standard deviation
SEM	Standard error mean
SHM	Somatic hypermutation
SHP1	SRC-homology-2-domain-containg protein tyrosine phosphatase 1
SLAM7	Signaling lymphocytic activation molecule family member 7
STAT	Signal transducer and activator of transcription
T_{CM}	Central memory T cell ($CD62L^{HI}$)
TCR	T cell receptor
T_{EM}	Effector memory T cell ($CD62L^{LO}$)
TGFβ	Transforming growth factor β
T_H	Helper T cell
TLR4	Toll-like receptor 4
TMB	Tetramethylbenzidine
TNF	Tissue necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg(s)	Regulatory T cells
TSC	Tuberous sclerosis complex
TTBS	Tris-buffered saline with Tween-20
TxCsA	Cyclosporine group (Allogeneic transplant with C3H/HeJ recipients and cyclosporine protocol)
TxRej	Rejecting group (Allogeneic transplant with C3H/HeJ recipients without induction protocol)
TxSyn	Syngeneic group (Syngeneic transplant with C3H/HeJ recipients without induction protocol)
$TxTLR4^{+/+}$	$TLR4^{+/+}$ control group (Allogeneic transplant with C3H/HeOuJ recipients and induction protocol)
TxTol	Tolerant group (Allogeneic transplant with C3H/HeJ recipients and induction protocol)

1. Introduction

1.1. The immune response

The immune system has evolved a complex network of cells and regulatory pathways that allows for the discrimination and protection of self tissue, while maintaining the ability to recognize and react to diverse and potentially harmful pathogens. The immune response to these pathogens is broadly divided into two separate, but interconnected components. The innate immune system is the first line of defense, providing non-specific recognition of foreign antigens (Ags) through receptors that are germline-encoded with broad specificity. These receptors recognize danger-associated molecular patters (DAMPs), such as cellular components released after tissue damage, and evolutionarily conserved pathogen-associated molecular patterns (PAMPs)[1, 2]. The innate immune system therefore provides early detection and control over pathogens, but is mostly insufficient at clearing them. Instead, the innate system is crucial in activating and regulating adaptive immunity, the second component of the immune response[3].

Adaptive immunity is Ag-specific, requires activation as well as time to develop, and ultimately clears most pathogens. Ag-specificity is achieved through the random recombination of the V, D, and J genes of the T cell receptor (TCR) and B cell receptor (BCR) in T and B cells respectively. This is capable of producing a repertoire of more than 10^8 different receptors[4]. While T and B cells with potentially self-reactive receptors are eliminated or inactivated through central or peripheral tolerance mechanisms described in chapter 1.4, the remaining cells participate in the immune response specifically targeting foreign antigens. The differences between innate and adaptive immunity are described in table 1-1. Together, these mechanisms provide efficient

protection from most pathogens, but also present obstacles in clinical settings where it may be advantageous to introduce foreign Ags such as in transplantation.

	Innate Immunity	Adaptive Immunity
Evolutionary Origin	Early (vertebrates)	Recent (jawed fish)
Receptor Encoding	Germ-line	Somatic
Receptor Recombination	No	Yes
Receptor Repertoire	Limited	Very Large
Target of Receptors	Invariable	Variable
Type of Response	General, Low-Specificity Response	Targeted, Antigen-Specific Response
Onset of Response	Fast	Slow
Memory	No	Yes
Components	Cellular: Neutrophils Basophils Mast Cells Eosinophils Macrophages Natural Killer Cells Soluble: Complement Interferon	Cellular: T-cells B-cells Soluble: Antibodies

Table 1-1. Differences between innate and adaptive immunity. The differences in innate and adaptive immunity are listed. Innate immunity is the first line of defense against pathogens, but is non-specific and often ineffective at clearing pathogens on its own. Instead, innate immunity is also important in activating and priming adaptive responses that are antigen-specific. Adaptive immunity is also capable of developing memory. Together, these two interconnected components of the immune system are efficient at controlling and clearing most pathogens. Adapted from Janeway, C.A., *et al*.[5]

1. 2. Transplantation

1.2.1. Current state of transplantation

The first successful organ transplant occurred in 1954, in which a kidney was removed from a healthy individual and transplanted into his identical twin who had renal failure[6, 7]; since, improvements in the pre- and post-operative care have allowed this treatment to be applied to allogeneic transplants with non-identical donors and recipients. These improvements include more effective strategies for the procurement and storage of organs as well as improvements in surgical techniques. Importantly, the immune response that normally occurs in recipients of allogeneic graft (reviewed in section 1.2.2.) could be inhibited with the advent of novel immunosuppressant agents which are described in section 1.3. The early immunosuppressants, including the anti-proliferative drug azathioprine, corticosteroids that prevent cytokine production necessary to mediate inflammation, and drugs that target T-cells such as antilymphocyte globulin, allowed for one-year graft survival rates between 40-50%[6, 7]. With the introduction of cyclosporine, a calcineurin inhibitor that prevents T-cell activation, graft-survival rates further increased to above 80% in the 1980s[6, 7]. These early innovations led to the increased reliance for transplantation as a treatment for organ-failure and also allowed for the development of transplantation techniques for other solid organs.

The rate of transplantation continues to increase. Between 1993 and 2002, the rate of solid organ transplantation increased in Canada from 49.5 per million population (PMP) to 56.8 PMP[8]. Patient survival rates also continue to improve. For instance, between 1991 and 2000, the 5-year survival rates were approximately 75% for adult receiving liver or

heart transplants[8]. These increases are a result of further advances in immunosuppression therapies, organ preservation, donor-recipient matching techniques and surgical techniques. Of note, recent developments have made pancreatic and small intestine transplantation a viable surgical option[9]. Together, these achievements have significantly improved the survival and quality-of-life of patients receiving transplantations. It has further allowed transplantation to be accepted as the standard-of-care for various end-stage organ failures.

Solid organ transplantation currently remains the primary and most effective treatment for patients with various end-stage organ failures. In 2008, 2,080 patients received a kidney, liver, lung, heart or pancreas transplant in Canada[9], while 27,965 solid organ transplants were performed in the United States[10]. These figures remain near the historical records achieved in the early and mid-2000s and represent a significant increase in the utilization of transplantation as a therapy compared to any other decade since the practice began.

Despite this progress in the field of transplantation, several obstacles remain that prevents its application to greater numbers of patients. Suitable organs for donations have become scarce as a result of increased demand coupled with donation rates that are static[8, 11]. The yearly gap between transplants performed and the patients on the waiting list has grown in Canada from 927 in 1992 to 2230 in 2001[8]. This occurred while the rate of deceased organ donation in Canada fluctuated from a high of 15.3PMP to a low of 13.0PMP between 1993 and 2002, with 1,660 Canadians dying while waiting for a transplant during this time[11]. While obtaining organs remains challenging, the immunosuppression used to prevent their rejection and sustain their long term function

also presents problems. The consequences of these drugs, which are discussed in greater detail in chapter 1.3, include susceptibility to infections, toxicity, and development of cancer. Moreover, immunosuppressants remain ineffective at preventing chronic rejection and patients must be considered for re-transplantation in the long-term after graft function is lost, further exacerbating the strain on the organ donation pool. These current difficulties in transplantation could be alleviated by strategies that reduce or eliminate the requirement for immunosuppression while preventing all types of graft rejection.

1.2.2. Immunobiology of graft rejection

The surgical transplantation of a graft from a non-identical donor of the same species elicits a response from the recipient's immune system. Termed an allogeneic response, this is a result of polymorphic proteins, particularly the major histocompatibility complex (MHC), that are different in the graft than those found in the recipient. The recipient immune system recognizes these differences as foreign which ultimately leads to the rejection of the graft. The specific immune mechanisms involved in the rejection of the graft can vary based on many factors, such as the genetic disparity between the donor and recipient and the organ transplanted. Similarly, treatments have been established that can control certain mechanisms of graft rejection. A summary of the different mechanisms of graft rejection and their treatments is provided in Table 1-2. Here, these immune mechanisms along with the strategies utilized to control them will be discussed.

	Hyperacute Rejection	Acute Humoral Rejection	Acute Cellular Rejection	Chronic Allograft Dysfunction
Onset	Minutes to Hours	Weeks to ~ 3 months	Weeks to ~ 3 months	> 3 months to ~10+ years
Histological Features	- Intestinal hemorrhage - Edema - Neutrophil Infiltration - Fibrin deposition and thrombosis - Rapid graft necrosis	- Interstitial hemorrhage - Edema - Neutrophil Infiltration - Fibrin deposition and thrombosis	- Infiltration of inflammatory cells, particularly T cells and Macrophages - Vasculopathy - Graft cell apoptosis	- Replacement fibrosis in graft parenchyma - Vaculopathy, particularly through vascular stenosis by proliferative lesions - Atherosclerotic lesions
Pathophysiology	Preformed anti-donor antibodies that activate complement upon graft ligation	Anti-donor antibodies that developed after transplant activate complement upon ligation to graft	Recognition of foreign MHC and peptides. APCs activate T cells to promote a $CD8^+$ cytotoxic T lymphocyte response and a delayed-type hypersensitivity reaction	Unclear. Involvement from non-immune (ex/ graft age, severity of ischemia/ reperfusion injury) and immunological (chronic graft rejection through allorecognition) sources possible
Treatments examples	- Pre-screening to determine sensitivity - Depletion of antibodies - Inhibition of complement	- Removal of antibodies - Inhibition/ depletion of B cells - Complement inhibition	- Immunosuppression that targets and prevents components of T cell activation	- No effective treatments

Table 1-2. Mechanisms of graft rejection and their treatment. Different immune mechanisms lead to the development of different types of graft rejection, which are summarized here. The prevalent type of rejection depends on numerous factors, including type of organ transplanted and genetic disparity between donors and recipients. Often, multiple types of rejection are evident in a graft. Left untreated, this will ultimately lead to the loss of graft function.

1.2.2.1. Hyperacute rejection

Hyperacute rejection occurs immediately upon the perfusion of recipient's blood into the donor organ and is the result of preformed cytotoxic antibodies that target the vascular endothelium of the graft[12]. It is more commonly seen in xeno-transplantation (transplantation of organs and tissues between species) but can also be rarely seen in allogeneic transplantation (transplantation of organs and tissues within a species). Upon binding to donor antigens in the vasculature, these antibodies are capable of activating complement through the classical pathway. As a result, C1q, C2, and C4 components of the classical pathway are nearly always found in the endothelium of grafts with hyperacute rejection. Complement activation results in endothelial cell injury through endothelial dysfunction, retraction and sloughing. This promotes interstitial hemorrhage, edema, and neutrophil infiltration. Moreover, complement promotes platelet adhesion and activation, the amplification of the coagulation cascade, and the loss of thromboregulatory function in the vessel, which together leads to accelerated necrosis of graft tissue through fibrin deposition and the occlusion of graft vessels through thrombosis[12-14]. These processes result in the complete loss of graft function within minutes to a few hours after the initial reperfusion of the recipient's blood into the donor organ[13, 14].

The preformed antibodies, which are responsible for the initiation of the events that lead to hyperacute rejection, arise as a result of the prior sensitization of the host towards donor graft antigens. These antibodies can be formed both in allogeneic transplants, where donor and recipient are from the same species with genetic differences, and xenografts where the graft is from a different species and greater genetic disparity exists

between the donor and recipient. In allogeneic conditions, prior sensitization to donor Ags can occur as a result of previous blood transfusions, pregnancies, or previous organ transplantations[12]. This leads to the development of plasma cells and memory B cells capable of producing anti-donor antibodies that recognize. In particular, the presence of immunoglobulin (Ig) G that recognize donor class I and class II MHC (called Human Leukocyte Ag (HLA) in patients) prior to transplantation correlates with an increased risk of developing hyperacute rejection[15]. Xenografts carry a more profound risk of hyperacute rejection since the prevalence of preformed anti-graft antibodies is greater as a result of the larger genetic discordance between the graft and donor. One percent of the circulating antibodies found in healthy individuals are believed to react with xenoantigens. The antibodies are predominantly IgG, but IgA and IgM antibodies are also present[16, 17]. These antibodies primarily target carbohydrate epitopes that are specific to donor graft antigens not found in the recipient, with antibodies developed against α-galactosyl having particular importance in the hyperacute rejection of porcine grafts in humans[18]. It is believed these anti-α-galactosyl antibodies develop in humans as a response to intestinal flora, as the bacteria in the gut may provide a continuous source of α-galactosyl antigenic stimulation[19]. Ultimately, it is the prior exposure to graft antigens that leads to the development of preformed antibodies responsible for the hyperacute rejection of the transplanted organ.

The occurrence of hyperacute rejection has remained low as a result of the ability to identify patients with preformed anti-graft antibodies; new advances also have the potential to prevent hyperacute rejection even in sensitized patients[20]. The current clinical standard is to determine the patient's sensitization towards different HLA alleles in a

panel reactive antibody (PRA) test. Here, recipient antibodies are challenged with a panel of cells with different HLA alleles. If preformed anti-HLA antibodies are present, they would bind the cell, and lyse them upon addition of complement[20,21]. If possible, patients would receive a graft after determining that they are not reactive to donor HLA. However, due to time constraints in obtaining in certain organs, this is not always possible. In these patients, and patients with high PRA positive results, therapeutics measures to decrease the likelihood of hyperacute rejection can be applied. These strategies, which can also be applied to xeno-transplantation, could include the depletion of antibodies through plasmapheresis[22], or the depletion of complement through therapies such as Yunnan-cobra venom factor[23]. To prevent hyperacute rejection in xenografts, transgenic pig organs that lack xenoantigens and over-express complement regulatory proteins are being studied for implementation into the clinic[24]. Together, these screens and therapeutics have minimized the occurrence of hyperacute rejection and thus the focus of intensive study has remained on the prevention of other forms of rejection.

1.2.2.2. Acute vascular rejection

Antibodies that develop after organ transplantation are responsible for the development of acute vascular rejection (AVR; also known as acute humoral rejection). These antibodies primarily target donor HLA class I, but can also target ABO blood Ags if blood types are not matched, graft endothelial cell Ags, and/or other polymorphic proteins called minor histocompatibility Ags (mH) that are expressed on the extracellular components of the graft[25]. These antigens are bound by B cell Receptors, activating B cells which then differentiate into IgM antibody producing plasma cells. Alloantigen activated T-helper (T_H)-2 cells (described in section 1.2.2.3) then provide further signals

to B cells that promote the processes of somatic hypermutation (SHM) and class switch recombination (CSR) that produces higher affinity IgG antibodies. These signals provided by T_H2 cells occur through the cognate interaction between TCR and Ag presentation on B cell MHC class II, co-stimulation such as through the interaction of T cell bound Cluster of Differentiation (CD)-40 ligand (CD40L) with B cell bound CD40, and cytokine production[26-30]. T_H2 cells could alternatively provide help to B cells in the absence of a cognate TCR:MHC interaction with B cells. This occurs if both the T_H2 cell and B cell are in close proximity such as when both are activated by the same graft antigen presenting cell (APC). While B cells engage graft antigen on this APC, T cells can provide costimulation and cytokines for this B cell as the T cell recognizes the MHC on the graft APC. This pathway appears to aid in B cell activation and IgM secretion, but not in promoting conversion to IgG antibody production[31]. Since T cell help is required for the efficient production of alloantibodies, acute vascular rejection is often seen together with acute cellular rejection. In addition to this process, xenografts can elicit an effective B cell response without the need for T cell help. This T-independent process produces IgM antibodies against the xenograft, and in experiments these antibodies caused the rejection of hamster skin grafts in athymic nude rat models that had the same kinetics as rejection in wildtype rats[32]. After developing, these antibodies then begin the process of acute vascular rejection.

The precise mechanisms by which donor-specific antibodies cause AVR are poorly understood. C4d, a complement activation byproduct, has been found to correlate with AVR in renal and cardiac grafts and is utilized as a diagnostic indicator of AVR in biopsies[25, 33]; this strongly suggests a role for complement activation in AVR similar to

that in hyperacute rejection. The initiation of the complement cascade could then lead to the pathophysiological and histological features of AVR, including capillary endothelial cell swelling, interstitial edema, interstitial hemorrhage, neutrophil infiltration, and formation of thrombi[34], which together cause the loss of graft function.

The incidence of AVR is relatively low and effective treatments for its management or prevention are available. Estimates attribute 5% to 25% of graft losses to AVR in patients that had a negative crossmatch to donor MHC prior to transplantation[33]. Removal of donor-specific antibodies after their development is the most common and effective treatment to manage AVR. This involves plasmapheresis that filters blood nonspecifically, removing both proteins and antibodies. This technique can be modified to specifically remove antibodies through immunoadsorption techniques[25]. Moreover, residual antibodies could be inhibited through the administration of intravenous Ig (IVIg). Although the precise mechanism of IVIg is unclear, it is believed that these polyclonal Ig preparations contain anti-idiotypic antibodies that target and neutralize patient antibodies, including donor-specific antibodies. Also, IVIg is believed to bind to BCRs resulting in their down-regulation and the subsequent inhibition of antibody synthesis. Other reported effects of IVIg in blocking pathways important in AVR development include the blocking of endothelial activation, inhibiting complement, and blocking constant fragment (Fc)-γ receptors (FcγR)[25]. Complement inhibition through solubilized complement regulatory protein (CD35) and the monoclonal antibody Eculizumab that targets C5 in the complement cascade are undergoing clinical trails to temporarily treat patients with AVR until alloantibody removal can be initiated[25]. AVR could also be treated, or possibly even prevented, through the inhibition and/or depletion of B-cells. B-

cells can be specifically targeted, inhibited and depleted through Rituximab, a monoclonal antibodies against the B cell surface marker CD20. This antibody has been shown to efficiently decrease the pool of circulating B cells in several trials[25]. Immunosuppressants that target T-cells, such as calcineurin inhibitors, also help to decrease circulating alloantibody because of the importance of T_H2 help in B cell antibody production[27]. Indeed, many of the therapies utilized to treat and prevent acute cellular rejection have shown efficacy in managing AVR.

1.2.2.3. Acute cellular rejection

The central role of T-cells in acute cellular rejection (ACR) has long been appreciated. Early experiments showed the prevention of rejection in neonatally thymectomized mice and athymic nude mice. Moreover, these early studies also demonstrated that adoptively transferring T-cells into lethally irradiated host mice was sufficient to cause rejection of skin and cardiac grafts in the absence of alloantibodies[34]. Specifically, $CD8^+$ cytotoxic T lymphocytes (CTLs) are the major effector cells responsible for graft loss in ACR. These cells become activated through recognition of foreign MHC class I molecules on donor APCs, and subsequently target and lyse donor graft cells expressing the same foreign MHC class I[35, 36]. Activated CTLs are capable of inducing the apoptosis of donor cells through several mechanisms, including through Fas – Fas ligand (FasL) interactions and perforin and granzyme B secretion[37-39]. Although it has been shown experimentally that $CD8^+$ are sufficient to mediate rejection alone, such as in irradiated rodent strains reconstituted with pure $CD8^+$ cells[34] and in interferon (IFN)-$\gamma^{-/-}$ mice where a $CD8^+$ response can still develop and reject the graft[40], it has also been shown in other strains that $CD4^+$ T cell help can increase or is absolutely necessary for $CD8^+$ activity[34]. $CD4^+$ T

cells can differentiate into either T_H1 or T_H2 phenotype after activation through cognate interactions with MHC class II on APCs. T_H2 cells interact with B cells and participate in the AVR response described in section 1.2.2.2, whereas T_H1 cells releases interleukin (IL)-2 that promotes CTL survival and proliferation, and also release IFNγ that increases CTL activity by increasing MHC class I expression on cells[36]. By promoting a proinflammatory state through the release of IFNγ and tissue necrosis factor (TNF)-α, T_H1 cells affect the permeability of vessels, can initiate platelet aggregation, and promote a delayed-type hypersensitivity (DTH) reaction that relies on monocytes and macrophages. In the DTH response, monocytes mature into macrophages that can directly destroy graft tissue through a number of proteolytic enzymes and reactive oxygen species. Indirectly, macrophages can also promote graft destruction by acting as APCs to further activate T cells. They also release a wide variety of proinflammatory cytokines and growth factors including platelet-activating factor and fibroblastic growth factor that can lead to thrombi formation[41,42]. In experimental models, this DTH response alone was shown to be sufficient to mediate graft rejection[43,44]. It has also been proposed that $CD4^+$ cells can be also directly cytotoxic to donor cells through Fas expression and perforin mediated method, although the relevance *in vivo* is not understood[45, 46]. The relative contributions of $CD8^+$, $CD4^+$, and DTH responses in the development of ACR are not well defined, but together these are responsible for the infiltration of inflammatory cells, graft cell apoptosis, and vasculopathy[47,48]. Thus, the induction of these responses after allogeneic MHC (alloMHC) recognition will lead to the loss of graft function through ACR.

The precise mechanisms by which recipient T cells recognize foreign MHC also remain speculative. Defining the molecular basis for this recognition has been difficult because the ability for TCRs to recognize alloMHC is counterintuitive, since recipient thymocytes are restricted to self-MHC through T lymphocyte development[49]. Specifically, self-MHC restriction occurs through positive selection in the thymus, where only T cells with the ability to react to peptides presented on self-MHC progress through development. Despite this restriction, it is estimated that up to 10% of a recipient's T cell repertoire can recognize foreign MHC[50]. This response, which is larger than those elicited by nominal Ags, is thought to be achieved through several proposed molecular mechanisms.

Direct allorecognition of foreign MHC on donor APC by recipient T cells is involved and is sufficient for rejection. This was shown in studies utilizing mice that lacked MHC class II expression on a strain that also lacked T and B cell development due to recombination-activating gene (RAG) deficiency. Here, reconstitution with syngeneic $CD4^+$ T cells from $RAG^{+/+}$ mice led to rejection of allografts that expressed MHC II[51]. Processing of graft MHC was not possible since host APCs lacked MHC class II, and T-lymphocytes must have therefore recognized graft MHC directly. There are two models that could explain this direct recognition. The high determinant density model proposes that different MHC have different amino acids exposed at the peptide binding grove while also presenting self or foreign antigens. Therefore, certain recipient TCR clones will recognize peptides, of either self or foreign origins, as foreign because of the different amino acids that remain exposed on the binding groove of allogeneic MHC. In this model, a greater number of T cells will be activated because all alloMHC would

present this amino acid difference, and this high density would allow for the robust stimulation of T cells that recognize it[52, 53]. Conversely, the multiple binary complex model proposes that different MHCs display a different set of self-peptides. In recipient mice, tolerance may not have been established towards the peptides displayed by allogeneic MHC. Many different recipient T cells may react with the new peptides presented by alloMHC resulting in a robust reaction[52,54]. Both models of direct allorecognition rely on molecular mimicry, such that the allogeneic MHC with a self or allo-peptide resembles a self-MHC molecule presenting a foreign antigen[55-57]. It is also likely that that both models contribute to robust direct allorecognition.

Indirect allorecognition also contributes to graft rejection, although it is unlikely to produce as quick or robust response as direct allorecognition. Indirect recognition occurs after foreign MHC peptides are processed and presented on host MHC on host APC. This process is similar to the recognition of nominal antigens, and results in $CD4^+$ responses because of presentation on self-MHC class II[52]. The need for antigen processing makes the indirect recognition pathway slower than direct recognition. Moreover, fewer T cells would also respond in the indirect recognition pathway since a limited number of antigenic peptides can be derived from the polymorphic foreign MHC[52]. Thus, it is generally regarded that the direct pathway dominates acute responses with indirect pathways contributing to longer term alloantigen presentation. However, indirect recognition alone is sufficient in causing acute rejection, and other groups indicate that in certain TCR transgenic models, indirect activation may be favoured[52, 58] . Therefore the relative contribution of direct versus indirect pathways has not been fully elucidated.

Other mechanisms of allorecognition have been described, although the relevance of these *in vivo* has not been established. In semi-direct allorecognition, host dendritic cells (DCs) are able to obtain intact allogeneic MHCs from donor APCs and endothelial cells through cell-cell contact or through the release and uptake of vesicles. Thus, host DCs would then be able to activate host T cells utilizing alloMHC[59]. In a different recognition pathway, non-hematopoietic graft cells have been reported to activate host T cells. While all previous mechanisms required host T cell activation through APCs, graft vascular endothelial cells in this model have been reported to activate recipient $CD8^+$ T cells through direct allorecognition and by providing costimulation through B7-CD28[60, 61]. These allorecognition pathways, whose relative contribution remains to be elucidated, may also contribute to the overall robust allogeneic response.

As a result of the critical role of T cells, therapeutic strategies to manage or prevent ACR have focused on preventing T cell interactions with foreign MHC and inhibiting T cells activation. This is currently accomplished through a cocktail of immunosuppressant agents, reviewed in section 1.3, which block different targets in the T cell activation pathway. As a result of the development and utilization of these pharmaceuticals, graft loss as a result of ACR has been well managed and the one- and five - year survival rates of transplanted organs have been steadily increasing as described in section 1.2.1.

1.2.2.4. Late graft loss and chronic graft rejection

While the one-year survival rates of organs have steadily improved with the advent of new immunosuppressive therapies, the survival of grafts over the long-term has not changed substantially. Late graft loss can take months, years, and even a decade or longer

to develop in some organs. Among the most common causes of late graft loss is chronic allograft dysfunction, which is defined as the progressive decline in graft function occurring 3 months after transplantation[62], and it is estimated that this accounts for 44% of all kidney losses after one year post-transplantation[62]. Chronic allograft dysfunction is usually accompanied with some common pathophysiological and histological features, such as replacement fibrosis that is often found within the grafts' parenchyma. Vascular pathology is also common in chronic rejection. Proliferative vascular lesions, caused by intimal hyperplasia and neointimal proliferation of smooth muscle cells, can progressively cause vascular stenosis. Similarly, atherosclerotic lesions can occur within graft vessels contributing to graft vasculopathy[63]. These processes lead to the progressive loss of organ function that can be assessed through certain organ specific tests, such as measuring serum creatinine for kidneys or blood glucose for the pancreas. Over time, chronic allograft dysfunction will lead to the complete loss of organ function.

The precise pathophysiological mechanisms that lead to chronic allograft dysfunction are obscure, multifactorial and tend to vary between patients and organs. Non-immunological factors have been described in contributing to its development. For instance, the severity of chronic allograft dysfunction correlates with factors that cause graft injury at the time of transplantation, including donor age, whether the donor was brain dead, method of graft preservation, and the severity of ischemia-reperfusion injury. Factors thought to cause chronic allograft dysfunction after transplantation include the development of subsequent viral infections and whether the recipient was hypertensive or had developed hyperlipidemia. The amount of immunosuppressive drugs, especially calcineurin inhibitors and steroids that the recipient received, was found to exacerbate

chronic allograft dysfunction. Its development was also associated with the ability of the graft to heal following acute rejection episodes[62-65]. Immunological factors are also thought to contribute to chronic allograft dysfunction. The recognition of alloantigen, which in this context is termed chronic rejection, was shown to promote chronic allograft dysfunction. For example, in an experimental heart transplantation model, a more rapid onset of graft arteriosclerosis is observed after pretransplant immunization with donor splenocytes[66]. In patients, T cell infiltration and/or alloantibodies can be found in chronic allograft dysfunction[63, 65]. Although these factors have been identified, the precise cause of chronic allograft dysfunction remains ill-defined.

There are currently no effective strategies available to circumvent or treat chronic allograft dysfunction. Immunosuppressive agents that are effective at preventing acute rejection episodes fail to prevent it, and can possibly exacerbate this by mediating further graft damage. The toxicity associated with immunosuppression and steroids can lead to cell death and an elevation of reactive oxygen species that can lead to further graft dysfunction[62, 64]. Although the 1 and 5 year survival rate of grafts have steadily increased with the advent of new immunosuppression strategies, the lack of therapies targeting chronic rejection results in 10 year survival rates which have remained nearly static at 50% for most organs[62, 64].

1.3. Immunosuppression

Immunosuppression is critical to prevent acute rejection episodes. Many pharmaceuticals are available for this purpose and most target and impair different aspects of T cell allorecognition and activation to achieve this goal. These agents and

their targets are summarized in Figure 1-1. The precise combination and dosing of these agents will vary between patients. However these immunosuppressive agents are also known to cause severe adverse effects. These side effects may lead to their minimization or discontinuation despite the risk of rejection. Nevertheless, the advent of these therapies and the optimization of their use have lead to an increase in the one-year survival rate over 90% for the majority of transplanted organs.

Early immunosuppressive treatments in the 1950s and 1960s were developed on the observations that proliferation of lymphocytes was an important feature of graft rejection; however, the therapies lacked the specificity to solely target these cells. Rather, these drugs affected many cell types and conversely had many side effects. This included the anti-proliferative agent 6-mercaptopurine and azathioprine. These purine analogues prevent further deoxyribonucleic acid (DNA) synthesis when inserted during DNA replication[67]. In 1995, a mycophenolate mofetil (MMF) was registered to replace azathioprine. MMF is more stable, can be taken orally, and is more effective at preventing rejection. This newer drug acts by reversibly inhibiting inosine monophosphate dehydrogenase that blocks de novo purine synthesis. Therefore, DNA synthesis would be impaired in proliferative lymphocytes that rely on de novo purine synthesis, whereas cells that can more effectively acquire purines through the scavenger pathway could, in part, escape its effects[68]. Corticosteroids have also been utilized to complement anti-proliferative drugs since the 1960s. These prevent transcription of cytokines, many of which are necessary for T cell survival and proliferation. Corticosteroids can also inhibit the activity of dendritic cells and B cells, but also adversely effect lipid and glucose metabolism as well as blood pressure regulation[69].

These drugs proved some efficacy in preventing rejection, but lacked specificity for lymphocytes.

The use of polyclonal antibodies to T cells (thymoglobulin) was the first attempt at a therapy that targeted T lymphocytes. These polyclonal cytotoxic antibodies generated in rabbits, goats and horses recognize many different antigens on human T cells[70]. Subsequently, more selective agents were generated to overcome some of the problems associated with the use of polyclonal reagents. Muromonab-CD3, also known as Orthoclone OKT 3, is a monoclonal antibody that works similarly by recognizing the CD3 component of the TCR[71]. Currently these antibody therapies remain in use perioperatively in order to deplete all T cells from recipients before further immunosuppression[70, 71]. This is intended to remove donor reactive T cell that contribute to graft rejection. However this therapy lacks this specificity as all T lymphocytes are depleted in addition to donor-reactive T cells.

Further advances were made in immunosuppression with the advent of pharmaceuticals that could specifically inhibit the function and proliferation of activated T cells. The first such therapy was Cyclosporine A (CsA) which revolutionized transplantation by greatly increased the 1-year survival rates of grafts in the 1980s. CsA is a calcineurin inhibitor (CNI) that blocks the down stream TCR signaling following ligation with the cognate MHC:peptide complex (signal 1)[72]. Due to the high nephrotoxicity of CsA, similar pharmaceuticals continued to be explored. Tacrolimus, a new generation CNI, is one such drug[73]. Other drugs being tested for use as alternatives or in combination with CNIs include agents that block the costimulatory signals (signal 2) required for T cell activation. For example, the drug Belatacept, which is Cytotoxic T-

lymphocyte Antigen (CTLA)-4Ig, outcompetes T cells for B7.1/B7.2 required for T cell activation. CTLA-4Ig binding to these APC molecules can also prevent DCs from maturing[74]. Recently, sirolimus (rapamycin) has gained greater use clinically to prevent T cell activation by inhibiting the mammalian target of rapamycin (mTOR). In T lymphocytes, mTOR activation occurs downstream of IL-2 ligation to IL-2R and constitutes the 3rd signal required for T cell activation. This signal occurs in an autocrine manner, with T cells producing IL-2 after being activated through the TCR and costimulation. Activation of mTOR promotes entry into the cell cycle for proliferation. Rapamycin blockade of mTOR arrests activated cells before the G_0 or $G_1 \rightarrow$ S phase transition[75]. Although these newer immunosuppressive agents are capable of effectively blocking activated T cells, they remain incapable of discriminate between alloactivated T cells and those activated in response to other stimuli.

These immunosuppressive agents required to prevent graft rejection are conversely responsible for many adverse effects that result in greater morbidity and even contribute to the mortality in transplant patients. Since they non-specifically target activated T lymphocytes, the host immune system is impaired in mounting a normal response to infections, even those that are commonly found and easily cleared in the general population. These opportunistic infections include Cytomegalovirus (CMV)[76] and Epstein-Barr Virus (EBV)[77]. EBV infection has also been linked to promoting lymphoproliferative disorders in transplant patients[78]. Other cancers become prevalent with immunosuppression since tumor-specific T cells are also inhibited. Immunosuppressants can also promote cancer development through immune-independent mechanisms. For instance, CsA is been shown to make non-transformed cells more

invasive and can increase their motility[79]. Other complications of immunosuppression unrelated to their immune effects also exist. For instance, CsA is highly nephrotoxic that leads to kidney failure and promotes cardiovascular complications such as hypertension[80]. Tissue injury that results from the toxicity of immunosuppression has also been proposed to promote chronic graft rejection. Together, these effects severely limit the long term success of transplants, contributing to the lower 10-year survival rates of transplanted grafts. This emphasizes the need to reduce or eliminate immunosuppression, which can be accomplished through the development of therapeutic strategies that specifically inhibit donor reactive lymphocytes or promote immunological tolerance towards donor Ags.

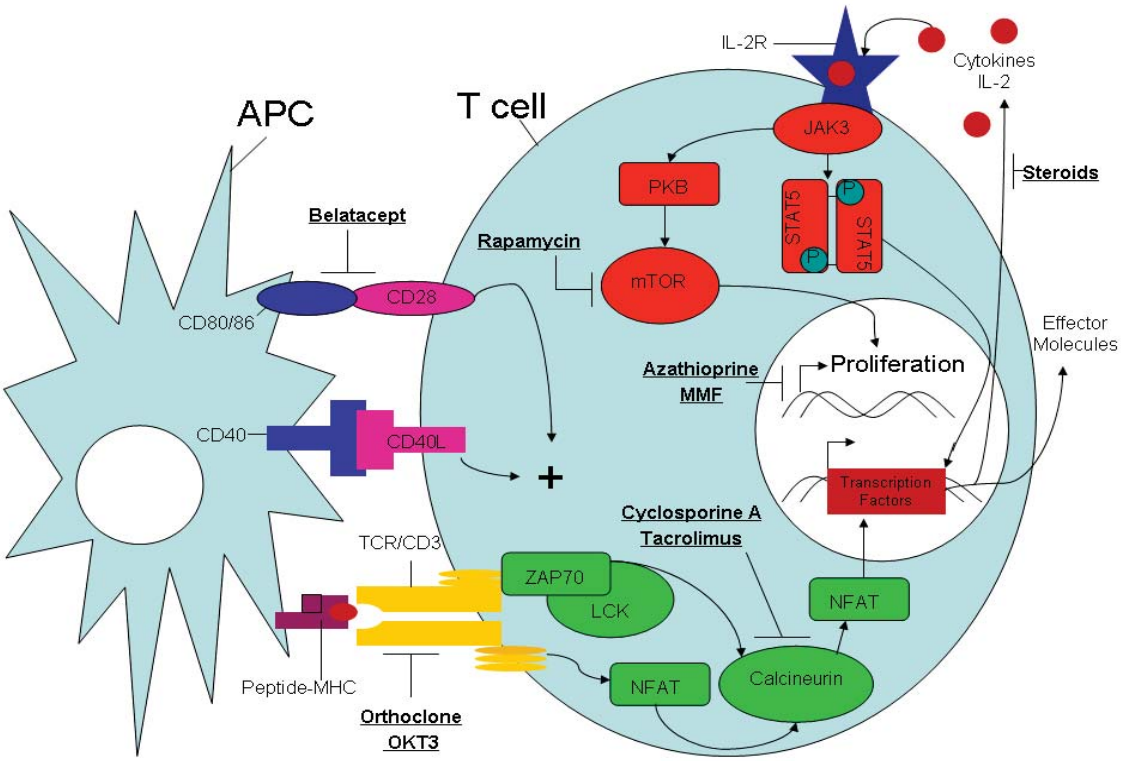


Figure 1-1. Immunosuppressive agents prevent or inhibit different aspects of T-cell activation. Acute cellular rejection is well controlled with various immunosuppressive agents. Examples of common immunosuppressive agents and their targets in the T cell activation pathway are shown. Under normal conditions, T cells become activated following a cognate interaction between TCR and MHC:peptide interaction along with optimal costimulation. These signals can be prevented through the CD3 monoclonal antibody Orthoclone OKT 3 and Belatacept (CTLA-4-Ig), respectively. Signals downstream of these interactions can also be inhibited. Cyclosporine A and Tacrolimus are calcineurin inhibitors that ultimately prevent transcription of cytokines and effector molecules, while steroids prevent cytokine synthesis. Proliferation can be blocked by several pathways as well. It can be directly inhibited with the purine analogue azathioprine or the purine synthesis inhibitor mycophenolate mofetil (MMF). Proliferation can also be blocked by rapamycin which works by inhibiting mTOR, which is downstream of the IL-2 and IL-2R signaling pathway. Adapted from Kahan, B.D., *et al*.[81]

1.4. Immunological tolerance

1.4.1. Overview

Developing tolerance towards a transplanted graft would allow for transplantation to be performed without the need for long term immunosuppression. In a healthy individual, the immune system exhibits tolerance towards self-antigen, which prevents the development of autoimmune diseases; it is believed that underlying mechanisms that account for this could be manipulated to promote graft tolerance. Since T cells have a prominent role in mediating graft rejection, the mechanisms that account for T cell self-tolerance are of particular interest in transplantation. Autoreactive T cells are normally deleted during their development in the thymus in a process termed central tolerance[82]. However, autoreactive T cells can develop and exit into the periphery. These cells are deleted or prevented from activating through many different processes collectively contributing to peripheral tolerance[83]. The results of the deletion and/or inhibition of T cells are summarized in Figure 1-2, as are the consequences of manipulating these tolerance pathways. If these mechanisms could be applied to donor-reactive T lymphocytes, graft tolerance might be achieved.

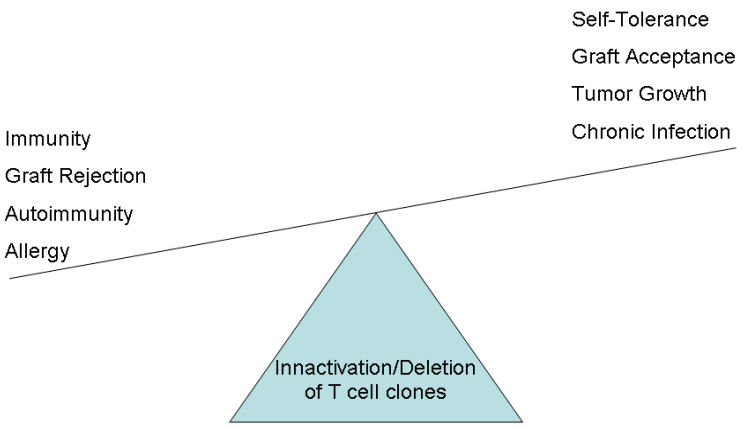


Figure 1-2. Consequences of the inactivation or deletion of T cell clones. Normal tolerogenic mechanisms are in place to prevent autoimmunity while maintaining the ability to recognize and respond to foreign pathogens. Altering the mechanisms of central and peripheral tolerance can have both positive and negative consequences for several disease states.

1.4.2. Central tolerance

Central tolerance is achieved as self-reactive T cell clones are eliminated during T lymphocyte development in a process known as negative selection. Before this selection occurs, precursor T lymphocytes must be positively selected for the ability to recognize self-MHC. This occurs as thymic seeding progenitors, the precursors to T cells, enter the thymic cortex. Here, these cells lack the expression of TCR and lack both CD4 and CD8[84]. After successful V(D)J recombination and subsequent low-level expression of the TCR, the cell upregulates both CD4 and CD8 co-receptors and are thus termed double positive T cells. Then, in the process of positive selection, these double positive T cells interact with the self-MHC of thymic epithelial cells that present self-peptide. Cells that can bind and interact with this MHC with low to intermediate affinity obtain survival

factors and continue to develop, whereas cells that do not bind are eliminated by neglect. Thus, cells capable of recognizing self-MHC survive[85]. The co-receptor that bound to the MHC:TCR complex at this time also remains and the developing T cell eliminates the expression of the other to become a single positive T cell. This ensures T cells are restricted to self-MHC recognition.

Negative selection then removes cells with potential to recognize self-antigens in the periphery. While T cells in the cortex that had a high affinity reaction to self-peptide:MHC on thymic epithelial cells may receive a strong signal that triggers apoptosis, negative selection also occurs in the thymic medulla[86-88]. Here, single positive lymphocytes interact with thymic stromal cells and bone marrow derived APCs, particularly DCs. On self-MHC, these APCs present self peptide that could be derived from proteins in the extracellular media or proteins expressed within APCs. Moreover, certain peripheral tissue-specific antigens, such as insulin, can be expressed and presented on the MHC of thymic stromal cells in the medulla. This expression is achieved through the function of the transcription factor known as autoimmune regulator (AIRE) and allows these peripheral antigens to participate in the negative selection of T cells[89]. Interaction between the TCR of developing single positive thymocytes with these MHC:peptide complexes would signal these cells for apoptosis and results in clonal deletion. Not every peripheral antigen is expressed by AIRE and a subset of autoreactive T cells will escape into the periphery where they are regulated by peripheral tolerance mechanisms. Nevertheless, central tolerance could be manipulated to induce tolerance towards transplanted antigens.

Bone marrow chimerism is an extensively studied technique used to exploit central tolerance mechanisms to confer tolerance towards transplanted cells and tissues. Several animal[90, 91] and clinical[92-94] studies reported tolerance induction following mixed chimerism induction. Successful mixed chimerism in these studies was induced through various protocols. Before the solid-organ transplant, these protocols included low-dose nonmyeloablative irradiation of the recipient, which partially ablates the host's immune system to provide a niche for the engraftment of donor hematopoietic cells[95]. Full chimerism through total lymphoid irradiation has been explored, but presents considerable toxicity and severely impairs normal host immune function; therefore this was not used clinically. An immunosuppressive treatment was also administered to prevent rejection of the bone marrow and to prevent graft-versus-host disease[95]. After engraftment, donor hematopoietic stem cells are capable of differentiating and repopulating cells of the lymphoid and myeloid lineage, including thymic APCs that participate in negative selection. As the recipient's immune system develops, T cells that strongly react with alloMHC on donor APCs will be eliminated. Therefore, tolerance can be induced in these recipients towards subsequent grafts from the same donor.

Utilizing this strategy, a group at Massachusetts General Hospital was able to induce mixed chimerism in all 5 patients in one preclinical study[96, 97]. Following transplantation of a subsequent kidney from the same donor (which contained only a single MHC haplotype mismatch), 4 patients exhibited long-term acceptance of the graft after immunosuppression was withdrawn, which occurred 9-14 months following surgery. Only transient chimerism was achieved in this study, therefore it was speculated that clonal deletion of alloreactive cells was required to induce tolerance, but peripheral

mechanisms may have promoted graft acceptance over the long-term. Nevertheless, this study demonstrated the ability to manipulate the mechanisms of central tolerance to induce graft tolerance.

Despite this success, mixed chimerism presents several obstacles that currently limit its widespread use clinically. The variability of success in establishing mixed chimerism may be a barrier in establishing subsequent tolerance in patients. The percentage of chimerism and the time period that is required to establish tolerance to subsequent solid-organ transplant would need to be determined before its clinical use. Toxicity is still a concern in partial immune ablation through irradiation and may be toxic to some patients. Although irradiation could be minimized to reduce this effect, an insufficient dose could prevent the engraftment of donor hematopoietic cells. Therefore the optimal dose of irradiation must be determined. As a result, other strategies that induce tolerance have been explored, particularly strategies that exploit peripheral tolerance mechanism.

1.4.3. Peripheral tolerance

1.4.3.1. Anergy and activation-induced cell death

Certain autoreactive T-lymphocytes that exit the thymus can be prevented from activation by becoming quiescent or deleted following suboptimal costimulation. Under normal pathogenic conditions, APCs upregulate costimulatory molecules that are required for efficient activation of naïve T lymphocytes in addition to the ligation of TCR with the appropriate MHC:peptide complex[98]. Examples of these upregulated costimulatory molecules on APCs include the well characterized CD80 and CD86 molecules (B7.1 and B7.2, respectively) which bind to CD28 on T cells, and inducible

costimulator ligand (ICOS-L) that binds to the T cell membrane molecule, ICOS. These costimulatory interactions transmit signals into the T cells that stimulate the function of transcription factors such as nuclear factor of activated T-cells (NFAT) and subunits of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB). After costimulation, these transcription factors increase expression of pro-survival molecules such as B-cell lymphoma-extra large (Bcl-x_L), prevent the expression of FasL that could be used to trigger apoptosis, and increase expression and release of IL-2 required for sustained proliferation[99, 100]. However, in the absence of pathogenic or danger signals when Ags are likely to be derived from self-molecules, APCs down-regulate the expression of these costimulatory molecules. Subsequent TCR ligation with MHC:peptide complexes without costimulation will prevent T cell activation as these cells will enter a quiescent state called anergy or become clonally deleted through activation-induced cell death (AICD). Thus, the lack of costimulation is important in promoting peripheral tolerance by preventing the activation of certain autoreactive T lymphocyte clones.

In the absence of costimulation, the factors which determine whether a T cell will undergo anergy or AICD are poorly understood. It is believed that the Ag dose and the frequency of TCR stimulation are critical in determining this outcome. T cells that are repeatedly stimulated with low doses of Ag or continuous Ag stimulation are thought to undergo AICD[101]. TCR stimulation in this context increases the expression of FasL and the interaction of this molecule with Fas on the same T cell can trigger apoptosis[99-100]. Subsets of T cells also respond to a single high dose of Ag through AICD, but others have been shown to become anergic[101]. Anergy can also be actively induced through cytokines such as transforming growth factor β (TGFβ)[102]. Together, these mechanisms

ensure that T cell responses are prevented in the absence of costimulation when Ags are likely to be self-molecules.

Anergy or AICD induction in alloreactive cells has been attempted experimentally and clinically to promote tolerance towards transplanted grafts. Costimulatory blockade has been used in animal models to prolong graft survival, and even induce graft tolerance across full MHC mismatched barriers in certain models[103]. The blockade utilizes a monoclonal antibody that binds and blocks CD40L on T cells, thereby blocking its interaction with CD40 for costimulation. This protocol also utilized CTLA-4-Ig, which binds to and prevents T cell access to the costimulatory molecules CD80 and CD86 on APCs[103]. Together, this effectively prevents costimulation from occurring and promotes anergy or AICD in alloreactive cells. CTLA-4-Ig has also been developed into a pharmaceutical for use in patients called Belatacept. Unlike the animal models, this strategy has proven to be only slightly more beneficial at preserving graft function compared to other immunosuppressants such as cyclosporine and has not led to the development of tolerance in patients[104, 105]. Furthermore, Belatacept lacks specificity towards alloreactive cells and compromises immune responses to all antigens. Also, this drug would require continuous administration since inactivated alloreactive cells can be regenerated in the thymus. As a result, other tolerance inducing strategies in the periphery have also been explored.

1.4.3.2. Immunoprivileged sites and ignorance by immune cells

Immune responses towards antigens can be prevented in the periphery if these antigens are located in immunoprivileged sites. In humans, this mechanism is particularly

important in maintaining the integrity of tissues that have limited capacity for self-renewal. This includes the brain, the anterior chamber of the eye and the cornea[106]. Immunoprivileged sites are also important in order to allow for reproduction and to prevent abortion of fetuses. Since both sperm and fetuses express antigens that could be recognized as foreign, the testes and pregnant uterus have developed mechanisms that allow it to escape normal immune surveillance[106]. New insights into how these immunoprivileged sites are established can provide novel mechanisms to exploit to similarly establish tolerance to transplanted grafts.

The understanding of the features that account for immunoprivileged sites has evolved greatly. Once, these sites were thought to be a phenomenon that occurred because of physiological and anatomical structure of these organs that allowed for Ags to be sequestered from immune cells. For instance, these sites lack conventional lymphatic drainage and the blood-brain barrier was thought to prevent lymphocyte migration into the brain[107]. This theory has been widely discredited in part due to studies that showed antigens injected into the anterior chamber of the eye and the brain entered peripheral lymph nodes[108]. Alloantigen antibodies found in multiparous females also indicates the maternal immune system has access to fetal antigens[106]. Therefore, sequestration of antigens could not solely account for the establishment of immunoprivileged sites. Instead other mechanisms to escape immune recognition have been described at these sites. MHC class 1a molecules are reduced or absent in the eye, brain, and trophoblasts, allowing these tissues to avoid recognition by activated CTLs[109]. These tissues also increase expression of nonclassical MHC class 1b molecules which inhibit lysis that would be mediated by natural killer (NK) cells in response to the missing MHC class 1a

molecules[110, 111]. Furthermore, molecules that induce apoptosis in inflammatory cells, such as FasL and TNF-related apoptosis-inducing ligand (TRAIL) are upregulated on tissues in many of these sites[112, 113]. Complement regulatory proteins, like decay-accelerating factor and membrane cofactor protein, are also elevated here and prevent the activation of complement that would normally lead to lysis of cells through the formation of membrane attack complexes[114]. Soluble inhibitory factors can also be found at these sites, such as TGFβ, α-melanocyte-stimulating hormone, vasoactive intestinal peptide and calcitonin gene-related protein found in the eye[106, 115]. Together, these mechanisms may account for the development of immunoprivileged sites by effectively preventing certain responses.

Sites devoid of any immune response would be particularly susceptible to infections and it is therefore unlikely that the above mechanism prevent all immune responses from occurring. Rather, these can prevent the development of both CTL and DTH responses, the latter which is known to result in ischemic necrosis and excessive injury to bystander cells. This collateral damage would be devastating to tissues with limited regenerative ability such as terminally differentiated corneal endothelial, retinal cells, and many cells of the central nervous system[116, 117]. Instead, these sites can skew and mount less destructive immune responses, such as the T_H2 response. While skewing towards T_H2 response may be sufficient at mediating responses towards certain infectious pathogens, alone it may be insufficient at mediating other responses such as transplant rejection.

Immunopriviledged sites have been utilized extensively to achieve both clinical and experimental transplantation tolerance. For instance, allogeneic corneal transplantations are routine clinical procedures. Immunosuppression is not required

because tolerance is maintained through this immunoprivileged site[106]. Studies also attempt to induce tolerance towards immunocompetent tissues by mimicking the properties of immunoprivileged sites. For example, transplantation of microencapsulated pancreatic islet cells is studied as a therapy for patients with type 1 diabetes[118, 119]. In this strategy, islets are surrounded by a semipermeable membrane allows for cells to sample host blood glucose and release appropriate amounts of insulin, while being protected from the host immune system through a physical barrier. Currently, several limitations prevent the widespread use of this strategy. For instance, during the production of material used for the encapsulation, many contaminants were found to also be produced such as endotoxin and polyphenols[120]. Immune responses also develop against the material itself[119, 121]. However further innovations to this technology, such as inducing stable expression of immunoregulatory molecules, could foreseeable increase its efficacy. Nevertheless, exploiting the properties of immunoprivileged sites remains a promising mechanism to achieve tolerance to transplanted tissues.

1.4.3.3. Regulatory T cells

Peripheral tolerance can also mediated by certain subsets of immune cells that are capable of negatively regulating effector cells. Although many cells with potential regulatory capability have been described, their role *in vivo* remains to be clearly defined. Examples of these cells include a population of TCRαβ or TCRγδ T lymphocytes that express CD8αα found within the epithelial compartment of the small intestine. These CD8αα T cells have oligoclonal TCRs. They mostly recognize self-peptides, which is consistent with a role for mediating tolerance towards gut tissue antigens[122]. However, the precise role and function of these cells remains speculative. Natural Killer T cells

(NKT cells) are cells which possess CD3 and NK marker, are restricted by CD1d antigen-presenting molecules, and have been shown to have both inflammatory and regulatory functions[123]. The best understood of these is the invariant NKT cell (iNKT), which are named because of its nearly invariant TCR-α chain rearrangement. Several have been shown to recognize self-Ags, such as lyso-phosphatidylcholine[124]. To promote tolerance, iNKT cells have been shown to secrete IL-10 (although studies revealed this may be a dispensable mechanism) and iNKT cells can also induce immature DCs to mature into tolerizing or non-inflammatory DCs that produce IL-10[125,126]. However, the mechanisms that determine whether iNKT cells initiate a pro- or anti-inflammatory response are not understood. Although the function and role of all these regulatory cells remains to be defined, this indicates that the maintenance of peripheral tolerance through regulatory cells is complex and likely involves many different cell subsets.

Double negative (DN) T cells are a regulatory cell type that has been more extensively defined. These cells are identified as TCRαβ^{+}CD3^{+}CD4^{-}CD8^{-}NK1.1^{-}, comprise 1-2% of peripheral T lymphocytes in humans, and can developed in the thymus or periphery[127]. It has been shown that DN T cell mediated suppression occurs through cell-cell contact. This is mediated by direct acquisition of MHC-peptides from APCs. DN T cells can then interact with and suppress other T cells that recognize this MHC-peptide complex[127]. FasL expression on DN T cells has also been described as a mechanism to suppress these other T cells[128]. Chronically activated DN Tregs may also be able to mediate suppression through soluble factors[128]. As a result of their suppressive abilities, DN T cells are an attractive target for allogeneic Ag (alloAg) tolerance induction.

The most extensively studied cell with immunomodulatory capabilities are regulatory T cells (Tregs). They are identified by the expression of CD4 and CD25 surface molecules, as well as the expression of the forkhead box P3 (Foxp3) transcription factor. Several observations have confirmed the role of $Foxp3^+$ as a master regulator of Treg development and function[129]. In patients, mutated Foxp3 causes IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) that is characterized by the development of various autoimmune diseases[130]. The *Foxp3* gene is also the defective in the Scurfy mouse strain and leads to hyperactive $CD4^+$ T cells and increased proinflammatory cytokine production in these mice[131]. The systemic inflammation in Scurfy mice can be prevented by introducing $CD4^+CD25^+$ cells from normal mice. Ectopic retroviral induction of FoxP3 into $CD4^+CD25^-$ also promotes the Treg phenotype in these cells, which were then shown to be capable of inhibiting T cell responses *in vitro* and prevent the development of autoimmunity *in vivo*[132]. Together, this evidence supports a role for $CD4^+CD25^+Foxp3^+$ cells in regulating peripheral tolerance.

The development of these $CD4^+CD25^+Foxp3^+$ cells occurs both in the thymus and the periphery. After normal T cell development in the thymus, a subset of cells express $CD4^+CD25^+Foxp3^+$ and exit into the periphery. These cells are called natural Tregs (nTregs)[133]. Conversely, Tregs that develop in the periphery are termed induced Tregs (iTregs). This occurs as naïve $CD4^+$ peripheral T cells acquire the Treg phenotype. For instance, TCR:MHC ligation in the presence of TGFβ and IL-10 will induce iTreg formation[134]. After development, these Tregs account for 5-10% of the adult immune compartment and can promote tolerance to Ags in the periphery[135].

Tregs can mediate tolerance through several different mechanisms to prevent the activation of naïve T and B cells, as well as inhibit effector cells such as differentiated $CD4^+$ and $CD8^+$ T cells, NK cells, NKT cells, macrophages, osteoclasts and DCs[136]. Activation of Tregs occurs in regional lymph nodes and the spleen. Since Tregs do not require as high concentrations of Ag as conventional T cells to be activated, the interaction of Tregs with cognate MHC class II:peptide complex on APCs, including immature DCs, is sufficient for their activation[137]. After activation, Tregs can promote tolerance by targeting APCs through interactions of CTLA-4 on Tregs that binds to and sends a negative regulatory signal through CD80/86 on APCs. This interaction has been shown to induce DCs to express indoleamine 2, 3-dioxygenase (IDO), a molecule that has regulatory functions[138]. Tregs can also outcompete conventional T cells for MHC binding. Lymphocyte activating gene 3 (LAG3) on Tregs has been shown to bind to MHC class II on MHC. This binding induces a negative regulatory signal in immature DCs through the MHC that prevents DC maturation. This is accomplished through an immunoreceptor tyrosine-based activation motif (ITAM) mediated pathway involving FcγRγ and extracellular-signal-regulated kinase (ERK)-mediated activation of SRC-homology-2-domain-containg protein tyrosine phosphatase 1 (SHP1)[139]. Efficient Ag presentation and costimulation is prevented through this inhibition of DC maturation and thus subsequent activation of conventional T cells is prevented (Figure 1-3A). Conventional T cells can also be directly targeted by Tregs when they are in close proximity to each other, such as when both recognize Ag on the same APC. Since APCs can present different peptides on the MHC of the same cell, Tregs can inhibit the activation of T cells that do not necessarily recognize the same antigen. Treg inhibition of

T cells can be mediate through direct cell-to-cell contact. Through gap junctions, Tregs can induce the upregulation of intracellular cyclic adenosine monophosphate (cAMP) that prevents T cell proliferation and IL-2 production[140]. Tregs can similarly regulate T cells through soluble effector molecules independent of cell contact. CD39 and CD73 expressed on the surface of Tregs generates pericellular adenosine that ligates to adenosine receptor 2A ($A_{2A}R$) on T cells thus inducing their suppression[141]. Ligation of IL-2 to CD25 (high affinity IL-2 Receptor-α) on Tregs can deprive surrounding T cells of this important growth factor for proliferation[142] (Figure 1-3B). Tregs can release perforin and granzyme that bind to the membrane of surrounding cells, destroying the integrity of the membrane leading to the cytolysis of the cell[143] (Figure 1-3C). Cytokines that negatively regulate the immune response, such as TGF-β, IL-10, and IL-35, are also secreted by Tregs after their activation. After binding to their respective receptors, these cytokines promote pathways that inhibit T cell activation (Figure 1-3D). Fibrinogen-like protein (FGL)-2, a known immunoregulatory cytokine, is also secreted by Tregs and inhibits APC maturation and promotes B cell apoptosis after ligation to FcγRIIb[144-146]. Moreover, TGF-β and IL-10 can further promote the conversion of activated $CD4^+$ into iTregs. Together, these processes create a microenvironment surrounding activated Tregs that ultimately promotes tolerance to the Ags recognized by Tregs.

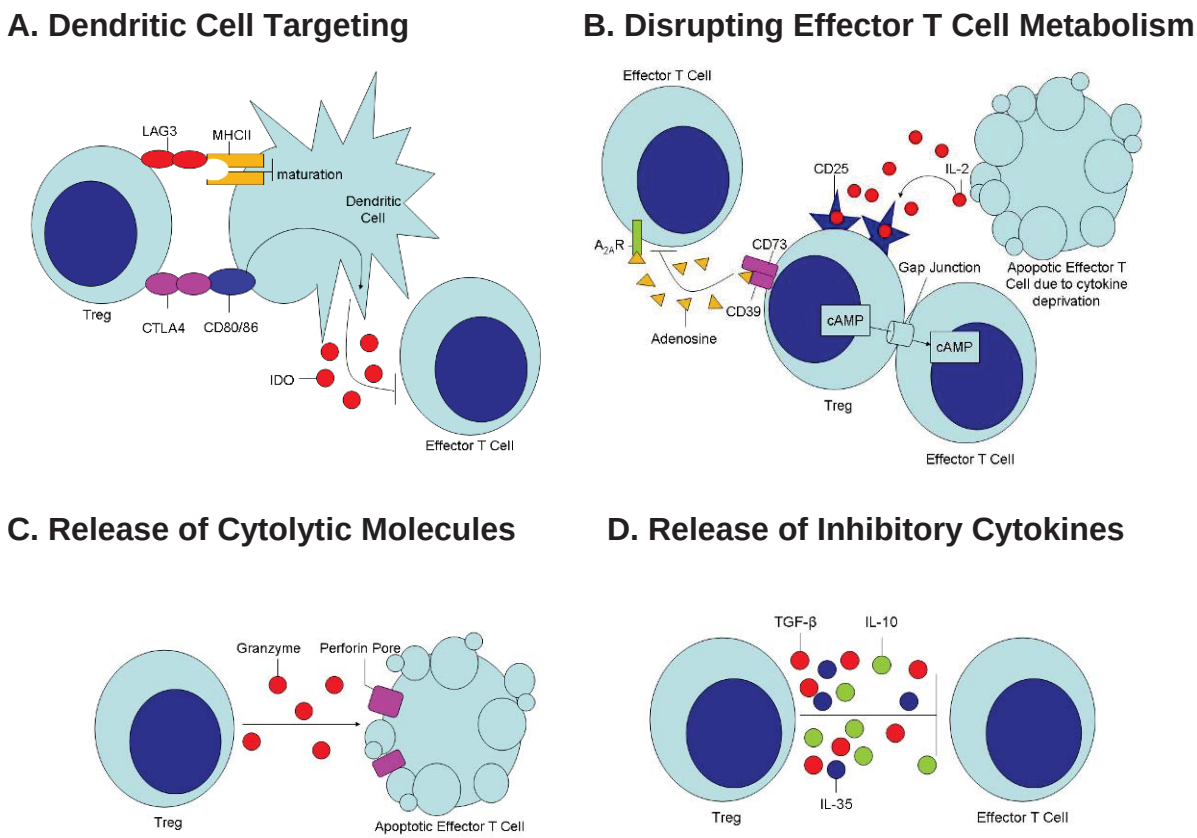


Figure 1-3. Mechanisms of Treg-mediated immune suppression. Examples of well-described immunoregulatory pathways utilized by Tregs are illustrated. A) Dendritic cells (DCs) can be targeted by Tregs. DC maturation and function can be inhibited by interactions between LAG3 on Tregs and MHC class II on DCs. CTLA-4 on Tregs can bind to DCs through CD80/86 (B7.1/B7.2) which promotes DC production of the immunosuppressive cytokine, IDO. B) Effector T cells are inhibited by metabolic disruption promoted by adenosine receptor 2A ligation of adenosine, generated by Tregs through CD39 and CD73. Similarly, cAMP can disrupt effector T cell metabolic functions after it is transferred from Tregs through gap junctions. Binding of IL-2 to Treg high affinity IL-2R (CD25) can prevent access of this cytokine to effector T cells, depriving them of this important cytokine for proliferation and survival. C) Tregs can release perforin and granzyme A and B resulting in lysis of effector cells. D) IL-10, TGF-β, and IL-35 are inhibitory cytokines released by Tregs that can directly inhibit effector T cells. Figure adapted from Vignali, D.A., *et al*.[146]

Regulatory T cells hold tremendous potential in establishing tolerance to transplanted grafts since they are specific to antigen. Therefore, many experimental procedures have explored the possibility of promoting the differentiation, proliferation, and maintenance of regulatory T cells specific for the graft. Experimental models in mice have shown their efficacy in accepting grafts after these Tregs are expanded *ex vivo* or *in vivo* and injected into the host prior to transplant[147, 148]. However, obstacles exist in the translation of these Treg based therapies into transplant patients. For instance, patients who maintain graft function with minimal immunosuppression often have limited Tregs present[149, 150]. To account for this observation, regulatory T cells appear to be more heterogeneous in humans and thus Foxp3 may not be a reliable marker in patients[151]. Although some regulatory T cells in humans express $Foxp3^+$, it is clear that better understanding of the subsets involved in maintaining peripheral tolerance in humans is required. This understanding will then aid with the translation of regulatory cell research from animal models into creating therapies for patients to establish graft tolerance.

1.5. Biomarkers for graft tolerance

1.5.1 The need for tolerance biomarkers

Tolerance towards transplanted grafts is actively sought since this will eliminate the need for immunosuppression while also preventing graft rejection. However, it is currently difficult to differentiate patients who have achieved tolerance versus those who require immunosuppression to maintain graft function. Retrospective studies have suggested that as many as 20% of liver transplant patients have developed tolerance to their graft[152]; but presently there are no mechanisms to identify these patients and thus

physicians are hesitant to remove immunosuppression blindly and risk the often fatal consequences of graft rejection. Currently, these patients are only serendipitously discovered when immunosuppression must be withdrawn because of severe infections, the development of cancer, or as a result of the non-compliance of the patients with their immunosuppressive regimen[153]. Furthermore, as tolerance induction protocols are developed in rodent models, the success of these therapies in inducing tolerance in patients will not be realized unless immunosuppression can be reliably removed without the risk of rejection. The challenge therefore remains to dependably identify patients tolerant to their grafts in order to be able to remove immunosuppression.

Biomarkers that correlate with tolerance to grafts could be used to achieve this goal. The National Institutes of Health Biomarkers Definitions Working Group describes biomarkers as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention"[154]. Many biomarkers have been in clinical use to diagnosis various diseases or to assess a patients response to therapies. For instance, blood glucose levels are used to diagnosis and assess treatments for diabetes. In transplantation, these biomarkers could identify patients that are tolerant to their grafts and can have immunosuppression reduced or withdrawn.

1.5.2. Current advances in transplantation biomarker discovery

To date, the majority of groups have focused on identifying biomarkers that correlate with rejection. These markers are useful in modulating immunosuppressive

therapy in response to worsening graft function. However, the absence of markers of graft rejection during immunosuppression is solely unreliable to identify tolerant patients.

Therefore tolerant biomarkers are required. To discover these, the focus has been on the few liver and kidney patients found to be tolerant to their grafts by chance as discussed above. Gene profiles from peripheral blood mononuclear cells (and urine in kidney transplant patients) have been analyzed from these patients. One study examined 25 tolerant kidney patients and identified 3 genes that predicted tolerance with 100% accuracy. These genes are *IGKV1D-13, IGKV4-1,* and *IGLL1*, and these are involved in the differentiation of B cells from pre- to mature B cells or involved in B cell activation[155]. Also, CD20 messenger ribonucleic acid (mRNA) was found to be increased in tolerant patients. Another study by Sánchez-Fueyo and colleagues identified transcripts associated with NK cells, NK T cells and γδT cells as elevated in the peripheral blood of tolerant liver recipients. Of these markers, killer cell lectin-like receptor subfamily F member 1 (*KLRF1)* and signaling lymphocytic activation molecule family member 7 (SLAM7) predicted tolerance in 87% of patients[156]. These studies have provided putative markers of transplant tolerance.

However, many obstacles in the search for biomarkers of transplantation tolerance are present. A major complication of the above studies is that only a few patients exist to develop this panel. Without a large cohort of patients, it is unknown whether this panel could be broadly applied to other patients. Similarly, these studies utilized non-invasive methods to prevent unnecessary harm to the graft, examining peripheral blood or urine of patients instead. These tissues may not accurately reflect the immune deviation occurring within the graft to promote tolerance and more reliable biomarkers may be found here.

As a result, the biomarkers of graft tolerance thus far identified are not adequate for widespread use clinically.

1.5.3. Rationale for biomarker discovery in an animal model

To avoid the obstacles inherent in studies with tolerant patients, our studies sought to identify these biomarkers in an established and robust murine model of transplantation where tolerance towards the graft can be induced. This approach is advantageous in that it is possible to clearly define a group of tolerant and rejecting mice. The approach also affords easy access to the graft and secondary lymphoid organs for subsequent genomic and/or proteomic analyses, with sufficient numbers to obtain statistical significance. After identifying and confirming potential candidate biomarkers, it will then be possible to test the panel of biomarkers for relevance in patients before broad application clinically.

1.6. Hypothesis and aims

Through the establishment of a robust mouse model of transplantation tolerance, where graft function is maintained without the need for long-term immunosuppression, it will be possible to identify biomarkers that correlate with graft tolerance. It will then be possible to confirm the relevance of these biomarkers in patients. These biomarkers could then be used to identify patients who achieve tolerance and can have their immunosuppression reduced or removed.

To achieve this, the aims of the present study were:

a) to first establish and confirm that graft function is maintained over the long-term without the need for immunosuppression in our mouse model;

b) confirm that specific tolerance towards donor Ags has been achieved;

c) determine the mechanisms that promote tolerance in this model, which will guide in the discovery of biomarkers; and

d) using genomic techniques, determine which biomarkers correlate with graft tolerance.

Finally, a future aim of the study will be to determine the validity and applicability of these biomarkers to patients.

2. Materials and Methods

2.1. Mice

Female C3H/HeJ (MHC haplotype H-2^k; *toll like receptor 4 (tlr4)*$^{-/-}$), C3H/HeOuJ (MHC haplotype H-2^k; *tlr4*$^{+/+}$), BALB/cJ (MHC haplotype H-2^d), and C57BL/6J (MHC haplotype H-2^b) mice 6-8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). Mice were housed in a sterile animal facility at the Toronto General Hospital and treated according to policies provided by the University Health Network in accordance with guidelines from the Canadian Council on Animal Care. All mice transplanted were between 8 – 10 weeks old.

2.2. Heterotopic Cardiac Transplantation

Heterotopic cardiac transplantations were performed by Dr. Wei He according to the protocol previously described by Correy and colleagues[157]. Donor hearts were removed from BALB/cJ or C3H/HeJ mice by first ligating the inferior vena cava with 6-0 silk sutures below the heart and dividing it distally to the ligature. The right and left superior vena cava were then similarly ligated and cut between the heart and azygos or hemiazygos veins. After cutting the posterior descending artery ligament and separating the aorta and pulmonary artery, the main pulmonary artery is mobilized and transected at the point of bifurcation. Pulmonary veins were then ligated en masse and divided distally. The heart was then excised and immersed in cold saline.

Recipient C3H/HeJ or C3H/HeOuJ mice were anesthetized with an intraperitoneal (i.p.) injection of pentobarbital. The gut was carefully pulled to the left side and covered with moist gauze. The abdominal aorta and the inferior vena cava were then mobilized

from the bifurcations of the renal vessels to the bifurcations of the common iliac vessels. Lumbar veins behind the inferior vena cava were then ligated with 7-0 silk sutures. All small branches of this segment were cauterized. The abdominal aorta and inferior vena cava between the two bifurcations were clamped. The anterior wall of abdominal aorta and inferior vena cava were then punctured with a 30-gauge needle and then incised longitudinally using a pair of iris scissors. The length of this aortotomy was adjusted to the width of donor aorta, and the length of venotomy was equal to the width of the donor pulmonary artery.

While observing through a microscope at 25X magnification, donor grafts were implanted into the recipient through end-to-side anastomoses of donor aorta and pulmonary artery to the recipient's abdominal aorta and inferior vena cava, respectively. The front and back wall of arteries required 10 continuous stitches to complete the anastomosis with 11-0 sutures. The anastomosis of the veins was carried out in a similar manner. Afterwards, the clamps on the recipient's abdominal aorta and inferior vena cava were removed and the transplanted heart then begins to beat spontaneously. The total warm ischemic time of the procedure is approximately 30min. The gut was reinserted into the peritoneum and the skin incision was then closed with 6-0 silk sutures.

Daily assessment of graft function was conducted through transabdominal palpation and a score from 0-4 was given based on the strength and rate of beats. Rejection was defined as a score of 0, which was awarded upon complete cessation of palpable beats. This was confirmed by direct visual examination of the graft.

2.3. Tolerizing Protocol and Control Groups

The tolerizing protocol was adapted from that described by Li, Y., *et al.*[103] Rapamycin (Wyeth-Ayerst, Princeton, New Jersey, USA) was purchased from the Toronto General Hospital pharmacy. After dilution in 1X Phosphate-Buffered Saline (PBS), 0.4mg/kg rapamycin was injected i.p. into mice on the first 3 days after transplantation, followed by 7 more i.p. injections, every-other-day. The last injection was on d.16 post-transplantation. This protocol was administered to C3H/HeJ mice in the tolerant group that received a cardiac allograft (BALB/cJ → C3H/HeJ). Both C3H/HeJ mice that did not receive a transplant and C3H/HeOuJ mouse recipients of BALB/cJ heart transplants also received this protocol and served as the rapamycin-only and the $TLR4^{+/+}$ control groups, respectively. Cyclosporine A (CsA) control group mice were given an allogeneic transplant (BALB/cJ → C3H/HeJ) followed by administration of 20mg/kg CsA (Novartis, Basel, Switzerland) diluted in 1X PBS. This was administered subcutaneously every day for 16 days in a protocol similar to that described by Li, Y., *et al.*[103] An allogeneic transplant group (BALB/cJ → C3H/HeJ) that did not receive any immunosuppressive protocols served as the rejecting control. Other controls included a syngeneic transplant (C3H/HeJ → C3H/HeJ) which did not receive the tolerizing protocol, and a naïve C3H/HeJ group that did not receive a transplant and the tolerizing protocol.

2.4. Skin Grafts

Full thickness dorsal skin, $1cm^2$ in size, from donor (BALB/cJ) or 3rd party (C57BL/6J) mice were grafted to the dorsum of tolerant group C3H/HeJ mice (which had an equivalent section of full thickness skin removed immediately prior to the grafting) 30

days after the initial cardiac transplant. Skin graft rejection was assessed as the complete necrosis of the graft.

2.5. Histology and Immunohistochemistry

Transplanted cardiac grafts or naïve hearts were removed and dissected into < ⅓cm sections by cutting perpendicular to the vertical axis of the heart. The peri-suture area and the apex of the heart were discarded and not analyzed. The remainder was processed for either hematoxylin & eosin (H&E) staining or immunohistochemistry.

Cardiac tissue was embedded in paraffin prior to H&E staining. This was done by first immersing tissue in 10% formalin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 48 hours while shaking. Tissue was then taken to the Centre for Modeling of Human Diseases (CMHD) Pathology Core at Toronto's Mount Sinai Hospital for further processing. Tissue was embedded in paraffin, cut into 5µm thick sections and stained with H&E.

For immunohistochemical staining, frozen tissue sections were prepared by embedding cardiac tissue in Optimal Cutting Temperature Compound (Tissue-Tek, Sakura Finetek, Torrence, California, USA)-filled cryomolds. These molds were then placed in liquid nitrogen and processed at the CMHD Pathology Core. Tissue was cut into 5µm thick sections and stained using rat anti-mouse CD4 IgG2b antibody (Clone GK1.5; eBioscience, San Diego, California, USA) or rat anti-mouse/rat Foxp3 IgG2a antibody (Clone FJK-16s; eBioscience). Tissue was then incubated with a secondary anti-rat Ig antibody, conjugated with Horse Radish Peroxidase (HRP) that allowed for colour development after addition of the substrate 3,3'-diaminobenzidine (DAB) (Zymed, San

Francisco, California, USA). After digitally scanning and copying stained sections, positive cells identified by a brown stain were enumerated using the computerized morphometry program, Spectrum version 10.2.2.2317 (Aperio Technologies Inc., Vista, California, USA).

2.6. Splenocyte isolation

Spleens were dissected from mice, washed with 1XPBS diluted in double distilled water, and cut into small pieces less than ½ cm. Using a plunger from a 10mL syringe, spleens were mashed against a 40μm nylon filter which separated splenocytes from connective tissue. Lympholyte M density separation medium (Cedarlane, Burlington, Ontario) allowed for isolation of mononuclear cells from erythrocytes.

2.7. One-Way Mixed Lymphocyte Reaction

In a 96-well U-bottom suspension cell plate (SARSTEDT AG & Co., Nümbrecht, Germany), $2x10^5$ responder splenocytes from tolerant or control group mice were co-cultured in triplicate with $8x10^5$ stimulator splenocytes from naïve donor (BALB/cJ), 3rd party (C57BL/6J), or syngeneic (C3H/HeJ) mice. Stimulator splenocytes were previously exposed to a 2000rad dose of γ-irradiation from a ^{137}Cesium (Cs) source Gammacell-40 Exactor irradiator (Nordion International Inc., Kanata, Ontario, Canada) to introduce DNA double strand breaks that prevented cell division. Co-cultures were incubated at 37°C with 5%CO_2 for 3 days in 200μL α – Modified Eagles Media (MEM) (Invitrogen, Carlsbad, California, USA) solution supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Scientific HyClone, Logan, Utah, USA) and 0.5μM 2- Mercaptoethanol (2-ME) (Sigma-Aldrich, St. Louis, Missouri, USA). Afterwards, 1μCurie (1μCi) per well of ^{3}H-

thymidine (Amersham Biosciences, Buckinghamshire, UK) was added. Cells were harvested 18 hours later using the UNIFILTER-96 Filtermate Harvester (PerkinElmer, Boston, Massachusetts, USA) and counted by Packard Microplate Scintillation Counter (PerkinElmer).

2.8. Cytotoxic T-Lymphocyte Lysis Assay

Responder splenocytes from tolerant or control group mice were co-cultured with $5x10^6$ stimulator splenocytes from naïve donor (BALB/cJ), 3rd party (C57BL/6J), or syngeneic (C3H/HeJ) mice in a 1:1 ratio in a flat-bottom 24-well plate for suspension cells (SARSTEDT AG & Co.,). Stimulator cells were irradiated as previously described. Co-cultures were incubated for 5 days at 37°C with 5% CO_2 in 3mL of supplemented αMEM. Cells were removed from culture through rigorous pipetting, and adherent cells were removed after 10min incubation with 5mM ethylenediaminetetraacetic acid (EDTA) (Applied Biosystems, Foster City, California, USA) at 37°C. Cells were washed three times with supplemented αMEM and viable cells were counted.

A20 (MHC haplotype H-2^d) and EL4 (MHC haplotype H-2^b) cell lines were used as target cells and were incubated with 1mCi/mL sodium chromate (Na_2CrO_4) (PerkinElmer) at 37°C. After a 90min incubation, target cells were washed 3 times with 1XPBS, counted, and mixed with the appropriate responder cells as follows: Responder cells co-cultured with donor (BALB/cJ) splenocytes were mixed with 10^4 chromium labeled A20 cells, and responder cells co-cultured with 3rd party (C57BL/6J) splenocytes were mixed with 10^4 chromium labeled EL4 targets. Naïve C3H/HeJ responders incubated with syngeneic (C3H/HeJ) stimulator cells were incubated with A20 or EL4 targets separately as a negative control. Effector-to-target ratios included 25:1, 10:1, 5:1

and 1:1 and were performed in triplicate. A20 and EL4 cells were cultured alone to assess background levels. Max lysis was determined by separately incubating EL4 and A20 cells alone and adding 5uL 9% Triton-X100 (Sigma-Aldrich) after 4hrs in culture.

These cultures were maintained in 96-well U-bottom suspension cell plates (SARSTEDT AG & Co.,) with 200µL supplemented αMEM for 5 hours. Supernatants were then extracted and transferred to LumaPlate-96 (PerkinElmer) plates and left overnight to dry**.** Released chromium was then counted using a Packard Microplate Scintillation Counter (PerkinElmer)

2.9. Flow Cytometry and Reagents

Antibodies and reagents utilized for flow cytometry. Detection antibodies recognized mouse antigens and included: Fluorescein isothiocyanate (FITC)- CD4, allophycocyanin (APC*) – CD8α, phycoerythrin (PE) – Foxp3, FITC – CD44, and PE-CD62L. Isotype control antibodies included: FITC- rat IgG2a, APC* – rat IgG2a, PE- rat IgG2b, FITC- rat IgG2b, and PE- rat IgG2a, respectively. Propidium iodide (PI) was utilized as a viability marker in assays that did not require cell fixation. All antibodies and reagents were obtained from eBioscience.

Cell Suspensions. Single-cell suspensions of 10^6 splenocytes, thymocytes or lymph node cells were made in flow cytometry staining buffer (eBioscience) containing 1XPBS supplemented with 1%FBS and 0.09% sodium azide. 10^6 cells in 100µL of flow cytometry staining buffer in a polypropylene test tube were used for staining.

Treg labeling. The protocol provided by the manufacturer (eBioscience) was followed. While on ice, anti-mouse CD16/32 (eBioscience) was added to cells to block

binding of subsequent antibodies to Fc receptors (Fc block). After 15 min, antibodies staining CD4 and CD8 surface antigens were added and allowed to incubate for 30 min. Cells were then washed and incubated overnight with fixation and permeabilization solution. Cells were then washed, incubated again with Fc block for 15min, followed by the addition of antibody staining for intracellular Foxp3 and 30min incubation. After 3 washes, cells were analyzed on a flow cytometer.

CD8 Memory T Cell Staining. While on ice, single cell splenocyte suspensions were incubated with Fc block for 15min, followed by incubation with antibodies against surface antigens (CD44, CD62L) and 5μL of PI. Cells were washed before analysis on a flow cytometer.

Analysis. Stained single cell suspensions were assessed using a BD FACSCalibur or BD LSR II flow cytometer (BD Bioscience, Franklin Lakes, New Jersey, USA). Data was analyzed using FlowJo software version 8.8.4 and version 8.8.6 (Tree Star Inc, Ashland, Oregon, USA). Forward and side scatter were used to gate viable lymphocytes, and PI positive populations were also used to exclude nonviable cells when appropriate.

2.10. RNA isolation

Total RNA was extracted from frozen grafts or naïve hearts using TRIzol (Invitrogen) according to manufacturer's instruction (Invitrogen). The RNA precipitate was completely dissolved in water, and its quality and quantity were analyzed by RNA Bioanalyzer (Agilent Technologies, Santa Clara, California, USA). RNA was stored at -80^{o}C for later use in multiplex polymerase chain reaction (PCR) and quantitative reverse transcriptase PCR (qRT-PCR) studies.

2.11. Multiplex PCR

The expression of multiple genes was analyzed through multiplex PCR utilizing GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter, Brea, California, USA). Stored RNA was thawed and processed according to manufacturer's protocols. Briefly, 5µL of 5-20ng/µL RNA from transplanted or naïve hearts was added to a 96-well microplate (Beckman Coulter) in triplicate. To each well, 5µL of 2.5ng/µL kanamycin resistance gene (KAN^r) RNA was added as a standard. A reverse transcriptase cycle followed by PCR was completed using primers that allowed for the analysis of 23 Treg related genes. The primers for this custom panel of genes were generously provided by Beckman for use in our lab. Expression values were normalized to the house keeping gene hypoxanthine phosphoribosyltransferase (HPRT) and subsequently further normalized to expression in naïve mouse hearts. Values were expressed as the mean of three measurements.

2.12. Quantitative reverse-transcriptase polymerase chain reaction

Previously prepared RNA from transplanted grafts or naïve hearts were used for qRT-PCR to validate results from multiplex PCR studies. The complementary DNA (cDNA) samples for qRT-PCR analysis were synthesized with oligo-deoxythymidine (dT) primers using the Superscript III First Strand Synthesis System for qRT-PCR (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions. qRT-PCR and data analysis were performed using the LightCycler480 system (Roche, Basel, Switzerland). Expression values were normalized to house keeping genes hypoxanthine phosphoribosyltransferase (HPRT) and actin-related protein (ARP). Values

were further normalized to expression in naïve mouse hearts and were expressed as the mean of three measurements.

2.13. FGL-2 Sandwich Enzyme Linked Immunosorbent Assay

Collection of plasma samples. Heart punctures were performed using EDTA (Applied Biosystems) coated syringes on mice previously anesthetized with pentobarbital. The blood collected was then transferred to a 1.5mL eppendorf tube (Eppendorf, Hamburg, Germany) and spun at 1000g for 10 min. The plasma layer was removed and transferred to another tube where it was stored at -80^{o}C until use.

Sandwich enzyme linked immunosorbent assay (ELISA). Costar 96-well plates (Corning Inc., Corning, New York, USA) were coated with capture antibody by adding 2µg/mL monoclonal IgG1 anti-FGL-2 antibody (clone 6H12), and left to incubate overnight at 4^{o}C. After blocking with bovine serum albumin (BSA) (Sigma-Aldrich) and washing with Tris-buffered saline with Tween-20 (TTBS) (BioShop Canada Inc, Burlington, Ontario, Canada), 50µL of 1:10 diluted plasma was added to wells in triplicate and incubated for 1 hour at 37^{o}C. Wells were washed again with TTBS before incubating with 2µg/mL polyclonal rabbit anti-FGL2 antibody at 37^{o}C for 2 hours. Following another wash, an HRP-conjugated anti-rabbit secondary antibody was added to detect polyclonal anti-FGL-2 binding. Following addition of the HRP substrate, tetramethylbenzidine (TMB) (Sigma-Aldrich), absorbance was measured at 450nm using a Multiskan Ascent ELISA plate reader (Titertek Instruments Inc., Huntsville, Alabama, USA).

2.14. Statistics

Log-rank tests were performed to assess the statistical significance of survival data plotted on Kaplan-Meier curves. Unless otherwise specified, statistical significance was assessed using the Analysis of Variance (ANOVA) test followed by a Tukey's Honestly Significant Difference (HSD) test as a post-hoc analysis for group comparisons. Differences with $P \leq 0.05$ were considered significant.

* $P \leq 0.05$ and ** $P \leq 0.01$, unless otherwise noted.

2.15. Contributions by others

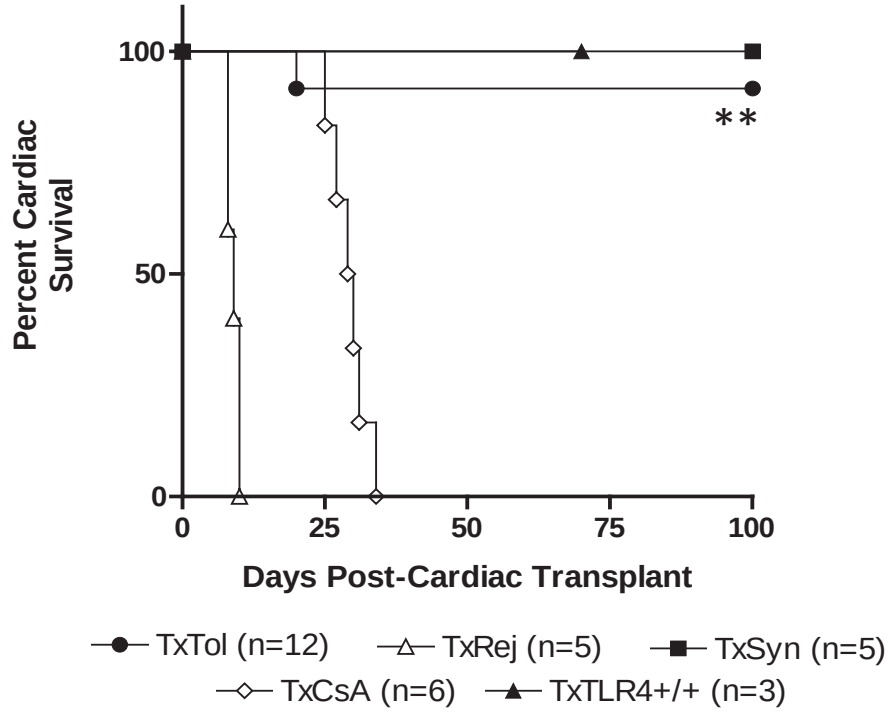
Heterotopic cardiac transplants were performed by Dr. Wei He. Processing of paraffin embedded and frozen tissues, as well as H&E and immunohistochemical staining, was completed by the CMHD Pathology Core at Mount Sinai Hospital in Toronto, Ontario, Canada. Expert pathological advice was provided by Drs. Oyedele Ayedi and M. James Phillips of the Pathology Departments at University Health Network and Hospital for Sick Children, respectively. Multiplex PCR and qRT-PCR studies were completed and analyzed by Dr. Jihong Wang.

3. Results

3.1. Long-term cardiac allograft function and morphology are preserved following rapamycin induction therapy.

Heterotopic cardiac transplantations were performed in female C3H/HeJ (*tlr4*$^{-/-}$) mice which received an allogeneic heart (BALB/cJ → C3H/HeJ) with either a 16 day rapamycin induction treatment (Tolerant Group (TxTol)), with a 16 day CsA treatment (CsA control group (TxCsA)), or no treatment (Rejecting Group (TxRej)). C3H/HeJ recipients that received a syngeneic graft (C3H/HeJ→ C3H/HeJ) were used as a control (Syngeneic Group (TxSyn)). Three C3H/HeOuJ mice (*tlr*$^{+/+}$) also received BALB/cJ hearts with the rapamycin treatment and served as the TLR4-positive control group (TxTLR4$^{+/+}$). Cardiac grafts were monitored for beating through transabdominal palpation following heterotopic transplantation and a cessation of beating indicated graft rejection. All five grafts without therapy rejected at 9.0 ± 1.0 days, whereas 11 out of 12 allogeneic grafts in C3H/HeJ recipients treated with rapamycin as per the tolerizing protocol continued to beat for ≥100 days until time of sacrifice ($p=8.2x10^{-6}$). Treatment with CsA, however, led to graft rejection between days 26 and 35 post-transplantation. Furthermore, when *tlr*$^{+/+}$ C3H/HeOuJ mice were used as recipients and given the rapamycin induction protocol, grafts continued to beat for ≥70 days until time of sacrifice (Figure 3-1A). Histological examination of rejecting group grafts showed a pronounced mononuclear cell infiltration in the endothelium (vasculitis) as well as throughout the endocardium, myocardium, and epicardium at day 7 post-surgery (Figure 3-1Bi). Grafts in CsA treated control groups 30 days following transplantation resembled grafts in the rejecting group at day 7 post-transplantation (data not shown). Conversely,

cardiomyocyte structure in the myocardium remained largely preserved in tolerant group grafts 100 days after transplantation (Figure 3-Bii) and the heart resembled the syngeneic grafts at day 100 post-surgery and naïve heart controls (Figures 3-Biii and 3-Biv, respectively). Nevertheless, compared to 100 day syngeneic grafts and naïve hearts, an increase in mononuclear cell infiltrates was evident in tolerant grafts at day 100 following transplant, but remained noticeably less than the infiltration in day 7 rejecting group grafts.



** $p=8.2x10^{-6}$ between TxRej and TxTol

Figure 3-1A. Heterotopic cardiac allograft function is maintained indefinitely following rapamycin induction treatment. C3H/HeJ (*tlr4*$^{-/-}$) mice were given a heterotopic cardiac graft from an allogeneic source (BALB/cJ) and treated for 16 days with either rapamycin (TxTol), cyclosporine (TxCsA) or untreated (TxRej). Five C3H/HeJ mice were given a syngeneic graft from another C3H/HeJ mouse (TxSyn). BALB/cJ hearts were also transplanted into C3H/HeOuJ (*tlr4*$^{+/+}$) recipients, which were given the rapamycin induction protocol (TxTLR4+/+). Grafts were monitored daily for beating and the cessation of graft beating indicated rejection. The graph represents Kaplan-Meier cumulative survival and statistical significance between TxTol and TxRej was determined by the log-rank test.

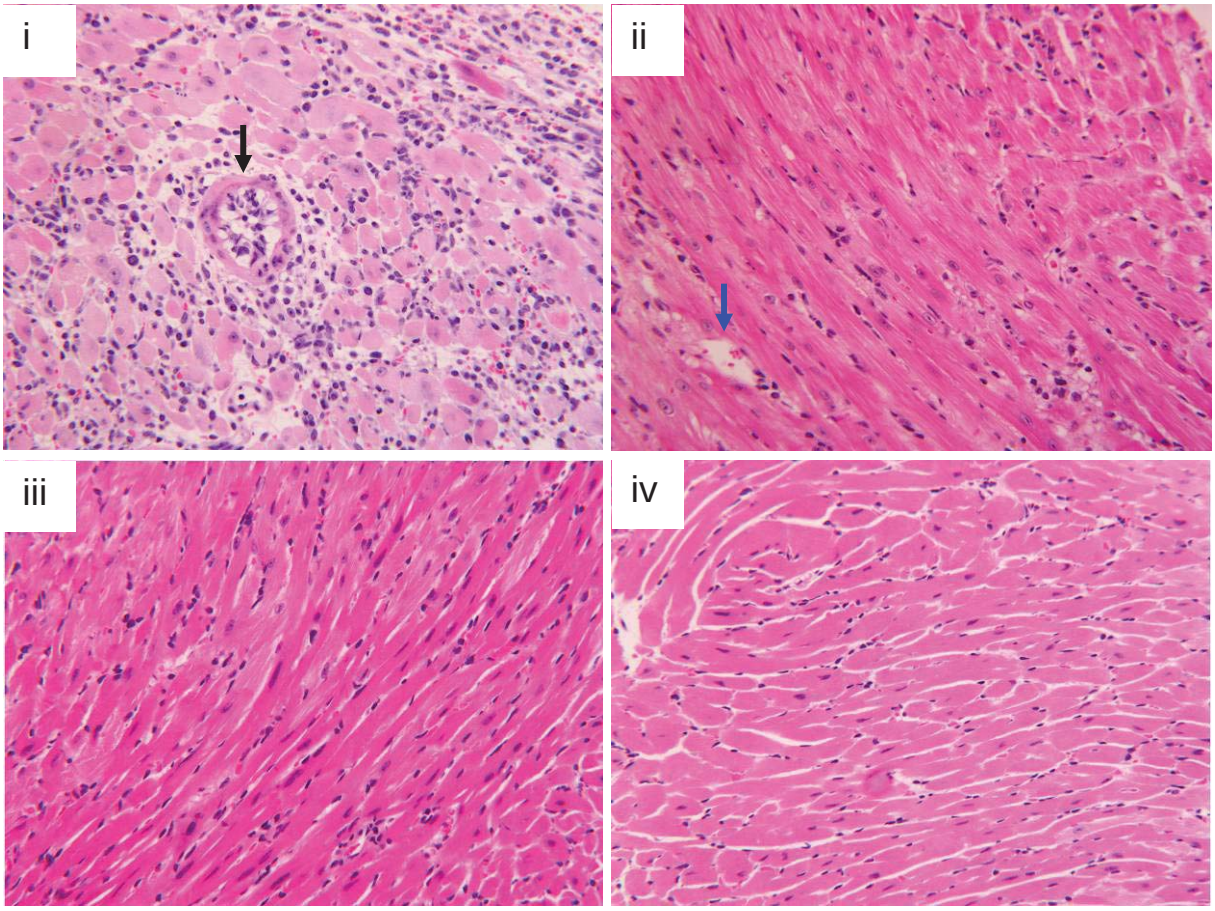
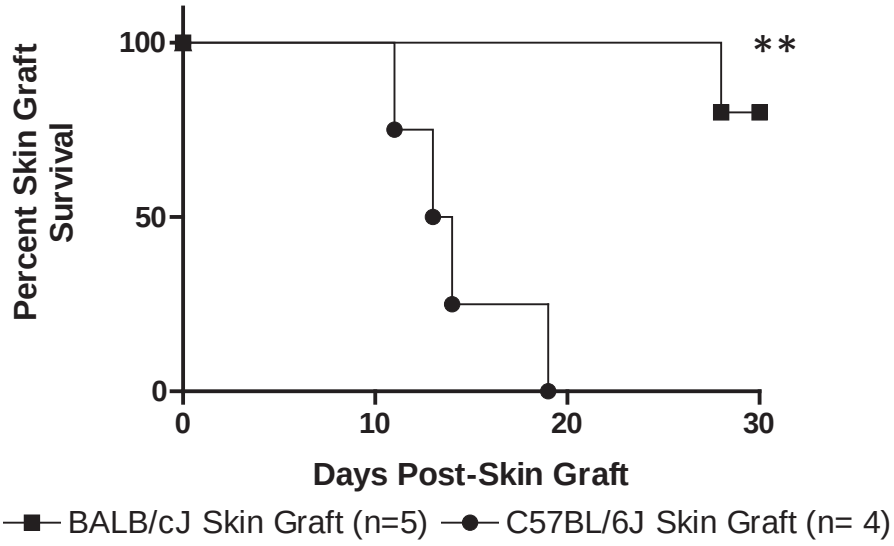


Figure 3-1B. Heterotopic cardiac allograft morphology is preserved following rapamycin induction treatment. Cardiac grafts were sectioned, stained with H&E, and representative photographs are shown at 300X. (i) Cardiac grafts from rejecting group mice (TxRej) that received allogeneic transplants without rapamycin treatment show an increase in mononuclear cell infiltrates at day 7 post-transplantation. Vasculitis (black arrows) is also prominent. (ii) In contrast, grafts from tolerant group (TxTol) mice that received the rapamycin induction protocol showed fewer infiltrating cells and a marked reduction in the development of vasculitis (normal blood vessel indicated by blue arrow) at 100 days following transplantation. Moreover, myocardium structure remained well-preserved in grafts of the tolerant group at this time point and resembled (iii) 100-day syngeneic grafts and (iv) naïve hearts.

3.2. Skin graft survival is prolonged on tolerant group mice when the graft is of donor but not 3rd party origin.

To determine whether tolerance was donor-specific, full-thickness skin grafts from either donor-strain (BALB/cJ) or 3rd party (C57BL/6J) mice were transplanted onto tolerant group recipients 30 days after the initial heterotopic cardiac transplant. The survival was prolonged indefinitely (≥30 days) in four of five skin grafts of donor origin, whereas skin grafts from 3rd party mice were rejected at 14.25 ± 3.40 days (p=0.003) (Figure 3-2).



**p=0.003

Figure 3-2. The survival of skin grafts from donor mice, but not 3rd party mice, is prolonged on C3H/HeJ recipients that previously received a BALB/cJ cardiac graft and rapamycin induction treatment. Skin grafts from either donor strain (BALB/cJ) or 3rd party (C57BL/6J) origin were engrafted onto tolerant group C3H/HeJ recipients, which had an allogeneic BALB/cJ cardiac transplant 30 days prior with rapamycin treatment. Skin grafts were monitored daily by visual inspection with complete necrosis of the skin graft indicating rejection. Kaplan-Meier curves were used to plot skin graft survival data and statistical significance was calculated using the log-rank test.

3.3. Lymphocyte activity is reduced specifically towards donor antigens in tolerant group mice.

Splenic lymphocytes were next isolated from mice that had accepted heart allografts and assessed *in vitro* for their proliferative and cytotoxic responses towards donor (BALB/cJ) and 3rd party (C57BL/6J) antigens. These responses were compared to splenic lymphocytes isolated from rejecting mice, naïve mice that received rapamycin treatment (rapamycin-only group (RPM)), and naïve mice. Lymphocytes were isolated from all groups at 100 days following transplantation (84 days after rapamycin withdrawal).

Lymphocyte proliferation was assessed in a standard mixed lymphocyte reaction. Responder lymphocytes isolated from spleens of tolerant, rejecting, or control mice ($2.0x10^5$ cells/well) were added to co-cultures with irradiated splenocytes ($8.0x10^5$ cells/well) from donor (BALB/cJ), 3rd party (C57BL/6J), or syngeneic (C3H/HeJ) origin. Lymphocytes from mice that had rejected their heart graft showed significantly enhanced proliferation to donor antigens compared to tolerant group mice, as assessed by ^{3}H-thymidine uptake (8651.67 ± 113.54 counts per minute (cpm) compared to 5883.33 ± 255.04 cpm, respectively; $p=6.82x10^{-4}$). The proliferation of tolerant mouse splenocytes was also comparable to both naive and rapamycin-only controls. Proliferation was not statistically different in any group when challenged with 3rd party splenocytes (Figure 3-3A).

To assess cytotoxic T lymphocyte (CTL) activity, a ^{51}Chromium (^{51}Cr) -release assay was performed. Mononuclear cells were isolated from spleens of tolerant, rejecting, and control mice were isolated and co-cultured for 5-days with irradiated

splenocytes from donor (BALB/cJ), 3rd party (C57BL/6J), or syngeneic (C3H/HeJ) mice. Cells from cultures were then removed and challenged with chromium labelled target cells. A20 cells (derived from BALB/c mice) were utilized if effectors were co-cultured with irradiated BALB/cJ splenic mononuclear cells and EL4 targets (derived from C57BL/6 mice) were used if effectors were co-cultured with splenic mononuclear cells from C57BL/6J mice. A20 and EL4 cells were utilized as targets for splenic mononuclear cells isolated from naïve mice that were previously co-cultured for 5 days with syngeneic C3H/HeJ splenic mononuclear cells (naïve anti-C3H) to assess background cytotoxicity. At all effector-to-target ratios tested, the cytotoxicity of lymphocytes from tolerant mice towards A20 donor targets was equivalent to rapamycin-only and naive controls, and the cytotoxicity of all these groups was less than the cytotoxicity of rejecting group splenocytes towards A20 targets (Figure 3-3Bi). The increased cytotoxicity of the rejecting groups towards A20 targets compared to the tolerant group was statistically significant at all ratios examined. When challenged with 3rd party EL4 targets, cytotoxicity did not differ between responder groups at all effector-to-target ratios (Figure 3-3Bii).

The results for both the mixed lymphocyte reaction and ^{51}Cr release assay were equivalent when tolerant and rejecting mice were used at 30 days post-transplant and rapamycin-only control mice were used at 30 days post-first rapamycin injection.

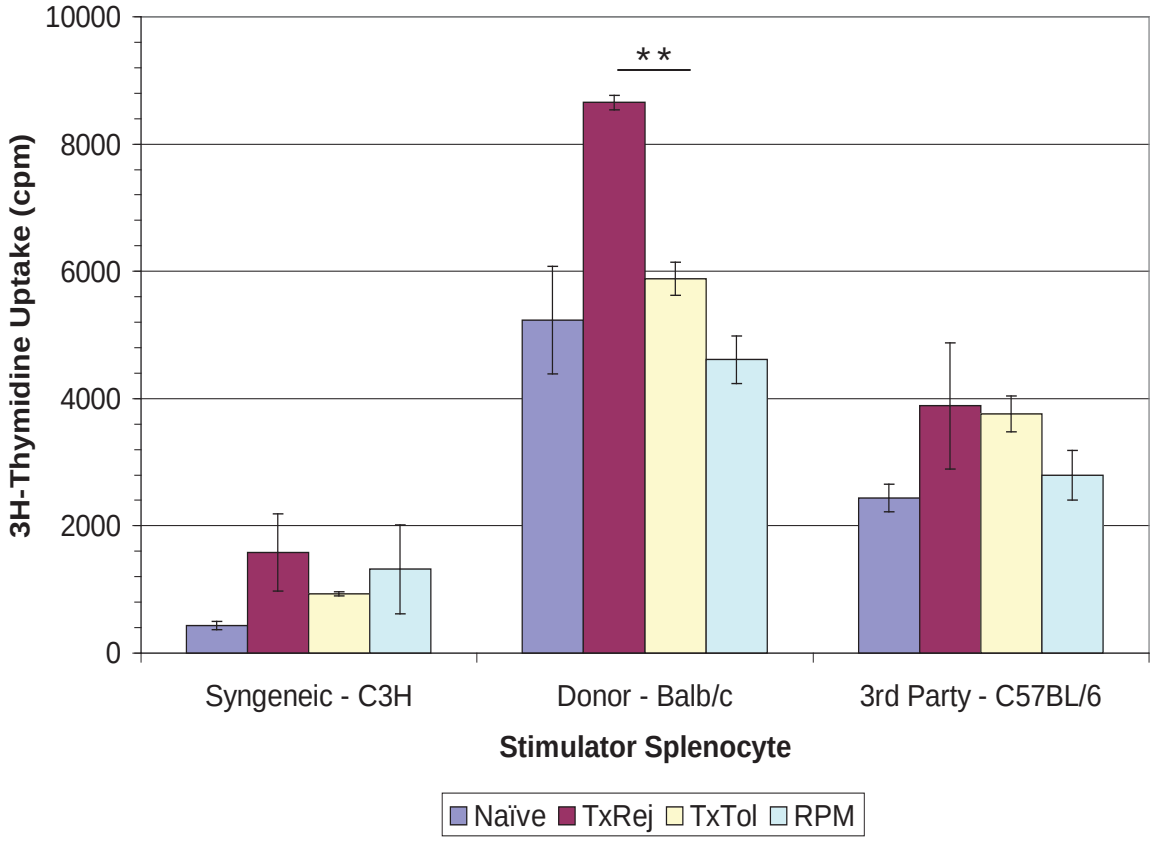


Figure 3-3A. Reduced lymphocyte proliferation in response to donor antigens in tolerant group mice compared to rejecting mice. The proliferation of responder T cells is shown through [^{3}H]-thymidine (1μCi) incorporation. Responder lymphocytes ($2.0x10^5$cells/well) are isolated from spleens of a naïve, rejecting (TxRej), tolerant (TxTol) or rapamycin-only mice (RPM) (at 100 days post-transplant or 84 days after rapamycin withdrawal) and co-cultured in triplicate for 3 days with irradiated splenocytes ($8.0x10^5$ cells/well) from donor (BALB/cJ) or 3rd party (C57BL/6J) mice. Background proliferation levels were assessed by the incubation of isolated lymphocytes from all groups with irradiated syngeneic (naïve C3H/HeJ) splenocytes ($8.0x10^5$ cells/well). Values are shown as means ± Standard Deviation (SD). This graph is representative of three independent experiments. Similar results were obtained when splenocytes were taken at day 30 post-transplant (14 days after rapamycin withdrawal).

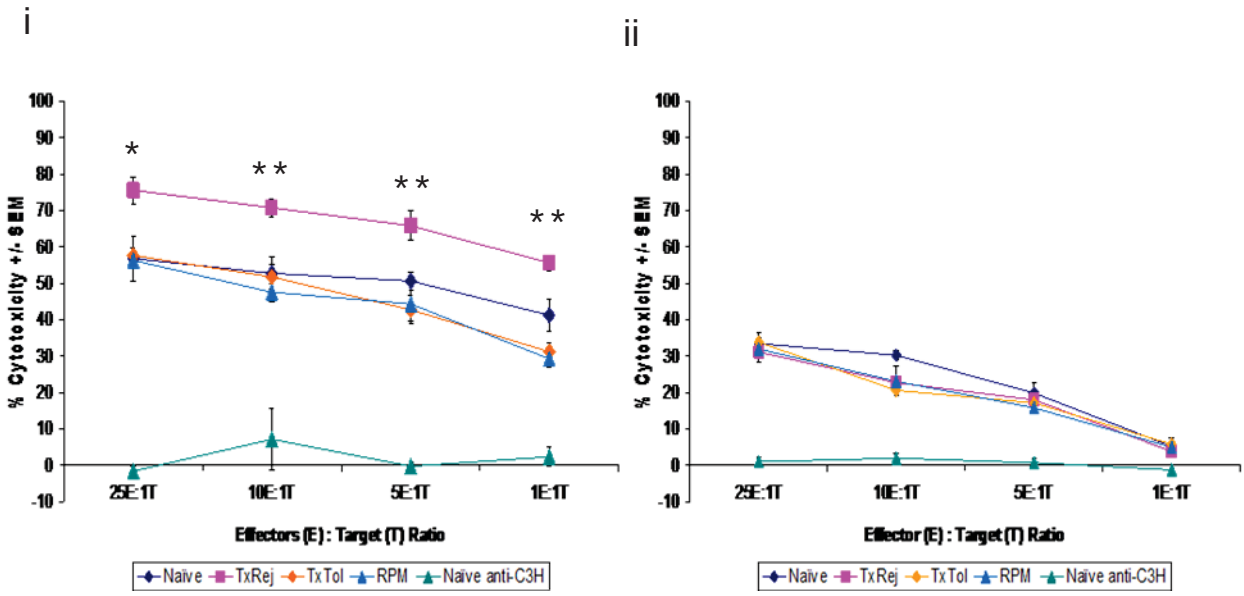


Figure 3-3B. Tolerant mice lymphocytes have reduced cytotoxicity specifically towards donor antigens compared to rejecting mice. Splenocytes were isolated from naïve, rejecting (TxRej), tolerant (TxTol), and rapamycin-only control (RPM) mice at 100 days post-transplant (84 days after rapamycin withdrawal). i) Responder splenocytes were co-cultured with irradiated donor BALB/cJ (H-2^d) splenocytes at a 1:1 ratio for 5 days. Naïve splenocytes were also incubated with irradiated C3H/HeJ (naïve anti-C3H) at a 1:1 ratio. After co-culture, cells were removed and incubated in triplicate with $1.0x10^4$ ^{51}Cr-labelled A20 cells (BALB/c derived; H-2^d) for 5 hrs at various effector-to-target ratios. ^{51}Cr released into supernatants was counted and calculated as a percent of max lysis. Compared to rejecting mice, tolerant mice had decreased cytotoxicity to donor antigens that was similar to naïve and rapamycin-only controls. The increased cytotoxicity of rejecting group splenocytes achieved statistical significance compared to the tolerant group from effector-to-target ratios of 25:1 through to 1:1. ii) Response to 3rd party antigens was similarly assessed, except responder cells were co-cultured with irradiated C57BL/6J (H-2^b) splenocytes for 5 days prior to challenging with ^{51}Cr-labelled EL4 target cells (C57BL/6J derived; H-2^b). No statistical difference was observed. Values are represented as means ± standard error mean (SEM). Graphs are representative of three independent experiments. Similar results were obtained when splenocytes were taken at day 30 post-transplant (14 days after rapamycin withdrawal).

3.4. Tolerant grafts have increased Foxp3$^+$ regulatory T-cell infiltrates compared to controls.

Previous studies identified a role for rapamycin in promoting Treg differentiation[158]. To investigate whether rapamycin-induced Tregs could promote tolerance to cardiac allografts in this model, we first assessed for the presence of Tregs in grafts through Foxp3$^+$ immunohistochemical (IHC) staining. No Foxp3$^+$ staining was observed in naïve hearts (Figure 3-Ai) and syngeneic grafts at 100 days post-transplantation (Figure 3-4Aii). Although Foxp3$^+$ staining was observed in day 7 rejecting grafts (Figure 3-4Aiii), tolerant mice at day 100 following transplantation had noticeably greater numbers of Foxp3$^+$ cells (Figure 3-4Aiv). Morphometric analysis determined Foxp3$^+$ cells as a percentage of CD4$^+$ cells within the graft. Foxp3$^+$ cells accounted for less than 1% of CD4$^+$ cells in syngeneic grafts at all time points measured. Rejecting grafts were positive for Foxp3$^+$ cells at day 7 and day 30 post-transplantation ($0.83 \pm 0.28\%$ and $1.74 \pm 1.28\%$, respectively) but no viable cells of any origin were observed at day 100 post transplant (data not shown). Conversely, percentages of Foxp3$^+$ cells were elevated in tolerant grafts at day 30 post-transplantation ($3.80 \pm 1.23\%$) and increased to $4.61 \pm 2.11\%$ by day 100 post transplant, although these increases did not reach statistical significance compared to rejecting grafts (Figure 3-4B).

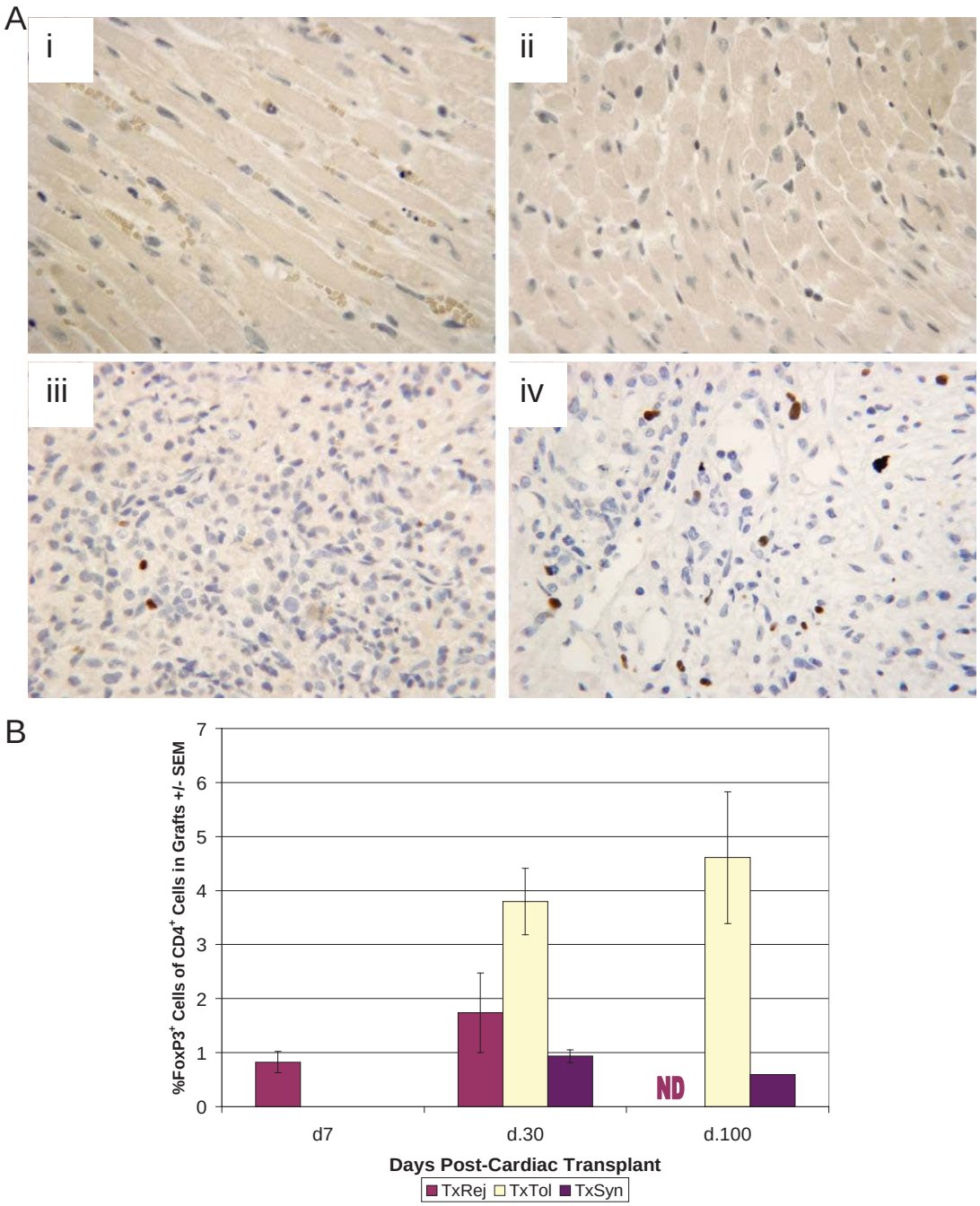


Figure 3-4. Increased Foxp3$^+$ infiltrates in tolerant grafts compared to controls. (A) Representative Foxp3$^+$ immuno-peroxidase staining of i) naïve hearts, and grafts from ii) day 100 syngeneic group, iii) rejecting group at day 7 post-transplantation, and iv) day 100 tolerant group. 400X magnification. (B) Through morphometric analysis, cells that stain positive for Foxp3 are shown as a percentage of CD4$^+$ graft-infiltrating cells from rejecting (TxRej), tolerant (TxTol) and syngeneic mice (TxSyn) at different time points after transplantation. Tolerant and syngeneic grafts were not analyzed at day 7. Day 100 rejecting grafts had no detectable viable cardiomyocytes or lymphocytes (ND = not detectable). Data represents means ± SEM of 3-6 mice in each group at each time point.

3.5. $CD4^+Foxp3^+$ regulatory T cell percentages and absolute numbers are increased in the spleens but not lymph nodes and thymus of tolerant mice compared to control groups.

Differences in Treg populations in lymphoid organs of tolerant and control mice were assessed by flow cytometry on day 30 post transplant (14 days after rapamycin withdrawal). Tregs were identified through the cell surface expression of CD4 and the coexpression of intracellular Foxp3. In spleens, tolerant mice had an increase in the proportion of Tregs as a percentage of $CD4^+$ cells and an increase in the absolute number of Tregs compared to rejecting, syngeneic, rapamycin-only, and naïve controls. (Figure 3-5 A and B). Since no draining lymph nodes (LNs) have been clearly defined in this model, the inguinal, axillary, brachial, and mesenteric lymph nodes were pooled and similarly assessed for Tregs. Although the absolute number of Tregs was elevated in rejecting controls, this did not reach statistical significance (Figure 3-5Ciii). No statistical differences were also observed in lymph node Treg populations taken as a percent of total LN lymphocytes or a percent of $CD4^+$ LN lymphocytes (Figure 3-5C i and ii). Numbers of thymic Tregs were also assessed by flow cytometry as a percentage of $CD4^+CD8^-$ single positive thymocytes. No statistical difference was seen among any group when comparing proportions or absolute numbers of Tregs from the thymus (Figure 3-5D).

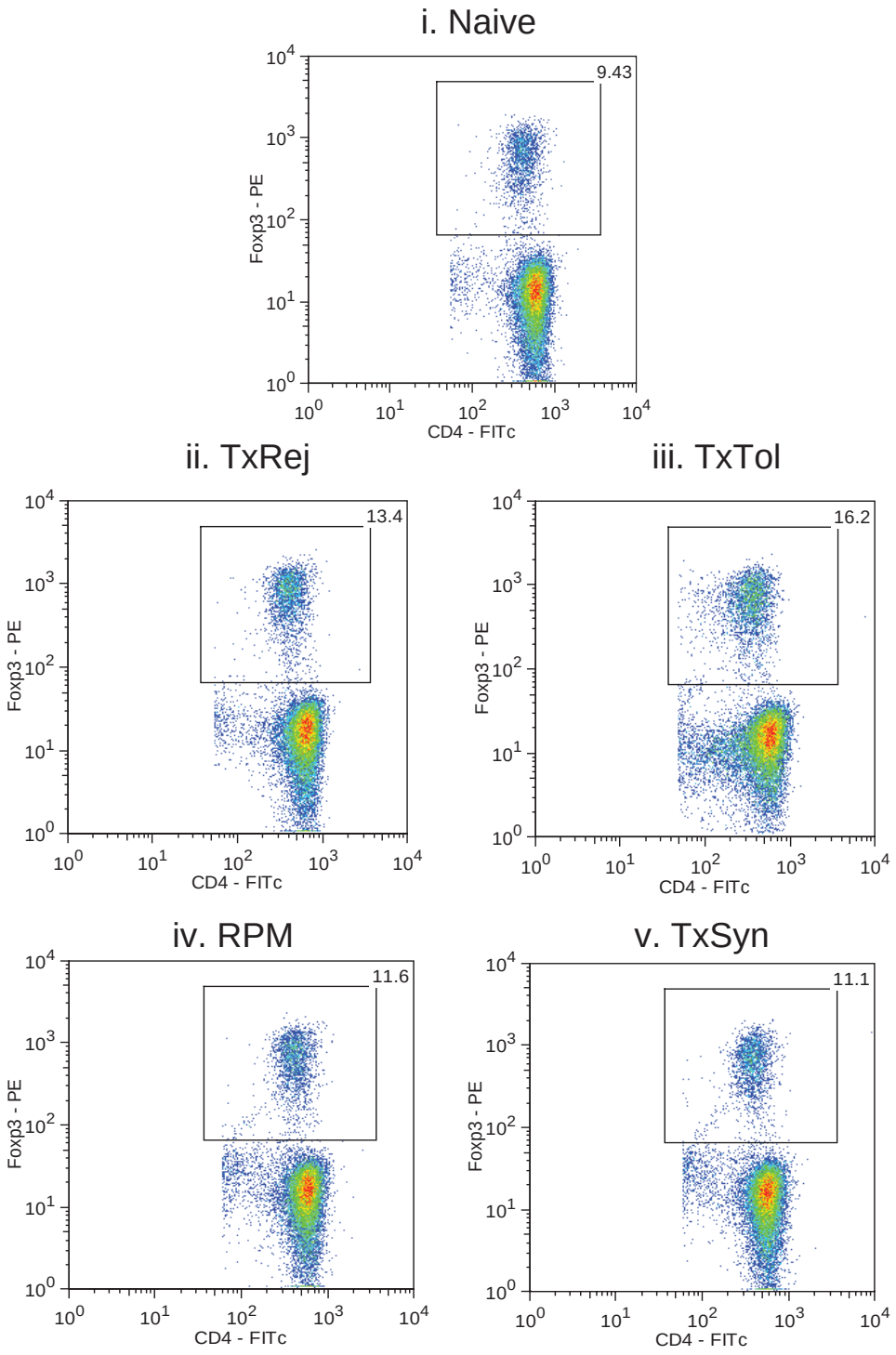


Figure 3-5A. Splenic CD4$^+$Foxp3$^+$ regulatory T cell proportions are increased in tolerant mice. Representative flow plots displaying splenic Treg population as a percent of CD4$^+$ splenocytes from i) naïve, ii) rejecting (TxRej), iii) tolerant (TxTol), iv) rapamycin-only (RPM), and v) syngeneic (TxSyn) mice. Data was collected on day 30 following transplantation (14 days after rapamycin withdrawal) and is representative of 3-4 mice per group.

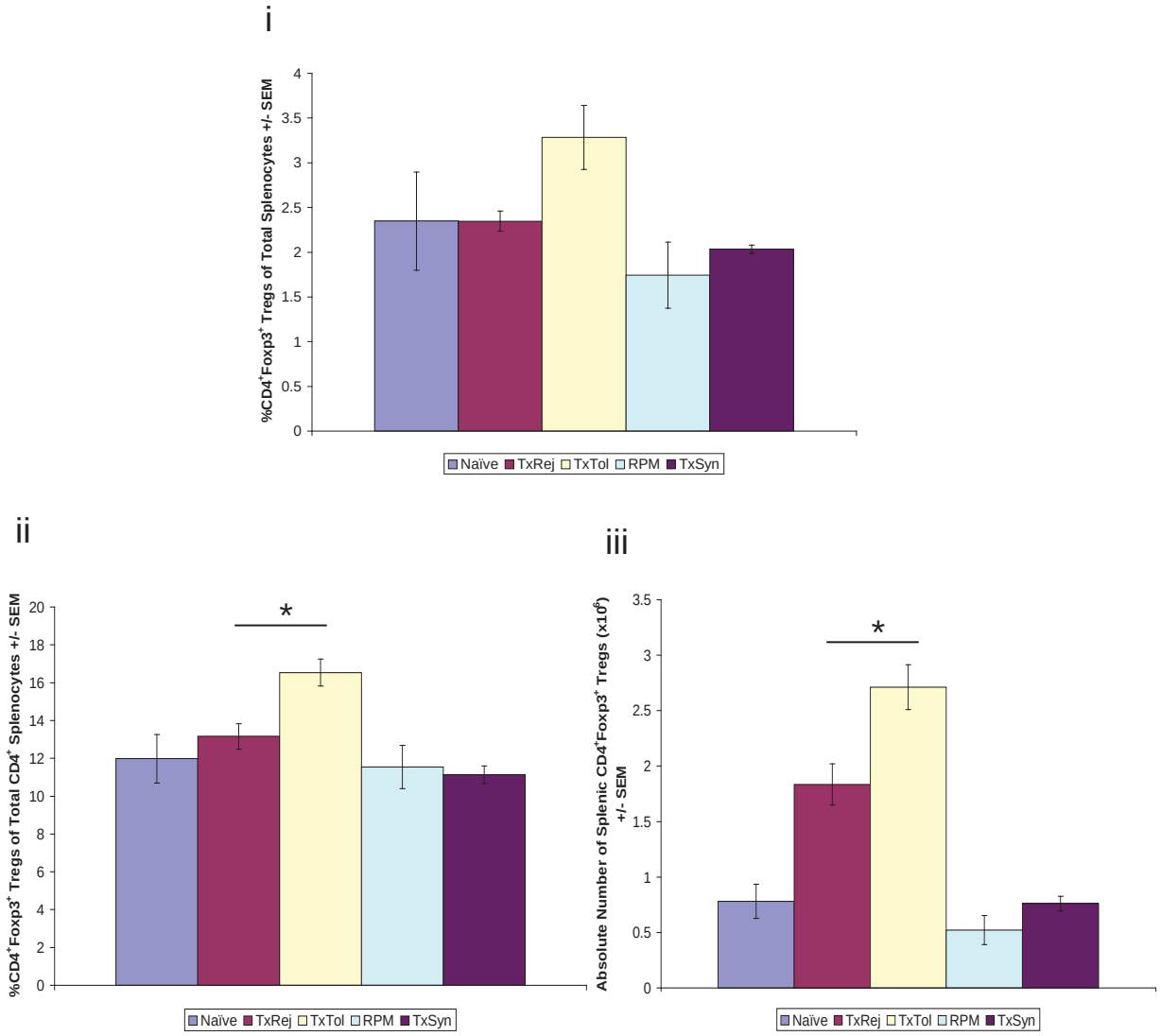


Figure 3-5B. Tregs are elevated in the spleens of tolerant mice compared to controls. Spleens from naïve, rejecting (TxRej), tolerant (TxTol), rapamycin-only (RPM) and syngeneic (TxSyn) mice were examined by flow cytometry for Treg cells as determined by co-expression of CD4 and Foxp3. Treg populations are represented as (i) a percentage of total splenocytes, (ii) as a proportion of $CD4^+$ splenocytes, and (iii) absolute numbers of Tregs calculated by multiplying the percentage of Tregs as determined by flow cytometry by the total number of splenocytes recovered in each mouse. Spleens were harvested 30 days after transplantation (14 days after rapamycin withdrawal). Data was collected from 3-4 mice in each group and represented as means ± SEM.

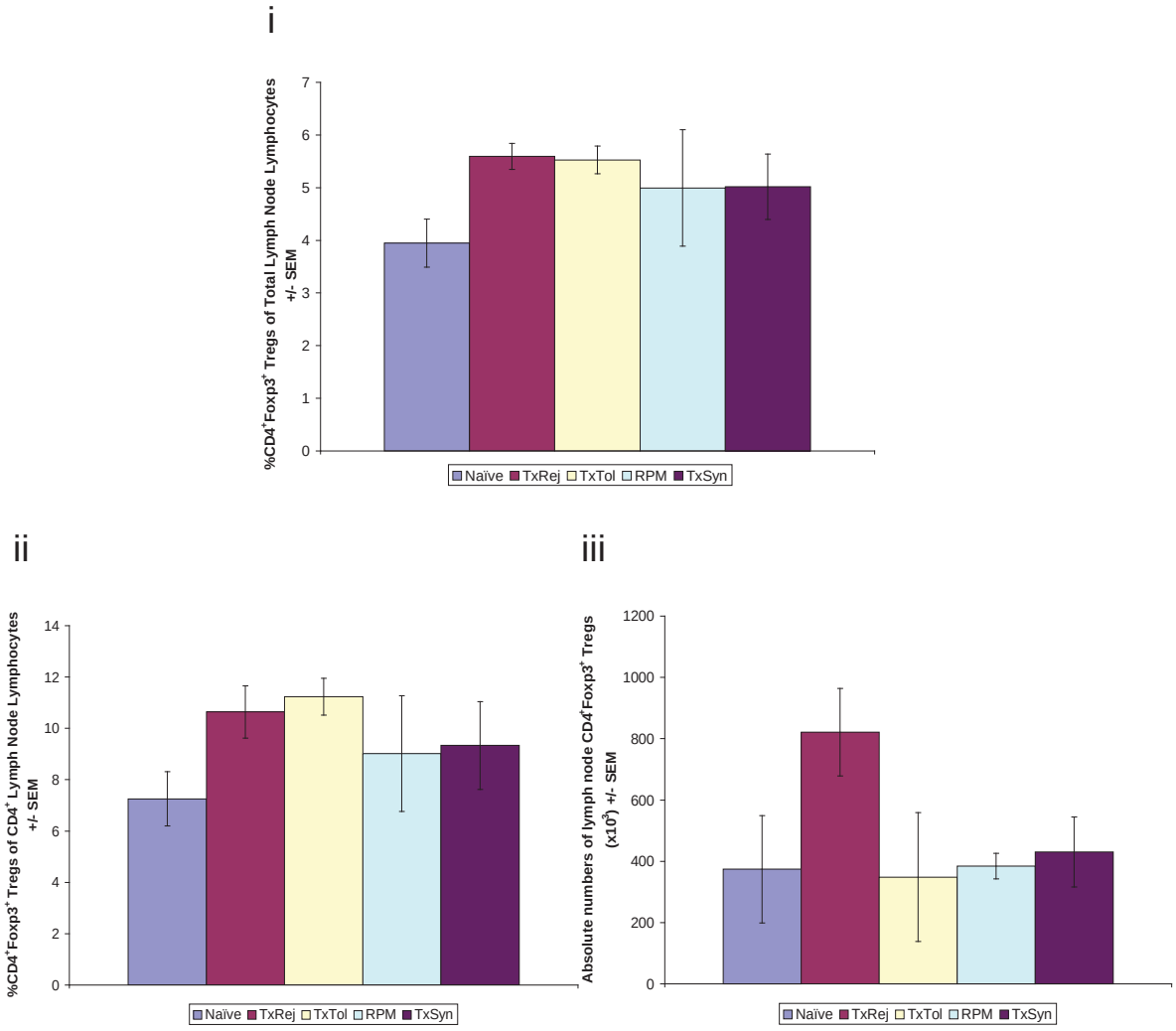


Figure 3-5C. Treg populations in the lymph node do not statistically differ between groups. LNs lymphocytes were isolated and pooled from naïve, rejecting (TxRej), tolerant (TxTol), rapamycin-only (RPM) and syngeneic (TxSyn) mice and were examined by flow cytometry for Treg cells as determined by co-expression of CD4 and Foxp3. Treg populations are represented as (i) a percentage of total LN lymphocytes, (ii) as a proportion of $CD4^+$ LN lymphocytes, and (iii) absolute numbers of Tregs calculated by multiplying the percentage of Tregs as determined by flow cytometry by the total number of lymphocytes recovered in each mouse. Lymph nodes were harvested 30 days after transplantation (14 days after rapamycin withdrawal). Data was collected from 3-4 mice in each group and represented as means ± SEM.

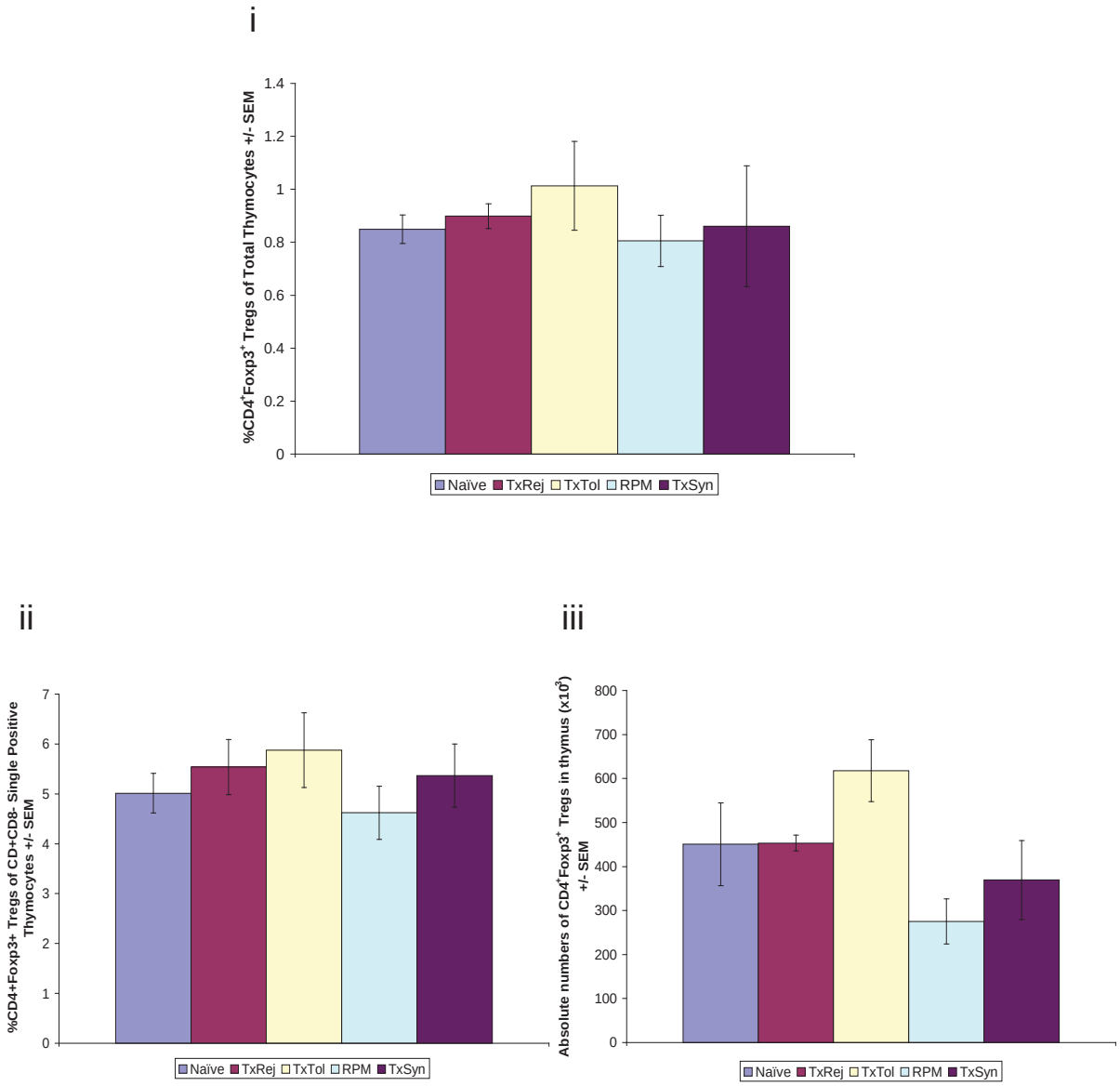


Figure 3-5D. Thymic Treg output did not statistically differ between mice. Thymocytes from naïve, rejecting (TxRej), tolerant (TxTol), rapamycin-only (RPM) and syngeneic (TxSyn) mice were examined by flow cytometry for Treg cells as determined by co-expression of CD4 and Foxp3. Treg populations are represented as (i) a percentage of total thymocytes, (ii) as a proportion of $CD4^{+}CD8^{-}$ single positive lymphocytes, and (iii) absolute numbers of Tregs calculated by multiplying the percentage of Tregs as determined by flow cytometry by the total number of thymocytes recovered in each mouse. Thymi were excised 30 days after transplantation (14 days after rapamycin withdrawal). Data was collected from 3-6 mice in each group and represented as means ± SEM.

3.6. Tolerant mice have decreased splenic $CD8^+CD44^+$ memory T cells with increased $CD62L^{LO}$ proportions compared to rejecting mice

To assess whether differences in memory T cell development could account for the acquisition of tolerance and the decreases in *in vitro* proliferation and cytotoxicity observed in tolerant group mice, we first determined proportions of $CD8^+CD44^+$ memory T cells in the spleen by flow cytometry 30 days after transplantation (14 days after rapamycin withdrawal). Spleens from rejecting mice had increase proportions of memory cells compared to tolerant mice (2.66 ± 0.33% versus 2.16 ± 0.25%, respectively), although this trend has not reached statistical significance (Figure 3-6i). Absolute splenic $CD8^+$ memory T cell numbers were significantly increased in rejecting versus tolerant mice ($2.21x10^6 \pm 2.74x10^5$ $CD8^+CD44^+$ cells compared to $1.49x10^6 \pm 4.71x10^5$ $CD8^+CD44^+$ cells, respectively; p=0.038). Absolute $CD8^+CD44^+$ cell numbers were increased in tolerant mice compared to naïve controls, but did not differ significantly from rapamycin-only and syngeneic mouse groups (Figure 3-6ii). Through flow cytometry, splenic $CD8^+CD44^+$ memory T cells were also assessed for the expression of CD62L, which differentiates effector memory cells (T_{EM}; $CD62L^{LO}$) from central memory cells (T_{CM}; $CD62L^{HI}$)[159]. Out of total $CD8^+CD44^+$ memory T cells, the proportion of T_{EM} cells in the spleen was significantly lower in rejecting mice (15.3 ± 2.4%) compared to tolerant mice (22.6 ± 4.3%) (p=0.028) whereas the proportions of T_{EM} cells in tolerant mice did not significantly differ from the proportions in all other control groups. (Figure 3-6iii).

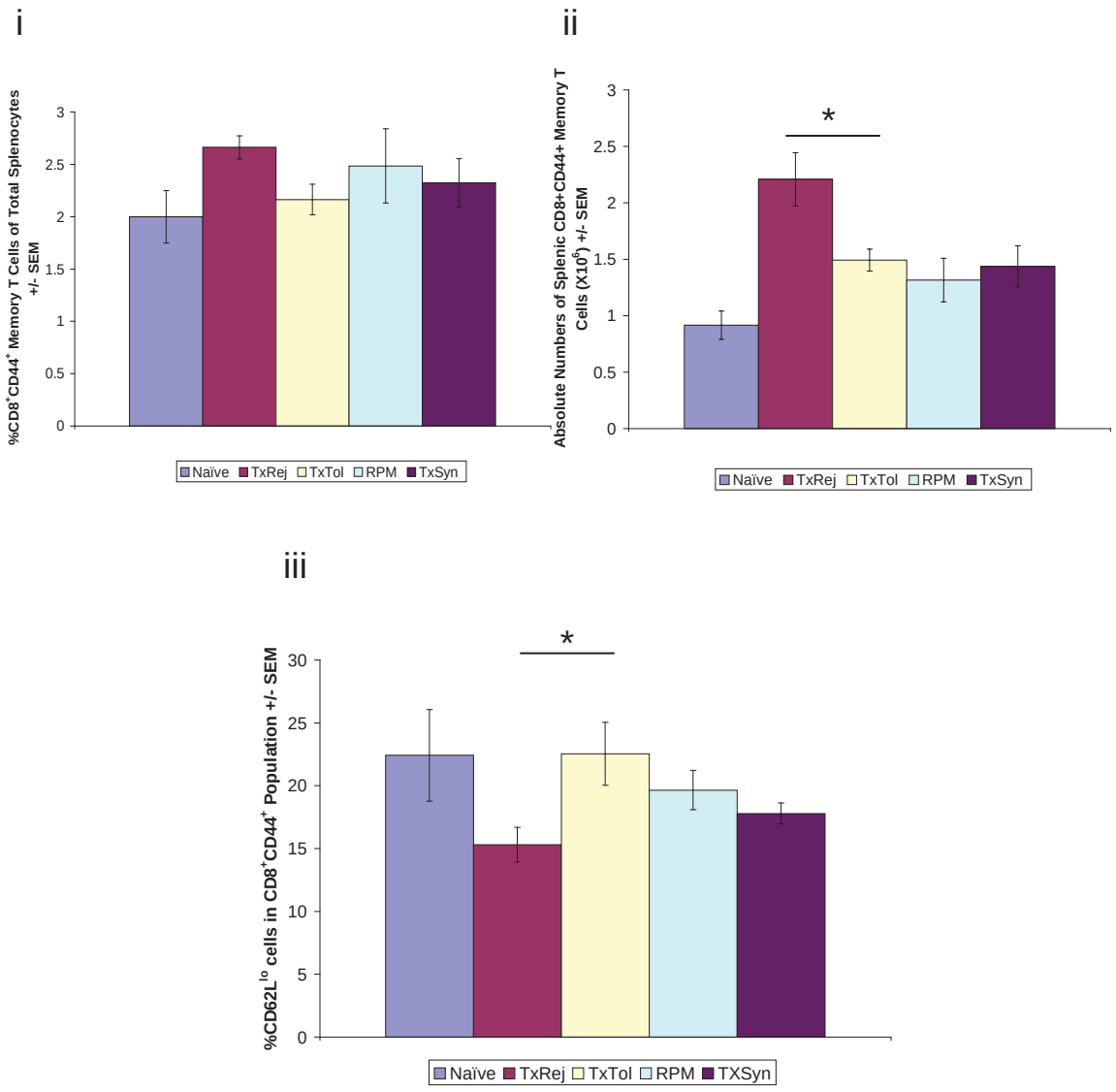


Figure 3-6. Decreased $CD8^+CD44^+$ memory T cells with increased $CD62L^{LO}$ proportions in the spleen of tolerant mice compared to rejecting mice. (i) The proportion and (ii) absolute numbers of splenocytes coexpressing CD8 and CD44 surface molecules from naïve, rejecting (TxRej), tolerant (TxTol), rapamycin-only (RPM) and syngeneic (TxSyn) mice were assessed by flow cytometry. Absolute numbers were obtained by multiplying the total splenocytes recovered by the percentage of $CD8^+CD44^+$ cells as determined by flow cytometry. (ii) The proportion of splenic $CD8^+CD44^+$ cells expressing $CD62L^{LO}$ was also assessed by flow cytometry. Splenocytes were collected at day 30 post-transplant (14 days after rapamycin withdrawal). Data were collected from 4-8 mice per group and means ± SEM are shown.

3.7. Differentially expressed regulatory T cell related genes in the cardiac allograft correlate with tolerance.

To identify putative biomarkers of tolerance, we assessed the mRNA expression of 23 regulatory T cell related genes by multiplex PCR at different time points following transplantation in tolerant and rejecting grafts, and in naïve hearts. Differentially expressed genes were confirmed by qRT-PCR. The regulatory genes *fgl-2, foxp3, tgfβ*$_1$, and *lag3* were up-regulated in tolerant grafts compared to rejecting and naïve controls (Figure 3-7i). Conversely *IFN-γ* and *granzyme B (gzmB)*, which are genes associated with inflammation, were up-regulated significantly in rejecting grafts, but not in tolerant grafts or naïve hearts (Figure 3-7ii). The remaining 18 genes were not differentially expressed between the rejecting group and day 17 or day 30 tolerant groups (data not shown).

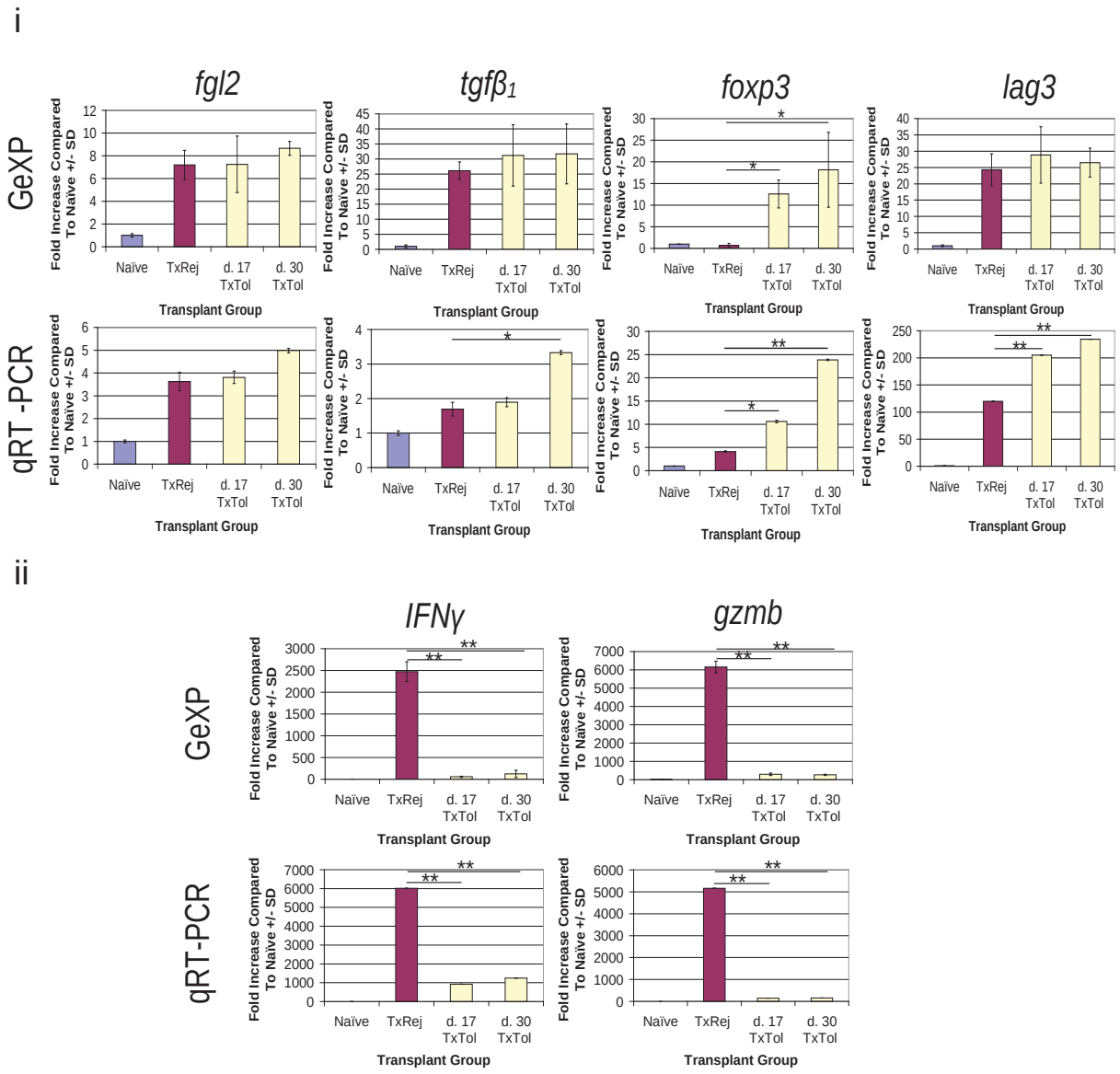


Figure 3-7. Differentially expressed regulatory T cell-related genes in the graft serve as putative biomarkers of tolerance. Graphs display genes that were differentially expressed from a panel of 23 Treg-related genes. This panel included genes associated with regulatory (i) and pro-inflammatory (ii) functions. mRNA Expression was assessed through multiplex PCR (GeXP; top rows) and trends were confirmed utilizing qRT-PCR (bottom rows). The expression of a gene was normalized to the housekeeping gene, HPRT (and also to ARP in qRT-PCR) and expression was then calculated as a fold-increase over the expression in naïve hearts. Tolerant grafts (TxTol) were obtained at day 17 and day 30 post-transplant and rejecting grafts (TxRej) were assessed at day 5 post-transplant. Three mice were used at each time point per group and data is represented as means ± SD. The remaining 18 genes analyzed were not differentially expressed between groups and graphs were not included.

3.8. Sustained elevated expression of Fibrinogen-like Protein 2 (FGL2), a known regulatory T cell effector molecule, in plasma samples correlates with graft acceptance.

An ELISA to detect plasma levels of FGL2 was utilized to determine whether biomarkers of graft tolerance could be identified in peripheral blood samples. Plasma levels of FGL2 in naïve mice were 2.45 ± 0.36 ng/mL, and represented baseline values. FGL2 plasma levels were next measured in rejecting and tolerant mice at day 5, 10, 17, 30, and 100 post-transplantation and plasma from rapamycin-only control mice were analyzed at day 30 and 100 after the first rapamycin injection. At day 5, levels of FGL2 were significantly increased in rejecting mice compared to tolerant mice (13.20 ± 1.65 ng/mL versus 6.66 ± 1.34 ng/mL, respectively; p=0.002), but returned to baseline levels at day 17. In comparison, levels of FGL2 remained elevated in tolerant mice to day 100 post-transplantation coincident with increased numbers of $Foxp3^+$ Treg in heart grafts of tolerant mice. Levels of FGL2 in tolerant mice were significantly higher than in rejecting and rapamycin-only controls at day 30 and day 100 post transplant Plasma levels did not differ significantly from baseline levels at all time points in rapamycin-only treated mice (Figure 3-8).

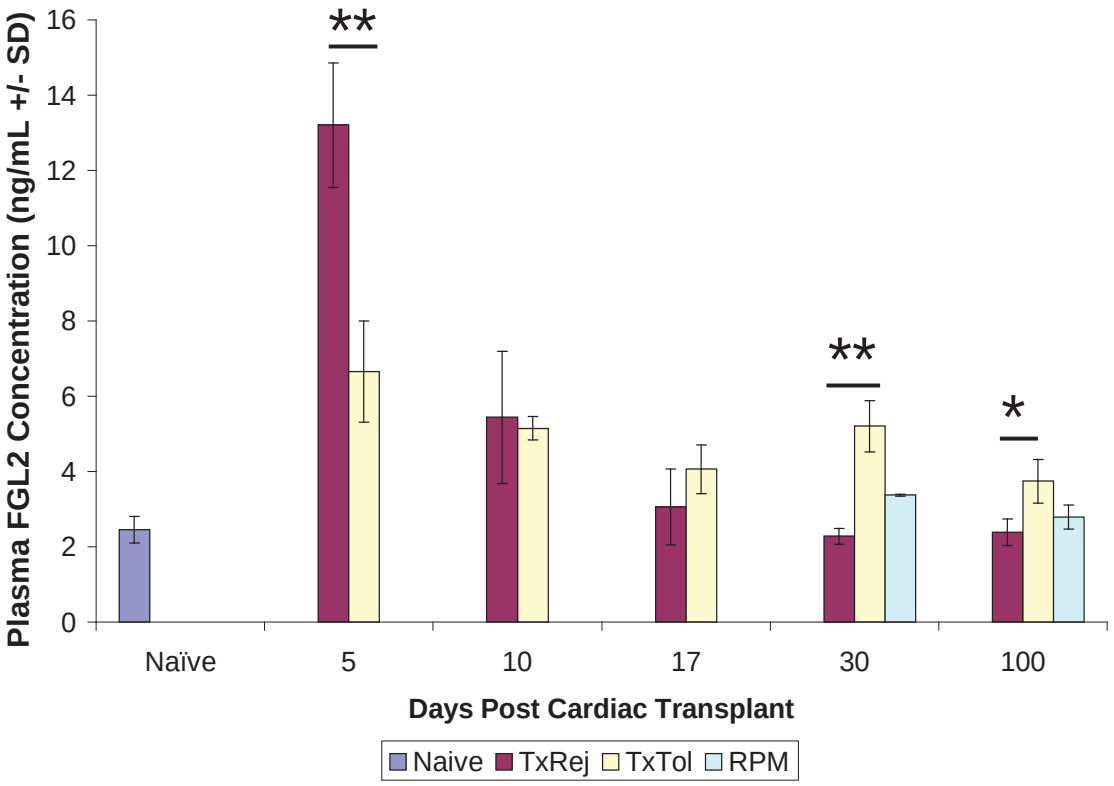


Figure 3-8. Elevated FGL2 plasma concentration is sustained in tolerant mice. Plasma levels of FGL2 in naïve, rejecting (TxRej), tolerant (TxTol), and rapamycin-only control (RPM) mice were measured by sandwich ELISA at different time points following transplantation or the first rapamycin injection. Graph shows means ± SD; n=2-3 mice per group at each time point.

4. Discussion and Conclusions

Transplantation has evolved into the most effective treatment for patients with various end-stage organ failures; however its long-term success is greatly limited by the continuous requirement for immunosuppressive therapy to prevent graft rejection. These immunosuppressant agents are often responsible for many side effects that greatly increase the morbidity and mortality of transplant patients, as reviewed in section 1.3. As a result, strategies which would allow for the minimization or elimination of immunosuppressive agents in patients have been actively pursued.

The discovery of biomarkers that correlate with transplantation tolerance might allow for the identification of patients that have achieved a state of tolerance to their graft and can have their immunosuppression appropriately reduced or withdrawn. Current attempts to describe potential biomarkers in patients have been limited due to the scarcity of known tolerant patients and a lack of appropriate control groups to study. While most biomarker studies therefore focused on identifying markers of graft rejection, a few studies took advantage of a small cohort of known tolerant kidney and liver transplant patients. The results of these studies have been reviewed in section 1.5.2. However, due to the low sample size and lack of adequate controls in these studies, it remains to be determined if these markers can be widely applied to transplant patients.

In order to overcome these inherent obstacles in biomarker identification and validation in patients, our group has approached transplantation tolerance biomarker discovery by analyzing a robust model of solid-organ allograft tolerance in mice. To accomplish this, we adapted a mouse model of tolerance previously described by Yi and

colleagues[103]. Hearts were excised from a BALB/cJ donor (MHC haplotype H-2^d) and transplanted heterotopically into the peritoneal cavity of a C3H/HeJ recipient (MHC haplotype H-2^k). This model was selected because graft function and survival could be easily assessed through transabdominal palpation of the donor heart and the full MHC mismatch replicates full mismatches observed in most patients. A short regimen of the immunosuppressant agent rapamycin over the first 16 days following transplantation established long-term graft function that was maintained indefinitely without the need for further immunosuppression (Figure 3-1A). Without this treatment, grafts from transplanted mice rejected consistently between day 8 and 10 following surgery. These findings were confirmed through histology of the transplanted grafts. After 100 days following transplantation, the structure of the myocardium of grafts from the tolerant group was well preserved and was similar to the structure of naïve hearts or grafts from day 100 syngeneic controls. In comparison, rejecting group grafts at day 100 had no viable cardiac tissue (data not shown). Rejecting heart allografts at day 7 showed large mononuclear cell infiltration within the interstitium of the graft as well as within the graft's endothelium that resulted in marked vasculitis. Although an increase in mononuclear cell infiltrates was observed in tolerant grafts, the cellular infiltrates were markedly reduced compared to acutely rejecting grafts. There was also an absence of vasculitis in tolerant grafts (Figure 3-1B). Thus, these findings illustrate that cardiac allograft function was indefinitely maintained in this mouse model following a 16 day course of rapamycin.

C3H/HeJ mice, which were utilized as graft recipients, possess a defective *tlr4* gene which could impair the immune response towards the graft, thus allowing for the

acquisition of long-term graft survival following the induction protocol. TLR4 is cell surface receptor on APCs that recognizes lipopolysaccharide (LPS), which is evolutionarily conserved on gram-negative bacteria. Upon ligation to LPS, downstream signals of TLR4 initiate the transcription of NF-κB that then allows for the activation and maturation of macrophages and DCs. While macrophages can then promote inflammatory responses, DCs elevate the expression of MHC class II and co-stimulatory molecules allowing for the efficient activation of T-cells[1-3]. Tolerant group mice may be unresponsive to bacterial infections that occur during or after transplantation, and this impaired response could have significant impact on graft survival. In order to assess whether the impaired *tlr4* gene contributes to the phenotype in tolerant mice, a control group was utilized with $tlr4^{+/+}$ C3H/HeOuJ mice (that are otherwise genetically identical to C3H/HeJ mice) as recipients. Despite the presence of functional TLR4, allogeneic BALB/cJ cardiac grafts were accepted and beat indefinitely in C3H/HeOuJ recipients that received the induction protocol (Figure 3-1A). This indicates that the *tlr4* deficiency in C3H/HeJ does not contribute to the acquisition of long-term graft survival following rapamycin treatment.

It was further investigated whether the phenotype in tolerant group mice could be achieved using different immunosuppressive agents. CsA, a calcineurin inhibitor, was administered over the first 16 days following transplantation of BALB/cJ hearts into C3H/HeJ recipients. All six grafts in this group rejected between days 26 and 35 post-transplantation (Figure 3-1A), indicating long-term graft survival cannot be induced utilizing CsA. This suggests that rapamycin, but not CsA, promotes distinct pathways

that allow for grafts to remain functional over the long-term after the withdrawal of immunosuppression.

The immunosuppressive effects of rapamycin are well known and it was therefore critical in our study to ensure that long-term graft survival was not a result of generalized immunosuppression. Rapamycin exerts these effects by targeting mTOR, which is a serine/threonine protein kinase that promotes a number of cellular events including cell growth and proliferation, transcription, and mRNA translation[75]. mTOR exists in two complexes, the rapamycin-sensitive mTORC1 and the rapamycin-insensitive mTORC2. In mTORC1, mTOR is in a complex with regulatory associated protein of mTOR (RAPTOR) which is critical for its activity. While lymphocytes remain inactivated, mTORC1 is inhibited by a complex composed of tuberous sclerosis complex 1 (TSC1) and TSC2. Following the activation of the phosphatidylinositol 3-kinases (PI3K) – AKT pathway that occurs after lymphocyte activation, mTOR is released from its inhibition[160]. Rapamycin can prevent mTOR-dependent processes by first creating a complex with FK506-binding protein 1A, 12kDa (FKBP12), which then binds to the FKBP12-rapamycin-binding (FRB) domain of mTORC1. This binding is proposed to disrupt the interaction between mTOR and RAPTOR that is critical for mTOR's function[75]. As a result, rapamycin prevents the mTOR dependent activation and proliferation of lymphocytes that would normally occur after antigen engagement with costimulatory and cytokine signals. In our study, it was important to ensure that the long-term graft survival was not a result of this immunosuppressive effect, but rather of tolerance induction.

Donor-antigen specific tolerance was first demonstrated *in vivo* in this model by adding an orthotopic skin graft onto C3H/HeJ mice that, 30 days prior to the skin graft,

were transplanted with a BALB/cJ cardiac graft and given the rapamycin protocol. Four out of five skin grafts from BALB/cJ mice, which are the same strain as the heart donor, lasted for ≥30 days, whereas skin grafts from a 3rd party C57BL/6J mice were rejected between day 11 and 19 following skin grafting (Figure 3-2). Therefore, mice that accepted their cardiac allografts remained immunocompetent by mounting an immune response towards 3rd party alloAgs, while remaining tolerant specifically towards donor alloAgs.

Similarly, donor-specific tolerance was confirmed through *in vitro* assays. Lymphocytes from C3H/HeJ recipients were challenge with irradiated donor (BALB/cJ) or 3rd party (C57BL/6J) splenocytes in a mixed lymphocyte reaction to assess CD4^{+} T cell proliferation (Figure 3-3A). Also, a ^{51}Cr- release assay assessed the ability of recipient CD8^{+} lymphocytes at killing radio-labeled target cells that were derived from either donor or 3rd party mice (Figure 3-3B). When challenged with donor alloAgs, rejecting mice exhibited significantly increased proliferation and cytotoxicity compared to all other groups. The elevated responses in rejecting mice were expected since the previous exposure to graft Ags would promote the development of a more robust secondary immune response. Despite the antigen exposure through the graft in tolerant mice, the proliferation and cytotoxicity of their lymphocytes in response to donor Ags were impaired compared to rejecting mice and were equivalent to the responses in naïve and rapamycin-only controls. This suggests that tolerant mice achieve a state of split tolerance, whereby graft function is maintained *in vivo* although tolerant mice remain capable of responding to donor alloAgs in these *in vitro* assays (albeit reduced in comparison to rejecting mice). Furthermore, when challenged with 3rd party alloAgs, the

proliferation and cytotoxicity responses in all groups were statistically equivalent indicating that tolerant mice remained fully immunocompetent to these antigens. These assays were also performed 30 days after transplantation and similar results were attained (data not shown). Together, these studies strongly suggest that donor-specific tolerance was achieved in tolerant mice and not generalized immunosuppression.

The immune mechanisms responsible for establishing donor-specific tolerance in this model were then investigated. The development of split tolerance in transplanted mice receiving the rapamycin induction protocol suggests that central tolerance mechanisms are not contributing to graft acceptance. This is because negative selection would be expected to clonally remove most donor-reactive cells and no responses would then be expected from lymphocytes from tolerant mice in these *in vitro* assays. Therefore, the contribution of peripheral tolerance mechanisms was examined.

The role of Tregs in promoting tolerance in this model was first investigated. Tregs could account for the *in vitro* responses observed in tolerant mice as an increase in donor-specific Treg proportions and/or activity could suppress the function of donor-reactive T cells. Previous reports also associated rapamycin treatment with an increase in Treg activity. In studies by other groups, as long as rapamycin was being administered, an increase in the proportion of Tregs compared to other $CD4^+$ T cells was observed in all lymphoid compartments in mice and patients[161]. *In vitro*, Treg populations were also increased when T cells were cultured with rapamycin. Moreover, rapamycin treated Tregs from mice and humans were able to retain their ability to suppress proliferation of activated T cells[158, 162]. Therefore, the administration of rapamycin in this model could

promote Treg numbers and function that would account for the development of donor-specific split tolerance.

Tregs are proposed to be resistant to the immunosuppressive effects of rapamycin since they are thought to be less reliant on the rapamycin-sensitive PI3K-AKT-mTOR pathway for cell cycle progression and activation. Evidence for a role of a different activation pathway include the observation that phosphatase and tensin homologue (PTEN) expression is sustained following Treg TCR activation, which is normally down-regulated in conventional T cells[163]. This maintained PTEN expression would prevent activation of the PI3K-AKT-mTOR pathway, and other signaling pathways would therefore be required for Treg cycling and activation. It is further proposed that normal Treg function can occur as a result of the serine/threonine-protein kinase PIM2[164]. PIM2 is induced in activated Tregs independent of mTOR through the synergistic effects of the Foxp3 transcription factor and signal transducer and activator of transcription 5 (STAT5) that are activated after TCR and IL-2 ligation. It has known anti-apoptotic functions and is suggested to have a role in cell growth through the observation that it can cooperate with other oncogenes to promote malignant transformations of cells[165]. Therefore, unlike conventional T cells, rapamycin treatment does not appear to affect the survival or function of Tregs.

Evidence also suggests that rapamycin can promote Treg differentiation from naïve $CD4^+CD25^-Foxp3^-$ T cells. *In vitro*, initial activation through TCR and costimulatory molecules results in the exposure of the *foxp3* gene locus through chromatin remodeling in a process dependent on the PI3K-AKT-mTOR pathway. Normally, mTOR-dependent pathways would then inhibit the expression of Foxp3. With

rapamycin, the mTOR-dependent inhibition of Foxp3 is prevented, and normal STAT5 activation through IL-2 or IL-15 binding to IL-2R could then promote Foxp3 expression and the subsequent conversion of the cell to a Treg phenotype[166, 167]. While rapamycin could also prevent the mTOR dependent exposure of the *foxp3* locus, this process only requires limited TCR and costimulatory signals. Therefore, the efficiency of chromatin remodeling and *foxp3* exposure in a naïve T cell following its activation would depend on the strength of the dose and the time at which rapamycin inhibition is initiated. Together, this process provides another explanation for enhanced Treg cell numbers and activity following rapamycin.

Tregs were identified as potentially important mediators in establishing tolerance to cardiac allografts in this model. Their populations were found elevated both in the graft and spleens of tolerant mice compared to all other controls (Figures 3-4 and 3-5A, B). The increase could be a result of the rapamycin-mediated effects on Tregs previously described, and constant alloAg stimulation could maintain this population. Moreover, there were no differences in thymic outputs of Tregs in tolerant mice compared to other groups, which is consistent with rapamycin's role in promoting the peripheral conversion of naïve $CD4^+$ T cells into Tregs (Figure 3-5D). Compared to background levels, Tregs were also increased in the grafts and spleens of rejecting mice, possibly as a response to control the inflammation associated with rejection (Figure 3-5A, B). Without this counter-regulatory mechanism, inflammation could prove fatal to the animal. However, rejecting mice had lower percentages and numbers of Tregs compared to tolerant mice, and these Tregs may also lack specificity for alloAgs. Conversely, the increase in Treg populations in tolerant mice may be sufficient in maintaining tolerance specifically to the

graft. To confirm the role of Tregs in maintaining graft tolerance, a Treg depletion assay using anti-CD25 antibodies (clone PC61) is currently being performed. In preliminary studies, *in vivo* administration of 250μg of anti-CD25 antibody, injected i.p. into tolerant mice on days -2, 0, 3, 6, 9, 12, and 15 following transplantation has resulted in graft loss in 67% of mice (data not shown). To complete these studies, more mice receiving this protocol and a control group receiving an IgG1 isotype control antibody are required.

Impaired memory T cell development and function might also be contributing to allograft acceptance in tolerant mice. Under normal conditions, memory T cells develop after initial exposure to Ags and are responsible for a quick and robust secondary response after a subsequent challenge with the same Ag. This secondary immune response was observed in rejecting mice in the *in vitro* MLR and CTL assays, but absent in tolerant mice. Despite being primed with alloAgs, the responses of tolerant mice were similar to naïve and control mice which mount primary responses. Thus, this observation in tolerant mice could be a result of impaired memory T cells.

Memory T cells in mice can be either $CD4^+$ or $CD8^+$ and both express $CD44^{HI}$. They are further divided into central memory (T_{CM}) or effector memory (T_{EM}) subsets based on $CD62L^{HI}$ or $CD62^{LO}$ expression, respectively. T_{CM} cells reside in secondary lymphoid organs whereas T_{EM} cells circulate throughout the periphery where they encounter antigens[159]. The unique properties of these cells allow them to have robust responses to antigens that can significantly contribute to graft rejection.

Memory T cells have been identified as a potent barrier to achieving tolerance to allografts since they require a lower activation threshold and can mediate robust anti-graft

responses. Direct cytotoxicity towards targets is rapidly acquired in memory T cells as they have been shown to preferentially lyse cells through granzymes and perforin stored in granules ready for release instead of Fas/FasL interactions that require time in order to be expressed on cell surfaces[168]. They also contain higher levels of preformed IFN-γ and other proinflammatory cytokines and chemokines that allow them to quickly initiate delayed-type hypersensitivity responses[168]. The expression of different adhesion molecules and chemokine receptors may also allow for rapid migration of memory T cells to sites of inflammation[169]. Furthermore, they have been found to be more resistant to many immunosuppressive agents compared to naïve T cells[170, 171]. As a result of their potential role in mediating rejecting and preventing tolerance, the proportions and phenotype of these cells were investigated in this model.

Differences in memory T cell populations were found in tolerant mice that may contribute to the development of allograft tolerance. $CD8^+CD44^+$ memory T cells were found elevated in the spleens of rejecting mice compared to all other groups. Conversely, tolerant mice had equivalent proportions of these T cells as naïve mice (Figure 3-6i). The absolute splenic $CD8^+CD44^+$ memory T cell number was also lower in tolerant mice compared to the rejecting group. Although the absolute number of splenic $CD8^+CD44^+$ memory T cells was higher in tolerant mice compared to naïve controls, it was similar to syngeneic and rapamycin-only controls (Figure 3-6ii). No differences were observed in $CD4^+CD44^+$ memory T cells (data not shown). Further examining the splenic $CD8^+CD44^+$ populations, the expression of $CD62L^{LO}$ on these cells was found to differ between groups. Tolerant mice had similar $CD62L^{LO}$ proportions compared to naïve mice, and both were higher than the proportion found in rejecting mice (Figure 3-6iii).

This observation may suggest that these normally circulating $CD62L^{LO}$ T_{EM} cells in tolerant mice instead remain in secondary lymphoid organs and are incapable of trafficking to the periphery where they would mediate a response against the graft. This hypothesis could be further supported after examining the CD62L expression level on circulating peripheral blood $CD8^+$ memory cells. Nevertheless, it is likely that this observed phenotype is a result of contributions from multiple mechanisms. This could be a result of T_{EM} cell intrinsic defects that prevent their efficient migration. For instance, rapamycin has been reported to increase the expression of CD62L, which may prevent T_{EM} cells from escaping secondary lymphoid organs[172]. Dysfunctional proinflammatory cytokine and chemokine release from the graft may not signal T_{EM} cells to migrate to this organ, also resulting in their accumulation in secondary lymphoid organs. To determine the cause of the differences in splenic $CD8^+$ memory T cells in tolerant mice and to determine the functional consequences of these differences on graft survival, further studies are required. For example, this could be addressed, in part, by an adoptive transfer of carboxyfluorescein succinimidyl ester (CFSE) labeled $CD8^+$ memory T cell between tolerant and rejecting hosts to monitor cell migration and to observe the effects on graft survival.

The observed differences in $CD8^+$ memory T cells in tolerant mice appear to contradict recent reports that mTOR inhibition in fact increases the quantity and quality of these cells in other models. This observation was first made in mice that were infected with an acute form of lymphocytic choriomeningitis virus (LCMV) that concurrently received low doses of rapamycin (75μg/kg/d)[173]. Rapamycin administration during the expansion phase of T cell activation (from days 0 to 8 of infection) counter-intuitively led

to an increase in memory T cells after viral clearance. When rapamycin was administered from days 8-30, during the contraction phase of the T cell response, the numbers of memory T cells were not affected, but a greater proportion expressed markers of highly active memory T cells ($CD127^{hi}$, $CD62L^{hi}$, $KLRG1^{lo}$, $CD27^{hi}$, $Bcl2^{hi}$). When administered throughout the infection, the result was both an increase in the number and quality of $CD8^+$ memory T cells[173]. This effect was shown to be intrinsic to antigen-specific $CD8^+$ T cells, as RNA interference (RNA_i) knockdowns of mTOR and RAPTOR in these cells caused an increase in memory cell markers[173]. This study indicates that mTOR is important in the decision between effector and memory cell fates. $CD8^+$ effector lymphocyte differentiation is promoted through STAT4 signaling downstream of IL-12, which sustains mTOR transcription that then causes T-bet expression. T-bet allows for $CD8^+$ effector differentiation and prevents expression of eomesodermin, a transcription factor implicated in memory cell development[174]. It is therefore proposed that low mTOR activity, as a result of environmental cues or mTOR inhibition through rapamycin, prevents T-bet expression that results in increased eomesodermin levels, thus promoting a memory cell phenotype. These findings strongly indicate a role for mTOR inhibition in increasing $CD8^+$ memory cell numbers and quality.

Our findings do not directly contradict the above reports but rather suggest that additional factors are involved in tightly regulating $CD8^+$ memory T cell differentiation. In fact, $CD8^+CD44^+$ memory T cells are increased in rapamycin-only control mice compared to naïve controls, which is in agreement with the reported data. The lower numbers of these memory cells in rapamycin-treated versus untreated allograft recipients does not necessarily oppose the previous findings, as our observations may be the result

of different doses of rapamycin and the different antigens utilized in our model. A low dose of rapamycin was utilized in the viral model so that T cell responses would not be entirely inhibited. In comparison, a higher dose was utilized in our transplant model that efficiently prevented rejection during its administration. This elevated dose could prevent the reported effects on memory cells by completely inhibiting both effector and memory cell development. Furthermore, it is well recognized that alloAgs elicit a more robust T cell response compared to viruses and most other antigens. This elevated response could result in the development of a larger $CD8^+$ memory T cell population in untreated allograft recipients than untreated virally infected mice. Finally, other cell types in the tolerant allograft model may cause a decrease in $CD8^+$ memory cells. For instance, Treg differentiation following rapamycin treatment is well documented. In this allograft model, the higher rapamycin doses and robust signaling induced by the alloresponse may produce more Tregs in tolerant mice that then could efficiently prevent both T cell activation and $CD8^+$ memory T cell differentiation. Therefore, Tregs could be responsible for the reduction in memory T cells in tolerant mice, whereas the Treg numbers are insufficient to prevent memory differentiation in the viral model. The findings in the mouse allograft model thus suggest memory cell differentiation in response to rapamycin is dependent on the strength of mTOR inhibition and the type of antigen.

After identifying Tregs as potentially important mediator in promoting tolerance to these cardiac allografts in mice, perhaps by preventing both the activation of alloreactive T cells and the development of $CD8^+$ memory T cells, we investigated the possibility of using Treg-associated genes as biomarkers of transplantation tolerance. A panel of 23 Treg associated genes were analyzed through multiplex PCR on naïve,

rejecting (day 5 post-transplantation) and tolerant (day 17 and day 30 post-transplantation) hearts. The sensitivity of this assay was low when examining transplanted cardiac tissue since the primers for multiplex PCR were optimized on splenocytes. Therefore, genes that appeared differentially expressed between groups were further validated and confirmed through qRT-PCR. Four genes associated with Tregs were found elevated in tolerant grafts compared to naïve or rejecting hearts. Among these genes was *foxp3*, the transcription factor considered the master regulator of Tregs in mice[131]. Tolerant grafts also had elevated expression of the genes *lag3,* which encodes a Treg cell surface protein that prevents APC maturation upon binding to MHC[139], and *tgfβ$_1$,* which encodes the immunomodulatory cytokine TGF-β[146]. Similarly, tolerant grafts had an increase in *fgl2* expression, which encodes a cytokine released by Tregs that inhibits immune responses by binding to Fc$_\gamma$RIIb causing apoptosis of B cells and preventing APC maturation[144] (Figure 3-7i). Conversely, the expression of certain pro-inflammatory mediators was greatly reduced in tolerant grafts and naïve hearts compared to rejecting grafts. This included the gene for IFN-γ, a cytokine released by T_H1 that causes a delayed-type hypersensitivity response by promoting the differentiation of monocytes into macrophages. Macrophages can further recruit $CD8^+$ T cells and IFN-γ also helps $CD8^+$ T cells acquire efficient cytotoxic activities[36]. Similarly, the gene for granzyme B, a soluble cytolytic molecule released by activated $CD8^+$ T cells and NK cells, was increased in rejecting grafts[38, 39] (Figure 3-7ii). Together, this genomic data highlights that Treg activity is increased in grafts of tolerant mice while inflammatory mechanisms are inhibited. This distinct expression pattern in tolerant grafts serves as a putative biomarker of transplantation tolerance. Following a biopsy of a heart or other

transplanted organ, the presence of this genomic profile could potentially identify patients that may have achieved tolerance to their graft.

While biopsies could be used to determine the genomic profiles of transplant patients, a less invasive method to identify biomarkers of transplantation tolerance would be desirable. The expression of the immunoregulatory cytokine FGL2, which we previously found elevated in tolerant grafts, was therefore examined as a putative biomarker in peripheral blood samples. The plasma of naïve, rejecting, and tolerant mice at various time points following transplantation was therefore assessed for this protein. Rejecting mice were found to have higher levels of FGL2 in the plasma than tolerant mice 5 days after transplantation. We speculate that this increase is meant to counter-balance the excessive inflammation associated with rejection, which would be detrimental to mice if left uncontrolled. However, the concentration of plasma FGL2 in rejecting mice declined and returned to baseline levels by day 17. Although the increase over baseline was not as dramatic in tolerant mice at day 5 following transplant, the increase in plasma FGL2 levels was maintained over 100 days (Figure 3-8). Therefore, the sustained elevation of plasma FGL2 could be utilized as another biomarker of transplantation tolerance.

Further studies are required in order to ensure the applicability of these biomarkers to patients and to identify other biomarkers of transplantation tolerance. While the described Treg expression pattern may be most beneficial in identifying subsets of patients who develop tolerance to their grafts through Treg-dependent mechanisms, it would be expected to be less effective at identifying patients who achieve tolerance through different mechanisms. A more inclusive biomarker panel could be

created by further identifying biomarkers related to different tolerance induction pathways. This can be accomplished by determining other pathways that may contribute to graft tolerance in our cardiac allograft model. For instance, impaired $CD8^+$ memory T cell development has been described in this model, and a decrease in memory cell makers could provide additional biomarkers of tolerance. Rapamycin has also been implicated in promoting other tolerizing mechanisms which have yet to be evaluated in this model. Rapamycin has been shown to inhibit DC differentiation, maturation, antigen-uptake, antigen presentation, migration, and cytokine production, while promoting DC apoptosis[175, 176]. Together, these effects impair DC cells from effectively interacting and activating T cells, potentially reducing or prevent efficient alloresponses. Biomarkers associated with this, and other rapamycin-sensitive pathways, could strongly correlate with tolerance. An unbiased, genome-wide cDNA microarray analysis of tolerant cardiac allografts may identify the involvement of these different pathways and the resulting expression pattern found could also serve as biomarkers. Nevertheless, tolerance in patients could be acquired through a number of different mechanisms, many of which may not be involved in this particular model. The addition of biomarkers identified from other models of graft tolerance would provide a more extensive screen for tolerance in patients. Furthermore, before their clinical use, biomarkers discovered in mice from our current work or from other models would have to be validated for their applicability in patients. Once verified, the combination of these biomarkers could provide a comprehensive screen to identify patients that have achieved a degree of tolerance to their allografts and could have their immunosuppression minimized or withdrawn.

The panel of biomarkers that we have identified for transplantation tolerance differs from those described in studies that have examined a small cohort of patients who have been found to be tolerant to their graft. These latter studies have identified that B cells, NK cells, NK T cells, and γδ T cell markers correlate with graft tolerance[155, 156]. These markers were not directly tested in this study as the scope was limited to only assessing Treg-associated markers since these cells were identified as potentially important mediators of tolerance in this model. However, Treg markers did not associate with tolerance in studies from patients. This could be a result from the different tissues utilized in patient studies. With the aim to minimize the invasiveness of the assay, urine or blood samples were studied, while a critical role for Tregs may still exist within the grafts of these patients. Differences in identified biomarkers may also reflect differences in the organ studied and the mechanisms by which tolerance is established. Finally, the importance of different markers may also be exaggerated in these patient studies because of the relatively small patient sample size. The approach of biomarker discovery in mice is advantageous because it avoids this problem, and easily allows for large sample sizes, appropriate control groups, and is reproducible.

This study demonstrates the feasibility of identifying biomarkers that correlate to transplantation tolerance after establishing a robust model of allograft tolerance in mice. The short rapamycin induction protocol was shown to be successful in establishing long-term donor-specific tolerance towards a fully mismatched heterotopic cardiac allograft in mice. Although it is known that rapamycin could increase Treg proportions, this study is the first to demonstrate that *in vivo* rapamycin-induced Tregs are potentially capable of establishing tolerance to a mouse cardiac allograft. Lower splenic populations of $CD8^+$

memory T cells were also observed with an increase in $CD62L^{LO}$ proportions suggesting that impaired $CD8^+$ memory T cell development and trafficking could be contributing to tolerance induction. However, it remains to be determined whether these differences are a result of $CD8^+$ memory T cell intrinsic defects, or a result of extrinsic effects, such as the influence of Tregs. Nevertheless, a targeted genomic approach has indicated that Treg genes may be used as biomarkers that correlate with transplantation tolerance when examining graft tissues. Sustained elevated plasma levels of the Treg effector molecule FGL2 may also be useful as a biomarker of graft tolerance identified through peripheral blood samples. After validating these biomarkers for their applicability to patients, it may then be possible to identify those who have achieved tolerance to their transplanted graft and can have their immunosuppression accordingly reduced or withdrawn. This will improve the long-term success of transplantation by decreasing the morbidity and mortality of these patients.

5. References

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