Transforming Growth Factor β, Allograft Acceptance and Chronic Allograft Rejection

by

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Dedication

To my parents, Dennis Alton and Dorothy Louise Faust.

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AAV	adeno-associated virus
Ab	antibody
Adβgal	adenovirus that encodes βgal
AdDec	adenovirus that encodes rat decorin
AICD	activation induced cell death
ALK	activin receptor like kinase
APC	antigen presenting cell
AP	activator protein
ATP	adenosine triphosphate
AZA	azathioprine
BM	bone marrow
CD4-DNR	transgenic mice with a T cell specific dominant negative TGFβ receptor II
CD40L	CD40 ligand
CR	chronic allograft rejection
CREB-ATF	cAMP response element binding protein/activating transcription factor
CNS	conserved noncoding sequence
CSA	cyclosporin A
CTGF	connective tissue growth factor
CTL	cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte associated antigen 4
DC	dendritic cell
DNMT	DNA methyltransferase
DP	double positive
DST	donor-specific transfusion
DTH	delayed-type hypersensitivity
ECM	extracellular matrix
ELISPOT	enzyme linked immunosorbent spot
FasL	Fas Ligand
FGF	fibroblast growth factor
FKBP	FK-binding protein
G-CSF	granulocyte colony stimulating factor
GIC	graft infiltrating cells
GITR	glucocorticoid-induced TNFR
H2	histocompatibility locus
HAR	hyperacute allograft rejection
HO	heme oxygenase
HLA	human leukocyte antigen system
Ig	immunoglobulin
IGF	insulin growth factor

IFN	interferon
IL17-/-	IL-17 deficient
iTreg	induced Treg
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
Jak3	janus kinase 3
JNK	jun N-terminal kinase
LAG	lymphocyte-activation gene
LAP	latency associated protein
LLC	large latent TGFβ complex
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLR	mixed lymphocyte reaction
MMF	mycophenolate mofetil
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
NK	natural killer cell
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NRP	neuropilin
nTreg	natural or thymically derived Treg
OX40L	Ox40 ligand
PDGF	platelet derived growth factor
PD-L1/2	programmed cell death ligand 1 or 2
PGE	prostaglandin E
SCID	severe combined immunodeficiency
SMC	smooth muscle cell
SLC	small latent TGFβ complex
SP	single positive
STAT	signal transducer and activator of transcription
TAV	transplant associated vasculopathy
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGFβ	transforming growth factor β
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIMP	thiolnosinic acid
TLR	toll-like receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRAF	TNFR-associated
Treg	T regulatory cell
WT	wild type

Abstract

Chronic allograft rejection (CR) is the main barrier to long-term transplant survival. CR is a progressive disease defined by interstitial fibrosis, vascular neointimal development, and graft dysfunction. The underlying mechanisms responsible for CR remain poorly defined, although transforming growth factor β (TGFβ) has been strongly implicated in promoting fibrotic diseases and CR. However, TGFβ is a suppressive cytokine, which may be beneficial in the transplant setting. Hence, an in depth assessment of the fibrotic and anti-inflammatory activities of TGFβ in cardiac transplant was performed.

In this study, the role of TGFβ on graft-reactive cellular and humoral responses, T regulatory cell (Treg) function, allograft acceptance and the progression of CR are assessed. These studies identify TGFβ dependent and independent pathways to allograft acceptance, and investigate the contribution of TGFβ-induced IL-17 in the progression of CR. Since TGFβ exhibits exacerbating or ameliorating characteristics depending on the site of action, TGFβ neutralization within the allograft addresses local TGFβ inhibition on fibrosis and graft-reactive T and B cell responses. Studies in this dissertation provide insight into the underlying causes of CR and identify therapeutic targets for treatment of this disease.

Chapter 1: Introduction

Following 50 years of pioneering work by multiple researchers in animal models of heart transplant(1-6), Christian Barnard performed the first successful human orthotopic heart transplant at Groote Schuur Hospital in Cape Town, South Africa on December 3, 1967 (1). The transplant recipient received an immunosuppressive cocktail of high dose prednisone, hydrocortisone, azathioprine, actinomycin C as well as local irradiation, and succumbed to pneumonia 18 days post-transplant (1). One year later, over 100 cardiac transplants were performed in 17 countries with a mean survival rate of 29 days (3). Most institutions abandoned the procedure by 1970 (2). However, experimental work that focused on immunsuppression, infection, donor management and surgical technique boosted the one-year survival rate from 22% to 65% between 1968 and 1978 and resulted in a resurgence of interest in cardiac transplant (3). The introduction of the immunosuppressant, cyclosporine, in 1981, further improved the one-year survival rate to 80% (4). As of 2008, the Registry of the International Society for Heart and Lung Transplantation calculated that over 81,000 transplants worldwide have been performed, with a one-year survival rate of 81% and a patient half-life (time to 50% survival) of 10 years (5). Early mortality is due to primary graft failure, while chronic rejection (CR) is the main cause of death following the first year (5).

Prior to work performed by Sir Peter Medawar and colleagues, the mechanism by which a transplant recipient rejects an allograft was unknown (6). Medawar's initial work in a burn unit began to elucidate the role of the immune system in allograft rejection and methods of inducing tolerance (7). Medawar and Gibson performed serial biopsies and examined the skin graft histology from a severely burned woman who received both autografts and allografts (6, 7). Within a week, high numbers of infiltrating leukocytes were observed within the allografts and they were rejected. In contrast, the autografts remained normal, with minimal signs of inflammatory cell infiltration. A second set of skin grafts from the allogeneic donor underwent accelerated rejection, accompanied with a more intense inflammatory response, indicating a recall response. Medawar postulated that "…the mechanism by which foreign skin is eliminated belongs to the general category of actively acquired immune reactions" (7). The discovery of histocompatibility genes (8) and the realization that animals recognize their own tissues ("self") and do not react against them while responding rapidly to foreign ("non-self") stimuli (9), led Medawar to realize that genetic variations between donor and host resulted in differential immune responses toward transplants (10). This work fueled his interest in acquired tolerance, also referred to as the 'Holy Grail,' which is "...a state in which an animal or even a patient can be made selectively unresponsive to the antigens of a given graft, while the remainder of the immune defense mechanisms remain intact" (3). As evidenced by the heart transplant statistics from the Registry in 2008, the Holy Grail of transplant tolerance has yet to be realized, requiring further refinements in our understanding of the interactions between the immune system and the graft.

1.1 T cells

Medawar and Gibson's skin graft experiment (7) demonstrated immunologic specificity of allograft rejection: donor-specific response, systemic immunity and immunologic memory. Most solid organ transplants do not occur between genetically identical individuals, so the major barrier to transplant acceptance is the recipient's immune system recognizing and responding to the genetic disparity in the major histocompatibility complex (MHC) (HLA in humans; H2 in mice) (11). T cells are the are the mediators of graft rejection (12). The importance of T cells in graft rejection is illustrated in transplanted mice that lack T cells, due to genetic mutation or neonatal thymectomy (13). These mice fail to reject grafts unless reconstituted with T cells. CD4+ T cells initiate this response following interactions with antigen presenting cells (APCs) by becoming activated and assuming effector functions (11). CD4+ T cells enhance the immune response toward an allograft by producing cytokines and aiding in the activation of alloreactive CD8+ T cells and B cells (12). In the absence of immunosuppressants, activated CD8+ T cells infiltrate cardiac allografts and mediate graft destruction during acute rejection (12). Conversely, CD4+ T cells can acutely reject allografts by mounting a Th2-dominant response characterized by an aggressive eosinophil infiltration (14). To make an effective immune response, however, T cells must be able to respond to their specific antigen and undergo activation and effector cell differentiation. The following four sections will review this process.

1.2 T cell activation: signal one

Activation of a T cell is initiated by engagement of the T cell receptor (TCR)/CD3 complex with the peptide:MHC complex of an APC (11) (Figure 1). The TCR only

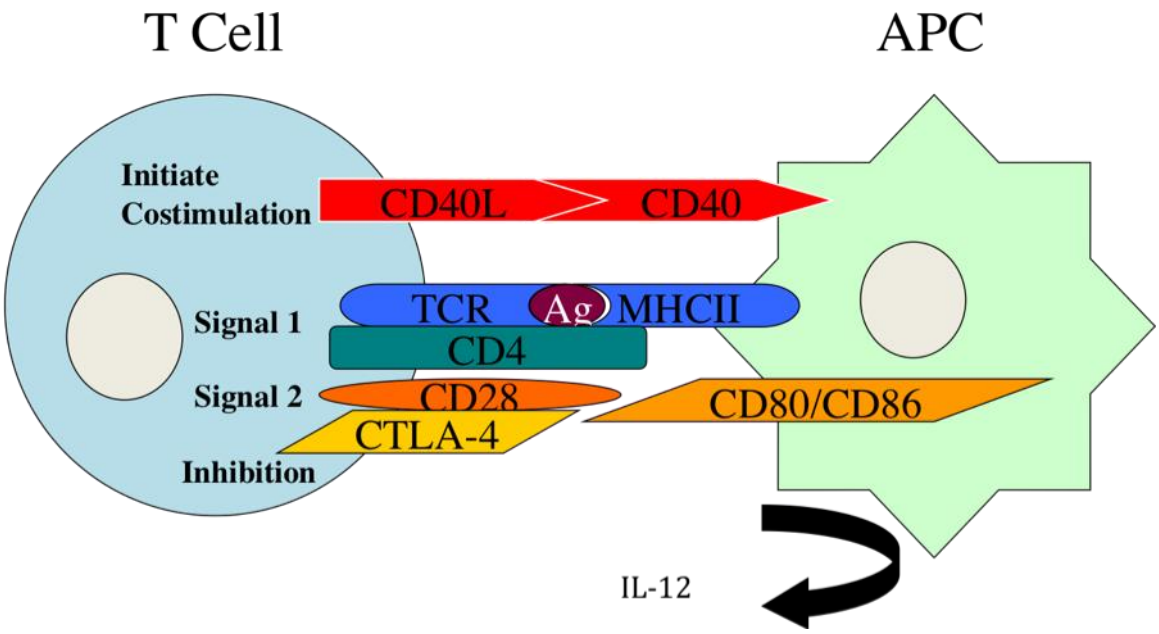


Figure 1: Initial steps in T cell activation. APCs, which express CD40, present Ag to the T cell receptor in the context of peptide-MHC. This is referred to as signal 1. Antigen recognition by the T cell induces CD40L expression. Stimulation of the APC through CD40 induces maturation, including IL-12 production and CD80/CD86 expression. CD80/CD86 delivers co-stimulation to the T cell through CD28, which is signal 2. T cells proliferate and assume effector function. A CD28-related protein termed CTLA-4 is also induced on activated T cells and serves to deliver an inhibitory signal to dampen the proliferative response. CTLA-4 binds CD80/CD86 with at least a log fold higher affinity than CD28.

recognizes foreign antigen in the context of MHC molecules, and the sole function of the MHC molecule is to associate with peptides from "non-self" in order to present them on the cell surface for T cell recognition (11). There are two groups of MHC loci involved in antigen presentation: Class I and Class II (reviewed in (15)). MHC I molecules are composed of a 45 kDa heavy chain, comprised of the α1, α2 and α3 domain and the non-covalently associated light β2-microglobulin (β2m) (16, 17). Class I molecules associate with peptides derived from the cytosol and are presented on the surface of most cells and tissues to facilitate efficient immune surveillance by CD8+ T cells (18). These peptides are short (8-11 amino acid in length) created by the proteasome and trimmed by aminopeptidases in the endoplasmic reticulum (19). MHC II molecules consist of an α

and β chain, which form a heterodimeric glycoprotein. The length of the peptides are typically larger, between 12-19 amino acids long, and are derived from extracellular sources, endosomes and lysosomes (15). MHC class II is restricted to APCs, such as dendritic cells (DCs), macrophages, and B cells (20).

The cell surface co-receptors of T cells, CD4 and CD8, are necessary for an effective immune response toward antigen. CD8 is expressed by CD8+ T cells, which can produce cytokines and differentiate into cytotoxic T cells and directly kill cells, while CD4 is expressed by T "helper" cells (Th), whose primary function is to activate additional cells, such as B cells and CD8+ T cells (12). During antigen recognition, the CD4 and CD8 molecules associate with the TCR on the T cell and bind to conserved sequence on the MHC portion of the peptide:MHC complex, away from the peptide-binding site. The CD4 molecule on T cells binds to MHC II and the CD8 molecule binds to MHC I (20). The differential expression of CD4 and CD8 on T cells dictates the nature of the antigens to which these cells respond.

There are multiple pathways in which antigen recognition can occur in the transplant setting (11). In the direct allorecognition pathway, recipient T cells can recognize intact MHC alloantigens displayed on the surface of donor APCs (passenger leukocytes) that are carried within transplanted organs (21). These passenger leukocytes migrate from the graft into the recipient spleen and lymph nodes and activate T cells that are capable of cross-reacting. The indirect allorecognition pathway involves a self-MHC restricted response in which alloantigen shed by dying cells from the graft or passenger leukocytes is processed and presented by recipient APCs, such as DCs and/or

macrophages (22). Either of these pathways is capable of providing the first signal of activation to alloreactive T cells.

1.3 Allorecognition

The strength of a T cell response provoked by MHC-incompatible cells is vigorous (23). This can be observed *in vitro* in a mixed lymphocyte reaction (MLR) (24) or *in vivo* in the rejection of solid organ transplants (reviewed in (11)). Polymorphisms at the MHC locus are referred to as alloantigens (20). MHC class I and II molecules are particularly polymorphic in the antigen-binding cleft, which creates a vast diversity in the number of potential T cell receptor binding sites (11). Alloreactivity refers to the stimulation of T cells by "non-self" MHC molecules, and can occur through either direct or indirect allorecognition (20).

Direct Allorecognition

In direct allorecognition, a high frequency of recipient T cells can recognize intact MHC alloantigens displayed on the surface of donor APCs that are carried within transplanted organs (21). T cell precursor frequencies against foreign MHC can be as high as $1:10^3$-10^4 compared with $1:10^5$ or less for antigen specific self-MHC restricted cells (21, 23). This indicates that T cells in the recipient can react with self-MHC and peptide as well as cross-react with foreign MHC as a result of conserved sequences within the locus (25). In direct allorecognition, host T cells are primed by donor APCs that migrate from the graft into the spleen and draining lymph nodes of the recipient (23). T cells with receptors that cross-react with allogeneic class I and class II molecules, in combination with peptide, are activated and acquire effector function. Alloreactive CD8+ T cells migrate into the graft and mediate rejection, while CD4+ T cells secrete cytokines

and initiate antibody (Ab) production by alloreactive B cells (12). Direct allorecognition is primarily responsible for acute rejection (22).

Indirect Allorecognition

Indirect allorecognition comprises a second mechanism of alloreactivity that results in graft rejection (23). Indirect allorecognition is a self-MHC restricted response in which alloantigen shed from the graft by dying cells is processed and presented by recipient APCs (22). Peptides derived from foreign MHC molecules make up a significant source of polymorphic peptides that are recognized by recipient T cells. T cells (primarily CD4+)--activated through the indirect allorecognition pathway--contribute to rejection by stimulating macrophages, which can induce graft injury (22). In addition, the indirect pathway plays an important role in the induction of a humoral response toward the graft. Indirect allorecognition is thought to contribute significantly to CR (26).

1.4 T cell activation: signal 2

T cell activation was initially postulated to require only one signal (reviewed in (27)). However, investigations by Lafferty and Cunningham lead them to realize that a second signal—a co-stimulatory signal--was required for T cell activation and rapid expansion of T cells in response to antigen (27). There are a constellation of co-stimulatory molecules that are crucial to drive clonal expansion, survival and T cell differentiation, including CD28:CD80 (B7-1)/CD86 (B7-2), OX40:OX40L, PD1/2:PD-L1/2, and CD40L:CD40 (28). CD40L:CD40 and CD28:CD80/CD86 will be discussed further.

CD40:CD40L

CD40 and its ligand, CD154 (CD40L), are members of the tumor necrosis factor receptor (TNFR) and TNF superfamily, respectively (28). Similar to many co-stimulatory molecules, CD40L is expressed only on a T cell following activation, while CD40 is expressed constitutively at a low level on APCs, but can be significantly upregulated upon activation. CD40 is expressed by B cells, macrophages, DCs, and endothelial cells (29). In addition, inflammatory cytokines can induce parenchymal cells, such as fibroblasts and endothelial cells, to express CD40 (30). CD40L is expressed on a variety of cells, including activated CD4+ and CD8+ T cells, natural killer (NK) cells, eosinophils and human platelets (29). The main effects of CD40:CD40L interaction is to prime and expand CD4+ T cells, activate APCs to upregulate co-stimulatory activity and cytokine production, induce B cell isotype switching, and stimulate macrophages and endothelial cells (31).

CD40 is a transmembrane protein with a cytoplasmic tail that contains two domains. The first domain associates with TNFR-associated (TRAF) binding, while the second domain independently activates NF-κB (reviewed in (31)). In addition, a proline-rich sequence in the intracellular membrane proximal region of CD40 interacts with Janus Kinase 3 (Jak3). Upon CD40L binding, CD40 forms a trimeric complex that activates NF-κB. Simultaneously, the intracellular region interacts with JAK/signal transducer and activator of transcription (STAT) pathway, as well as members of the TRAF family, which phosphorylates STAT3, releasing it to translocate into the nucleus to regulate gene transcription (31). B cell CD40 ligation induces proliferation and differentiation, the secretion of IL-6 and IL-10, and antibody class-switching from IgM to

IgG (32). CD40 stimulation of macrophages induces these cells to produce nitric oxide and IL-12 to enhance macrophage cytotoxicity (33). CD40 ligation in DCs induces a prosurvival signal, upregulates CD80/CD86, enhances antigen presentation, and induces the secretion of TNFα, IL-8, IL-12 and macrophage inflammatory protein -1α (MIP-1α) (31).

CD40L signaling has been demonstrated to activate jun N-terminal kinase/mitogen activated protein kinase (JNK/p38MAP kinase), sphinogmyelinase, and PKC, inducing the activation of NF-κB (reviewed in (34)). However, the entire mechanism of action still remains to be elucidated. In addition to T cell priming, cross-linking of CD40L induces the secretion of many cytokines including IL-4, IFNγ, IL-10 and TNFα (35). The importance of this co-stimulatory molecule in T cell activation was demonstrated in CD40L deficient mice, which exhibit a lack of T cell priming in response to antigen (36). In humans, CD40L dysfunction is associated with X-linked hyper-IgM syndrome, characterized by an inability to produce IgG, IgA and IgE isotypes (37).

In multiple rodent transplant models, anti-CD40L mAb treatment, which disrupts the CD40/CD40L pathway, has been very effective in preventing acute allograft rejection (38). Inductive treatment with anti-CD40L mAb can prevent rejection of heart, kidney, and islet allografts (reviewed in (39)). CD40L deficient mice also exhibit prolonged heart and islet graft acceptance. CD40L blockade in a stringent model of transplant, skin allografts, is not sufficient therapy itself and must be combined with additional therapies, including donor specific transfusion (DST), CTLA-4 IgG or anti-45RB in order to induced transplant tolerance (37, 38). The remarkable ability of CD40L blockade to

prolong allograft survival in rodents encouraged translation studies. Primate trials employing Rhesus monkeys showed promise when 5 monthly doses of anti-CD40L mAb permitted prolonged survival of kidney and pancreatic islet allografts (39). However, once the therapy was withdrawn, the monkeys rejected their grafts, indicating that anti-CD40L mAb induced immunosuppression, rather than classical tolerance in primates. Even more disappointing were the results of phase 1 clinical trials, in which anti-CD40L mAb treatment was associated with thromboembolic events as a result of CD40L expression on platelets and subsequent aggregation (40). Research is currently being conducted to try and circumvent problems associated with anti-CD40L mAb therapy.

CD28:CD80/CD86

In the early 1980's, a T cell membrane receptor, CD28, was identified to enhance TCR-induced proliferation and differentiation of naïve T cells (41). CD28 is constitutively expressed both on mouse and human T cells (42), and its ligands, CD80 (B7.1) and CD86 (B7.2), are upregulated on APCs by CD40 ligation and the cytokines, IL-4 and IFNγ (41). Even in conjunction with low TCR occupancy, CD28 is potent at inducing a synergistic signal in naïve T cells to activate transcription factors including NF-κB, nuclear factor of activated T cells (NFAT), and activator protein-1 (AP-1) (43-46), which control cell cycle, survival and differentiation. Following binding of CD80/CD86 on APCs, CD28-mediated signals also upregulate IL-2 production (47), IL-2R expression (48) and the secretion of IFNγ, granulocyte macrophage colony-stimulating factor (GM-CSF), TNFα, IL-1, IL-3 and IL-4 by T cells (41). T cell death signals and inhibition of cell cycle progression can be reversed by CD28 co-stimulation. This is partially through the expression of the anti-apoptotic protein, Bcl-xL dependent

on NF-κB activation (49, 50). In addition, the pro-apoptotic transcription factor p73 is inhibited in T cells following CD28 signaling, further facilitating T cell survival (51).

CD28 deficient mice exhibit reduced immune responses toward infections (52) (53), allograft antigens (54, 55), graft-versus-host-disease (GVHD) (56), contact hypersensitivity (57) and asthma (58). These mice exhibit impaired T cell proliferation and IL-2 production (41). Consistently, CD80/CD86 deficiency also results in impaired T cell responses (48, 59) and prolonged allograft survival in the absence of immunosuppressive agents (42, 60).

Although CD28 generates a strong co-stimulatory signal for naïve cells after TCR stimulation, T cell derived cytotoxic T lymphocyte associated antigen 4 (CTLA-4) provides a blunting signal to contain the immune response (61). CTLA-4 exhibits greater than one log higher affinity for CD80/CD86 than CD28 and generates an inhibitory signal to the T cell (61). CTLA-4 is present on naïve cells at a very low level but is rapidly upregulated following T cell activation and is expressed at its highest level during the late stages of T cell priming (62). This co-inhibitor acts through a variety of mechanisms. Firstly, CTLA-4 signaling actively suppresses CD28 co-stimulation (63). Additional mechanisms include the inhibition of cell-cycle progression (62), a direct inhibitory interaction with CTLA-4 and the TCR-ζ chain (64), induction of immunosuppressive cytokines, such as TGFβ (65), and activation of Treg cells (66). Ligation of CTLA-4 has been demonstrated to induce peripheral T cell tolerance and anergy (67, 68). CTLA-4 deficient mice exhibit fatal lymphoproliferative disorders that can be eliminated with the introduction of CD28 deficiency, providing further support that CTLA-4 suppress T cells primarily through the inhibition of CD28 (63).

1.5 Helper T cell differentiation

Following engagement of the TCR with the appropriate peptide-MHC complex and co-stimulatory signals, clonal expansion of the responding T cell population and rapid differentiation into CD4+ helper T (Th) cells occurs (reviewed in (69, 70)). These Th cells are critical mediators in the immune response. Th cells mount a response contingent on the polarizing conditions that exist when the T cells first encounters antigen, including the type of APC that presents the antigen, the co-stimulatory pathway signaling employed, the cytokines present, and the concentration of antigen (71). During an immune response, naïve Th cells can differentiate into 4 lineages of effector T cells: Th1 cells secrete IFNγ and combat intracellular infections that require cell-mediated immunity, Th2 cells secrete IL-4 and defend against extracellular parasites, Th17 cells secrete IL-17 and combat extracellular bacteria, and Treg secrete a variety of suppressor molecules and inhibit immune responses (Figure 2) (reviewed in (72)).

Th1

Naïve CD4+ T cells activated through TCR engagement and exposed to APC-derived IFNγ and IL-12 differentiate into Th1 cells (72). This is thought to occur in two steps with two corresponding waves of expression of the transcription factor, T-box expressed in T cells (T-bet) (73). In the early polarizing phase, IFNγ produced by cells of the innate immune system, such as NK cells, DCs and macrophages, synergistically acts with TCR signaling to induce T-bet expression in addition to IFNγ (73). The critical role T-bet plays in Th1 differentiation and effector responses was revealed in T-bet deficient

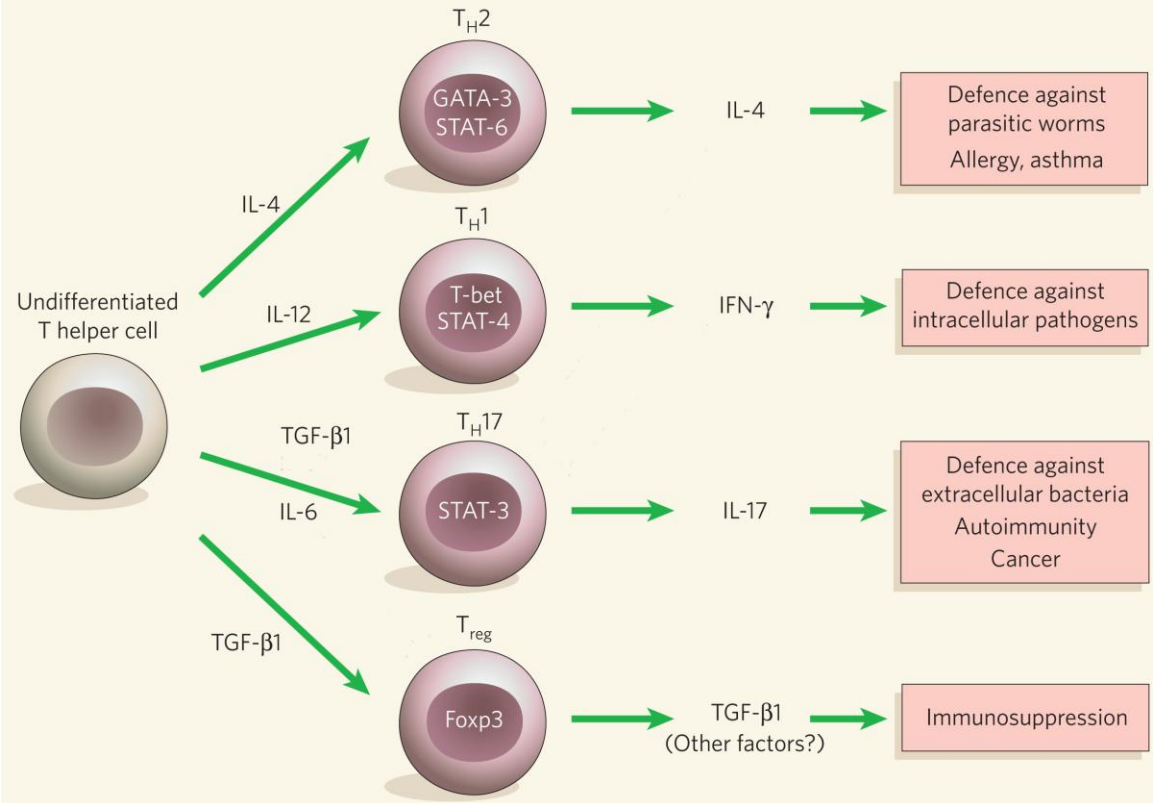


Figure 2: Pathways of Th Development. Th cells are critical mediators in the immune response. Th cells mount a response contingent on the polarizing conditions that exist when the T cells first encounter antigen, including the type of APC that present antigen, the co-stimulatory pathway signaling employed, the cytokines present, and the concentration of antigen. During an immune response, undifferentiated Th cells can differentiate into 4 lineages of effector T cells: Th1, Th2, Th17 and Treg. Figure adapted from (74).

mice, which do not exhibit a functional CD4+Th1 response (75). These mice demonstrate a significant shift in Th1 to Th2 lineage development and an increased susceptibility to intracellular pathogens (75). Following termination of TCR signaling, IL-12Rβ2 expression is upregulated and innate immune cell-derived IL-12 maintains the second phase of T-bet expression, stabilizing the Th1 phenotype and promoting further expansion of Th1 cells. The early wave of T-bet expression is induced by STAT1, while STAT4 is required for the second wave of T-bet expression. Upon recognition of antigen, effector Th1 cells will secrete abundant IFNγ, further reinforcing the differentiation of

more Th1 cells (76). This two step process requires naïve T cells to receive pro-inflammatory signals beyond the acute TCR signaling phase of the response before complete commitment to the Th1 lineage occurs.

Th1 cells promote the activation of macrophages and mobilize the cellular arm of the immune response to infiltrate into sites of inflammation and combat intracellular pathogens (72). These cells promote delayed type hypersensitivity (DTH) and cytotoxic T lymphocyte (CTL) responses (described later in the chapter) (69). Th1 cells generate the pro-inflammatory cytokines IFNγ, TNF-β, IL-2 and lymphotoxin-α. Th1 generated cytokines activate the microbicidal activity of macrophages to efficiently destroy intracellular pathogens. In addition, Th1- derived IFNγ stimulates B cells to generate antibodies that enhance opsonization by specifically upregulating the subclasses IgG2a and IgG2b in the mouse and IgG1 and IgG3 in humans (77). Further, IFNγ enhances antigen presentation by upregulating MHC expression on cell surfaces (78). The importance of IFNγ in the Th1 response is underscored in mice that are deficient in the IFNγ receptor (79). These mice are unable to control viral and intracellular bacterial infections.

Th2

If a naïve Th cell is exposed to IL-4 following TCR engagement, signaling through STAT6 and activation of the transcription factor GATA3 promotes Th2 cell differentiation (80). GATA3 initiates chromatin remodeling of the IL-4, IL-5 and IL-13 locus, permitting additional transcription factors involved in Th2 development to activate the loci (81-83). High levels of IL-4 production further induce GATA3 expression establishing a positive feedback loop and reinforcing the development of Th2 cells (84).

The source of IL-4 that initiates the development of a Th2 response was initially hypothesized to originate from DC signals in the form of ligands for the T cell Notch receptor (85). Notch signaling in T cells was believed to trigger IL-4 production by developing Th2 cells. Support for this was derived from experiments in Notch 1 and 2 deficient mice, which demonstrate impaired Th2 cell differentiation. However, mounting evidence suggests that both mast cells and/or basophils are the original generators of IL-4 (86). The importance of GATA3 in promoting Th2 differentiation was demonstrated when T cells were infected with a retroviral vector that expresses the GATA3 transcription factor (87). The transduced cells exhibited rapid expansion into Th2 effector cells. GATA3 suppresses STAT4 and IL12Rβ2 expression, thereby inhibiting Th1 lineage development (88), while the Th1 transcription factor, T-bet, interacts with GATA3 and inhibits Th2 differentiation (75).

Th2 cells secrete cytokines including IL-4, IL-13, IL-5, IL-9, and IL-10, which are essential for optimal antibody production and elimination of extracellular pathogens (69). Cytokines produced by Th2 cells drive B cells to become activated, differentiate and produce Th2-dependent antibody isotypes such as IgE and IgG1 (89). In addition to stimulating B cell proliferation and differentiation, IL-4 upregulates MHC class II induction. IL-4 deficient mice fail to induce Th2 cells, exhibit reduced IgG1 and IgE production and mount inappropriate Th1 responses when challenged with extracellular pathogens (89). IL-5 enhances the generation of eosinophils and basophils by the bone marrow, while IL-9 stimulates mast cell production and activation and further amplifies the Th2 response (90). Th2 responses are characterized by tissue infiltration by eosinophils and basophils, in addition to significant mast cell degranulation (89). Th2

cytokines, specifically, IL-4, IL-5, IL-9 and IL-13 are strongly implicated in allergic reactions and airway inflammation.

Th17

The Th17 lineage is a recently identified T helper subset of the immune system that produces the cytokine, IL-17 (reviewed in (91)). There are six IL-17 family members, termed IL-17A-F. CD4+ memory T cells were initially identified as the generators of IL-17, but CD8+ T cells, γδ T cells, neutrophils, eosinophils and monocytes, have also been demonstrated to produce this cytokine (74). A diverse array of tissues expresses the IL-17R and recently cardiomyocytes have been demonstrated to generate significant amounts of this cytokine (92). TGFβ in association with IL-6 or IL-21 favors the commitment of CD4+ T cells to the Th17 lineage and induces RORγT, which promotes IL-23 receptor and IL-17 gene expression (93-95). STAT3, activated by both IL-6 and IL-23, binds to the IL-17 promoter and activates transcription (74). IL-21, which is also produced by Th17, is important for amplifying the response, while IL-23 is critical for maintenance of the lineage and continued secretion of IL-17 (96). Cytokines produced by Th1 and Th2 cells inhibit Th17 lineage differentiation as do the transcription factors, T-bet and FoxP3 (74).

IL-17 stimulates stromal cells, such as fibroblasts, endothelial cells, and epithelial cells to produce IL-6, IL-8, granulocyte colony stimulating factor (G-CSF), and prostaglandin E2 (PGE2) (91, 97, 98). IL-17 also up-regulates critical chemoattractants, such as CXCL1 and CXCL2 (91, 97, 98). These chemokines are important in granulopoiesis and in the recruitment of monocytes and neutrophils to sites of inflammation. Indeed, over-expression of IL-17 in transgenic mice results in massive

systemic hematopoiesis and inflammation in response to high expression of G-CSF and stem cell factor (99). Conversely, IL-17RA knockout mice demonstrate deficiencies in G-CSF, granulopoiesis, and increased susceptibility to *Klebsilla pneumoniae* (100). IL-17 deficient mice exhibit reduced delayed-type hypersensitivity, airway hypersensitivity and contact hypersensitivity responses, but normal host versus graft reaction (HVGR), indicating that donor CD4+ T cell produced IL-17 is not necessary for allo-specific CD8+ T cell activation (101).

Th17 lineage induction is strongly associated with autoimmune diseases (102-104). Increased IL-17A transcript levels are detected in patients with inflammatory bowel disease (102). In rheumatoid arthritis, IL-17 mediates matrix metalloproteinase 1 (MMP1) activity in the joint synovium and is implicated in tissue destruction (103). Excessive IL-17 production has also been implicated in asthma and experimental allergic encephalomyelitis (105). IL-17 has also recently been identified as a pro-fibrotic cytokine and IL-17 production is associated with both pulmonary fibrosis and scleroderma (104). The mechanisms by which IL-17 may mediate fibrosis will be discussed at length in Chapter 5.

Regulatory T Cells

Treg overview

Over a century ago, Paul Ehrlich recognized that goats could generate antibodies against the blood group antigens of other goats but did not generate a response against their own (106). Ehrlich proposed that the immune system is programmed to avoid the induction of autoreactive responses, and his studies became the first experimental evidence of immunological self-tolerance. Immunological tolerance is mediated by two

separate maintenance mechanisms: recessive and dominant tolerance (reviewed in (107)). Recessive tolerance involves the elimination of self-reactive thymoyctes or chronically stimulated peripheral T cells by apoptosis or anergy. Dominant tolerance is mediated by a specialized subset of immune cells, most commonly by Treg, which are dedicated to suppressing immune responses and maintaining immune homeostasis. Treg can be generated in the thymus and are referred to as natural Treg (nTreg) or induced in the periphery (iTreg) from naïve CD4+ T cells (reviewed in (107)). iTreg include type 1 regulatory cells T regulatory 1 (Tr1) and T helper 3 (Th3). The Tr1 cells express high levels of IL-10, TGFβ, and IL-5. Naïve CD4+ T cells in the presence of chronic inflammation, immunosuppressive drugs, and soluble proteins develop into Tr1 cells that secrete IL-10 and TGFβ (108). Th3 cells are induced through oral antigen administration and suppress through TGFβ production. All Treg, regardless of the location of generation, express the forkhead-winged helix transcription factor gene (FoxP3), which has become the most definitive marker for Treg in both mice and humans (109) in addition to the IL-2 receptor alpha chain, CD25 (110). Regulatory cells also reportedly express glucocorticoid-induced TNFR (GITR), OX40, and/or CTLA-4 on their cell surface (111). Both Treg subsets suppress pathogenic immune responses via a variety of mechanisms including: 1) through the production of anti-inflammatory cytokines; 2) through direct Treg-T effector or Treg-naïve T cell interactions; and 3) through modulating the activation state and function of APCs (112). Treg are important in autoimmunity, cancer, allergy, asthma and transplantation.

FoxP3

Sustained FoxP3 expression in mature Treg is requisite for Treg cell maintenance and suppressor function (113). Stable FoxP3 expression is required for Treg differentiation for suppressor function, proliferation, and metabolic fitness (107, 113). Deficiency in FoxP3 expression, as observed in humans suffering from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome or in the *scurfy* mouse, results in an early onset lymphoproliferative disease affecting multiple organs and tissues and results in death (114-118). This pathology is due to T cell acquisition of effector cytokine production, such as IL-2, IL-17, IL-4, IFNγ and self-targeted immune-mediated tissue destruction (119, 120). FoxP3 is an X-linked gene in both humans and mice that is heavily regulated (reviewed in (107)). Several transcription factors interact with and activate the *FoxP3* locus through conserved noncoding sequence 1 (FoxP3-CNS1) and the conserved noncoding sequence 2 (FoxP3-CNS2). NFAT, NF-κB, AP-1, CREB-ATF-1 and STAT5 have all been demonstrated to bind to the FoxP3 promoter and upregulate its expression (121). FoxP3 expression is induced in nTreg by STAT5 in response to combined signaling by the TCR, the IL-2R and other gamma chain (γc) cytokine receptors, including IL-7, and IL-15 (107, 122). STAT5 binds to the FoxP3 locus and alters chromatin characteristics in the FoxP3-CNS2 element, directly driving FoxP3 transcription (123). It has been demonstrated that STAT5 plays a non-redundant role in promoting survival or expansion of nTreg: In the absence of STAT5, there is a significant reduction in FoxP3+CD4+ nTreg (123).

Unlike nTreg, which do not require TGFβ signaling to induce FoxP3 expression (124), iTreg require TGFβ-induced Smad-mediated activation of the FoxP3 locus at the

FoxP3-CNS1 response element (125). TGFβ cooperates with TCR signaling in naïve T cells to induce FoxP3 partially by inactivating the cell-cycle dependent recruitment and maintenance of a negative regulator of FoxP3, DNA methyltransferase (DNMT1) (126). There are additional binding sites within the FoxP3-CNS1 element for NFAT and STAT5 dependent regulation of FoxP3, which has been demonstrated in vitro (reviewed in (107)).

Thymically-derived, nTreg

Insight into the existence of the thymically derived subset of Treg that are capable of mediating immune tolerance arose from neonatal thymectomy experiments (127). Thymectomy of mice between days 2-4 post-natal resulted in T cell-mediated autoimmunity that could be alleviated through thymus engrafting or adoptive transfer of thymocytes or splenocytes from genetically identical adult mice (127). These experiments indicated that a subset of cells generated a few days post-natal in a mouse thymus mediates tolerance in a dominant manner. In 1995, Sakaguchi described a population of CD4+ cells expressing CD25 with the ability to suppress immune responses in multiple experimental models (128). Further studies revealed the critical role of FoxP3 expression in this subset of cells for Treg differentiation (113, 119, 129), maintenance and suppressor function (130, 131).

FoxP3 induction in nTreg occurs late in differentiation when thymocytes have differentiated into single positive (SP) CD4+ thymocytes (107). During thymic T cell development, bone marrow precursor cells migrate to the thymus and interact with thymic stroma (reviewed in (132)). Distinct thymic microenvironments and precursor cell migration through these microenvironments is necessary for thymic development and T

cell maturation. The first major checkpoint in T cell development occurs at the positive selection phase, where CD4CD8 double positive (DP) thymocytes have reached the cortical region of the thymus and randomly generated a TCR repertoire (132). At this point, the TCR of DP thymocytes interacts with self peptide-MHC complexes displayed by thymic cortical epithelial cells. The DP thymocytes that exhibit weak affinity interactions for self peptide-MHC differentiate into immature SP CD4 and CD8 thymocytes (132). The selected SP thymocytes proceed to the thymic medullary region, where they undergo negative selection. Negative selection eliminates SP thymocytes that demonstrate high-affinity interactions with self peptide-MHC expressed primarily on thymic medullary epithelial cells and dendritic cells (133). Hence, negative selection eliminates the majority of T cells bearing TCRs with high affinity for self, and this process constitutes central tolerance. However, some SP CD4+ thymocytes that manifest very strong TCR signaling undergo selective survival. These self-reactive T cells upregulate FoxP3 expression and mature into nTreg (133). Further investigation revealed that there is a narrow range of affinity of self-reactive peptide/TCR interactions between negative selection and positive selection that satisfies the conditions for FoxP3 induction (107). The importance of FoxP3 in nTreg differentiation and suppressive capacity at this step is illustrated in FoxP3-/- mice. In these mice, the cells that would normally differentiate into nTreg would, in the absence of this key transcription factor, fail to mature into regulatory cells. Upon release into the periphery, the T cells become activated and assume effector function, resulting in autoimmune disease (reviewed in (107)).

In addition to TCR engagement, co-stimulatory signals (134) and cytokines play a pivotal cell-intrinsic role in nTreg differentiation (135). The importance of CD28 in nTreg differentiation was revealed in CD28 and CD80/CD86 deficient mice that show a significant reduction in nTreg (134). The CD28 cytoplasmic tail Ick-binding domain is critical for the induction of FoxP3 in nTreg. Downstream mediators of CD28 co-stimulatory signals include NFAT, AP-1 and NF-κB, all three of which can bind and activate the FoxP3 promoter (reviewed in (107)). An additional signal in the form of γc cytokines, including IL-2, IL-7 and IL-15 are also critical for nTreg differentiation. Mice lacking IL-2 or the IL-2R exhibit 50% less nTreg compared to WT, while mice with a combined deficiency of IL-2, IL-7 and IL-15 are completely devoid of nTreg (111, 112) . TCR engagement in nTreg precursor cells results in upregulation of CD25 and enhanced responsiveness to IL-2 signals (136). STAT5, activated downstream of IL-2 and the aforementioned γc cytokines, induces FoxP3 expression through changes in chromatin structure at the locus, promotes survival and expansion of nTreg (112, 113).

Peripheral iTreg

iTreg were first identified in mice treated with very small doses of antigen by osmotic pump delivery (137). The conversion of alloantigen specific CD4+ T cells into FoxP3+ iTreg was also observed in tolerized mice treated with non-depleting CD4+ mAbs (138). While the majority of Treg in the periphery are of thymic origin (107), FoxP3 expression and suppressive capacity can be induced in peripheral naïve CD4+CD25- T cells by: 1) a combination of strong TCR signal and high concentrations of TGFβ (126) ; 2) co-stimulation in the presence of inhibitory cytokines, such as TGFβ; and 3) activation in the absence of optimal antigen exposure (139). An analysis of TCR

repertoires comparing CD4+FoxP3- and CD4+FoxP3+ iTreg revealed only partial overlap in TCR resemblance, providing support that iTreg cell differentiation requires a distinct TCR specificity that promotes efficient FoxP3 induction (reviewed in (135)). In addition to TCR signal, CTLA-4 is required for TGFβ-mediated iTreg in vitro. However, CD28 and CD28 associated NF-κB signaling is dispensable for iTreg differentiation (135). In fact, CD28 cross-linking prevents FoxP3 induction in peripheral naïve CD4+ T cells upon stimulation with TGFβ (reviewed in (133)). *In vitro,* TGFβ-induction of Treg requires IL-2 (140), which not only directs STAT5-dependent regulation of the FoxP3 promoter and promotes cell survival, but also opposes differentiation of activated CD4+ T cells into Th17 cells in the presence of TGFβ (100). TGFβ mediated Smad2 and NFAT binding to FoxP3CNS1 is critical in peripheral FoxP3 induction and, in collaboration with TCR signaling, induces FoxP3 through DNMT1 inactivation and chromatin remodeling (reviewed in (107)).

The generation of iTreg occurs in diverse microenvironments. iTreg have been observed in the lymph nodes during oral tolerance, the lamina propria of the gut in response to microbiota and food antigens, tumors, chronically inflamed tissue and transplanted tissue (reviewed in (107, 141)). In these iTreg cell-dominated environments, infectious tolerance, defined as the spread of tolerance to new groups of T cells is thought to occur through the recruitment and induction of FoxP3-CD4+CD25- T cells into FoxP3+CD4+CD25+ with suppressive capacity (142). It was recently demonstrated that TGFβ is expressed on the cell surface of activated, but not resting Treg, and that Treg derived TGFβ is essential for inhibiting proliferation of activated, but not naïve T cells (143). Further, Treg were capable of inducing FoxP3 expression and suppressor function

to antigen-specific naïve T cells indicating that infectious tolerance can be conferred in a TGFβ-dependent manner (143).

Treg maintenance

The three main factors critical for homeostatic maintenance of both natural and induced Treg in the periphery include: IL-2, TGFβ and CD28 ligation (133). IL-2 plays an important role in Treg proliferation and contributes to Treg function (107, 111, 144). The requirement for TGFβ in Treg maintenance was revealed when its absence resulted in decreased peripheral Treg populations despite normal thymic output (124). While CD28 engagement is not necessary for the generation of iTreg, this molecule is important for both natural and induced Treg homeostasis in the periphery (reviewed in (112)). These factors induce signaling pathways that mediate permissive chromatin modifications within the FoxP3 promoter to enhance gene expression. In the absence of any of these factors, the number of Treg within the periphery decrease (107).

A variety of molecules can reverse FoxP3 and Treg suppressor activity (107). *In vitro* cross-linking of OX40, results in a loss of FoxP3 expression and suppressor function in mature Treg (107). In addition, GITR ligation specifically on responders, but not Treg, renders the effector cells unable to respond to Treg inhibition (145). Finally, in mouse Treg, engagement of the toll-like receptor 2 (TLR2) on Treg has been demonstrated to reverse the regulatory cell's suppressive effect (112).

Treg migration

The immune response requires efficient migration of lymphocytes throughout the body between lymphoid and non-lymphoid organs. This trafficking depends primarily on chemoattractants such as chemokines, cytokines, inflammatory lipid mediators

(leukotrienes and prostaglandins), defensins, the complement proteins, C3a and C5a, and anti-microbial peptides (146). Chemokines can be classified as inflammatory/inducible or homeostatic/lymphoid depending on the location of chemokine production and the type of stimuli elicited (146). Recognition of a chemoattractant by a lymphocyte initiates a process of vascular attachment, transendothelial migration, and tissue migration under chemotaxis (147). This process is mediated through lymphocyte cell surface adhesion and chemoattractant receptors (147). Cell adhesion molecules, such as integrins and selectins, are expressed on the tissue endothelium and are required for lymphocyte adhesion and transmigration. Selectins permit weak bonds to form between lymphocytes and activated endothelial cells, enabling the lymphocytes to roll and tether along the internal surface of the endothelial cell (148). Activation of the lymphocyte by chemokines induces a conformational change in lymphocyte integrins, which, in association with the appropriate cell adhesion molecules expressed on the endothelium, allows the lymphocyte to adhere with greater strength and transmigrate into the tissue (147). The extravasated lymphocyte follows chemoattractant gradients to the site of inflammation.

There is significant overlap between chemokine expression on CD4+CD25- T cells and CD4+CD25+ Treg, which is largely a function of Treg chemotaxis to sites of inflammation in order to control effector T cell function (149). CD25+ cells demonstrate elevated expression of the chemokine receptors CXCR3 (150), CCR4 and CCR8 (151) and migrate in response to their respective ligands, eotaxin, CCL17 and CCL1 (152). The importance of Treg expressed CCR4 in homing to sites of inflammation and suppressing effector function was illustrated in the enrichment of CCR4 expressing Treg in ovarian carcinoma tumors (153) and in the requirement for CCR4 in Treg migration within

cardiac allografts and tolerance induction in recipients treated with anti-CD40L mAb (154).

Selectin expression in Treg is crucial for Treg homing and suppressive capacity *in vivo*. In a skin inflammation model, Treg from VII-deficient mice, which lack E/P selectin ligands, were unable to migrate into the inflamed tissue and inhibit Th1-mediated delayed-type hypersensitivity (155). L-selectin (CD62L) is also important in homing to lymph nodes and migration into tissue in which acute or chronic inflammation is occurring (156). CD62L+ Treg are more potent suppressors than CD62L- Treg, and exhibit enhanced survival (157). *In vivo*, CD62L+ Treg demonstrate superior suppressive abilities in a mouse model of autoimmune diabetes (158). In addition, expansion of CD62L+ Treg preferentially migrate to the lymph node of tolerant allograft recipients and show alloantigen-specific suppressor function (159). Clearly, Treg migration is important for Treg function and peripheral tolerance induction.

Treg suppression

Treg are potent suppressors of T cell proliferation and effector function both *in vitro* and *in vivo* (reviewed in (112)). The cellular targets of FoxP3+Treg suppression include CD4+ and CD8+ T cells, DCs, B cells, macrophages, osteoblasts, mast cells, NK cells and NKT cells (112). There are multiple mechanisms by which Treg can directly and indirectly suppress CD4+CD25- T cells. Treg impact almost every feature of effector T cell function, including T cell activation, IL-2 production, proliferation, and trafficking (147). There are multiple mechanisms by which Treg can directly and indirectly suppress CD4+CD25- T cells. Treg can directly suppress CD4+CD25- T cells via IL-2 consumption, suppressor cytokine production (TGFβ, IL-10, IL-35), granzyme-mediated

cytolysis of target cells and cell surface molecule production that initiates cell cycle arrest (galectin-1) (112). Treg can indirectly inhibit CD4+CD25- T cells by suppressing the function and activation of APCs through CD80/CD86 down-regulation, binding of the lymphocyte activation gene-3 (LAG-3) to the MHC class II molecules on DCs thereby suppressing maturation, inactivation of pro-inflammatory extracellular ATP by CD39, and competing against CD4+CD25- T cells for DC interactions employing the Treg receptor, neuropilin (Nrp-1) (112).

IL-2

Both *in vivo* and *in vitro* studies have demonstrated that Treg cell-mediated inhibition of IL-2 can suppress both proliferation and cytokine production in CD4+CD25- T cells (160-162). Treg may inhibit IL-2 by directly competing with CD4+CD25- T cells for IL-2 consumption, resulting in diminished proliferation of the responder cells followed by BIM-mediated apoptosis (163). Studies also reveal that Treg can suppress IL-2 production in CD4+CD25- T cells by inhibiting the induction of IL-2 gene expression (160-162). IL-2 also enhances Treg suppressive function. At high levels of IL-2, Treg will become activated and expand (144). IL-2 also primes Treg to produce IL-10 upon restimulation, either upon migration into the secondary lymphoid organs or within inflamed tissue (144). Therefore, Treg uptake of IL-2 not only suppresses T cell activation but expands the Treg population and upregulates production of a suppressor cytokine, IL-10, to control T cells that may have escaped suppression initially and have now migrated into inflamed tissue.

1.6 TGFβ gene expression, signaling and suppressor function

TGFβ is a multifunctional cytokine that controls proliferation, differentiation, and other functions in diverse cell types (164). TGFβ belongs to a superfamily of proteins that include morphogenic proteins, activins, and growth differentiation factors (165). Mammals express three homologous isoforms of TGFβ, each of which is encoded by a different gene. TGFβ1 is the predominant isoform expressed in the immune system. TGFβ2 and 3 play important roles in development and are primarily expressed in mesenchymal tissues and bones (reviewed in (164)). TGFβ is synthesized as a prepro-TGFβ precursor (166). This precursor contains a signal peptide that is removed when the TGFβ precursor is processed in the Golgi (166). TGFβ is noncovalently associated with a latency-associated protein (LAP) forming the small latent TGFβ complex (SLC) (167). The SLC can be secreted or it can further associate with the latent TGFβ binding protein, forming a large complex (LLC). The SLC and LLC sequester TGFβ to the extracellular matrix, preventing the cytokine from interacting with its receptor (167). Activation of TGFβ requires liberation from the complexes through proteolytic cleavage or a conformational change (167).

TGFβ becomes activated following dissociation from LAP or LTBP and mediates signaling by binding TGFβ type I and II transmembrane serine/threonine kinase receptors (166). Following TGFβ receptor ligation, activin receptor like kinase (ALK5) phosphorylates Smad2 and Smad3, which translocate to the nucleus in complex with Smad4 (reviewed in (168)). In association with other transcription factors, the Smad complex binds target promoters of a multitude of genes and regulates expression. TGFβ signaling also activates PI3K and various MAPK signaling pathways (168). TGFβ

signaling is down-regulated by the expression of Smad7, which competes with Smad2 and Smad3 binding and degrades these factors through ubiquitin ligase complexes (168).

Unlike isoforms 2 and 3 gene expression, which are regulated primarily by developmental or hormonal signals, little is known about the transcriptional regulation of TGFβ1. The promoter of TGFβ1 contains binding sites for AP-1 where c-jun and c-fos induced by TGFβ signaling bind to and stimulate additional TGFβ1 production (164). However, AP-1 may have a dual role in regulating TGFβ1, depending on the context and the cell type, because it has been demonstrated that the AP-1 activators, c-Jun-NH2-terminal kinase 1 and 2 (JNK1, JNK2), inhibit TGFβ1 expression in fibroblasts (164). TGFβ1 gene expression may not correlate with secreted protein expression suggesting that TGFβ1 is post-transcriptionally regulated. Indeed, the TGFβ1 transcript found in abundance has a long 5' untranslated region that is associated with negative regulation of TGFβ translation (164). The exact mechanism is unknown.

TGFβ regulates cell proliferation

Mice with a systemic TGFβ deficiency (TGFβ-/- mice) (169) and mice that possess a T cell specific cell specific abrogation of TGFβ signaling (CD4-DNR mice) (170) manifest multi-focal autoimmune diseases, characterized by spontaneous T cell activation, hyperproliferation, effector cell induction and significant inflammatory cytokine production. TGFβ critically regulates Th and CTL differentiation, in addition to T and B cell proliferation, activation and survival (reviewed in (164)). TGFβ suppresses T cell proliferation by inhibiting IL-2 transcription (171) and by affecting cell cycle progression through selective upregulation of cyclin-dependent kinase inhibitors (p15, p21, and p27) and down-regulation of the cell cycle activators, c-myc, cyclin D2 and

cyclin E (164). However, TGFβ-mediated inhibition of cell proliferation depends on the activation status of the responder. CD28 co-stimulation over-rides TGFβ suppression in TCR-mediated naïve T cell proliferation (172). In addition, while TGFβ inhibits naïve T and B cells, it has minimal effects on activated cells as a consequence of reduced TGFβRII expression (141). The cytokine, IL-10, can ameliorate this by enhancing TGFβRII expression and conferring TGFβ responsiveness in activated T cells (141). Preferential TGFβ inhibition of naïve cells in the absence of co-stimulation is thought to dampen T cell responses toward self-antigen, while the limited ability of TGFβ to suppress TCR-activated cells that receive strong co-stimulatory signals, permits the development of cellular immunity toward infections (173).

TGFβ regulates differentiation of Th1 and Th2 cells

TGFβ is capable of opposing both Th1 and Th2 cell differentiation (Figure 3). Under polarizing conditions, activated naïve CD4+ T cells differentiate into helper T cells (69). TGFβ effectively blocks Th1 differentiation through the reduction of IL-12Rβ2 and T-bet gene expression (174, 175). TGFβ may achieve this by directly regulating IFNγ or T-bet transcription or by down-regulating STAT4 expression, which is a transcription factor activated by IL-12 that upregulates IFNγ expression upon T cell priming (174, 175). Gorelik and colleagues demonstrate that TGFβ directly inhibits GATA3 expression, thereby preventing Th2 differentiation (176). In addition, TGFβ interferes with TCR and co-stimulatory signaling by suppressing the activation of the Tec kinase, Ick, and by inhibiting calcium influx following TCR/CD28 stimulation (177). Impairments in calcium signaling negatively impacts NFAT, which is a transcription factor of GATA3 (177).

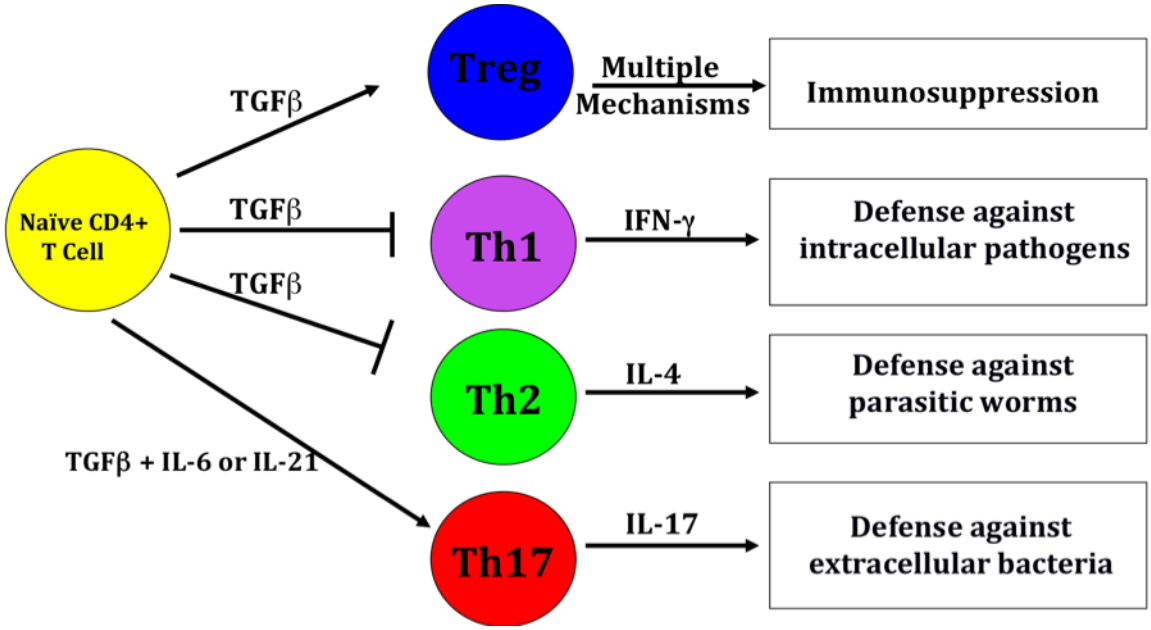


Figure 3: TGFβ and Th development. The pathways that are used by the host immune system to reject an organ evolved to combat intracellular and extracellular pathogens and TGFβ can play either an anti-inflammatory or pro-inflammatory role. TGFβ alone can drive an undifferentiated T helper cell to become a T regulatory cell, which is a T cell subset that actively suppresses immune responses and maintains immunological tolerance. TGFβ has been shown to inhibit differentiation of Th1 cells, which express IFNγ and mobilize the cellular arm of the immune system to combat intracellular pathogens. TGFβ also suppresses Th2 cells that secrete IL-4 and are essential for elimination of parasitic worms. TGFβ in conjunction with IL-6 or IL-21 drive the differentiation of the Th17 lineage, which produce IL-17 and defend against extracellular pathogens.

TGFβ inhibits CD8+ T cell differentiation

The suppressive effect of TGFβ on CD8+ T cells was revealed in experiments in which CD8+ T cells activated in the presence of TGFβ do not acquire cytotoxic T lymphocyte functions and weakly proliferate (reviewed in (164)). This is due to the inhibition of multiple effector molecules in CD8+ T cells (178). TGFβ suppresses perforin, T-bet and IFNγ gene expression in these cells. In addition, TGFβ affects the death receptor cytotoxic pathway of CD8+ T cells by inhibiting the expression of Fas ligand (164). However, similar to CD4+ inhibition by TGFβ, CD8+ T cell inhibition is

context dependent and co-stimulatory and cytokine signaling pathways can over-ride the suppressive effect of TGFβ (178).

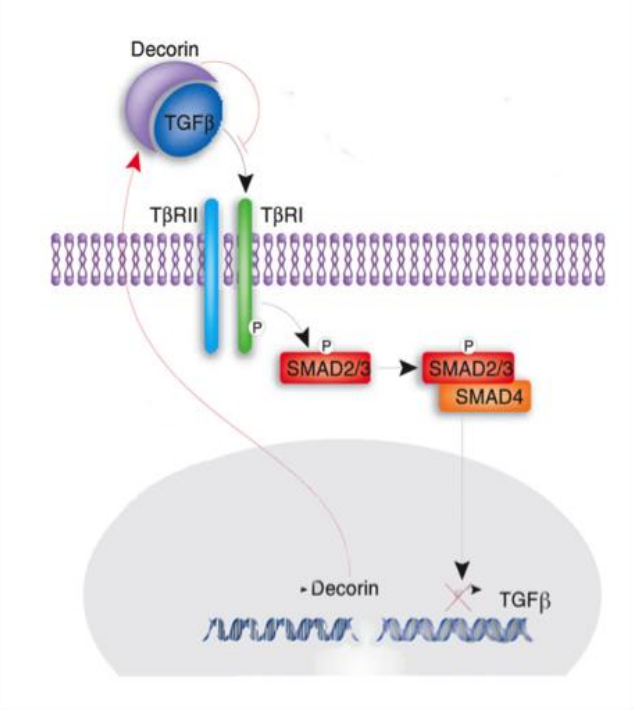
TGFβ regulation of B cells

TGFβ is an essential regulator of B cells as evidenced by the autoimmunity phenotype observed in mice with B cell-specific blockade of TGFβ signaling (179). Similar to its effect on T cell proliferation, TGFβ inhibits proliferation in B cells by repressing cyclin A expression, inactivating cdk2 and down-regulating c-myc (180). These alterations in cell cycle progression result in blockade at G1/S (164). TGFβ also inhibits B cell differentiation by preventing both light chain expression and the secreted form of the heavy chain (181, 182) and blocks class switching to most IgG isotypes. TGFβ negatively regulates B cell survival signals. TGFβ impairs IL-4 mediated STAT-6 activation, which is an important mitogenic survival factor for B cells (164). In addition, TGFβ upregulates pro-apoptotic members of the BCL-2 family and induces apoptosis in immature and resting B cells (183).

A negative regulator of TGFβ: Decorin

Decorin, an extracellular matrix protein, has received attention in cancer research and in fibroproliferative diseases for its ability to inhibit TGFβ (184). Decorin is a member of the small leucine rich proteoglycan (SLRP) family (185). The core protein of decorin contains 10 leucine rich repeats that interact with a number of extracellular proteins, including collagen, fibronectin, thrombospondin, complement component C1q, EGFR and TGFβ (186-188). The role of decorin in stabilizing the extracellular matrix (ECM) was revealed in decorin deficient mice, which exhibit abnormally fragile skin associated with skin collagen defects and irregularly shaped fibrillar networks (189).

Decorin also negatively impacts cell proliferation, an effect mediated through the induction of p21(184). Decorin has been demonstrated to down-modulate TGFβ bioactivity both *in vitro* and *in vivo* (190-193). Decorin binds all 3 isoforms of TGFβ, effectively inhibiting the cytokine's interaction with its receptor (Figure 4). In addition, decorin inhibits TGFβ gene expression by interrupting TGFβ/Smad-dependent transcriptional events (194, 195). Decorin gene transfer has attenuated TGFβ dependent fibrosis in models of glomerulonephritis (196), cardiac ventricular fibrosis (197), and pulmonary fibrosis (198).



Adapted from Akhurst, *Nature Genetics*, 2006

Figure 4: Decorin inhibits TGFβ. Decorin is an extracellular protein and a member of the small leucine-rich proteoglycan family. This ECM protein binds all 3 isoforms of TGFβ, thereby inhibiting TGFβ's interaction with its receptor. In addition to inhibiting the TGFβ bioactivity, decorin can also negatively regulate TGFβ gene expression (199).

1.7 Inflammatory response to allografts

The immune system evolved to defend against microbial pathogens, not transplanted organs. However, because organs are rarely transplanted between genetically identical individuals, the immune system recognizes the organ as "non-self" and mounts a response (11). This section reviews the pathology associated with acute rejection, delayed type hypersensitivity, humoral rejection, hyperacute rejection, and chronic rejection.

Acute allograft rejection

Acute cardiac rejection is characterized by a severe and localized inflammatory response within the allograft that results in destruction of graft tissue and loss of function (12). Initially, an alloantigen-independent inflammatory response occurs within hours post-transplantation (200, 201). This response is characterized by the activation of graft vascular endothelial cells and leukocyte infiltration (202). Vascular endothelial cells express adhesion molecules, such as ICAM-1, increasing accessibility to the graft by macrophages, neutrophils and dendritic cells (203). Macrophages infiltrate into the perivascular interstitium and express the cytokines, IL-1, IL-6, TNF and TGFβ (204). These cytokines serve to stimulate the migration of additional immune cells into the graft and, subsequently, into the recipient's draining lymph nodes for further immune activation (202).

This initial inflammatory response occurs regardless of whether the transplant is an isograft or allograft and is a function of ischemic injury and tissue hypoxia that is incurred during organ procurement and the operative period (205). Cold ischemic time following organ procurement induces allograft injury and enhances post-operative

inflammation (205). If the organ is recovered from a donor who has sustained brain death, hormonal dysfunction, proinflammatory cytokine release and hemodynamic instability contributes to graft damage prior to transplantation (206). During surgery, warm ischemia injury and delayed graft function serve to amplify inflammatory responses that occur post-operatively (207). These initial insults to the transplant result in graft injury and facilitate inflammatory cell recruitment into the allograft. In accepted cardiac allografts, these inflammatory events can take up to 20 days to resolve (200).

The initial inflammatory response toward a cardiac allograft evolves significantly within three days post-transplant (200). At this time, the alloantigen-dependent phase is initiated. T cells become the dominant infiltrating cells within the interstitium and eventually migrate throughout the graft in response to chemokines such as VCAM-1, MCP-1 and RANTES (207, 208). Host T cells are first activated by foreign or self-MHC in addition to simultaneous stimulatory signals from APCs (28). The activated T cells undergo clonal expansion and assume effector function. Once activated, recipient CD4+ T cells produce a variety of cytokines, including IL-2, IFNγ and IL-4 (12). IL-2 drives alloreactive CD8+ T cell differentiation into cytotoxic T lymphocytes (CTLs), which are capable of lysing allograft cells (209). CTLs kill through the release of perforin/granzyme and/or ligation of Fas with FasL, which signals the allogeneic cells to undergo apoptosis through a caspase mediated mechanism (12). The important role CTLs play in rejection was demonstrated in human transplant when donor-specific CTLs were eluted from rejecting kidney transplants (210), and in experiments in mice when CD8+ effector cells were adoptively transferred and mediated rejection of skin grafts in recipient mice (211). However, CD4+ T cells can also acutely reject allografts by

mounting a Th2-dominant response characterized by an aggressive eosinophil infiltration (14). CD4 helper T cells also facilitate donor-reactive Ab production in the spleen and lymph nodes through the activation of alloreactive B cells (described later in this chapter) (12). Circulating alloreactive IgG Ab are detected within 7 to 14 days post-transplant (200). Additional cytokines increase vascular permeability and enhance the accumulation of neutrophils and mononuclear cells into the graft. Histologically, acute cardiac rejection is associated with high numbers of infiltrating inflammatory cells, extracellular edema, cardiomyocyte lysis, hemorrhage and vascular damage (212). Acute rejection occurs rapidly in the absence of immunosuppression and results in graft failure within a matter of weeks post-transplantation.

Delayed type hypersensitivity

Another mechanism of graft rejection is delayed type hypersensitivity (DTH) (12). During DTH, alloreactive CD4+ and CD8+ Th1 cells are the main mediators of graft rejection. Alloreactive T cells differentiate into Th1 cells that produce the pro-inflammatory cytokines, IFNγ and TNF (12). IFNγ induces the expression of chemokines such as CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 to enhance the inflammatory response by facilitating the infiltration and activation of monocytes and macrophages (20). TNFα and TNFβ serve to enhance vascular permeability and mediate local tissue destruction. These cytokines activate monocytes and macrophages, which infiltrate the grafts and secrete cytokines and chemokines to attract additional inflammatory cell infiltration. Macrophages shape the local inflammatory response within the graft by producing proteolytic enzymes, nitric oxide and additional pro-inflammatory cytokines (12). These soluble inflammatory mediators directly impact the physiological functions

of the allograft through their effects on vascular integrity and tissue injury. DTH responses within an allograft are characterized by edema and intense cellular infiltrate (12). A DTH response is sufficient to mediate allograft rejection as demonstrated by CD4+ T cell adoptive transfer into SCID recipients of MHC Class I- or II-disparate skin grafts and subsequent graft rejection (213).

Humoral rejection

Advances in transvenous endomyocardial biopsies and histologic grading systems enhanced the diagnosis of cardiac rejection (214). However, some patients were diagnosed with 'biopsy negative' heart transplant rejection (215). Many of these patients were experiencing humoral graft rejection (215). Hammond first described humoral rejection as endothelial cell swelling and vasculitis associated with deposition of IgG and complement as observed by fluorescent microscopy of immunostained grafts (216). Antibody mediated rejection (AMR) occurs when Ab engages with graft antigen resulting in the fixation of complement and triggering of the complement cascade (77). The deposition of complement was first described in kidney biopsy specimens (217). Complement component C4d deposition within the peritubular capillaries of these biopsies correlated with severe rejection and graft loss (217).

In addition to complement-mediated cell lysis, Ab deposition and activation of complement in cardiac transplants is associated with both the accumulation and activation of leukocytes within the capillaries and arteries of the graft (77). The FcR on neutrophils and macrophages interacts with the Ab Fc tail and results in cellular activation. This interaction induces the production of various cytokines such as MCP-1, IL-8 and TNF-α, which promote inflammatory cell chemotaxis and cell survival signals

at the site of inflammation (12). Activation of the complement components, C3, and C5 also results in the formation of the split products, C3a and C5a, which are small diffusible fragments that mediate chemotaxis and activation through C3a and C5a receptors on leukocytes (218). Macrophages possess complement split product receptors for C5a, the stimulation of which induce the production of the pro-inflammatory cytokines, IL-1, IL-6, IL-8 and TNF-α that further amplify the immune response (12). Complement activation of antibodies also serves as a strong positive feedback loop for B cell activation, maturation and Ab production because the complement split product, C3d, is an activating ligand for CR2 on B cells (219).

Hyperacute allograft rejection

In humans, pre-formed anti-HLA antibodies occur in some patients as a result of previous transplant, pregnancy or transfusion (220). Major and minor blood group antigens can also elicit Ab responses in transplant recipients. Blood group antigens are expressed on endothelial cells within cardiac allografts and Abs can cross-react with these cells (220). Preformed alloantibodies to blood group antigens can result in a rapid—a matter of minutes—rejection of the graft mediated by a complement-dependent reaction referred to as hyperacute allograft rejection (HAR) (12). Activation of the complement and blood clotting cascades causes vascular blockage in a cardiac allograft resulting in hemorrhage, thrombosis, severe injury and graft death. HAR can be avoided by performing cross-matching to determine whether the recipient is sensitized (has antibodies) to donor antigen (12). HAR is a major barrier to xenotransplantation—the transplantation of organs between closely related species—as a result of pre-formed anti-αGal antibodies in humans that cross-react with xenografts (221). The anti-Gal

antibodies are predominantly directed against Galα1,3Galβ1, 4GlcNAc carbohydrate residues, which are expressed on endothelial cells of non-primate mammals and New World monkeys, but absent in humans, apes and Old World monkeys (222). As a result, anti-αGal antibodies are generated early in life through the encounter of αGal-expressing microorganisms in the intestinal tract and can compose up to 1% of antibodies in circulation (222).

Chronic rejection

While short-term survival rates of solid organ transplants continue to improve, long-term graft survival remains constant (5). In vascularized organ allografts, the primary cause for late graft failure is CR (reviewed in (223)). CR is characterized by proliferation of fibroblasts within the graft, which promotes interstitial fibrosis as well as thickening and narrowing of the coronary vessels, a process referred to as transplant associated vasculopathy (TAV) (224). CR may develop in response to multiple mechanisms and evolve from a cell and Ab-mediated phase that leads to allograft tissue damage to a tissue-remodeling phase driven by cytokines and growth factors (223).

TAV

Over 45% of cardiac transplants exhibit TAV (5) and this pathology is a key feature of CR (224). Mounting evidence indicates that TAV is the result of chronic inflammatory responses toward vessel walls, which is strongly associated with the accumulation of inflammatory cells, primarily macrophages and T cells (224). TAV does not develop in syngeneic transplants, providing evidence that this pathology is an immune mediated disease (225). In experimental models of TAV, T cells migrate into the intima of affected arteries and secrete cytotoxic enzymes that damage arterial walls (226).

These alloreactive T cells also secrete cytokines that induce the expression of endothelial cell adhesion molecules and chemokines, such as RANTES, which attract monocytes and macrophages. Macrophages, in particular, are implicated in the early phases of clinical and experimental TAV because these cells migrate into the perivascular areas and produce a number of cytokines, such as IL-6 and MCP-1 in addition to a variety of fibrogenic and matrix proteins (226). Cytokines and chemokines secreted by inflammatory cells results in smooth muscle cell (SMC) activation and migration from the media to the intima of the graft (224). SMCs proliferate and release additional cytokines and extracellular matrix proteins that lead to luminal narrowing and vascular dysfunction (224).

The development of alloantibodies and complement deposition also contributes to TAV (226). Evidence that Abs are necessary for the induction of CR stems from experiments in which B-cell-deficient mice employed as transplant recipients fail to develop the typical arterial lesions observed in TAV (227). In addition, cardiac transplants performed between mouse strains that produce Abs to donor antigen results in more severe TAV than transplants in the reverse combination, in which Abs were not detected (227). While Ab activation and complement deposition on vascular endothelial cells can result in cell lysis, sublytic amounts of complement can activate endothelial cells (ECs). Activated ECs release pro-fibrotic and inflammatory factors such as von Willebrand factor, P-selectin, IL-8, MCP-1, fibroblast growth factor (FGF), TGFβ, and platelet-derived growth factor (PDGF) (226). Immunohistochemistry staining of vascular endothelial cells in experimental models of TAV have revealed high expression of PDGF A and B (226), FGF and TGFβ, which are associated with fibrogenesis in native organs

(226). In addition to the pro-fibrotic factors, the secreted inflammatory mediators enhance innate and adaptive immune responses within the graft (226). Complement activation of platelets deposited on arterial endothelial cells induce the production of soluble CD40L, which is the ligand for CD40 on B cells, macrophages, and DCs leading to amplification of the immunity toward an allograft (226). Platelets also secrete growth factors that promote the proliferation of endothelial cells, smooth muscle cells and fibroblasts.

Interstitial fibrosis

Unlike the cardiomyocyte necrosis and cell death observed in acute rejection, CR is associated with progressive interstitial fibroproliferation and tissue remodeling (228). There is compelling evidence that this process is driven by alloantigen responses and may be the result of "smoldering, subacute rejection accumulating progressive subclinical damage" (228). CR is theorized to be the result of a chronic wound healing process. During tissue repair, fibroblasts proliferate and differentiate into myofibroblasts, which migrate into the wound bed. This cell type has the capacity to generate significant amounts of collagen and extracellular matrix proteins required to restore the tissue architecture (229). In normal repair, myofibroblasts appear transiently, perform their function in connective tissue restoration, and disappear, most likely due to apoptosis (229). It is the persistence of myofibroblasts within lesions that is responsible for the excessive collagen accumulation that leads to aberrant tissue remodeling and, in the case of CR, to organ failure.

Myofibroblast persistence within the interstitium of grafts has been observed in both clinical and experimental CR kidney and cardiac allografts (220). In human renal

allografts, myofibroblasts are associated with progressive interstitial fibrosis (229). Clinical CR cardiac allografts also exhibit myofibroblasts within the intima of vessels and the interstitial spaces (230). The abundance of myofibroblasts strongly correlates with collagen deposition, progressive fibrosis and graft dysfunction (220).

Role of TGFβ in CR

Multiple growth factors have been implicated in the activation and differentiation of fibroblasts into myofibroblasts, but TGFβ has been recognized as a pivotal cytokine in fibrotic diseases (231), including CR (232, 233). TGFβ has been implicated in fibrosis associated with rheumatoid arthritis, diabetic nephropathy, intestinal inflammation, and myocarditis (231). Further, a plethora of clinical and experimental evidence directly links TGFβ to cardiac fibrosis including: 1) collagen and ECM production in fibroblasts isolated from cardiac tissue is directly induced by TGFβ (234); 2) upregulation of TGFβ transcript and protein levels are associated with cardiac infarct scars, correlating with increased collagen deposition and elevated Smad 2, 3, and 4 expression (234); 3) in patients with cardiac fibrosis, TGFβ1 and LTBP levels are increased (233); 4) intragraft TGFβ transcript levels are only detected in CR cardiac allografts from recipients treated with anti-CD4 mAb but not in recipients treated with anti-CD40L mAb, that do not exhibit CR (232); and 5) gene transfer of TGFβ into cardiac allografts is capable of inducing CR (235).

TGFβ regulates fibroblast proliferation and differentiation into myofibroblasts and subsequent collagen synthesis (236, 237). However, additional factors must collaborate to promote the persistent fibrotic responses *in vivo* (236). In experimental cardiac transplantation, MHC disparity is required, as syngeneic grafts that over-express

TGFβ do not develop CR (232). Evidence points to connective tissue growth factor (CTGF) as the pivotal down-stream mediator of TGFβ-induced fibrosis of cardiac grafts in the presence of alloantigen stimulation (232). For fibroblasts to proliferate in response to TGFβ and CTGF, additional mitogenic factors, such as EGF and PDGF must also be present (236). In allogeneic transplants, activated endothelial cells and macrophages release PDGF and are most likely the cells responsible for providing fibroblasts this necessary mitogenic stimulus. For fibroblasts to subsequently differentiate into myofibroblasts, CTGF works in concert with insulin growth factor-2 (IGF-2), which is a growth factor expressed by cardiomyocytes (236). In this manner, TGFβ has the ability to induce cardiac fibroblast proliferation and differentiation into myofibroblasts through a CTGF-dependent pathway (233). However, sufficient co-mitogens must be present to amplify the fibrotic effect, and in CR grafts, alloantigen appears to play a key role in this process.

1.8 Transplant tolerance

For more than half a century, the question remains as to whether elements of self-tolerance can be exploited to achieve allograft tolerance in the absence of continued immunosuppression. Medawar and his colleagues demonstrated transplantation tolerance induced by adoptively transferring allogeneic bone marrow (BM) into neonatal mice (reviewed in (238)). Tolerance induction was achieved in these mice through the establishment of a state of mixed (recipient and donor) blood chimerism and subsequent purging of alloreactive cells (238). This was the first demonstration of acquired immunological tolerance, which exploits the recipient's natural tolerance mechanisms into accepting an allograft.

Tolerance in the transplant setting is defined as the indefinite acceptance of an allograft after a brief course of immunomodulatory treatment (238). Tolerance toward an allograft occurs in two phases: induction and maintenance. Induction of tolerance is established at the time of transplant primarily through immunosuppression. A minority of grafts are lost to acute rejection during the first year post-transplantation indicating that tolerance induction is achievable or that immunsuppression is successful (239). However, reprogramming the immune system to accept the allograft long-term and maintain tolerance in the absence of immunosuppression has proved to be a major challenge.

Transplant tolerance is composed of central and peripheral tolerance (reviewed in (240)). Deletion of the majority of thymocytes that exhibit high avidity for thymically expressed antigen constitutes central tolerance (107). Peripheral tolerance prevents alloreactive responses toward the graft through the extrathymic deletion of alloreactive cells by apoptosis, induction of anergy, and active suppression by Treg (240). There is most likely overlap between the two mechanisms in tolerance induction.

Central tolerance

Central tolerance in the transplant setting is primarily a function of thymocyte deletion analogous to self-tolerance (241). This mechanism involves the induction of programmed cell death (apoptosis) in thymocytes that react strongly to both self and donor antigen and subsequent removal of these cells from the T cell repertoire. Mixed hematopoietic chimerism and donor thymic transplantation are two strategies employed to induce central tolerance (reviewed in (242)). Mixed hematopoietic chimerism involves the transfusion of hematopoietic stem cells that engraft, co-exist with recipient stem cells, and generate cells of all hematopoietic lineages (243). The donor and recipient cells seed

the thymus and mediate negative selection (242). As a result, the T cell repertoire is tolerant toward the graft (243). Donor-specific tolerance toward cardiac allografts was achieved in BM chimeric rats (244). In the minor MHC antigen-disparate rat model of CR, no TAV was observed in F-344 rats that received cardiac allografts from Lewis rats and donor BM, while non-chimeric control rats exhibited significant TAV (245). Donor-specific tolerance to small bowel allograft can also be induced in this same model using BM chimerisms (244). Additional studies identified bone marrow cells, and not peripheral blood mononuclear cells (PBMCs) as the cell type critical for tolerance induction.

DST of spleen or bone marrow cells prior to transplant and followed by a drug therapy (cyclophosphamide or busulfan) that is toxic to proliferating cells, represents a twist on BM chimerism but also involves specific deletion of donor-reactive cells (246). Using this regimen, alloreactive recipient cells that respond to donor antigen would selectively undergo apoptosis. DST in conjunction with cyclophosphamide has been demonstrated to induce both long-term cardiac (247) and skin graft acceptance (248). In addition, DST in concert with co-stimulation blockade, such as CD40 or ICOS-B7h blockade, has been shown to prolong cardiac allograft survival (249).

Donor thymic transplantation has been less successful than mixed chimerism in inducing central tolerance toward a graft (242). Co-transplant of non-vascularized thymic tissue at the time of transplant often results in rejection of the thymus prior to the establishment of tolerance. To address this issue, revascularization of thymic tissue has been achieved using composite organs termed “thymokidneys” (250) and “thymohearts”(251). These composite organs constitute fully functional and vascularized

thymic tissue at the time of organ transplant. Class I MHC mismatched "thymokidneys" transplanted into swine treated with cyclosporine results in donor-reactive cellular and humoral hyporesponsiveness (252). Thymohearts transplanted across the same MHC barrier, however, induces alloreactivity and evidence of TAV (253). Improved techniques of vascularizing thymic tissue to cardiac transplants may improve this outcome.

Peripheral tolerance

Mechanisms of peripheral tolerance include extrathymic cell deletion by apoptosis, anergy and regulatory cell suppressor activity. All three mechanisms will be discussed below.

Treg in transplant

There is considerable evidence in both human or experimental transplant recipients, that Treg are requisite for tolerance rendered spontaneously or through immunomodulatory therapy (171, 254-256). Treg suppression of alloreactive cells has been observed both *in vitro* and *in vivo*. *In vitro*, Treg isolated from tolerized transplant recipients suppress alloreactive T cell activation and proliferation in MLR (257, 258). *In vivo*, pretransplant depletion of Treg by anti-CD25 mAb, results in CR in mouse models that normally exhibit spontaneous tolerance, and acute rejection in models that otherwise manifest CR (259-261).

The requirement for Treg as the main mediators in graft tolerance has been observed in a variety of tolerizing treatments and this tolerance is transferable to naïve T cells adoptively transferred into hosts in a process termed infectious tolerance (reviewed in (262)). There is considerable evidence that iTreg play a key role in infectious tolerance. *Ex vivo* generation of alloantigen-specific iTreg in the presence of TGFβ and

adoptively transferred into cardiac allograft transplant recipients can suppress alloreactivity and promote long-term graft survival (262). In addition, allograft acceptance in this model correlates with intragraft Treg migration. A similar correlation between favorable allograft outcome and a robust intragraft Treg population is frequently observed in human transplant (263-266), indicating the importance of Treg locally inhibiting alloreactive responses at the site of inflammation.

The development of Treg occurs through multiple mechanisms and most likely involves both natural and induced Treg. *In vitro* studies suggest that nTreg suppression may be allo-antigen specific or nonspecific (262), and that both the direct and indirect pathway of allorecognition can activate these Treg (263, 267). Likewise, induction of both CD4+ and CD8+ Treg in the presence of TGFβ and alloantigen *ex vivo* and subsequent transfer of these iTreg into recipients of cardiac allograft recipients, results in donor-reactive specific suppression and infectious tolerance (268). It was also recently demonstrated that TGFβ is expressed on the cell surface of activated, but not resting Treg, and that Treg derived TGFβ is essential for inhibiting proliferation of activated, but not naïve T cells (143). Further, Treg are capable of inducing FoxP3 expression and suppressor function to antigen-specific naïve T cells indicating that infectious tolerance can be conferred in a TGFβ-dependent manner (143). Most likely, a combination of nTreg and iTreg synergize to suppress alloreactivity and induce transplant tolerance.

Treg can suppress alloreactive cells through both contact dependent (66, 173) and independent pathways (143, 172). Contact independent-mediated suppression involves the secretion of the anti-inflammatory cytokines, IL-10 and TGFβ (143, 172). Contact dependent mechanisms include cross-linking of CTLA-4 on target cells in conjunction

with TCR engagement (66), granzyme B-mediated apoptosis (269) and cell surface bound TGFβ. Treg can inhibit both alloreactive Th1 and Th2 responses, prevent CTL development, suppress B cell proliferation, induce apoptosis, modify the function of APCs, convert naïve CD4+ T cells into Treg, and induce infectious tolerance (reviewed in (270)). While Tregs are primarily suppressive in function, in response to DC derived IL-6 and alloantigen, Treg can promote the development of Th17 cells (271). Therefore, in the transplant setting, Treg can suppress or activate alloreactive cells depending on the context.

Donor-reactive cell anergy and deletion

Both anergy and donor-reactive cell deletion by apoptosis are thought to work in concert to induce tolerance (272). Anergy is defined as a state in which donor-specific T cells are present within the periphery, but functionally inert, failing to proliferate both *in vitro* (273) and *in vivo* (274). As discussed previously, in order to become fully activated, T cells require two signals: 1) engagement of the TCR with peptide-MHC complex and 2) co-stimulation through the engagement of one or more T cell surface receptors with the corresponding molecules on APCs (ie. CD28:CD80/CD86 or CD40L:CD40) (249). TCR engagement with peptide-MHC with no additional co-stimulatory signal results in T cell anergy (275). Co-stimulatory blockade through anti-CD40L mAb, anti-CD28 mAb and CTLA-4-IgG treatment of transplant recipients results in prolonged allograft survival and tolerance induction (249, 276, 277). Evidence for anergy in bone marrow transplantation arises from primary MLR experiments that demonstrate allo-antigen specific hyporesponsiveness following anti-CD40L mAb treatment in vivo (278).

Donor-reactive T cells can undergo deletion or apoptosis by two mechanisms: activation induced cell death (AICD) or passive cell death (272, 279). AICD, which requires the presence of IL-2 and is mediated by FAS-FASL interactions, is important for the elimination of activated cells after repeated antigen stimulation (272, 279). The role for AICD in the induction of tolerance following co-stimulatory blockade was revealed in IL-2-/- mice and Bcl-xL mice (transgenic mice that express high levels of the anti-apoptotic gene, Bcl-xL), whose T cells are unable to undergo AICD and are resistant to induction of transplantation tolerance (272, 279). Passive cell death entails the elimination of activated cells due to cytokine or growth factor withdrawal (280). Both passive apoptosis and AICD are required for induction of peripheral transplantation tolerance in transplant models that employ co-stimulatory blockade (CTLA-4Ig or anti-CD40L mAb) or immunosuppression (rapamycin) (272). Additionally, in the absence of immunosuppression, significant levels of T cell apoptosis of graft infiltrating cells (GIC) into liver transplants has been shown to correlate with prolonged allograft survival (281). It is hypothesized that alloreactive cell death during the inductive phase of transplant is requisite to reduce the population of graft reactive cells to a level more conducive for control by anergy or regulation during the maintenance phase (272).

T cell depletion: anti-CD4 mAb treatment

A variety of immunomodulatory methods have been employed in transplantation to promote transplant tolerance. T cell depletion is successfully employed to treat acute rejection and can be used in induction regimens (reviewed in (282)). T cell depletion may target all T cells (ie. anti-lymphocyte globulins, described in Immunosuppressive Agents)

or subsets of T cells, such as CD4+ and CD8+ T cells following anti-CD4 mAb or anti-CD8 mAb therapy, respectively.

In the 1980's, Waldmann and colleagues tested a number of rat antibodies targeting mouse lymphocytes, all of which elicited neutralizing Ab responses except for anti-CD4 mAb (283). A brief course of anti-CD4 mAb in mice induced tolerance to aggregated human IgG (HGG), which is normally highly immunogenic in mice, but failed to induce tolerance toward chicken gamma-globulin or ovalbumin (283). The difference in these antigens was that HGG persisted, while chicken gamma-globulin and ovalbumin have very short half-lives. This indicated that tolerance induced by anti-CD4 mAb was not by default, but required antigen persistence to reinforce tolerance. Tolerance in mice treated with anti-CD4 mAb proved to be persistent, was CD4+ T cell-mediated, and was not reversible upon adoptive transfer naïve T cells into tolerized mice (284). This argued for a dominant active regulatory mechanism that did not require additional immunosuppression to confer antigen specific hyporesponsiveness to a new population of naïve cells.

Inductive anti-CD4 mAb treatment prevents acute rejection, inhibits donor-reactive responses and promotes tolerance in kidney, heart, and skin transplants (285-289). Tolerance induction is primarily through CD4+ cell depletion. Anti-CD4 mAb (GK1.5) is a complement fixing rat IgG2b and, *in vitro*, has been demonstrated to lyse CD4+ T cells through complement-fixation (285). However, experiments in anti-CD4 mAb treated complement component C1q-/- allograft recipients indicate that depletion occurs independently of C1q and the classical C pathway (285). It has been reported that anti-CD4 mAb preferentially depletes resting, naïve cells, while sparing effector or

memory CD4+ cells (290). Studies also suggest that CD4 ligation down-regulates both IL-2 and IL-2R expression, limiting T cell responsiveness to IL-2 (291). In addition, upon CD4+ T cell repopulation, one study indicates that there is a significant enhancement in the ratios of CD4+CD25+ and CD4+CD25+FoxP3+ T cells to CD4+ T cell in the periphery (292).

In skin transplant models, Waldmann and colleagues demonstrated that tolerance induced by a non-depleting anti-CD4 mAb treatment is dominant and mediated by CD4+ T cells (293). Adoptive transfer of naïve CD4+T cells into recipients exhibiting long-term allograft acceptance following inductive anti-CD4 mAb treatment, results in a spread of infectious tolerance to the naïve cells and no alloreactivy toward the graft is observed (293). It is postulated that in this model, Treg are less susceptible to apoptosis or inactivation in response to the anti-CD4 mAb and are able to proliferate and migrate into the graft in order to suppress alloreactivity. In addition, in skin transplant studies it was revealed that following CD4 blockade, Treg can be induced *de novo* through a TGFβ-dependent mechanism, and accumulate in tolerated grafts (138). These iTreg cells express FoxP3+ and can actively suppress graft destruction.

Anti-CD4 mAb treatment in vascularized mouse cardiac allograft model

In the vascularized mouse cardiac allograft model, transient depletion of CD4+ T cells results in prolonged graft survival and the development of CR (232) (Figure 5). Data generated in this experimental model reveals a strong correlation between TGFβ-induced connective tissue growth factor (CTGF) (a down-stream mediator of TGFβ-induced fibrosis) and CR. Adenoviral gene transfer of active TGFβ into allografts, but not syngeneic grafts, results in CR, indicating that immune recognition is required to induce

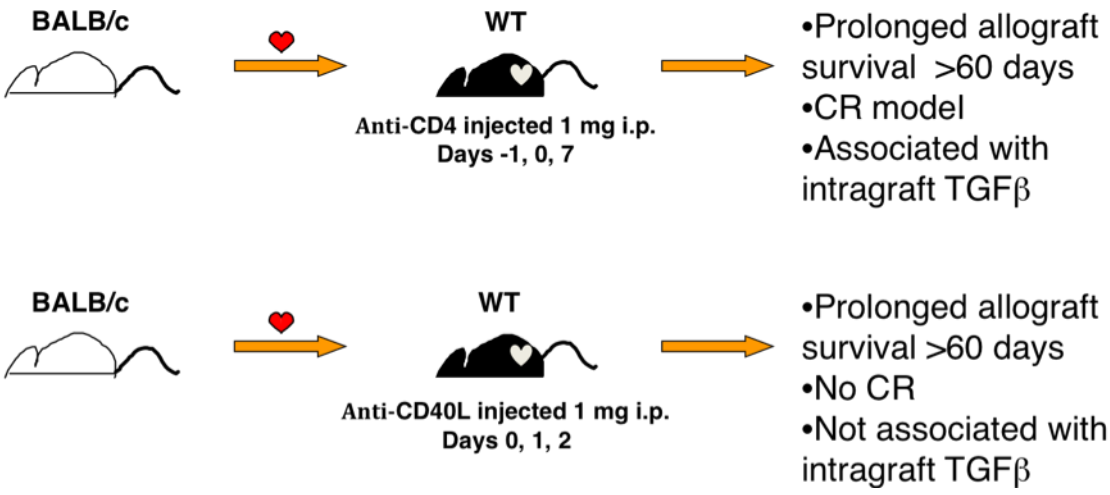


Figure 5: Experimental manipulations in CR. Transient depletion of recipient CD4+ T cells at the time of transplant promotes allograft acceptance but allows TGFβ expression within the graft and the progression of chronic rejection. In contrast, anti-CD40L mAb treatment of transplant recipients results in prolonged allograft survival but no evidence of CR.

CR (235). An additional cytokine, IL-6, has recently been implicated in driving fibrosis and hypertrophy in CR allografts in this model (294). Neutralization of IL-6 through anti-IL-6 mAb administration or by employing IL-6 deficient mice as transplant recipients ameliorated cardiomyocyte hypertrophy, graft fibrosis, and improved graft function (294).

Inductive anti-CD4 mAb treatment permits the CD4+ T cells to begin repopulating the periphery 3-4 weeks post-transplantation (285, 295, 296). As CD4+T cells return, donor-reactive T cells are functionally distinct from naïve cells in that these repopulating CD4+ exhibit a transient donor-reactive Th1 and Th2 priming that peaks around day 30 post-transplant (297). These graft-reactive cells progress to a hyporesponsive state toward the graft, but mount Th2 recall responses and demonstrate accelerated CR if transferred into allograft-bearing SCID mice (297). This altered functional T cell capacity is associated with intragraft expression of TGFβ and FoxP3.

TGFβ may perpetuate its suppressive activities by inducing TGFβ-producing Treg (298, 299). Indeed, flow cytometry of GICs and quantitative RT-PCR has been employed to detect the presence of FoxP3+ cells within the graft and an enriched population of intragraft Treg are detected in anti-CD4 mAb treated recipients compared to allografts from unmodified recipients. Maintenance of transplant tolerance and CR in anti-CD4 mAb treated recipients is strongly associated with intragraft TGFβ and the progression of graft-reactive cells to an altered state of differentiation (297).

Co-stimulatory blockade: CD40:CD40L disruption

In vivo co-stimulation blockade with anti-CD40L mAb has been shown to prolong allograft survival in several models of transplantation (reviewed in (38)). As discussed previously, failure to receive costimulatory signals following TCR ligation can result in T cell anergy or death, so inductive costimulatory blockade is a suitable therapeutic to selectively target donor-reactive cells prior to T cell effector function (28). The mechanism by which anti-CD40L mAb controls donor-reactive cells *in vivo* is still being elucidated and it is possible that this therapy has a multiple mechanisms of action.

The graft sparing effect of CD40:CD40L blockade transcends just blocking interactions with CD40, and reports indicate that anti-CD40L mAb may delete T cells through AICD, activate CD4+ T cells to secrete immunomodulatory cytokines, and induce Treg development (298, 300-302). Blocking CD40:CD40L interactions inhibits CD4+ and CD8+ T cell proliferation and induces anergy, unless exogenous IL-2 is applied. In addition to anergy induction, a cell cycle-dependent donor-reactive T cell apoptosis *in vivo* due to both AICD and passive apoptosis has been reported (279). In support of this, IL-2, Bcl-xL, and complement C3 are required for deletion of

autoreactive cells and knockout mice for these factors cannot maintain transplantation tolerance normally induced by CD40:CD40L blockade (279, 303). Anti-CD40L mAb also significantly inhibits alloantibody production by donor-reactive B cells due to the lack of T cell help (277).

Anti-CD40L mAb treatment also affects cytokine production and upregulates downstream mediators to induce and maintain tolerance. CD40:CD40L blockade stimulates secretion of IL-10, IFNγ, and TNFα but not IL-2 by CD4+ T cells *in vitro* (300). Cytokine secretion followed by apoptosis indicates early and direct effects of CD40:CD40L blockade on CD4+ cells (300). A deviation toward Th2 skewing has also been observed in CD4+ T cells in response to cross-linking of CD40L, which may, in fact, indicate Treg development since Treg have recently been identified to over-express a subset of Th2 gene transcripts in a model of skin transplant (35, 304). In addition, several downstream mediators, including CTLA-4, IFNγ, and heme oxygenase-1 (HO-1) were found to be responsible for graft survival, following CD40:CD40L blockade (38).

In addition to deletion of autoreactive cells, transplant recipients treated with anti-CD40L mAbs, manifest infectious tolerance, or the spread of tolerance to new groups of graft-reactive T cells, indicating suppression by Treg (301). It is postulated that these Treg may be resistant to anti-CD40L mAb therapy and would persist following graft-reactive T cell deletion, altering the ratio between regulators and aggressors in favor of tolerance (301). Alternatively, potentially donor-reactive T cells that fail to undergo apoptosis associated with AICD may become iTreg in response to antigen recognition in the absence of a co-stimulatory signal. In a model of skin transplantation, tolerance

induced by anti-CD40L mAb and DST, Treg were shown to be the essential mediators of tolerance (305).

Anti-CD40L mAb treatment in vascularized mouse cardiac allograft model

In the mouse cardiac allograft model, prolonged allograft survival can be achieved by disrupting CD40-CD40L interactions (232). Allografts in mice treated with anti-CD40L mAb remain free of CR and fail to express intragraft TGFβ (Figure 5). Gene transfer of active TGFβ into allografts of recipients treated with anti-CD40L mAb results in CR and elevated levels of the TGFβ induced downstream fibrotic factor, CTGF (232). A transient low but detectable Th1 and Th2 priming of donor-reactive cells are observed at day 10 post-transplant in anti-CD40L mAb treated recipients. However, these cells are eliminated since graft-reactive T cells in recipients with long-term allograft acceptance induced by CD40L blockade exhibit hyporesponsiveness (297). Adoptive transfer of splenocytes from long-term allograft recipients treated with anti-CD40L mAb into SCID donor-specific allograft recipients results in acute rejection mediated by a dominant Th1 response, similar to naïve cells (297). These observations provide support that one mechanism by which tolerance is induced in recipients treated with anti-CD40L mAb is through the maintenance of potentially alloreactive T cells in a naïve state (297).

1.9 Immunosuppressive drugs

Clinical immunosuppression in a transplant recipient is a balancing act between preventing rejection and losing protection against mutant cells and infectious agents as well as additional side effects described below. Immunosuppression is very intense immediately following transplantation but over the course of the first year diminishes as the allograft loses some of its immunogenicity and the recipient's immune system adapts

to the graft (306). Most acute rejection occurs in the first few months following transplantation and can be reversed by intensifying immunosuppression. Multiple drugs are administered to control transplant rejection and can be divided into immunosuppressive agents that inhibit of inflammation (corticosteroids) and proliferation (azathioprine, mycophenolate mofetil), calcineurin inhibitors (Cyclosporine and Tacrolimus), mTOR inhibitors (Sirolimus), and anti-lymphocyte Abs (anti-thymocyte globulin and OKT3) (306).

Corticosteroids

In 1963, Goodwin et al. demonstrated the effect of corticosteroids on reducing lymphocyte counts and reversing kidney allograft rejection (307). Corticosteroids have a multitude of immunosuppressive effects and can 1) suppress macrophage function and inhibit the release of TNF-α, IL-1 and IL-6; 2) inhibit T cell proliferation and induce apoptosis; 3) decrease MHC expression; 4) inhibit transmigration of immune cells through blood vessels; 5) inhibit adhesion molecule expression and 6) block the release of cytokines, including GM-CSF, IL-2, IL-4, IL-5, and IL-13 (306). Long-term use of corticosteroids can result in opportunistic infection, osteoporosis, diabetes, hypertension, diabetes, impaired healing of wounds, and weight gain (306).

Azathioprine

The imidazole derivative, Azathioprine (AZA), was first used as a primary immunosuppressive agent to control kidney rejection in 1963 (308). AZA is a purine analogue that has anti-proliferative effects on cells, including T and B cells. AZA is metabolized in the liver into the active drug, 6-mercaptopurine (6-MP) (306). 6-MP is further metabolized into thiolnosinic acid (TIMP). TIMP interferes with the synthesis of

guanylic and adenylic acids from inosinic acid and is incorporated into DNA strands during replication, thereby interfering with DNA synthesis (306). In addition, TIMP acts in a negative feedback loop for *de novo* purine synthesis--a pathway that lymphocytes are dependant on--leading to cell cycle arrest. Major side effects of AZA include bone marrow suppression: leucopenia, thrombocytopenia and anemia (306).

Mycophenolate mofetil

The active agent of Mycophenolate mofetil (MMF), mycophenolic acid (MPA), was initially isolated in 1896 from a penicillium culture (309). MMF selectively inhibits lymphocyte proliferation by interrupting the *de novo* purine synthesis pathway, which is essential for nucleotide synthesis since lymphocytes lack a purine salvage pathway (306). MPA inhibits inosine monophophate dehydrogenase (IMPDH), which is a critical enzyme in *de novo* purine synthesis, resulting in a guanine nucleotide deficiency. In addition to interfering specifically with lymphocyte DNA replication, MMF suppresses Ab induction, cytotoxic T cell differentiation, and reduces expression of adhesion molecules. Side effects include gastrointestinal disorders (306).

Cyclosporine

In the early 1970's, Jean Borel screened soil samples from Wisconsin and Norway to identify compounds with anti-microbial or anti-fungal properties (310). The fungus, *Tolypocladium inflatum Gams*, isolated from Norweigan soil, synthesized a compound termed cyclosporine that was identified to be immunosuppressive (310). The active metabolite, Cyclosporin A (CSA), complexes with cyclophilin and this complex binds to and inhibits the Ca2+-dependent serine phosphatase, calcineurin (306, 311). Calcineurin is critical for T cell receptor signaling transduction. CSA inhibits calcineurin-

dependent transcription factors, such as NFAT, which is important in the activation and transcription of many T cell cytokines and co-stimulatory molecules, which include IL-2, IFNγ, IL-4, TNFα, G-CSF and CD40L (311). A major side effect associated with CSA is nephrotoxicity (311).

Tacrolimus

Because of CSA's associated nephrotoxicity, Toru Kino tested Japanese soil for a compound more effective than CSA without the toxic side effects (306). A compound isolated from the bacteria, *Streptomyces tsukubaenis*, termed FK506 was found to be 100X as potent as CSA (311). The active agent of Tacrolimus, FK506, binds the cytoplasmic FK-binding protein (FKBP). This complex interacts with calcineurin and mode of action of FK506 is analogous to CSA (306). Treatment of transplant patients with Tacrolimus results in less rejection episodes but increased toxicity compared to CSA. Side effects of Tacrolimus include nephrotoxicity, neurotoxicity and diabetes induction (306).

Sirolimus

Sirolimus, also known as Rapamycin, was isolated from the actinomycete, *Streptomyces hygroscopicus,* found in soil samples from Rapa Nui, Easter Island (312). Similar in structure to FK506, Sirolimus also binds to FK binding protein which complexes with the mammalian target of rapamycin (mTor) (306). Sirolimus is capable of inhibiting T cell proliferation through Ca2+-dependent and independent mechanisms and does not affect calcineurin. Sirolimus suppresses protein synthesis in T cells and inhibits IL-2, IL-4 and IL-6 signaling and CD28-mediated events (306). T and B cell proliferation is blocked because Sirolimus prevents the progression of G1 to S phase of

the cell cycle. Side effects associated with Sirolimus are hyperlipidemia, thrombocytopenia and leucopenia (306).

Anti-lymphocyte antibodies

In 1960, it was observed that rabbit anti-rat lymphocyte serum prolonged skin graft survival in rats and upon this observation, humanized anti-lymphocyte and anti-thymocyte globulins were designed (313). These antibodies effectively depleted the patient's lymphoid tissue. OKT3 is a humanized mouse anti-human CD3 mAb that binds to the CD3εE chain of the TCR/CD3 complex (311). This Ab removes the TCR from the surface of the T cell through endocytosis or shedding, leaving the T lymphocytes nonfunctional and unable to reject a graft. Additional antibodies, OKT4 and OKT8, bind CD4+ and CD8+ T cells, respectively. Anti-thymocyte globulin (ATG) and anti-lymphocyte globulin (ALG) deplete circulating lymphocytes (311). A major side effect of these antibodies is cytokine release syndrome (CRS), which commonly occurs an hour post injection. CRS is the result of massive release of TNF and IFNγ when T cells undergo OKT3 induced activation. In addition, ATG and ALG initiate xeno-antibody responses, also referred to as "serum sickness." A final side effect is a significant impairment in cell-mediated immunity (313).

1.10 Dissertation overview

The purpose of these studies was to identify the role of TGFβ in graft-reactive cellular and humoral responses, Treg suppressor function, allograft acceptance and CR. Both TGFβ dependent and independent pathways to allograft acceptance are demonstrated, and the contribution of TGFβ-induced IL-17 in CR is investigated. TGFβ

neutralization within the allograft addresses local TGFβ inhibition on fibrosis and alloreactive T and B cell responses.

Chapter 2 describes the materials and methods employed in these studies.

Chapter 3 addresses the requirement for T cell responsiveness to TGFβ in allograft acceptance, T and B cell hyporesponsiveness, and the development of CR. TGFβ -induced IL-17 is implicated in graft fibrosis and may represent a therapeutic target for preventing CR.

Chapter 4 assesses the impact of intragraft TGFβ neutralization on CR, donor-reactive T cell responses, and allograft acceptance employing gene transfer of decorin into cardiac allografts.

Chapter 5 summarizes the findings presented herein and suggests additional studies to further elucidate the role of TGFβ in allograft acceptance and CR.

Chapter 2: Materials and Methods

2.1 Mice

C57BL/6 CD4-DNR (170), C57BL/6 wild type (WT) and BALB/c mice were purchased from the Jackson Laboratory. CD4-DNR mice express a dominant-negative form of the human TGFβ receptor II (TGFβRII) under the direction of the mouse CD4 promoter, which lacks a CD8 silencer. TGFβ signaling is abrogated in both CD4+ and CD8+ T cells in these transgenic mice. CD4-DNR were propagated by breeding C57BL/6 WT females with CD4-DNR males. The genotyping of transgene expressing CD4-DNR mice was carried out using the following PCR primers: primer WT forward figure5'CTAGGCCACAGAATTGAAAGATCT-3'; primer WT reverse 5'-TAGGTGGAAATTCTAGCATCATCC-3'; primer CD4-DNR transgene (TG) forward 5'-GCTGCACAT CGTCCTGTG-3'; primer TG reverse 5'-ACT TGACTGCACCGT TGTTG-3'. Primers WT forward and WT reverse were used to detect the internal control, IL-2 (324 bp). Primers TG forward and TG reverse were used to detect the transgenic allele (100 bp). CD4-DNR mice exhibit an autoimmune phenotype and immunopathology with age, resulting in the development of multi-focal inflammation best characterized by inflammatory bowel disease between 3-5 months of age (170). To avoid potential complications of age-related autoimmunity, mice were transplanted at 6 weeks of age and at the termination of each experiment the colons were examined macroscopically and histologicially for autoimmune manifestations. None of

the mice used in this study exhibited an autoimmune phenotype. IL-17-/- mice (101) were generated by Dr. Yoichiro Iwakura and provided by Dr. Weiping Zou in collaboration. All mice were housed under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan. These experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

2.2 Culture medium

Culture medium consisted of the following: RPMI 1640 supplemented with 2% FCS, 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomyin, 1.6 mM L-glutamine, 10 mM HEPES buffer (all from Invitrogen), 0.27 mM L-asparagine, 1.4 mM L-arginine HCl, 14 μM folic acid, and 50 μM 2-ME (all from Sigma-Aldrich).

2.3 Vascularized cardiac transplantation

CD4-DNR, IL-17-/-, and WT mice were transplanted with intact BALB/c cardiac allografts, as described (314). Briefly, the aorta and pulmonary artery of the donor heart were anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava, respectively. Upon perfusion with the recipient's blood, the transplanted heart resumes contraction. Graft function is monitored by abdominal palpation.

2.4 Adenoviral-mediated transduction of cardiac allografts

As described (315, 316), cardiac allografts were transduced by perfusion via the aorta with E1/E3 deleted adenoviral vectors (5×10^8 pfu) encoding the rat decorin (AdDec) or beta-galactosidase (Adβgal). AdDec was constructed in Dr. Elizabeth Nabel's lab using a cDNA kindly provided by Dr. Wayne Border (196). Following perfusion, grafts were recovered and placed in iced Ringer's for approximately 1 h prior to transplantation. Reporter gene studies with Adβgal have revealed that the distribution

of transgene expression within the cardiac allograft is patchy, and that both cardiac myocytes and cells of the vasculature express the transgene product (315).

2.5 In vivo mAb treatment

The hybridoma secreting anti-CD4 (clone GK1.5) was obtained from American Type Culture Collection. The hybridoma secreting anti-CD40L (clone MR1) was provided by Dr. Randy Noelle (Dartmouth, Lebanon, NH). Anti-CD4 and anti-CD40L mAb were purified and resuspended in PBS by Bio X Cell (West Lebanon, NH). Mice received 1 mg i.p. of anti-CD4 mAb on days -1, 0, 7 (232, 295, 296). In recipients transiently depleted of CD4+ T cells, CD4+ T cells begin to repopulate the periphery 3-4 weeks post-transplantation (285). Mice received 1 mg of anti-CD40L i.p. days 0, 1, and 2 (232). All doses are relative to day of transplant.

2.6 Histology

Allografts were recovered at the times indicated post-transplantation, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin-eosin (H & E) to assess myocyte viability (presence of cross striation and myocyte nuclei), and the nature and intensity of graft infiltrating cells.

2.7 Recovery of graft infiltrating cells (GIC)

Groups of three transplanted hearts were removed, pooled, minced, and digested with 1 mg/mL collagenase A (Roche) for 30 min at 37ºC. Tissue debris were allowed to settle at 1 x *g* and the suspension containing GIC was harvested by pipette. RBC were lysed by hypotonic shock, GIC were passed though a 30-µm pore size nylon mesh, and viable leukocytes were enumerated by trypan blue exclusion.

2.8 ELISPOT assays for cytokine-producing cells

ELISPOT assays were performed as previously described (317). Capture and detection antibodies specific for IFNγ (R4-6A2, XMG1.2), IL-4 (11B11, BVD6-24G2) and IL-17 (TC11-18H10.1, TC11-8H4.1) were purchased from Pharmingen (San Diego, CA). PVDF-backed microtiter plates (Millipore, Bedford, MA) were coated with unlabeled mAb and blocked with 1% BSA in PBS. Irradiated (1000 rad) donor splenocytes ($4x10^5$) and $1x10^6$ recipient splenocytes were added to the plates. After washing, a 1:1000 dilution of anti-biotin alkaline phosphatase (AP) conjugate (Vector Laboratories, Burlingame, CA) was added to IFNγ and IL-17 plates, and a 1:2000 dilution of horseradish peroxidase-conjugated streptavidin (SA-HRP; Dako, Carpinteria, CA) was added to IL-4 plates. Plates were washed and spots visualized by addition of nitroblue tetrazolium (NBT; Biorad, Hercules, CA) / 3 bromo-4-chloro-inolyl-phosphate (BCIP; Sigma) to IFNγ and IL-17 plates, or 3-amino-9-ethylcarbazole (AEC; Pierce, Rockford, IL) to IL-4 plates. Color development continued until spots were visible and stopped by adding H_2O. Plates were dried and spots quantified with an Immunospot Series 1 ELISPOT analyzer (Cellular Technology Ltd., Cleveland, OH).

2.9 RNA isolation and RT-PCR

Cardiac allografts were homogenized in 1 mL TRIzol® (Invitrogen Life Technologies, Carlsbad, CA) and RNA was isolated as per manufacturers protocol. 5 μg of total RNA were reverse transcribed using 10X PCR buffer (Roche), 10 mM dNTPs, Oligo (dt), M-MLV-RT (all from Invitrogen), and RNAsin (Promega). Products were then cleaned with 1:1 phenol/chloroform/isoamyl (25:24:1) and re-precipitated with 7.5 M NH_4OAC in pure EtOH overnight at -80ºC.

Real-time PCR was performed on cDNA using a Rotor-Gene 3000 TM (Corbett Life Science, CA). Primer binding to DNA was detected by SYBR Green ITM dye (Roche, Indianapolis, IN). Relative expression of the gene of interest was expressed as the comparative concentration of the gene product to the GAPDH product as calculated by accompanying Rotor-Gene software. Significance was determined with an unpaired Student t-test.

Primer sequences:

IL-17 sense: 5' GGACTCTCCACCGCAATGA

IL-17 anti-sense: 5'GACCAGGATCTCTTGCTGGA

FoxP3 sense: 5'CCAAGGTGAGCGAGTGTC

FoxP3 anti-sense: 5'AAGGCAGAGTCAGGAGAAGT

Rat Decorin: 5' AGCATAAATATGTCCAGGTCGTC

Rat Decorin anti-sense: 5'GAAGTCTTCCTAGTCTGGTATGAAGG

TGFβ sense: 5'CCTGAGTGGCTGTCTTTTGAC

TGFβ anti-sense: 5'CCTGTATTCCGTCTCCTTGGT

Collagen (pro-collagen 1a) sense: 5' TCCCTACTCAGCCGTCTGTGCC

Collagen anti-sense: 5' AGCCCTCGCTTCCGTACTCG

GAPDH sense: 5'CTGGTGCTGAGTATGTCGTG

GAPDH anti-sense: 5'CAGTCTTCTGAGTGGCAGTG

2.10 Donor-reactive Ab determination

As described (285, 296, 318), P815 cells (H-2^d) were stained for flow cytometric analysis using diluted (1:50) sera obtained from mice as the primary Ab, followed by FITC-conjugated isotype specific anti-mouse IgG, IgG1, or IgG2a secondary antibodies

(The Binding Site, San Diego, CA, USA) at a 1:50 dilution. Data are reported as the mean channel fluorescence determined on a Becton Dickinson FACSCaliber (San Jose, CA, USA).

2.11 Immunohistochemistry

To detect IgG deposition within the graft, frozen sections of grafts were fixed in cold acetone and incubated with 1:150 dilution of goat anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL) followed by AEC staining (285). To detect C3d and C4d deposition (285), sections of paraffin embedded tissue were fixed in methanol. A 1:20 dilution of goat anti-mouse C3d (R&D Systems, Minneapolis, MN) was added followed by secondary detection antibodies added as per R&D System's anti-goat cell and tissue staining kit. Slides were stained with rabbit anti-mouse C4d (kindly provided by Dr. William Baldwin, Cleveland Clinic) at a 1:500 dilution, followed by DAB development using the SuperPicTure^{TM} Polymer Detection Kit (Zymed). Specificity of staining was ensured by staining of native hearts.

2.12 Morphometric analysis of cardiac allograft fibrosis and hypertrophy

Graft fibrosis was quantified by morphometric analysis of Masson's trichrome stained tissues using iPLab software (Scanalytics Inc., Fairfax, VA) (294). Mean fibrotic areas were calculated from 10 to 12 areas per heart section analyzed at 200X magnification. A minimum of 5 individual hearts were analyzed per group. To quantify cardiomyocyte area as a measure of hypertrophy, digital outlines were drawn around 100 cardiomyocytes from views of H&E stained grafts at 200X magnification. Areas within outlines were quantified using SCION IMAGE Beta 4.0.2 software (Scion Corporation,

Frederick, MD) to measure cardiomyocyte cell size (294). Five individual hearts were analyzed per group.

2.13 Statistical analysis

Data were analyzed with GraphPad Prism 4.0c software using unpaired Student t-tests. p values of ≤ 0.05 were considered statistically significant.

Chapter 3: Role of T cell Transforming Growth Factor β Signaling and IL-17 in Allograft Acceptance and Fibrosis Associated with Chronic Rejection

3.1 Abstract

CR is the main barrier to long-term transplant survival. CR is a progressive disease defined by interstitial fibrosis, vascular neointimal development, and graft dysfunction. The underlying mechanisms responsible for CR remain poorly defined. TGFβ has been implicated in promoting fibrotic diseases including CR, but is beneficial in the transplant setting due to its immunosuppressive activities. To assess the requirement for T cell TGFβ signaling in allograft acceptance and the progression of CR, we used mice with abrogated T cell TGFβ signaling as allograft recipients. We compared responses from recipients that were transiently depleted of CD4+ cells (that develop CR and express intragraft TGFβ) to responses from mice that received anti-CD40L mAb therapy (that do not develop CR and do not express intragraft TGFβ). Allograft acceptance and suppression of graft-reactive T and B cells were independent of T cell TGFβ signaling in mice treated with anti-CD40L mAb. In recipients transiently depleted of CD4+ T cells, T cell TGFβ signaling was required for the development of fibrosis associated with CR, long-term graft acceptance, and suppression of graft-reactive T and B cell responses. Further, IL-17 was identified as a critical element in TGFβ driven allograft fibrosis. Thus, IL-17 may provide a therapeutic target for preventing graft fibrosis, a measure of CR, while sparing the immunosuppressive activities of TGFβ.

3.2 Introduction

With the advent of immunosuppressive therapies, a decline in graft loss due to acute rejection has established chronic allograft rejection (CR) as the leading cause of late graft failure (319). CR is an irreversible disease characterized by deteriorating graft function, interstitial fibrosis, and occlusive neointima (26, 223, 320, 321). Despite continued investigation, the underlying mechanisms responsible for these disease manifestations remain poorly defined and no therapies exist to prevent or treat CR except re-transplantation (322).

The immune system evolved to combat pathogens while maintaining tolerance to self, and TGFβ plays a pivotal role in regulating immune responses (164). The importance of TGFβ in immune regulation is underscored by the severe autoimmunity observed in knockout mice that lack TGFβ or are unable to signal through the TGFβ receptor (169, 170). TGFβ controls T cell mediated self-reactivity by regulating lymphocyte proliferation and survival, inhibiting Th1/Th2-cell differentiation, and dampening effector function (reviewed in (178)). TGFβ signaling in B cells inhibits proliferation and survival, prevents activation, and inhibits IgG class switching (178). TGFβ is also critical in both the development and function of T regulatory cells (Treg) (172, 173, 178, 323). TGFβ signaling in Treg is essential for peripheral maintenance of this cell subset (164, 324) and for the induction of FoxP3 expression and Treg function in CD4+CD25- T cells (325).

In addition to TGFβ's anti-inflammatory activities, TGFβ also mediates pro-inflammatory as well as pro-fibrotic activities. A reciprocal developmental pathway exists for the generation of pathogenic effector Th17 and Treg (93, 94, 326). TGFβ in

association with IL-6 or IL-21 favors the commitment of CD4+ T cells to the Th17 lineage (93, 94, 327-330). IL-17 stimulates stromal cells, such as fibroblasts, endothelial cells, and epithelial cells to produce IL-6, IL-8, granulocyte CSF (G-CSF), and PGE2 and up-regulates critical chemoattractants, such as CXCL1 and CXCL2 (91, 97, 98). IL-17 serves to amplify the inflammatory responses and has also recently been implicated as a pro-fibrotic cytokine (92, 231, 331-336).

A critical role for TGFβ in transplant acceptance has been described (337, 338), and early studies revealed the importance of TGFβ in donor-specific transfusions and allograft acceptance (339). Subsequent studies investigating skin allograft acceptance have also identified TGFβ as a protective factor against allograft rejection (299, 340). Further, transduction of cardiac allografts with active TGFβ prolongs graft survival (316) and is associated with the induction of graft-reactive Treg (235).

While TGFβ mediates many beneficial anti-inflammatory activities in the immune system (164), we have previously reported an association between TGFβ and fibrosis associated with CR using the mouse vascularized cardiac allograft model (232). Intragraft transcript levels of TGFβ were readily detectable in the CR grafts from recipients transiently depleted of CD4+ T cells, but not in the grafts of anti-CD40L treated recipients, which remain free of CR (232). Allograft transduction with active TGFβ resulted in CR in anti-CD40L treated recipients that do not normally exhibit CR, but was not observed in TGFβ transduced syngeneic grafts. This supported a critical role for TGFβ and alloantigen in the progression of CR. In this study, we sought to determine the role of TGFβ signaling on alloreactive effector cells, Treg function, and IL-17 induction by using T cell-specific dominant negative TGFβ receptor type II (CD4-DNR) (170) and

IL-17 deficient (IL-17-/-) mice (101) as transplant recipients. Further, we identify both TGFβ dependent and independent pathways to allograft acceptance.

3.3 Results

Experimental system

In the mouse cardiac allograft model, prolonged allograft survival can be achieved by transiently depleting recipients of CD4+ cells or by disrupting CD40-CD40L interactions. However, these two inductive therapies differ with respect to the development of CR. Allografts in mice treated with anti-CD40L mAb remain free of CR, while grafts in mice transiently depleted of CD4+ cells develop CR, which is associated with intragraft expression of TGFβ (232). While graft-reactive T cells remain in a hyporesponsive state in both settings, recall responses of cells from these groups differ in that dominant Th2 responses are mounted by mice that are depleted of CD4+ cells, while Th1 responses are mounted by recipients given anti-CD40L mAb therapy (297). The current study assessed the requirement for TGFβ signaling in T cells for allograft acceptance, graft-reactive T and B cell hyporesponsiveness, and graft fibrosis as a paramater of CR.

Effect of T cell TGFβ signaling on transplant acceptance

TGFβ mediates beneficial immunosuppressive activities in the transplant setting (337-339). To assess the requirement for T cell TGFβ signaling in allograft acceptance and the progression of graft fibrosis associated with CR, we used CD4-DNR mice with abrogated T cell TGFβ signaling (170) as allograft recipients. CD4-DNR mice express a dominant-negative form of the human TGFβRII under the direction of the mouse CD4 promoter, which lacks a CD8 silencer. Hence, TGFβ signaling is abrogated in both CD4+

and CD8+ T cells in these transgenic mice. To rule out allo-antigen independent cellular infiltration and tissue damage in CD4-DNR recipients, both WT and CD4-DNR mice were transplanted with syngeneic grafts. Grafts from both groups functioned until the experiment was terminated on day 50 post-transplant (Figure 6A). Histologically, sygeneic grafts from both groups were free of infiltrate, exhibited minimal fibrosis, normal arteries and viable myocytes (data not shown). Similar observations were made in CD4-DNR recipients of syngeneic grafts treated with inductive CD4+ T cell depletion (data not shown). The results indicate that there were no differences in graft survival or histology between the WT and CD4-DNR recipients of syngeneic grafts ruling out non-antigen specific inflammatory responses in graft loss in CD4-DNR mice.

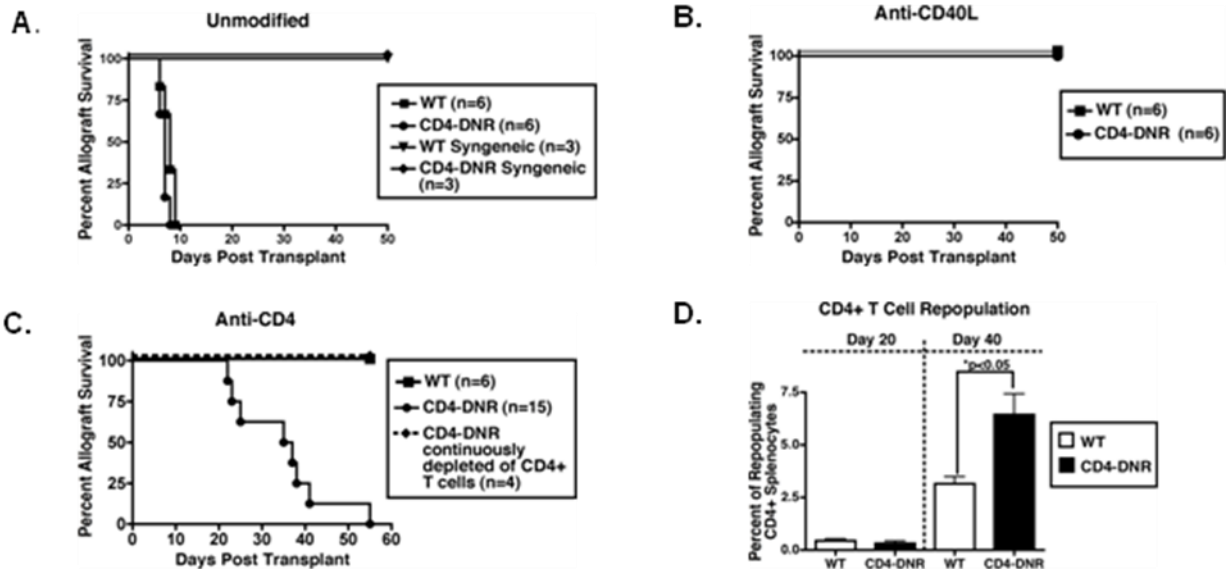


Figure 6: TGFβ dependent and independent mechanisms of allograft acceptance. WT (squares and triangles) and CD4-DNR (circles and diamonds) mice were transplanted with allogeneic or syngeneic cardiac allografts and were either left untreated **(A)**, given inductive anti-CD40L mAb **(B)**, or transiently depleted of CD4+ cells **(C)**. Graft function was monitored by palpation and recipients were recovered either at the time of rejection or 50 days post-transplant. **(D)** Flow cytometric analysis of CD4+ splenocytes that were harvested from WT or CD4-DNR on day 20 and day 40 post-transplant. Allograft recipients were treated inductively with anti-CD4 mAb.

Due to the immunosuppressive activities of TGFβ, we predicted that CD4-DNR recipients would mount exacerbated rejection responses when compared to WT mice. Allografts in both WT and CD4-DNR recipients were acutely rejected by day 9 post-transplant (Figure 6A) and histological examination revealed evidence of rejection (data not shown). While the tempo of rejection was not different between these two groups, a more intense infiltration of the grafts was observed in CD4-DNR recipients (total number of GICs per graft: WT = 0.33 x 10^6 +/- 0.01; CD4-DNR = 1.4 x 10^6 +/- 0.23 ($p<0.01$)) indicating that T cell TGFβ signaling dampens cell proliferation and/or cellular infiltration in unmodified recipients.

Treatment of both WT and CD4-DNR recipients with inductive anti-CD40L mAb resulted in long-term graft survival (Figure 6B), demonstrating that allograft acceptance following anti-CD40L therapy is independent of TGFβ signaling in T cells. WT allograft recipients treated with inductive CD4+ T cell depletion also exhibited long-term graft survival (Figure 6C). In contrast, 90% of the allografts from CD4-DNR mice transiently depleted of CD4+ T cells were rejected by day 40 post-transplantation (Figure 6C). The majority of the allografts were rejected between days 35-40, correlating with CD4+ T cell repopulation of the periphery (285, 296). A greater percentage of CD4+ T cells were present in the spleens of CD4-DNR recipients compared to WT suggesting that TGFβ signaling controls CD4+ T cell proliferation in recipients transiently depleted of CD4+ T cells (Figure 6D). Rejection could be attenuated in CD4-DNR recipients when CD4+ T cells were continuously depleted by weekly injections of anti-CD4 mAb (Figure 6C). These results indicate that T cell TGFβ signaling is essential for allograft acceptance in

recipients transiently depleted of CD4+ T cells and that repopulation of CD4+ cells is required for rejection in CD4-DNR mice.

TGFβ regulation of donor-reactive Th1, Th2 and Th17 responses

T cell TGFβ signaling inhibits differentiation of Th1 and Th2 cells through the suppression of T-bet/STAT4 and GATA3/NFAT (164, 178). To determine if T cell TGFβ signaling regulates donor-reactive cellular immune responses, we employed ELISPOT to quantify the number of *in vivo* primed donor-reactive Th1 (IFNγ), Th2 (IL-4) and Th17 (IL-17) responses (Figure 7). Elevated donor-reactive Th1 responses were observed in unmodified WT and CD4-DNR recipients indicating acute rejection is associated with a dominant Th1 response (Figure 7A). While abrogation of TGFβ signaling in recipients did not result in elevated Th1 responses compared to WT recipients, CD4-DNR recipients did exhibit enhanced IL-4 priming in response to donor antigen. However, this was not statistically significant. Donor-reactive Th17 responses were negligible in both WT and CD4-DNR recipients.

Donor-reactive Th responses were not observed in either WT or CD4-DNR recipients treated with anti-CD40L (Figure 7B). These findings indicate that T cell TGFβ signaling is not required for the suppression of donor-reactive responses (341) and is dispensable for allograft acceptance following anti-CD40L mAb treatment.

As previously reported (295-297), donor-reactive Th1 and Th2 responses are suppressed in recipients transiently depleted of CD4+ T cells. Consistent with these findings, inductive anti-CD4 mAb therapy resulted in hyporesponsiveness in WT recipients compared to unmodified recipients (Figure 7A and 7C). In contrast, elevated

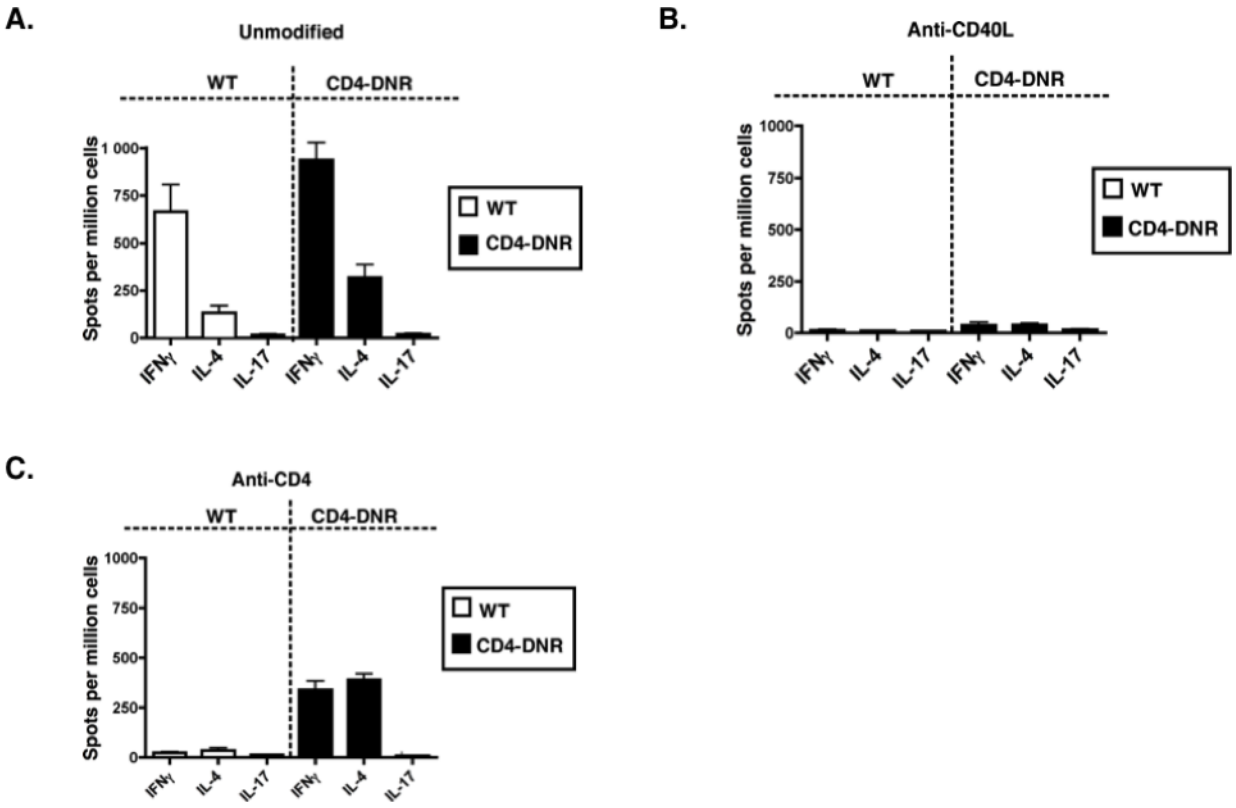


Figure 7: Donor-reactive Th1, Th2 and Th17 responses in WT and CD4-DNR recipients. WT (open bars) or CD4-DNR (shaded bars) allograft recipients were left untreated **(A)**, treated with inductive anti-CD40L mAb **(B)**, or treated with inductive anti-CD4 mAb **(C)**. Graft function was monitored by palpation and recipients were recovered either at the time of rejection, 50 days post-transplant (for anti-CD40L mAb treated WT and CD4-DNR recipients), or 40 days post-transplant (for anti-CD4 mAb treated WT recipients in order to directly compare to CD4-DNR recipients, which reject between days 35-40). At the termination of the experiment, splenocytes were harvested and processed for ELISPOT assays to quantify primed, donor-reactive IFNγ, IL-4, or IL-17-producing cells. Bars represent the mean number of cytokine producing cells (+/- S.E.M.) from at least six recipients per group.

donor-reactive Th1 and Th2 responses were detected in the CD4-DNR recipients transiently depleted of CD4+ T cells (Figure 7C).These data indicate that TGFβ plays a pivotal role in suppressing immune responses and in maintaining allograft acceptance in recipients initially depleted of CD4+ T cells.

Cardiac fibrosis is reduced in mice with abrogated T cell TGFβ signaling

TGFβ has been strongly implicated in many fibrotic diseases (231, 342), including CR (233, 320, 321, 343). To investigate the relationship between T cell TGFβ signaling and the development of graft fibrosis as a measure of CR, quantitative morphometric trichrome analysis was employed to evaluate allograft fibrosis in WT and CD4-DNR recipients treated with inductive anti-CD40L or anti-CD4 mAb. Allografts from WT and CD4-DNR recipients treated with inductive anti-CD40L mAb revealed minimal fibrosis (Figure 8A and 8B). Allografts from WT recipients transiently depleted of CD4+ T cells revealed significant fibrosis compared to allografts from CD4-DNR

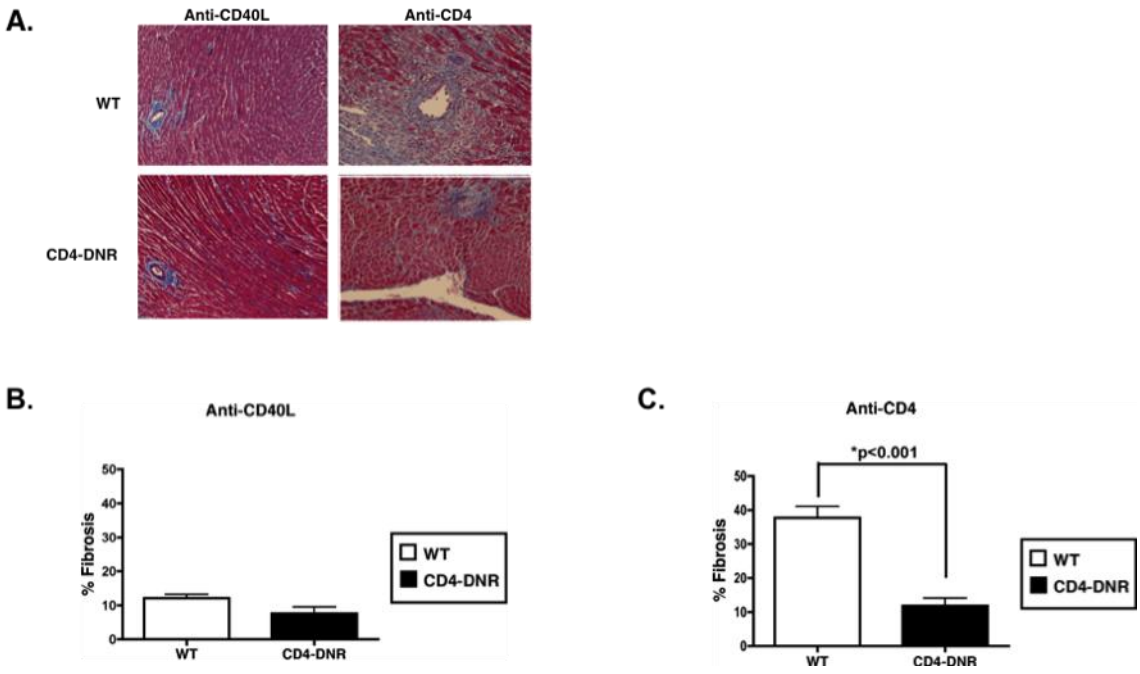


Figure 8: Allograft fibrosis in WT versus CD4-DNR recipients treated with anti-CD4 mAb. (A) Sections of grafts from recipients treated with inductive anti-CD40L (day 50 post-transplant) or anti-CD4 mAb (between days 35-40 post-transplant due to rejection in CD4-DNR recipients) were stained with Masson's trichrome, which stains fibrotic tissue blue. Frames are of grafts from WT and CD4-DNR recipients and are representative of at least 6 anti-CD40L mAb treated mice and 6-8 anti-CD4-treated mice. 200X magnification. **(B)** Quantification of mean fibrotic area by morphometric analysis in anti-CD40L and **(C)** anti-CD4 mAb treated WT and CD4-DNR recipients. Bars represent the average percentage (+/- S.E.M.) of graft area positive for fibrosis in 5 anti-CD40L treated recipients and 6 anti-CD4 treated recipients. WT (open bars) and CD4-DNR (shaded bars).

recipients (Figure 8A and 8C). These findings indicate that in recipients transiently depleted of CD4+ cells, allograft fibrosis is dependent on T cell TGFβ signaling.

Donor-reactive IgG production in WT and CD4-DNR recipients

Since donor-reactive antibodies have been implicated in both acute and chronic rejection (344, 345), we analyzed WT and CD4-DNR recipients for donor-reactive IgG antibodies (Figure 9). At the time of rejection, both WT and CD4-DNR unmodified

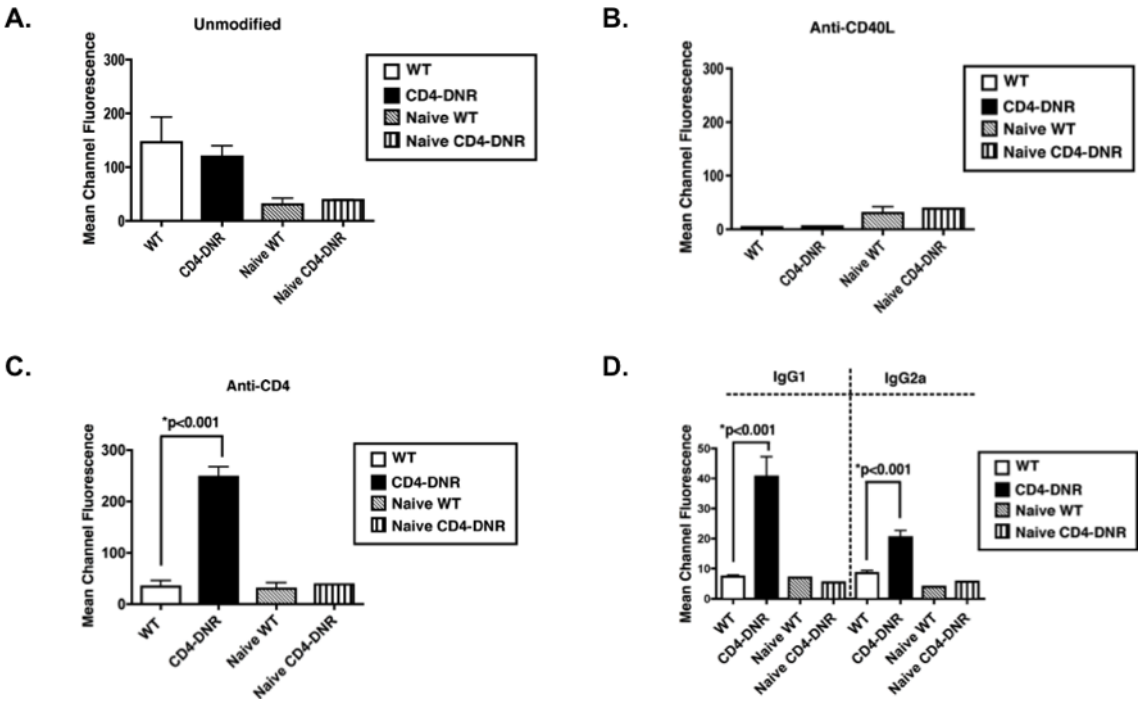


Figure 9: Effect of TGFβ unresponsiveness on donor-reactive alloantibody levels. Sera were obtained from WT (open bars) or CD4-DNR (shaded bars) allograft recipients that were left untreated **(A)**, treated with inductive anti-CD40L mAb **(B)**, or treated with inductive anti-CD4 mAb **(C)**. Sera were obtained at the time of rejection for unmodified recipients), 50 days post-transplant (for anti-CD40L mAb treated WT and CD4-DNR recipients), or 40 days post-transplant (for anti-CD4 mAb treated WT recipients). P815 (H-2^d) cells were incubated with 1:50 dilution of sera and bound donor-reactive Ab were detected by incubation with FITC-tagged anti-IgG, anti-IgG1 or anti-IgG2a Abs. The mean channel fluorescence is indicative of the relative amount of donor-reactive antibodies. Bars represent the average mean channel fluorescence of at least 6 WT and 6 CD4-DNR recipient samples (+/- S.E.M.). IgG1 and IgG2a donor-reactive Ab levels were analyzed in anti-CD4 mAb treated WT and CD4-DNR recipients **(D)**.

recipients exhibited comparable donor-reactive IgG production (Figure 9A). These results indicate that TGFβ signaling does not overtly influence IgG alloantibody production in unmodified recipients. It is well established that CD40-CD40L interactions are critical for Ab isotype switch (346). Consistent with these findings, both WT and CD4-DNR recipients treated with anti-CD40L mAb did not generate donor-reactive IgG production compared to unmodified recipients (Figure 9B). Hence, CD40-CD40L interactions are required for IgG isotype switching in the presence or absence of TGFβ signaling in T cells.

CD4-DNR recipients inductively depleted of CD4+ T cells produced significantly elevated levels of donor-reactive IgG compared to WT controls (Figure 9C). To further characterize the donor-reactive Ab production in these recipients, both Th1- and Th2-dependent IgG2a and IgG1 isotypes were measured (347). Consistent with an increase in total IgG level, the CD4-DNR recipients exhibited significantly elevated levels of both IgG1 and IgG2a alloAb production compared to WT recipients (Figure 9D). Collectively, these findings demonstrate that in the absence of TGFβ signaling, T cells acquire effector functions, secrete both Th1 and Th2 cytokines, and provide help for alloreactive B cells in recipients transiently depleted of CD4+ T cells.

Evidence of Ab-mediated rejection in CD4-DNR recipients treated with anti-CD4 mAb

The diagnostic criteria for Ab-mediated rejection include histologic evidence of acute tissue injury, serologic evidence of circulating donor-reactive antibodies, and the deposition of complement C3d and C4d within allografts (reviewed in (77, 219, 344)). Hence, we investigated the deposition of IgG and C3d and C4d within the grafts from WT and CD4-DNR recipients transiently depleted of CD4+ T cells (Figure 10). Intense

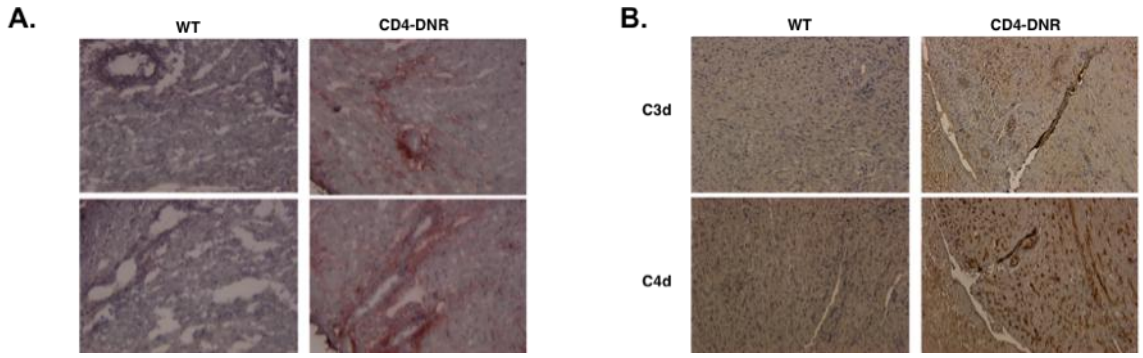


Figure 10: IgG, C3d and C4d deposition in allografts of WT and CD4-DNR recipients. WT (left column) or CD4-DNR (right column) allograft recipients were treated with inductive anti-CD4 mAb therapy. Grafts were recovered at either the time of rejection or 40 days after transplantation. Graft sections were fixed and incubated with goat anti-mouse IgG **(A)** or the goat anti-mouse C3d or C4d **(B)** followed by development with 3-amino-9-ethylcarbazole or DAB to visualize mouse Ab and complement deposition. Results are representative of grafts from 6-10 recipients. Magnification, 400X **(A)** and 200X **(B)**.

IgG staining was localized mainly to the capillaries and arteries of allografts from CD4-DNR recipients, while allografts from WT recipients were free of IgG deposition (Figure 10A). Further, both C3d and C4d were deposited within the vasculature structures of allografts from CD4-DNR recipients, but were not present in control allografts (Figure 10B). These data indicate that in recipients transiently depleted of CD4+ T cells, antibody mediated rejection of allografts occurs when T cells are unresponsive to TGFβ.

Anti-CD4 treated CD4-DNR recipients exhibit reduced intragraft FoxP3 and IL-17 levels compared to WT controls

TGFβ induces expression of FoxP3 (348), leading to the differentiation of CD4+CD25+ Treg cells from CD4+CD25- T cells (138, 325, 349). However, a dichotomy exists in which TGFβ in the context of IL-6 or IL-21 promotes pathogenic Th17 cell development (93, 94, 326-329). To investigate the role of TGFβ signaling in Treg and Th17 induction and the contribution of these subsets in CR, we assessed intragraft FoxP3 and IL-17 transcript levels from WT and CD4-DNR recipients inductively depleted of CD4+ T cells

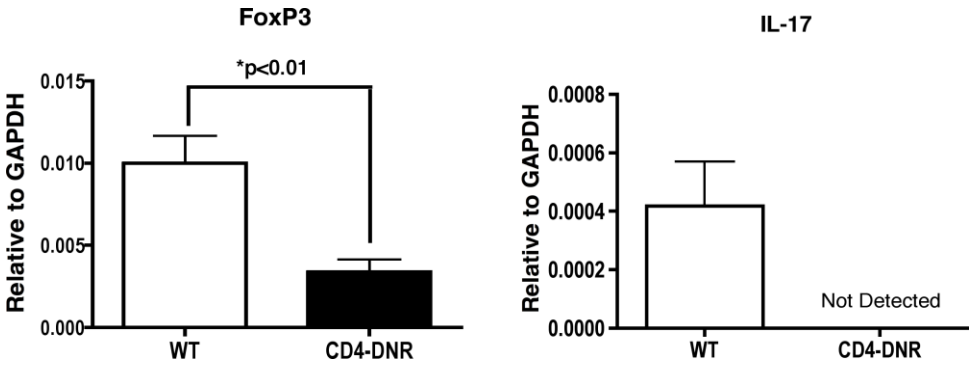


Figure 11: Reduction of TGFβ-dependent intragraft gene expression from recipients transiently depleted of CD4+ T cells.
RNA was harvested from allografts of WT and CD4-DNR recipient transiently depleted of CD4+ T cells. Intragraft transcript levels of FoxP3 and IL-17 were assessed by real-time RT-PCR. Allografts were recovered between days 35-40 post-transplant. Bars depict the means of RNA expression from 6 WT and 9 CD4-DNR grafts.

and showed that allografts from the CD4-DNR recipients exhibited significantly reduced intragraft FoxP3 gene expression compared to WT (Figure 11). This may indicate a failure to induce Treg from naïve CD4+ T cells in the absence of T cell TGFβ signaling, poor maintenance of peripheral Treg, and/or reduced Treg trafficking into the graft (reviewed in (298)). In addition, intragraft IL-17 was detected in allografts from WT but not CD4-DNR recipients (Figure 11). IL-17 was not detected by ELISPOT in the spleens of the WT mice (Figure 7), which may suggest a compartmentalized immune response against donor antigen. Indeed, similar polarization of Th17 responses solely within target tissue has been observed in murine models of allergic lung disease and hypersentitivity pneumonitis and lung fibrosis (336, 350). IL-17 is best recognized as a cytokine that mobilizes neutrophils and coordinates local tissue inflammation through the induction of various pro-inflammatory cytokines (91, 97). Numerous recent reports implicate IL-17 as a pro-fibrotic cytokine in the context of T cell dependent fibrosis (92, 331-336, 351), capable of inducing fibroblast proliferation and collagen deposition in cardiac tissue (92). Our observation that allografts from CD4-DNR recipients treated with anti-CD4

exhibited minimal fibrosis (Figure 8) led us to hypothesize that IL-17 plays a pro-fibrotic role in CR.

Allograft fibrosis is reduced in anti-CD4 mAb treated IL-17-/- recipients

To investigate the role of IL-17 production in fibrosis associated with CR, IL-17 -/- mice (101) were used as transplant recipients. Both WT and IL-17-/- allograft recipients treated with inductive anti-CD4 mAb therapy exhibited long-term graft survival, while unmodified recipients rejected their grafts by day 9 post-transplant (Figure 12A). In IL-17-/- recipients transiently depleted of CD4+ T cells, fibrosis was markedly reduced relative to WT counterparts (Figure 12B and 12C). This reduction of allograft fibrosis in IL-17-/- recipients implicates IL-17 as a cytokine responsible for TGFβ-mediated fibrosis and CR.

To investigate the effect of IL-17 deficiency on intragraft gene expression, we assessed both IL-17 and FoxP3 transcript levels from WT and IL-17-/- recipients that were transiently depleted of CD4+ t cells. Allografts from IL-17-/- recipients exhibited comparable FoxP3 gene expression to WT counterparts, while IL-17 transcripts were not detected in the IL-17-/- recipients (Figure 12D). These results suggest that reduced fibrosis in IL-17-/- recipients transiently depleted of CD4+ T cells is not a result of enhanced Treg number as assessed by intragraft FoxP3 transcript levels.

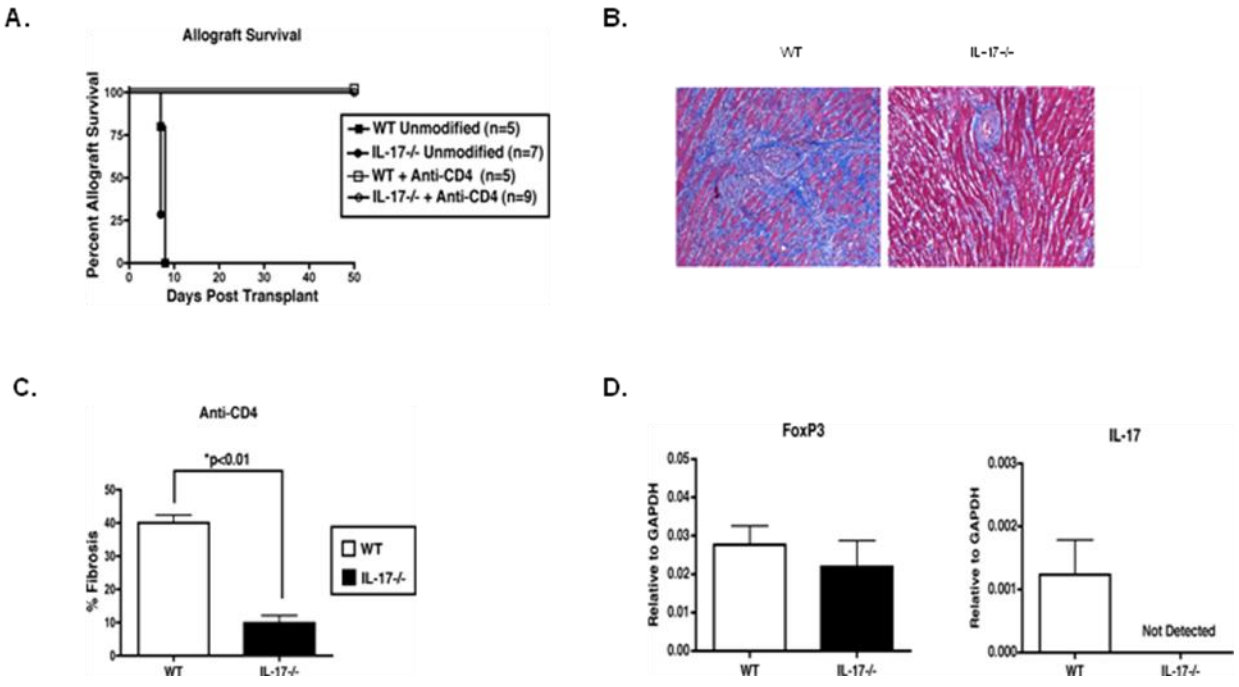


Figure 12: In recipients that fail to produce IL-17, allografts are protected from fibrosis. WT (squares) and IL-17-/- (circles) mice were transplanted with BALB/c cardiac allografts and were either left untreated (closed symbols) or transiently depleted of CD4+ cells (open symbols). Graft function was monitored by palpation. Unmodified recipients were recovered at the time of rejection, while inductive anti-CD4 mAb treated recipients were harvested at day 50 post-transplantation. Numbers in parentheses represent the number of recipients in each group **(A)**. Sections of grafts from recipients treated with inductive anti-CD4 mAb therapy (day 50 post-transplant) were stained with Masson's trichrome stain. Frames are of grafts from WT and IL-17-/- recipients and are representative of at least 6 WT and 9 IL-17-/- recipient allografts. 200X magnification **(B)**. Quantification of fibrosis by morphometric analysis. Bars represent the average percentage (+/- S.E.M.) of graft area positive for fibrosis in at least 5 WT and IL-17-/- recipients treated with anti-CD4 mAb. WT (open bars) and IL-17-/- (closed bars) **(C)**. On day 50 post-transplant, RNA was harvested from allografts of WT and IL-17-/- recipients transiently depleted of CD4+ T cells. Intragraft transcript levels of FoxP3 and IL-17 were assessed by quantitative RT-PCR. Bars depict the means of RNA from 5 WT and 9 IL-17-/- grafts **(D)**.

3.4 Discussion

We have previously reported an association between TGFβ and graft fibrosis associated with CR using the mouse vascularized cardiac model (232). Intragraft transcript levels of TGFβ are readily detectable in the CR grafts from recipients

transiently depleted of CD4+ T cells, but not in the grafts of anti-CD40L treated recipients, which remain free of CR. In the current study, we used inductive anti-CD4 or anti-CD40L mAb therapy and CD4-DNR recipients to evaluate the role of T cell TGFβ signaling in graft acceptance and the progression of fibrosis associated with CR. Collectively, our data suggests that in WT allograft recipients transiently depleted of CD4+ cells, allograft acceptance, T and B cell hyporesponsiveness, and fibrosis of the graft are dependent on TGFβ signaling in T cells. We further demonstrate that IL-17 is involved in the development of graft fibrosis in recipients transiently depleted of CD4+ T cells.

T cell TGFβ signaling is not required for long term allograft acceptance following anti-CD40L therapy (Figure 6). It is not fully understood how blockade of CD40L results in allograft acceptance, but a number of mechanisms, including donor-reactive T cell anergy and/or deletion, and the induction of Treg have been proposed (298, 305). We have reported that anti-CD40L mAb therapy allows for a transient appearance of primed donor-reactive cells (297). Hence, it is possible that these primed T cells express CD40L, which targets these cells for deletion and/or silencing by anti-CD40L mAb therapy. Our study demonstrates that these processes do not require T cell TGFβ signaling.

In recipients transiently depleted of CD4+ T cells, our findings support a TGFβ-dependent mechanism of graft acceptance. Inductive anti-CD4 mAb treatment of recipients results in transient depletion of CD4+ T cells at the time of transplant (285, 295, 296). CD4+ T cells begin to repopulate the periphery 3-4 weeks post-transplantation. As CD4+ T cells return, donor-reactive T cells are functionally distinct from naïve cells in that these repopulating CD4+ are hyporesponsive toward the graft but

mount Th2 recall responses (297). This altered functional T cell capacity is associated with intragraft expression of TGFβ. Inductive anti-CD4 mAb treatment of CD4-DNR recipients results in allograft rejection between day 35-40 post-transplantation (Figure 6). Th1 and Th2 were maintained in a quiescent state in WT recipients transiently depleted of CD4+ cells (Figure 7). In contrast, CD4-DNR recipients treated with inductive anti-CD4 mAb mount donor-reactive Th1 and Th2 responses, revealing that the induction of hyporesponsiveness requires that T cells be responsive to TGFβ (Figure 7). Repopulation of CD4+ T cells in the periphery is required for rejection in that CD4-DNR transplant recipients that are continuously depleted of CD4+ T cells did not reject their grafts (Figure 6) and did not mount Th1 and Th2 responses (data not shown). These observations support an essential role for TGFβ in cellular hyporesponsiveness and allograft acceptance in recipients inductively depleted of CD4+ T cells.

In the absence of TGFβ signaling, T cells differentiate into effector cells, secrete cytokines and provide help to B cells in CD4-DNR recipients transiently depleted of CD4+ cells. CD4-DNR recipients transiently depleted of CD4+ cells mounted significantly elevated donor-reactive alloantibody levels of both Th2 induced non-complement fixing, IgG1, and Th1 induced complement fixing, IgG2a, compared to wild type controls (Figure 9) (352). Both subclassses have been documented to synergize to cause rejection of cardiac allografts (352). IgG and complement split product C3d and C4d capillary deposition in both human and mouse myocardium is significantly associated with graft loss (353) and these products were detected in the vessels and surrounding the cardiac myocytes in the CD4-DNR recipient allografts that were rejected (Figure 10). Hence, T cells, which are unable to respond to TGFβ mount cellular

responses and provide help to activate alloreactive B cells in recipients inductively depleted of CD4+ T cells.

Donor-specific hyporesponsiveness is observed in both human and mouse transplant recipients that exhibit prolonged allograft acceptance (138, 270, 354). In many of these studies, allograft acceptance is strongly associated with Treg infiltration into the graft as detected by high FoxP3 transcript levels (138, 154, 270, 354). Treg are believed to play a critical role within allografts by inhibiting alloreactive T cell responses (270). Studies in skin allograft models reveal Treg enrichment in accepted grafts (355) is dependent on TGFβ and that this cytokine is important for long-term acceptance (138, 299). Consistent with these observations, inductive anti-CD4 mAb treatment of WT recipients exhibit enhanced intragraft FoxP3 transcript levels compared to CD4-DNR (Figure 11). In CD4-DNR recipients transiently depleted of CD4+ T cells, reduced FoxP3 expression may indicate impaired maintenance of peripheral Treg, reduced Treg localization within the graft, or a failure in Treg induction (reviewed in (298)). Donor-reactive Th responses observed in CD4-DNR recipients may result from decreased induction of Treg and/or the failure of Treg to control effector cells in the absence of TGFβ signaling (356). Our data suggest an active regulatory mechanism in which Treg migration into the grafts (Figure 11) and inhibition of alloreactive responses (Figure 7) require T cell TGFβ signaling.

While functional T cell TGFβ signaling prevented allograft rejection in recipients inductively depleted of CD4+ T cells, it promoted fibrosis associated with CR (Figure 8). In contrast, T cell unresponsiveness to TGFβ resulted in minimal fibrosis of grafts (Figure 8). It has been reported that sustained production of TGFβ in tissues is a main

contributor to the development of fibrosis (233, 357), but we have observed that gene transfer of TGFβ in syngeneic grafts fail to develop CR (232). Fibrosis was observed only in allografts that adenovirally expressed TGFβ and were transplanted into anti-CD40L recipients (232). This indicates that TGFβ alone is insufficient to induce fibrosis of the graft, and that alloantigen and elements of the immune system are required for fibrosis induction.

As T cells infiltrate the allograft and respond to TGFβ, they secrete multiple cytokines, which may influence the local environment to become pro-fibrotic. One TGFβ-induced cytokine that could potentially mediate fibrosis in this setting is IL-17 (92, 331). IL-17 is important in coordinating local tissue inflammation through the induction of various pro-inflammatory cytokines (91, 97). IL-17 has also been implicated as a contributor to fibrosis in a number of diseases (231, 332-334, 336). Allografts from IL-17-/- recipients transiently depleted of CD4+ T cells showed a significant reduction in fibrosis relative to their WT counterparts (Figure 12). These findings are consistent with TGFβ induced IL-17 promoting interstitial fibrosis in CR allografts.

IL-17 may induce collagen deposition within CR allografts through multiple mechanisms. IL-17 upregulates collagen gene expression in primary mouse cardiac fibroblasts (92). Indirectly, IL-17 induces the production of IL-6 (91, 97), which enhances the accumulation of collagen (358-360). IL-17 may also play a role in fibrosis by acting as a potent pro-inflammatory cytokine that induces endothelial cells and fibroblasts to secrete additional pro-inflammatory cytokines and chemokines (91, 97). These factors may enhance the recruitment of APC and alloreactive T cells into the graft

resulting in myocardial damage and extracellular matrix remodeling that favors fibrosis (321).

In summary, TGFβ is critical for the induction of fibrosis in this model of CR and in a number of fibrotic diseases, including diabetic nephropathy, rheumatoid arthritis, myocarditis, Crohn's disease and radiation-induced fibrosis (reviewed in (231)). As in most immune-mediated diseases, TGFβ can have both exacerbating and ameliorating actions making global inhibition of TGFβ unacceptable and local targeting of TGFβ or its downstream mediators an attractive therapy. This is evident in recipients transiently depleted of CD4+ cells in which T cell responsiveness to TGFβ is critical in maintaining alloreactive T cells in a hyporesponsive state. Our findings that TGFβ in CR grafts correlates with localized Th17 induction supports findings in other chronic inflammatory diseases (231) and provides a therapeutic target for preventing CR, while sparing the immunosuppressive activities of TGFβ.

Chapter 4: Transforming Growth Factor β Neutralization within Cardiac Allografts by Decorin Gene Transfer Attenuates Chronic Rejection

4.1 Abstract

CR is the leading cause of late graft failure following organ transplantation. CR is a progressive disease, characterized by deteriorating graft function, interstitial fibrosis, cardiac hypertrophy and occlusive neointima development. TGFβ, known for its immunosuppressive qualities, plays a beneficial role in the transplant setting by maintaining alloreactive T cells in a hyporesponsive state, but has also been implicated in promoting graft fibrosis and CR. In the mouse vascularized cardiac allograft model, transient depletion of CD4+ cells promotes graft survival but leads to CR, which is associated with intragraft TGFβ expression. Decorin, an extracellular matrix protein, inhibits both TGFβ bioactivity and gene expression. In this study, gene transfer of decorin into cardiac allografts was employed to assess the impact of intragraft TGFβ neutralization on CR, systemic donor-reactive T cell responses, and allograft acceptance. Decorin gene transfer and neutralization of TGFβ in cardiac allografts significantly attenuated interstitial fibrosis, cardiac hypertrophy and improved graft function, but did not result in systemic donor-reactive T cell responses. Thus, donor-reactive T and B cells remained in a hyporesponsive state. These findings indicate that neutralizing intragraft TGFβ inhibits the cytokine's fibrotic activities, but does not reverse its beneficial systemic immunosuppressive qualities.

Introduction

The accepted treatment for end-stage heart failure failure is transplantation (239). Immunosuppressive therapies diminish the incidence of graft loss due to acute graft rejection, leaving chronic rejection (CR) as the main impediment to long-term transplant survival (361). CR in cardiac allografts is characterized by interstitial fibrosis, vascular occlusion, cardiac hypertrophy and progressive dysfunction of the graft (224, 228, 321). The cellular mechanisms and cascade of events that lead to CR remains poorly defined and no effective therapies exist except retransplantation.

Transforming growth factor β (TGFβ) is a widely expressed cytokine that exerts pleiotropic effects on cell proliferation, migration, differentiation and survival (reviewed in (164)). TGFβ contributes to multiple biological processes, including tumorigenesis, development, wound healing, fibrosis and suppression of immune responses (164). The importance of TGFβ as an immune regulator was demonstrated in TGFβ deficient mice, which manifest a severe autoimmune phenotype that results in death at 3-4 weeks of age (169, 362). TGFβ controls T cell proliferation and survival and acts to inhibit Th1/Th2 differentiation and effector function (164, 178). B cells respond to TGFβ with decreased proliferation, survival and activation (363). TGFβ is also a critical cytokine in T regulatory cell (Treg) development and function (172, 173, 178). The Treg lineage-specific transcription factor, FoxP3, is induced by TGFβ, and results in the conversion of CD4+CD25- T cells into Treg (113). Furthermore, TGFβ signaling in Treg is essential for peripheral maintenance of this cell subset (164, 324).

TGFβ also contributes to wound healing and tissue repair (342). During normal wound healing, transient upregulation of TGFβ stimulates the production of factors that

act in concert to increase extra-cellular matrix (ECM) deposition, decrease matrix degradation, and restore normal tissue composition (357). While TGFβ is critical in wound healing and tissue repair, enhanced and prolonged TGFβ production is detrimental and observed in a number of fibrotic diseases, including pulmonary fibrosis (364), glomerulonephrtitis (365), scleroderma (366), and CR (232, 233).

Decorin, an ECM protein and member of the small leucine rich proteoglycan family, plays a role in TGFβ regulation (194). The core protein of decorin binds the active form of TGFβ, thereby inhibiting TGFβ's interaction with its receptor and sequestering the cytokine to the ECM (188, 193). In addition to inhibiting TGFβ's bioactivity, decorin negatively impacts TGFβ gene expression (195, 196). Decorin gene transfer ameliorated TGFβ-mediated fibrosis in a glomerulonephritis model (196) and in a pulmonary fibrosis model (367).

TGFβ mediates many beneficial anti-inflammatory effects in the transplant setting (299, 339) and we have previously reported an association with TGFβ and CR using the mouse vascularized cardiac allograft model (232). Intragraft TGFβ levels are readily detectable in the CR grafts from recipients transiently depleted of CD4+ T cells, but not in the grafts of anti-CD40L treated recipients, which remain free of CR. Adenoviral-mediated gene transfer of the active form of TGFβ into allografts induces fibrosis and results in CR in recipients treated with anti-CD40L that do not normally exhibit CR (232). This supports a critical role for TGFβ in the progression of CR.

In recipients transiently depleted of CD4+ T cells, CD4+ T cells begin to repopulate the periphery 3-4 weeks post-transplantation (285, 295, 296). Donor-reactive T cells in these animals are functionally distinct from naïve cells in that repopulating

CD4+ are hyporesponsive toward the graft but mount Th2 recall responses (297). The importance of TGFβ in allograft acceptance and suppression of graft-reactive T and B cells was revealed in transplant experiments employing mice with a dominant negative TGFβRII transgene (CD4-DNR) (Faust et al., submitted), which render the animals unresponsive to T cell TGFβ signaling (170). Transient CD4+ T cell depletion of CD4-DNR recipients resulted in both alloreactive cellular and humoral responses, which remained hyporesponsive in wild type (WT) recipients indicating that TGFβ is critical to suppression of T and B cell responses in this system. Graft rejection in these recipients correlated with CD4+ T cell repopulation of the periphery (Faust et al., submitted). These studies revealed that anti-CD4 mAb treatment is a TGFβ dependent model of allograft acceptance and that IL-17 is a critical element in TGFβ driven fibrosis (Faust et al., submitted).

In this study, we employed decorin gene transfer into cardiac allografts to assess the impact of intragraft TGFβ neutralization on CR, graft function, donor-reactive T and B cell responses, and allograft acceptance. We demonstrate that neutralizing intragraft TGFβ inhibits the cytokine's fibrotic activities, but does not reverse its beneficial systemic immunosuppressive qualities.

Results

Rationale

Prolonged allograft survival can be accomplished in the mouse cardiac allograft model by depleting CD4+ T cells transiently at the time of transplant. However, allografts in anti-CD4 mAb treated recipients develop interstitial fibrosis and CR, which

is associated with intragraft TGFβ expression (232). TGFβ is beneficial in the transplant setting, and has been associated with the progression of donor-reactive T and B cells to a hyporesponsive state in recipients treated with anti-CD4 mAb ((297), Faust et al., submitted). Conversely, T cell responsiveness to TGFβ can be deleterious for the graft by inducing fibrosis (Faust et al., submitted). Therefore, we explored the impact of local TGFβ neutralization employing decorin gene transfer into the allografts. We assessed the impact of intragraft TGFβ neutralization on allograft acceptance, graft function, T and B cell hyporesponsiveness and CR.

Decorin gene transfer into cardiac allografts

Prior studies utilizing adenoviral transduction of allografts revealed long-term transgene expression and negligible off-target tissue effects (315). To evaluate efficacy of decorin gene transfer, allografts were transduced with adenoviral vectors that encode rat decorin (AdDec) or βgal (Adβgal) and transplanted into recipients treated with inductive anti-CD4 mAb. Functioning allografts were harvested on day 7, day 14 and day 50 post-transplant (Figure 13). Employing rat decorin specific primers and quantitative RT-PCR, we verified that over-expression of rat decorin was detected predominantly within the cardiac allografts at day 7 and day 14 and not within non-target tissue, such as the spleen (Figure 13A). In addition, long-term rat decorin gene expression was detected in the AdDec infected allografts and not in the βgal controls at day 50 post-transplant (Figure 13B). These results demonstrate the efficacy and tissue localization of decorin gene transfer into allografts using adenoviral vectors, and the persistence of transgene expression.

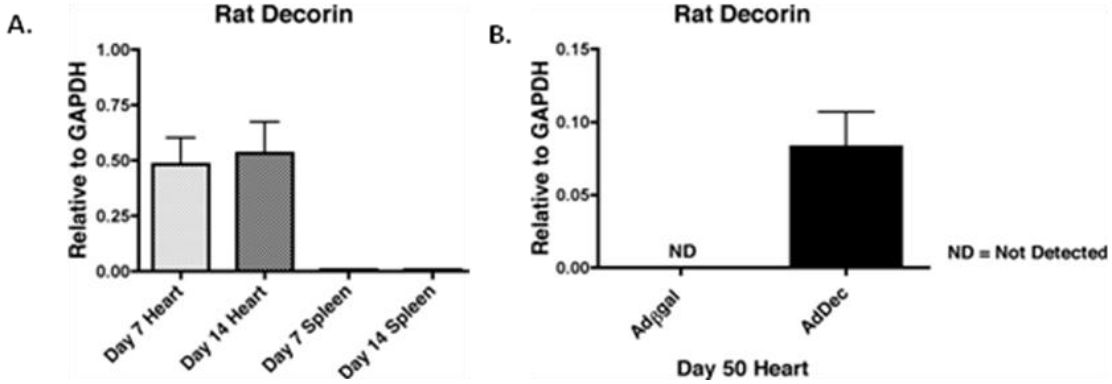


Figure 13: Decorin gene transfer and gene expression. BALB/c allografts were transduced with AdDec or Adβgal and transplanted into C57BL/6 mice that were given inductive anti-CD4 therapy on days -1, 0, and 7 relative to transplant. Spleens and allografts were recovered on day 7 (light shaded bars) and day 14 (dark shaded bars) for AdDec transduced allografts **(A)**. Functioning allografts were recovered on day 50 post-transplant for Adβgal (open bars) and AdDec (shaded bars) transduced allografts **(B).** Rat decorin expression relative to GAPDH was assessed by real-time RT-PCR from a minimum of 4 Adβgal (open bars) or 4 AdDec (shaded bars) transduced allografts (+/- S.E.M.).

Intragraft TGFβ neutralization by decorin gene transfer does not reverse T and B cell hyporesponsiveness in recipients treated with anti-CD4 mAb

While TGFβ is a known pro-fibrotic cytokine (368) it also has beneficial anti-inflammatory effects in the transplant setting and is frequently observed within accepted grafts (299, 337-339). Since TGFβ is critical in controlling donor-reactive responses following transient CD4+ T cell depletion (Faust et al., submitted), it was possible that intragraft neutralization of TGFβ might reverse immune hyporesponsiveness if decorin acted beyond the local confines of the allograft and inhibited TGFβ systemically (196). To determine if localized TGFβ neutralization within the allografts affected systemic donor-reactive immune responses, ELISPOT was employed to quantify the number of *in vivo* primed donor-reactive Th1 (IFNγ), Th2 (IL-4) and Th17 (IL-17) responses (Figure 14A). Gene transfer of βgal or decorin to allografts resulted in negligible T cell responses compared to untreated transplant recipients, indicating that TGFβ neutralization within

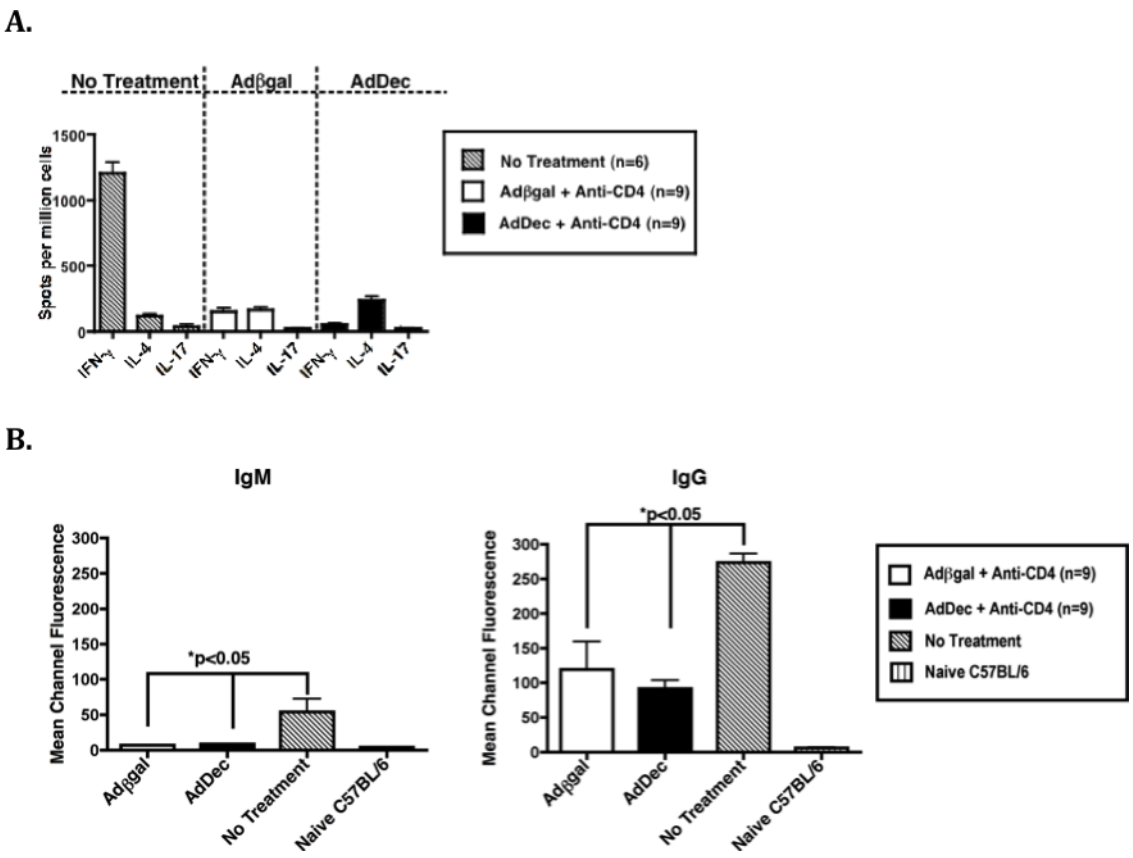


Figure 14: Intragraft TGFβ neutralization does not enhance donor-reactive T and B cell responses.

(A) On day 50 post-transplantation, splenocytes from Adβgal or AdDec transduced recipients were processed for ELISPOT assays to quantify primed, donor-reactive IFNγ, IL-4 or IL-17-producing cells. C57BL/6 recipients of BALB/c allografts that received no treatment (striped bars) acted as positive controls and splenocytes were harvested at the time of rejection. Bars represent the average number of spot forming cells (+/- S.E.M.). Numbers in parentheses represent the number of recipients in each group. **(B)** Fifty days post-transplant, sera were obtained from recipients transduced with Adβgal (open bars) or AdDec (shaded bars) and treated with inductive anti-CD4 mAb. P815 ($H\text{-}2^d$) cells were incubated with sera and bound donor-reactive Ab was detected by incubation with FITC-tagged anti-IgM or anti-IgG Abs. The mean channel fluorescence is indicative of the relative amount of donor-reactive Abs. Bars represent the average mean channel fluorescence of 9 Adβgal or 9 AdDec transduced recipient samples (+/- S.E.M.).

cardiac allografts did not reverse graft-reactive T cell hyporesponsiveness in recipients depleted of CD4+ T cells (Figure 14A). In addition, cardiac allograft contractions were noticeably stronger in AdDec transduced allografts when compared to βgal controls. These data demonstrate that intragraft inhibition of TGFβ had a beneficial effect on graft

function and did not reverse systemic donor-reactive T cell hyporesponsiveness normally observed in anti-CD4 mAb treated recipients

TGFβ also inhibits B cell responses by affecting B cell proliferation, survival signals, activation, and IgG class switching (164). In CD4-DNR recipients treated with anti-CD4 mAb, T cells differentiate into effector cells and provide help to B cells, which produce donor-reactive IgG (Faust et al., submitted). To examine the effect of intragraft decorin over-expression on donor-reactive antibody, we quantified donor-reactive IgM and IgG production by flow cytometry (Figure 14B). No difference in alloantibody production was observed in recipients whose allografts express decorin or the βgal. This demonstrates that B cell hyporesponsiveness is also not reversed in recipients that overexpress decorin within their grafts. This further indicates that there are no systemic effects on the alloreactive immune responses by local TGFβ neutralization.

Effect of intragraft decorin gene transfer on TGFβ-induced gene expression

TGFβ is a pleiotropic cytokine that exerts a variety of effects on many different cell types. A reciprocal developmental pathway exists for the generation of pathogenic effector Th17 cells and Treg in response to TGFβ, with IL-6 being the co-factor required for Th17 induction (93, 94, 327). In addition, TGFβ induces cardiac fibroblasts to differentiate into myofibroblasts, which produce significant amounts of collagen and contribute to fibrosis (368). To assay for the effect of decorin on TGFβ-induced genes such as TGFβ, collagen A1, FoxP3 and IL-17, RNA was isolated from the allografts and quantitative RT-PCR was performed (Figure 15).

In recipients transduced with AdDec, intragraft TGFβ ($p<0.05$), collagen A1 ($p<0.05$) and IL-17 ($p<0.05$) transcript levels were significantly reduced compared to

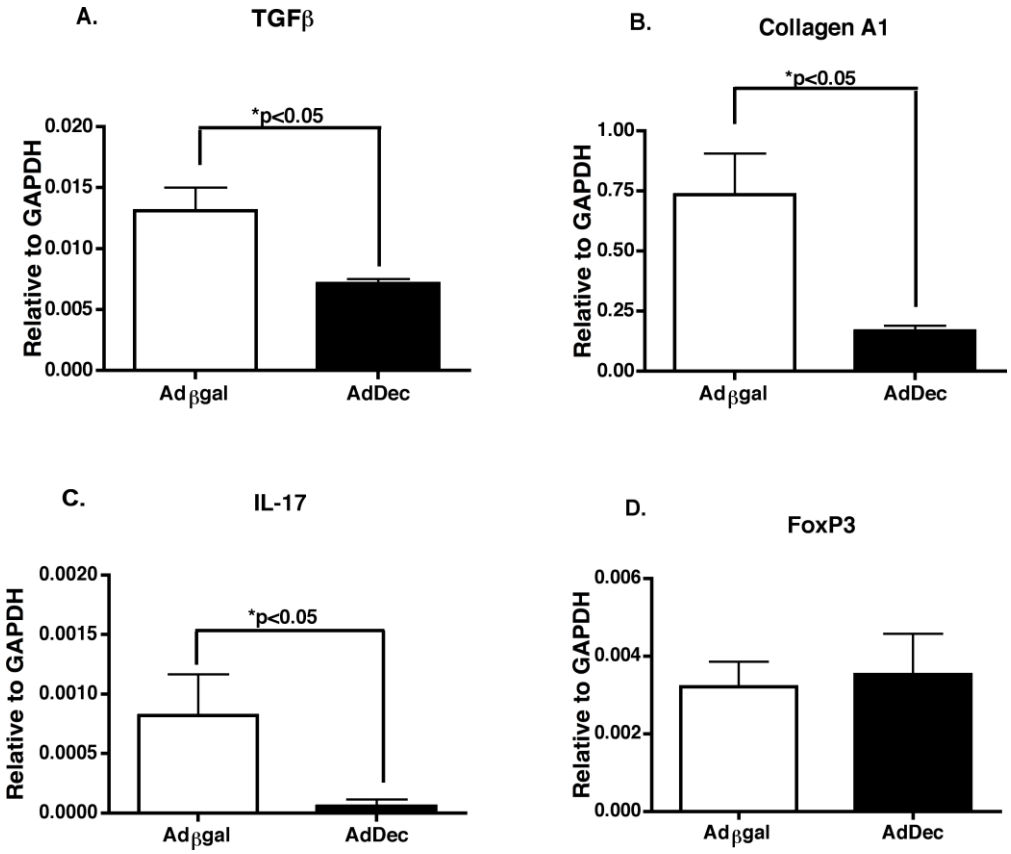


Figure 15: Effect of TGFβ neutralization on intragraft gene expression. RNA samples from grafts of recipients transiently depleted of CD4+ T cells and transduced with Adβgal or AdDec were recovered 50 days post-transplant. Intragraft expression of **(A)** TGFβ, **(B)** collagen A1, **(C)** IL-17 and **(D)** FoxP3 relative to GAPDH was assessed by real-time RT-PCR. Bars depict the means of RNA expression from 9 Adβgal or 9 AdDec transduced allografts.

control allografts (Figure 15A-D). In contrast, intragraft FoxP3 expression was comparable between AdDec and Adβgal transduced allografts. These observations indicate that localized TGFβ neutralization by decorin can significantly decrease gene expression associated with fibrosis, but does not affect FoxP3 expression. This data suggest Th17 polarization occurs within the site of inflammation--the graft--while Treg induction and maintenance occurs systemically in the secondary lymphoid tissues (147).

Intragraft TGFβ neutralization significantly attenuates graft fibrosis and hypertrophy

To investigate the effect of localized TGFβ neutralization on graft fibrosis,

quantitative morphometric trichrome analysis was performed (Figure 16). Assessment of allograft fibrosis revealed that intragraft decorin expression resulted in a significant

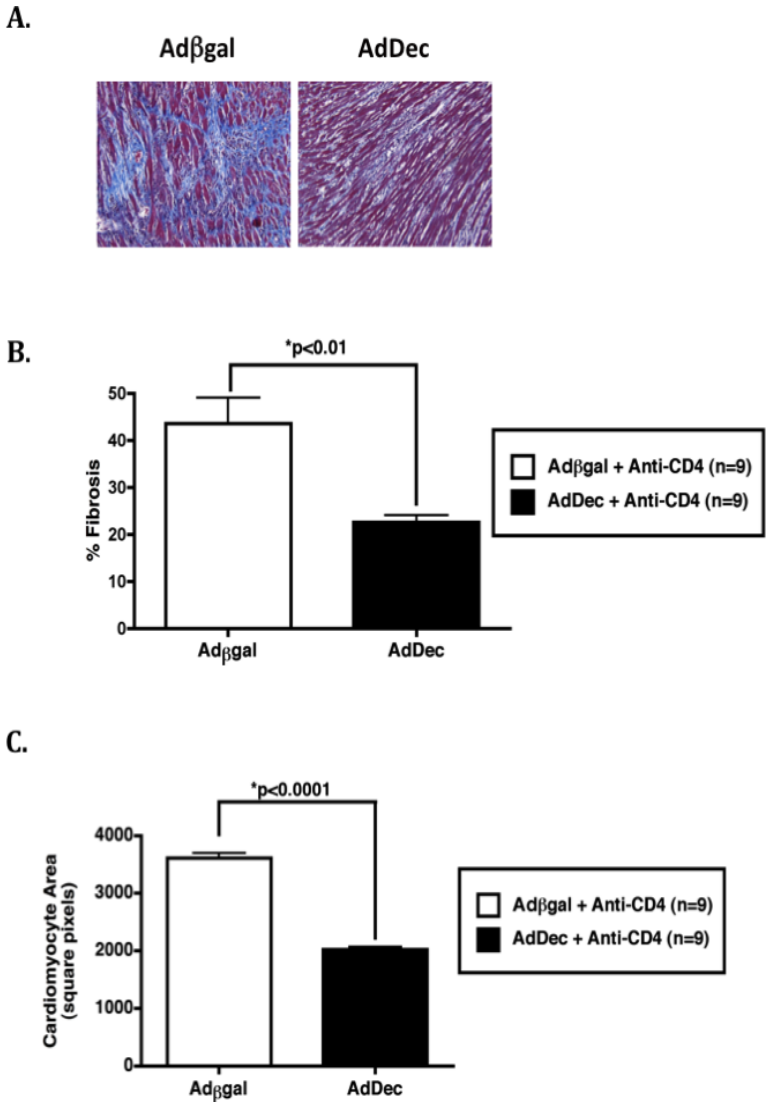


Figure 16: Decorin gene transfer and intragraft TGFβ neutralization attenuates fibrosis and hypertrophy.
(A) Sections of grafts from recipients transiently depleted of CD4+ T cells and transduced with Adβgal or AdDec were stained with Masson's trichrome stain on day 50 post-transplant. Fibrotic tissue appears blue. Magnification, 200X. **(B)** Morphometric analysis of trichrome staining. Bars represent the average percentage of (+/- S.E.M.) of graft area positive for fibrotic tissue in 9 Adβgal (open bars) or 9 AdDec (shaded bars) transduced recipients. **(C)** Cardiomyocyte area quantification of groups described in **(A)**. Bars represent mean (+/- S.E.M.) of area measurements from 100 cardiomyocytes per allograft at 200X magnification. Five individual hearts were analyzed per group.

reduction of fibrosis compared to control grafts ($p<0.01$) (Figure 16A and 16B). These data indicate that TGFβ promotes cardiac fibrosis and that localized neutralization of TGFβ can significantly attenuate CR.

Cardiac hypertrophy is defined as an increase in the heart mass (369). An increase in the size of the cardiac myocytes, as opposed to the number, is the primary basis of cardiac hypertrophy (369). TGFβ is critical in driving this process (reviewed in (370)). An upregulation of TGFβ in cardiac tissue increases cardiomyocyte size and leads to cardiac dysfunction (370-372). To evaluate the effect of intragraft TGFβ neutralization on cardiac hypertrophy, cardiomyocyte cell size was measured employing histologic analysis (294). Reduced cardiac hypertrophy was observed in AdDec transduced grafts (Figure 16C). These findings indicate that intragraft TGFβ correlates with both fibrosis and hypertrophy in CR allografts and that decorin gene transfer can attenuate both pathologies.

Discussion

CR is an intractable disease characterized by interstitial fibrosis, occlusive neointima development, and graft dysfunction (224, 228, 321). The etiology of CR is poorly understood and no therapies exist to block its progression. TGFβplays a beneficial role in the transplant setting because of its immunsuppressive qualities (299, 339), but has also been implicated in promoting graft fibrosis and CR (320, 343). In the mouse vascularized cardiac model, we have previously reported an association between TGFβ and CR (232). Intragraft TGFβ transcript levels were readily detected in the CR grafts from recipients transiently depleted of CD4+ T cells, but not in the grafts of anti-CD40L

treated recipients, which remain free of CR (232), suggesting the importance of TGFβ in this pathology. In this study, we employed decorin gene transfer and local neutralization of TGFβ in cardiac allografts to assess the impact of intragraft TGFβ neutralization on allograft acceptance, T and B cell hyporesponsiveness and CR. We demonstrate that local neutralization of TGFβ in cardiac allografts significantly attenuated interstitial fibrosis and improved graft function, but did not reverse the hyporeactive state of donor-reactive T or B cells.

Intragraft transcript levels of TGFβ are frequently detected in accepted grafts, including the cardiac allografts from inductive CD4+ T cell depleted recipients. TGFβ expression is believed to promote graft survival through the induction of Treg, which control graft-reactive Th1 and Th2 responses (299, 337, 339). Previous studies in recipients treated with inductive anti-CD4 mAb have revealed that repopulating CD4+ T cells are hyporesponsive toward donor antigen and mount Th2 responses upon re-challenge, while naïve T cells mount a dominant Th1 response (297). In this CR model, alloreactive T cells only progress to a hyporesponsive state in response to TGFβ (Faust et al., submitted). The critical role for TGFβ in this CR model was revealed when CD4-DNR mice were used as recipients and transiently depleted of CD4+ T cells. T cell TGFβ signaling was requisite for both long-term graft acceptance and suppression of graft-reactive T and B cell responses as well as graft fibrosis (Faust et al., submitted). Therefore, systemic strategies targeting TGFβ are not feasible since this could alter the hyporesponsiveness of graft-reactive T and B cells. In contrast, intragraft TGFβ inhibition would be beneficial in attenuating fibrosis. TGFβ neutralization within allografts, however, did not result in a reversal of T or B cell hyporesponsiveness (Figure

14). These findings demonstrate that localized TGFβ inhibition does not alter the systemic regulation of graft-reactive cells or lead to graft loss but was effective at reducing fibrosis associated with CR.

In transplantation, FoxP3+ Treg have been shown to play a central role in suppression of alloreactive T cells and in long-term allograft acceptance (reviewed in (270)). In both human and animal transplant, allograft acceptance strongly correlates with Treg infiltration into the graft as detected by enhanced intragraft FoxP3 transcript levels (138, 154, 354). Treg can be divided into two populations: natural Treg (nTreg), which arise in the thymus and do not require TGFβ to develop, and induced peripheral Treg (iTreg), which do require TGFβ to differentiate from naïve CD4+FoxP3- T cells into Treg (reviewed in (107)). In the current study, local neutralization of TGFβ did not affect intragraft FoxP3 expression, indicating that Treg migration and/or generation within the graft was unchanged between decorin and βgal transduced recipients (Figure 15D). However, these data argue against Treg induction within the grafts since intragraft TGFβ was neutralized by decorin gene transfer. Indeed, Treg have been reported to be generated in lymph nodes and subsequently migrate to the graft (147). While thymic-derived Treg do not require TGFβ for their generation, they do depend on TGFβ for their persistence in the periphery (133). Given that decorin gene transfer spares the systemic effects of TGFβ, it is also possible that nTreg may contribute in regulating alloreactive responses within the graft (373).

Interstitial fibrosis represents a hallmark of CR and results in pathogenic cardiac remodeling and graft dysfunction (233). The effector cells in this process are cardiac fibroblasts, which respond to TGFβ by inducing the expression of pro-fibrotic mediators

that upregulate extracellular matrix synthesis and down-regulate matrix degradation (233). During remodeling, cardiac fibroblasts located within the interstitium proliferate and produce proteins such as collagen (234), resulting in a significant increase in interstitial fibrosis (374). Cardiac fibrosis impairs contractility and reduces cardiac function.

Previous studies demonstrate that decorin gene transfer ameliorates TGFβ-induced fibrosis of multiple organs (196, 367). Decorin inhibits TGFβ bioactivity by sequestering TGFβ to the ECM (188, 193). In addition, decorin negatively impacts TGFβ gene expression by interrupting TGFβ/Smad-dependent transcriptional events (188, 193, 195). One mechanism in which decorin inhibits fibrosis is by reducing TGFβ-induced collagen transcript levels in cultured human cardiac fibroblasts (197). Gene transfer of decorin into allografts transplanted into recipients transiently depleted of CD4+ T cells significantly attenuated collagen deposition and fibrosis compared to control allografts (Figure 16A and 16B). Decorin over-expression also inhibited cardiac hypertrophy, demonstrating amelioration of an additional TGFβ-induced parameter correlated with CR (Figure 16C).

In addition to reduced TGFβ gene expression, decorin reduced intragraft transcript levels IL-17 (Figure 15C). IL-17 amplifies inflammatory responses (reviewed in (91, 93)) and has recently been identified as a cytokine with pro-fibrotic activities (92, 331-334, 336). Prior studies in IL-17-/- recipients treated with anti-CD4 mAb revealed that allografts from the deficient mice exhibited a significant reduction in fibrosis compared to WT (Faust et al., submitted). IL-17 may induce fibrosis though multiple mechanisms. Upregulation of collagen gene expression in direct response to IL-17 has

been observed in mouse cardiac fibroblasts (92). IL-17 may also induce endothelial cells and fibroblasts to secrete pro-inflammatory cytokines and chemokines (91, 97) that result in the recruitment of APC and alloreactive T cells into allograft. These inflammatory cells may secrete factors that lead to myocardial damage and tissue remodeling that favors fibrosis. The reduction of intragraft IL-17 expression in AdDec transduced grafts compared to WT counterparts further implicates this pro-inflammatory cytokine in CR.

Decorin has multiple molecular targets in cell growth in addition to its interaction with TGFβ (184). Decorin negatively impacts cell proliferation, an effect mediated through the induction of p21 (184). Decorin-induced cell cycle arrest might reduce fibrosis by suppressing cardiac fibroblasts from proliferating and differentiating into myofibroblasts. Decorin also interacts with complement C1q, inhibiting activation of the classical complement pathway (186). Hence, under inflammatory tissue damage and ECM remodeling, decorin may suppress complement activation and prevent further cardiomyocyte injury. Decorin may further inhibit the production of inflammatory chemokines and cytokines, including MCP-1 and IL-8 by preventing C1q from binding graft endothelial cells (186, 226). Therefore, in addition to decorin's inhibitory effects on TGFβ, suppression of complement activation may help to reduce fibrosis by limiting the damage inflicted on the allograft.

In summary, TGFβ is a critical cytokine in fibroproliferative disorders following inflammatory responses (reviewed in (342)). TGFβ can have both exacerbating and ameliorating effects in immune-mediated fibrotic diseases, making global inhibition undesirable and local neutralization of TGFβ an attractive therapy. As evidenced in this model of CR, systemic TGFβ production is requisite for T cell hyporesponsiveness (Faust

et. al., submitted), while local TGFβ production at the site of inflammation induces graft fibrosis. We demonstrate that neutralizing intragraft TGFβ inhibits the cytokine's fibrotic activities, but does not reverse its beneficial immunosuppressive qualities. These data provide insight into the underlying causes of CR, and identify intragraft TGFβ as a therapeutic target for treatment of this disease.

Chapter 5: Conclusion

The Holy Grail of transplant tolerance, defined as a brief course of immunosuppression (induction), followed by indefinite acceptance of an allograft (maintenance), has yet to be achieved (375). Currently, the therapies used in the clinic to prolong graft survival globally suppress the immune response, require life long compliance, and are associated with a multitude of side effects, including increased risk of malignancies and infections (311). These therapies do not specifically target donor-reactive cells and very often inhibit the induction of regulation, circumventing any development of tolerance induction (376). The failure to maintain tolerance leads to smoldering chronic inflammatory responses and may lead to CR (321). Therefore, while graft loss resulting from acute rejection is relatively rare due to immunosuppressive therapies, CR continues to challenge long-term graft survival and remains the primary cause of late graft failure.

TGFβ has been strongly implicated as a mediator of both prolonged allograft acceptance, due to its anti-inflammatory effects, and the causative agent of fibroproliferative changes within the allograft associated with CR (232, 233, 342). We have previously reported an association with TGFβ and CR using the mouse vascularized cardiac allograft model (232). Transient depletion of recipient CD4+ T cells results in prolonged graft survival, intragraft expression of TGFβ, and the development of CR. In contrast, when recipients are treated with inductive anti-CD40L mAb, allografts remain

free of CR and fail to express intragraft TGFβ. Further, adenoviral-mediated gene transfer of the active form of TGFβ into allografts, but not syngeneic grafts, induced fibrosis and results in CR in recipients treated with anti-CD40L indicating that alloantigen is necessary for the progression of CR (232). Studies in this thesis focused on the role of T cell specific TGFβ signaling in CR, the contribution of IL-17 to graft fibrosis, and the effect of intragraft neutralization of TGFβ on systemic alloreactive responses and CR.

5.1 Dissertation summary

Experiments performed in Chapter 3 investigated the role of TGFβ signaling on alloreactive effector cells, Treg and IL-17 induction. We compared responses from WT and CD4-DNR recipients that were depleted of CD4+ T cells to responses from recipients that received anti-CD40L mAb therapy. In recipients treated with anti-CD40L mAb, T cell TGFβ signaling is not required for long term allograft acceptance. In contrast, prolonged allograft acceptance and suppression of alloreactive T and B cells responses require T cell TGFβ signaling in mice transiently depleted of CD4+ T cells. Further, IL-17 is involved in the development of graft fibrosis and CR.

Chapter 4 addressed the impact of local TGFβ neutralization by intragraft decorin gene transfer on donor-reactive T and B cell responses, cardiac hypertrophy and fibrosis. Intragraft neutralization of TGFβ significantly attenuated graft fibrosis and hypertrophy, improved graft function and failed to induce systemic donor-reactive T cell responses in recipients transiently depleted of CD4+ T cells. Thus, neutralizing TGFβ within the allograft inhibits the cytokine's pro-fibrotic effects, but does not reverse its anti-inflammatory activities.

Our finding that TGFβ in CR grafts correlates with compartmentalized IL-17 production and participates in fibrosis is reminiscent of scleroderma, rheumatoid arthritis, allergic lung disease and hypersensitivity pneumonitis and lung fibrosis (231, 336, 351, 377). In CR, as in most chronic inflammatory diseases, TGFβ can have both exacerbating and ameliorating actions. Hence, data reported here indicate the effectiveness of locally neutralizing TGFβ, or selectively targeting a downstream mediator, such as IL-17, as providing therapeutic targets for the prevention of fibrosis.

5.2 Future directions

The data presented clearly associate TGFβ production with both allograft acceptance and CR in recipients transiently depleted of CD4+ T cells. Future directions in the CR model will include 1) identifying the cellular source of TGFβ; 2) elucidating the role of natural and induced Treg in controlling alloreactivity; 3) investigating the requirement for T cell-derived TGFβ in controlling donor-reactive cells and Treg development; and 4) determining if TGFβ-induced IL-17 is necessary and sufficient to induce fibrosis.

TGFβ and the graft

TGFβ is required for controlling alloreactivity and for inducing graft fibrosis in recipients transiently depleted of CD4+ T cells. The obvious question that remains is what cell type is generating this cytokine? A variety of experimental organ transplant models indicate that the graft, rather than passively provoking an immunological rejection response has the ability to modulate its own survival through cytokine production (reviewed in (378)). A cardiac graft in a recipient transiently depleted of CD4+ T cells may modulate its own survival and alter alloreactivity through TGFβ

production. Indeed, both cardiomycoytes (379) and cardiac endothelial cells (380) are capable of generating TGFβ. Graft-derived TGFβ production may generate survival signals and/or modulate alloreactivity by playing a role in initiating and maintaining tolerance in the allogeneic transplant setting. To assess the graft's role in TGFβ production, mice that are both immunodeficient and TGFβ1 deficient will be employed as cardiac donor animals in our transplant model (381, 382). As mentioned previously, TGFβ1 null mice exhibit multifocal inflammation and autoimmune-mediated lethality by 3 weeks of age (169). However, these animals can be rescued from the autoimmunity phenotype when crossed to SCID or RAG deficient mice (381, 383). Hence, these mice will provide a model to investigate the contribution of graft-derived TGFβ in CR.

WT C57BL/6 mice (H-2^b) will be transplanted with TGFβ1-/-SCID (H-2^d) cardiac allografts and transiently depleted of CD4+ T cells (Figure 17). Graft function will be monitored and functioning allografts will be evaluated for signs of CR fifty days post-transplant. Prolonged allograft acceptance and evidence of CR would indicate that infiltrating immune cells generate TGFβ, and that the graft responds to this cytokine with fibroproliferative changes. In a rodent model of CR, TGFβ staining is detected on some infiltrating cells (primarily macrophages and lymphocytes) (384). Therefore, the enhanced expression of TGFβ within the allograft may represent an immunosuppressive mechanism mediated by immune cells to self-limit destruction either directly, by suppressing alloreactivity or, indirectly, by inducing immunosuppressive Treg within the graft (164). If prolonged allograft acceptance and no evidence of CR are observed, this would suggest that the graft generates TGFβ1 in response to inflammation, but that graft-derived TGFβ is not necessary for allograft acceptance. Alternatively, prolonged allograft

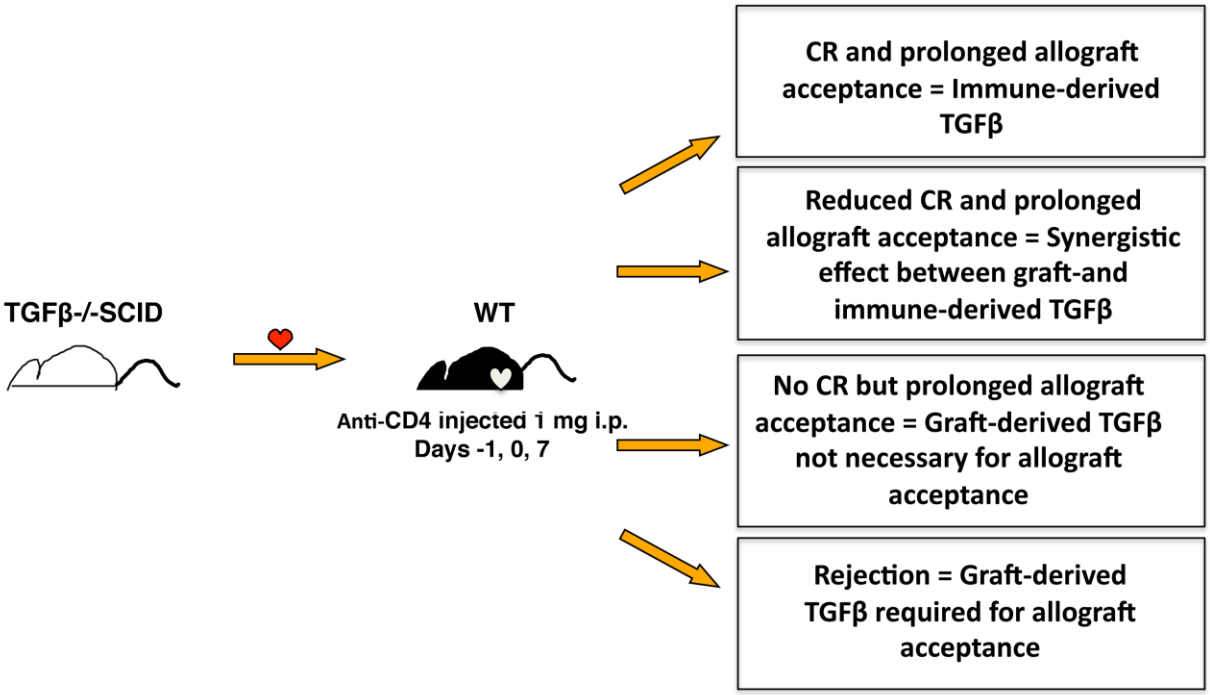


Figure 17: Identifying the source of TGFβ. WT C57BL/6 mice (H-2^b) will be transplanted with TGFβ1-/-SCID (H-2^d) cardiac allografts and transiently depleted of CD4+ T cells. Graft function will be monitored and functioning allografts will be evaluated for evidence of CR fifty days post-transplants

acceptance and a marked reduction in graft fibrosis would indicate a synergistic effect between graft-derived TGFβ and immune-mediated TGFβ in inducing fibrosis within the graft. Graft rejection would demonstrate that the graft plays an important role in early graft survival and tolerance induction by generating TGFβ. The graft may modulate the immune system through TGFβ1 production by inhibiting immune destruction and/or inducing the generation of Treg. A positive feedback loop may be established that involves the initial induction of Treg by graft-derived TGFβ, which is later augmented by TGFβ production by the Treg, themselves (143). These experiments will provide insight into the origin of TGFβ production and the effect of graft-derived TGFβ on prolonged allograft acceptance (143).

Role of nTreg and iTreg in controlling alloreactivity

In our model of CR, CD4+ Treg are postulated to be critical for the establishment of tolerance (297). Following transient depletion of CD4+ T cells, it is possible that nTreg are less susceptible to apoptosis or inactivation by anti-CD4 mAb. These regulatory cells may proliferate and accumulate within the graft and lymphoid tissue to maintain tolerance through TGFβ production (298). Indeed, Bromberg and colleagues recently reported that nTreg sequentially migrate into the allografts where these cells become activated and proliferate and subsequently proceed to the draining lymph nodes, where they suppress effector T cell proliferation and migration into the graft (373). In our mouse vascularized allograft study, as CD4+ T cells begin to repopulate the periphery and migrate into the graft or lymphoid tissues, these expanded nTreg may similarly suppress alloreactivity through TGFβ production, and in the absence of TGFβ signaling in T cells, allografts are rejected (Figure 18A).

There may also be a role for iTreg in prolonged cardiac allograft acceptance following transient depletion of CD4+ T cells. As the CD4+ T cells begin to repopulate the periphery, the healed-in allograft will continue to release antigens that are processed by the host APCs in a non-inflammatory environment (305). In the absence of inflammatory danger signals, quiescent APC may present alloantigen and generate TGFβ, resulting in the expansion of iTreg and suppression of alloreactive cells (Figure 18B) (262). Indeed, persistent incomplete signaling of T cells by alloantigen and TGFβ production can drive CD4+ T cells to become both anergic and regulatory (138, 262, 298). The iTreg may sustain and recruit additional iTreg through TGFβ production and establish and maintain tolerance (Figure 18C) (143, 262). The fact that TGFβ assists the

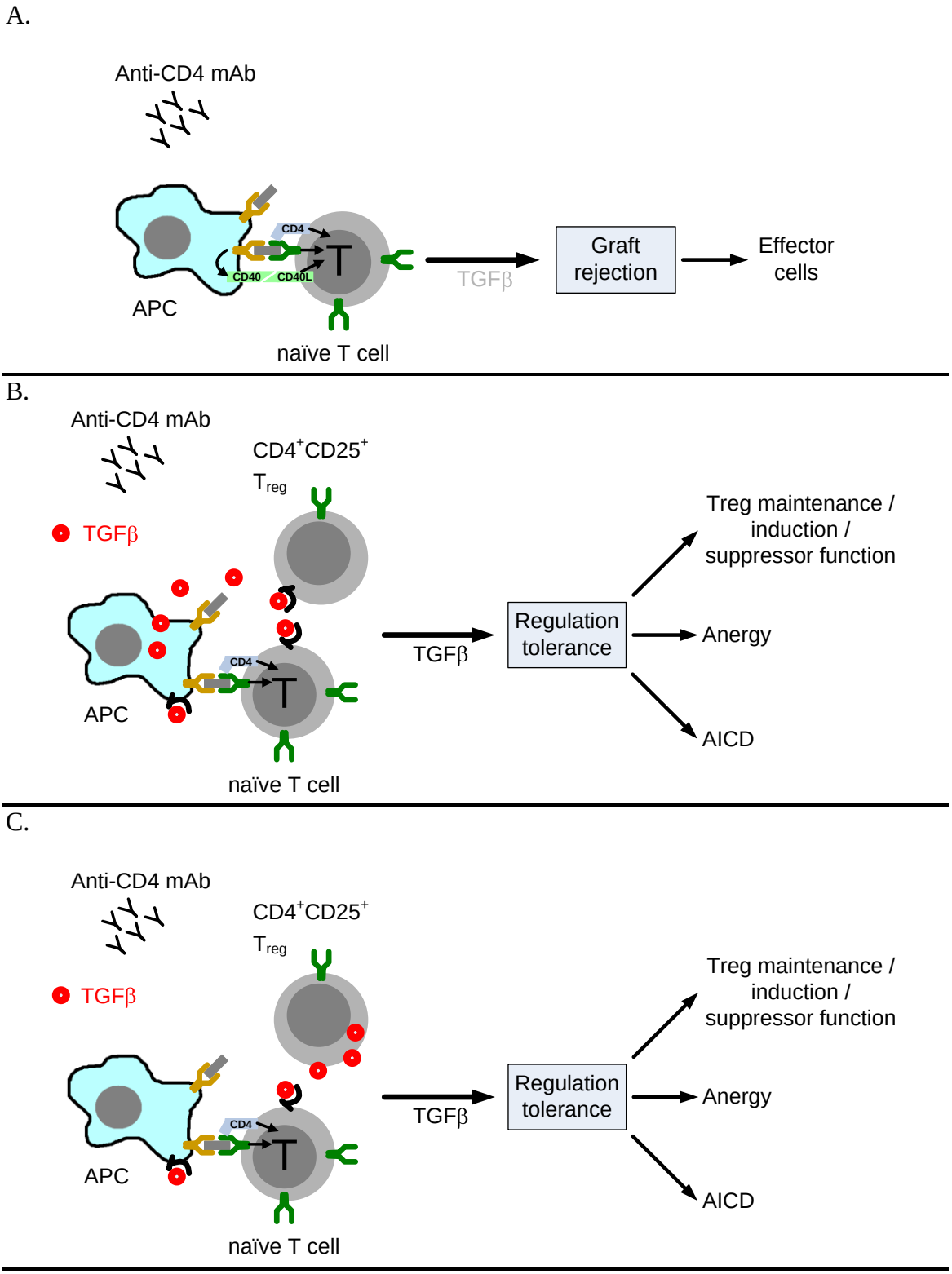


Figure 18: Mechanisms of tolerance induction following transient CD4+ T cell depletion. **(A)** Allograft acceptance in recipients transiently depleted of CD4+ T cells requires T cell responsiveness to TGFβ. In the absence of T cell TGFβ signaling, graft-reactive effector cells induce allograft rejection. **(B)** As the CD4+ T cells begin repopulating the periphery, the healed-

in graft will continue to release antigens that are processed by the host APCs in a non-inflammatory environment. In the absence of inflammatory danger signals, quiescent APC may present alloantigen and generate TGFβ, resulting in the expansion of iTreg and suppression of alloreactive cells. **(C)** As repopulating CD4+ T cells migrate into the graft or lymphoid tissues, Treg may suppress alloreactive responses through TGFβ production.

induction of naïve CD4+CD25- T cells into Tregs both *in vivo* and *in vitro* argues for the importance of this cytokine in the generation and maintenance of the peripheral Treg pool (138).

To investigate the contribution of natural and induced Treg in allograft acceptance in recipients transiently depleted of T cells *in vivo*, cardiac transplants will be performed in monospecific TCR-transgenic mice to determine the ability of inductive anti-CD4 mAb to induce *de novo* Treg. Female transgenic mice will be employed which generate CD4+ T cells that are monospecific for the male transplantation antigen *Dby (*presented by H-2E^{k}) on a RAG-/- background (A1(M).RAG-/-) (340). *Dby* encodes a dead box RNA helicase Y protein involved in diverse cellular functions, including RNA splicing, ribosomal assembly and protein translation (385). The *Dby* gene generates two transcripts: a long transcript that is ubiquitously expressed and a short transcript that is testes specific. The protein derived from the long *Dby* transcript is a class II restricted antigen and is highly expressed in cardiac tissue (340, 385). These mice have no prexisiting FoxP3+ Treg in the thymus or periphery (129, 340, 386) and are capable of rejecting a male skin graft acutely, within 15-20 days post-transplant (138).

Female A1(M).RAG-/- transgenic mice will be transplanted with male CBA.RAG-/- or female CBA.RAG-/- cardiac allografts and transiently depleted of CD4+ T cells (Figure 19). Graft function will be monitored and recipients with functioning grafts at day 50 post-transplant will be evaluated for peripheral Treg induction. iTreg

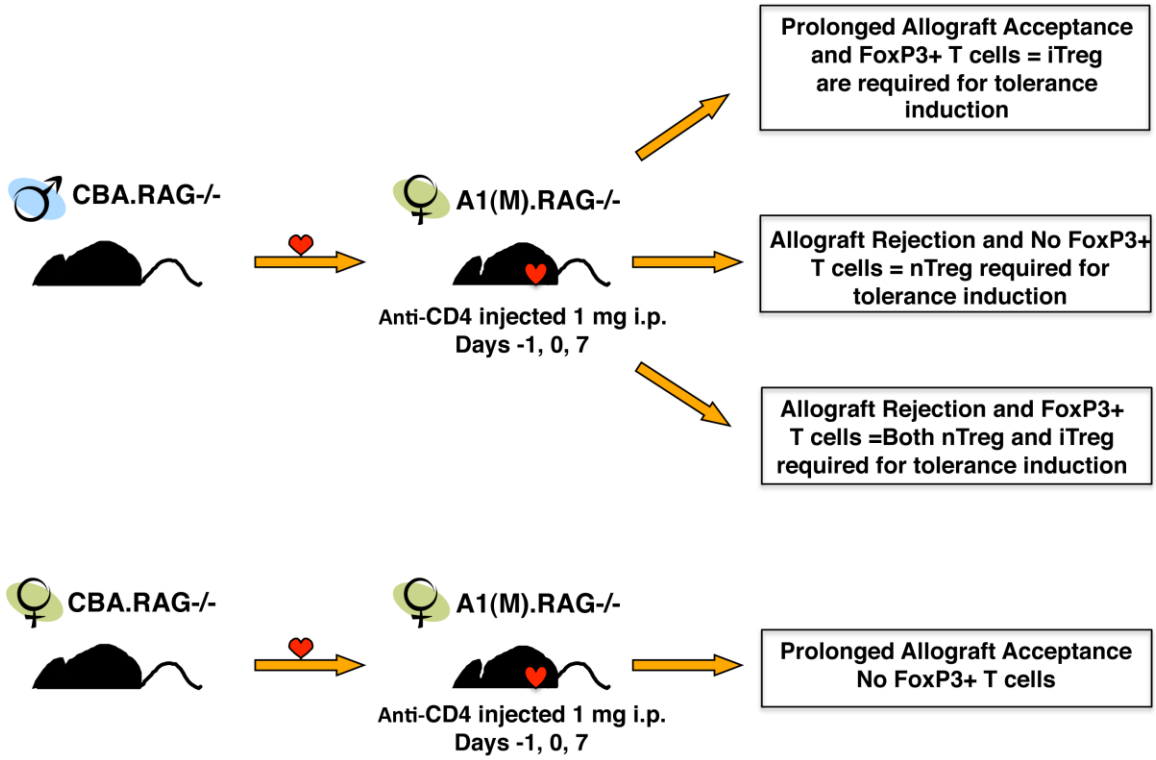


Figure 19: Assessing the contribution of natural and induced Treg in allograft acceptance. Female A1(M).RAG-/- transgenic mice that are monospecific for the male transplantation antigen *Dby* presented by H-2E^{k} on a RAG-/- background will be transplanted with a male or female allograft from CBA.RAG-/- donors and transiently depleted of CD4+ T cells. The Dby protein is a class II restricted antigen and is highly expressed in cardiac tissue. iTreg generation will be assessed by quantitative RT-PCR to assay for intragraft FoxP3 expression and by flow cytometry to quantify the percent of FoxP3+CD25+CD4+ GICs and splenocytes.

generation will be assessed by quantitative RT-PCR to assay for intragraft FoxP3 expression and by flow cytometry to quantify the percentage of FoxP3+CD25+CD4+ GICs and splenocytes. It is predicted that prolonged allograft acceptance will be observed in recipients of both male and female cardiac grafts. However, Treg will only be detected in recipients of male CBA.RAG-/- cardiac allografts. This will strongly support the hypothesis that FoxP3 expressing Treg can be induced in the periphery from naïve CD4+FoxP3- T cells following anti-CD4 mAb treatment. Alternatively, rejection of allografts will indicate that nTreg, or a synergistic relationship between nTreg and iTreg, are critical for prolonged allograft acceptance in anti-CD4 mAb treated recipients.

iTreg and infectious tolerance

Tolerant animals can maintain their grafts following adoptive transfer of naïve cells, and this is referred to as the acquisition of resistance, or infectious tolerance (238). Previous studies in our lab have investigated the ability of WT recipients treated with anti-CD4 mAb to maintain prolonged allograft acceptance following adoptive transfer of naïve splenocytes or splenocytes isolated from day 7 unmodified WT recipients, which will be referred to as effector T cells. Under both experimental conditions, prolonged allograft acceptance is observed, when cells are transferred to primary graft recipients on day 30 post-transplant. These data indicate an antigen specific regulation and maintenance of peripheral tolerance in the transplant setting. It is unknown, however, whether the adoptively transferred splenocytes undergo apoptosis or anergy, and/or whether a subset is induced in the periphery to become Treg, themselves.

To assay for iTreg induction of T cells following adoptive transfer in a fully MHC mismatched setting, WT C57BL/6 mice will be transplanted with BALB/c allografts and transiently depleted of CD4+ T cells. Allograft recipients will be infused with CD4+CD25-FoxP3- naïve T cells or CD4+CD25-FoxP3- effector T cells from FoxP3GFP transgenic mice (Figure 20). FoxP3GFP transgenic mice express an enhanced green fluorescent protein under the control of the mouse Foxp3 promoter (387). In order to track the transferred cells and verify that the cells survive, the cells will be labeled with a 675 nm-emitting proliferation dye (CellVue Claret). Graft function will be monitored and recipients with functioning grafts at day 50 post-transplant will be evaluated for peripheral Treg induction by quantitative RT-PCR to assay for intragraft GFP expression and by flow cytometry to quantify the percentage of GFP+CD4+CD25+ GICs and

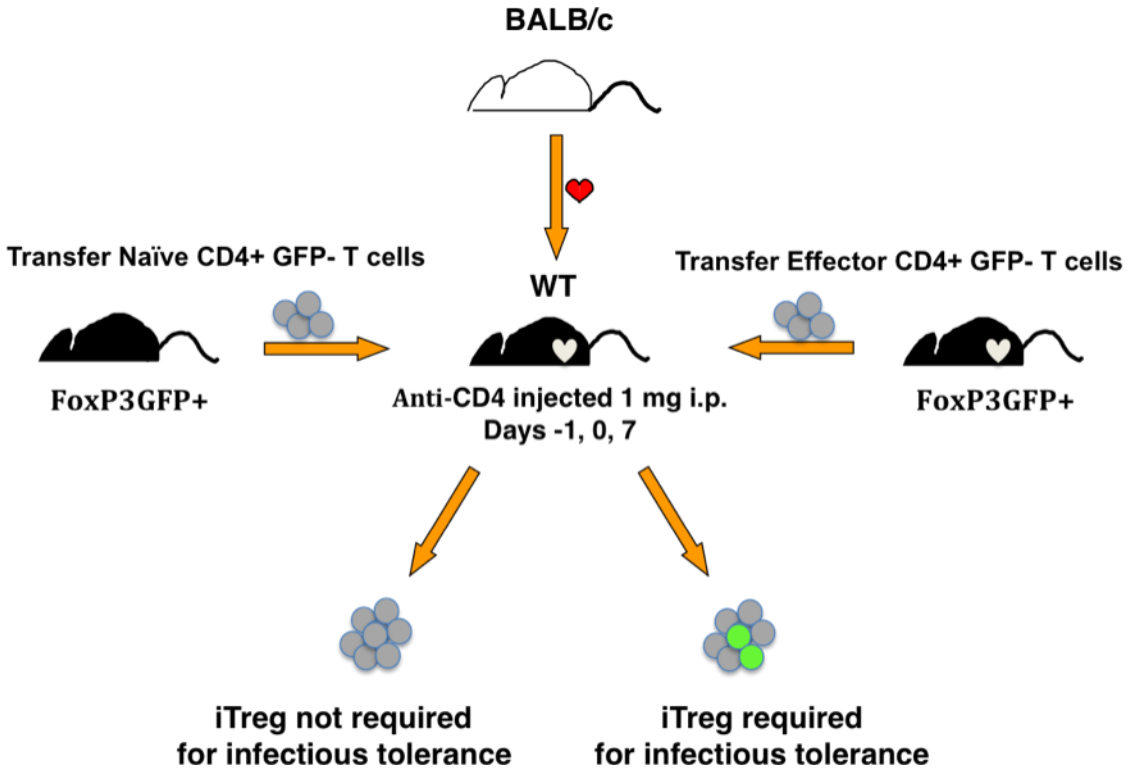


Figure 20: iTreg and infectious tolerance. WT C57BL/6 mice will be transplanted with BALB/c allografts and transiently depleted of CD4+ T cells. On day 30 post-transplant, recipients will receive CD4+CD25-FoxP3- naïve T cells or CD4+CD25-FoxP3- effector cells from FoxP3GFP transgenic mice. Graft function will be monitored and recipients with functioning allografts at day 50 will be evaluated for peripheral Treg induction by quantitative RT-PCR to assay for intragraft GFP expression and by flow cytometry to quantify the percentage of GFP+CD4+CD25+ GICs and splenocytes.

splenocytes. It is predicted that adoptive transfer of WT recipients with either naïve or effector CD4+ T cells from FoxP3GFP transgenic mice will result in prolonged allograft acceptance as observed in WT recipients. The detection of GFP+CD4+ T cells within the graft or spleen of primary graft recipients will indicate the *de novo* generation of potentially immunoregulatory T cells *in vivo*. These experiments will provide insight into the role of Treg in the maintenance of transplant tolerance in recipients transiently depleted of CD4+ T cells.

T cell-derived TGFβ and allograft acceptance

The requirement for T cell TGFβ signaling in controlling alloreactivity in recipients transiently depleted of CD4+ T cells was demonstrated in CD4-DNR mice (Chapter 3). Abrogation of T cell TGFβ signaling in this model results in donor-reactive Th1 and Th2, a significant reduction in Treg accumulation within the grafts and graft rejection dependent on CD4+ T cell population. Hence, TGFβ is crucial for regulation of T cell proliferation and survival, inhibition of Th1/Th2 differentiation and in Treg generation/maintenance in this model of transplant. However, it is unclear whether T cell-derived TGFβ or TGFβ produced by other cells within the environment is important for modulating alloreactivity and allograft fibrosis. Hence, experiments in this section will identify whether T cell generated TGFβ is required for allograft fibrosis and for controlling systemic immune responses in the transplant setting.

Studies conducted in TGFβ deficient mice have provided insight into the requirement for TGFβ in Treg maintenance and for suppressor function (124). Employing the TGFβ null mouse model, it was demonstrated that this cytokine is not required for the development of nTreg but is important for iTreg generation and maintenance of peripheral Treg. This was evident in that the TGFβ null mice exhibit normal thymic Treg numbers but a decline in the peripheral Treg population (124). Additional support for the role of TGFβ in peripheral Treg maintenance was provided in experiments performed in WT mice that received systemic neutralization of TGFβ by anti-TGFβ Ab administration (124). A dramatic decline in the number of peripheral Treg that was comparable to what is seen in TGFβ null mice was observed, corroborating the role of TGFβ in peripheral Treg maintenance (124). Interestingly, TGFβ deficient Treg are functional and can

suppress colitis in an adoptive transfer model in which the recipients express TGFβ (124, 356). This indicates that TGFβ produced by neighboring cells can sustain Treg suppressor function and phenotype, and that Treg may induce TGFβ expression from APCs or stromathymal and mesenchymal cells (388). Indeed, TGFβ -/- Treg combined with TGFβ -/- APCs are no longer capable of suppressor function, which is consistent with a requirement for exogenously supplied TGFβ1 to sustain the regulatory phenotype (124).

To assay for the requirement of T cell-derived TGFβ or TGFβ produced by non-T cells in prolonged allograft acceptance, T cell specific TGFβ null mice (389) will be employed as transplant recipients and transiently depleted of CD4+ T cells (Figure 21). These mice have a conditional knockout allele for TGFβ in which exon 6 of the gene is flanked with LoxP sites. When crossed to LCKCre transgenic mice, a T cell specific disruption of TGFβ is generated (389). T cell specific deletion of TGFβ does result in increased organ inflammation and Th1-effector cytokine production in the mice, but this phenotype is predominantly seen in older mice (5 months), permitting the use of this model in transplant experiments (389). Graft function will be monitored and functioning allografts will be evaluated for signs of CR fifty days post-transplant. ELISPOT will be employed to quantify donor-reactive Th1, Th2 and Th17 responses. Treg will be assessed by quantitative RT-PCR to assay for intragraft FoxP3 expression and by flow cytometry to quantify the percentage of FoxP3+CD25+CD4+ GICs and splenocytes. CR (as assessed by graft fibrosis) and prolonged allograft acceptance would indicate that non-T cell derived TGFβ is sufficient for fibrosis and allograft acceptance. Reduced CR and prolonged allograft survival would suggest that both T cell and non-T cell derived TGFβ

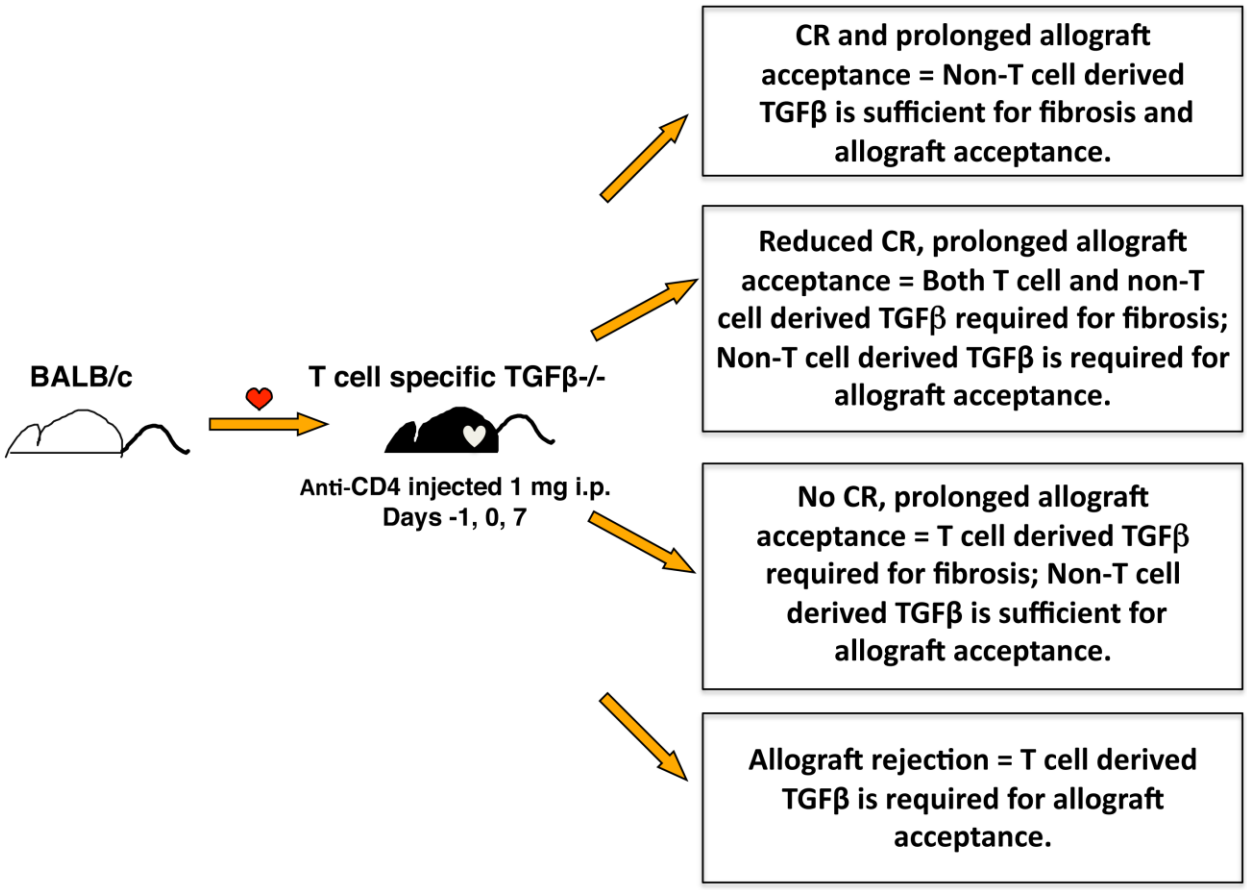


Figure 21: T cell derived TGFβ, allograft acceptance and fibrosis. T cell specific TGFβ null mice (H-2^b) will be employed as transplant recipients and transiently depleted of CD4+ T cells. Graft function will be monitored and functioning allografts will be evaluated for signs of CR fifty days post-transplant. ELISPOT will be employed to quantify donor-reactive Th1, Th2 and Th17 responses. Treg will be assessed by quantitative RT-PCR to assay for intragraft FoxP3 expression and by flow cytometry to quantify the percentage of FoxP3+CD25+CD4+ GICs and splenocytes.

is required for fibrosis and that non-T cell derived TGFβ is required for allograft acceptance. Prolonged allograft survival and no evidence of fibrosis would indicate that T cell derived TGFβ is required for fibrosis and that non-T cell derived TGFβ is sufficient for allograft acceptance. Finally, allograft rejection would indicate the requirement for T cell derived TGFβ in allograft acceptance.

TGFβ, IL-17 and CR

IL-17 is a pro-inflammatory cytokine with pleotropic functions that has been implicated in the development and progression of various autoimmune diseases (reviewed in (377)). Data presented in this dissertation implicate TGFβ-induced IL-17 as a mediator of graft fibrosis in recipients transiently depleted of CD4+ T cells. In chapter 3, IL-17 deficient recipients treated with anti-CD4 mAb exhibited a significant reduction in graft fibrosis compared to WT counterparts. In chapter 4, localized TGFβ neutralization by decorin gene transfer reduced intragraft IL-17 expression and fibrosis. Additional experiments will investigate whether IL-17 expression is both necessary and sufficient to induce graft fibrosis.

WT cardiac recipients transiently depleted of CD4+ T cells exhibit a compartmentalized Th17 response within the allograft. Th17 polarization within target tissue has also been observed in murine models of allergic lung disease and hypersentitivity pneumonitis and lung fibrosis (336, 350). This may indicate that, in response to the inflammatory milieu, Th17 cells are generated directly at the site of inflammation or within draining lymph nodes. Veldhoen and colleagues demonstrated that naïve CD4+ T cells could differentiate into Th17 cells in the presence of DC-derived IL-6 and Treg generated TGFβ (326). In the absence of Treg, the cells committed to a Th1 phenotype. This was corroborated by a number of researchers, including Bettelli et. al., who showed that naïve CD4+ T cells activated by anti-CD3 in the presence of TGFβ leads to the production of Treg, but that activation in the presence of IL-6 in addition to TGFβ diverted the naïve cells to develop into Th17 (93). Similar findings were reported in the allogeneic setting (271). CD4+ T cells stimulated by MHC class II mismatched

mature or immature BMDCs in the presence of Treg, enhanced, rather than suppressed, IL-17 production in the course of an alloreactive response through the production of TGFβ (271). DC-derived IL-6 in conjunction with Treg produced TGFβ were necessary and sufficient to drive Th17 development. This occurred simultaneously while Treg inhibited Th1 and Th2 differentiation through a TGFβ dependent mechanism (271).

The mechanism by which Th17 develop and induce fibrosis in cardiac allografts remains to be elucidated. CD4+ activated effector and memory T cells have been identified as the cell type that primarily secrete IL-17 (97). However, recent reports indicate that non-immune cells, such as cardiac fibroblasts, can express significant quantities of IL-17 (92). The functional IL-17 receptor (IL-17R) is a heteromeric complex of IL-17RA and IL-17RC (390). The IL-17R is present on a variety of cell types, including human and mouse cardiac fibroblasts (CF), which express both IL-17RA and IL-17RC. In addition, in cardiac fibroblasts, the IL-17R is upregulated in response to IL-17 (92).

Experiments in IL-17 deficient recipients that received WT BALB/c allografts and were transiently depleted of CD4+ T cells indicate that the cells primarily responsible for secreting IL-17 are immune cells, most likely CD4+ T cells. It is postulated that the polarized Th17 response is generated either within the cardiac allograft or in the draining lymph nodes (dLN), and that TGFβ-producing Treg accumulate within the grafts and/or lymph nodes and enhance the Th17 lineage development (271, 373). It may also be possible that a population of IL-17 secreting Treg are the mediators of IL-17 production (391). It has recently been reported that mouse FoxP3+CD25+ Treg can be converted into a hybrid T cell population that produce IL-17 and co-expresses FoxP3 and RORgt in

response to IL-23 signals from DCs (391). Therefore, Treg may play an unexpected pro-inflammatory role, depending on signals from innate cells. To investigate Th17 priming within the allografts and dLN, intracellular cytokine staining will be performed on GICs or T cells isolated from the dLN to examine IL-17, FoxP3, and CD4 expression in WT and IL-17 deficient recipients transiently depleted of CD4+ T cells. Allografts and dLNs will be recovered on day 30 post-transplant, since transient priming of alloreactive cells peaks at this time (297). It is predicted that a discrete population of IL-17 producing CD4+ T cells will be observed in the allografts and/or dLNs of WT recipients and that these are the cells responsible for inducing fibrotic changes associated with CR. IL-17 may induce myocardial inflammation, injury and tissue remodeling through a number of mechanisms and may act synergistically with TGFβ to induce cardiac fibrosis (Figure 22). Among its many functions, IL-17 stimulates matrix metalloproteinase 1 (MMP-1) expression and secretion in primary human CF through the activation of p38 MAPK- and ERK1/2-dependent c/EBPβ, NF-κB and AP-1 (331). In the myocardium, MMPs degrade ECM and release and activate ECM-bound factors, like TGFβ. Activated TGFβ can induce cardiac fibroblasts to generate and remodel ECM (233) and TGFβ induced Smad signaling has been reported to negatively regulate MMP expression by sequestering AP-1 proteins to the cytoplasm (392). This feedback loop ensures that during normal tissue remodeling, the rate of synthesis of ECM proteins is balanced by ECM degradation (342). However, excessive TGFβ production upsets this balance and leads to fibrosis (342). Indeed, diminished myocardial MMP activity can directly facilitate collagen accumulation (392).

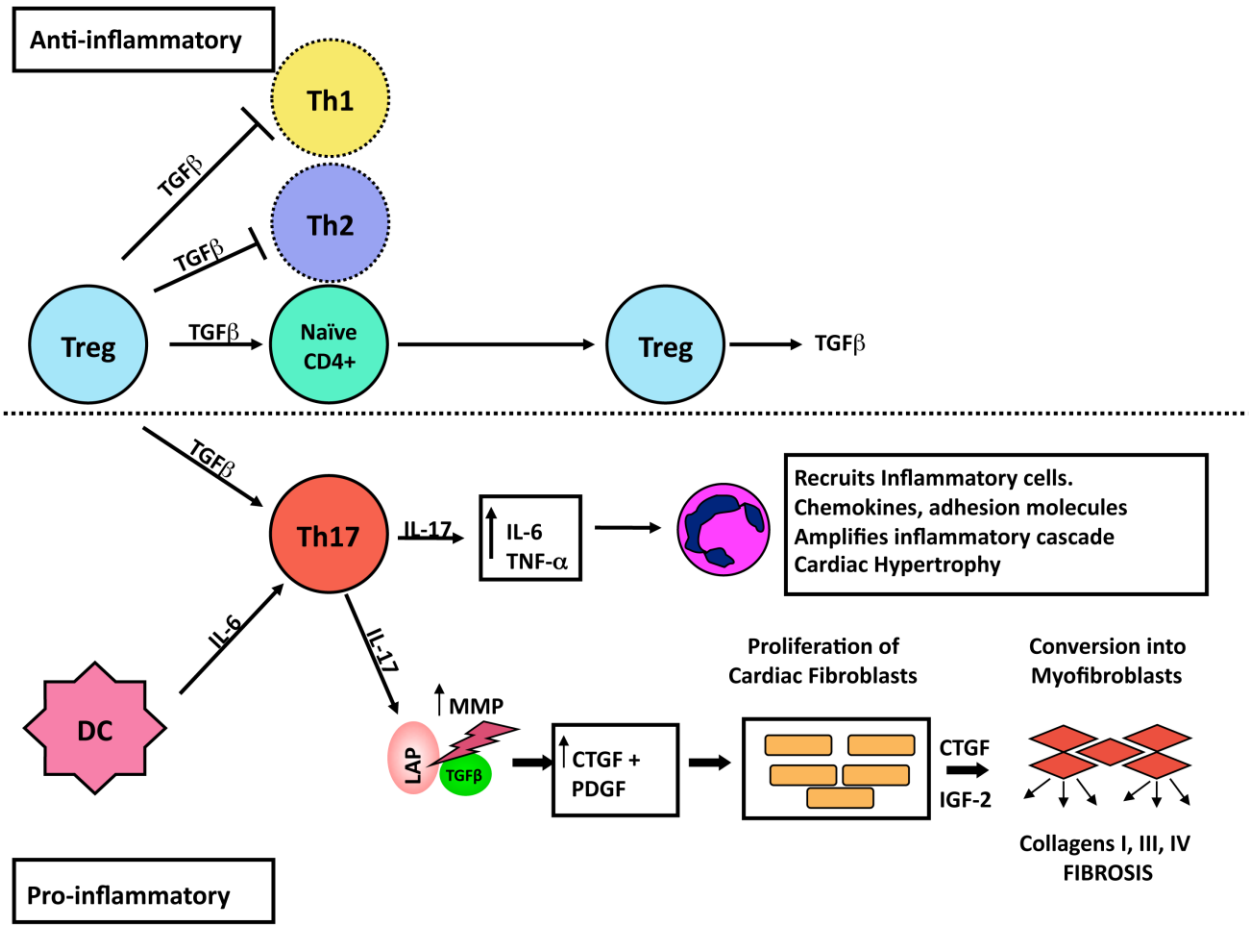


Figure 22: TGFβ, IL-17 and CR. Treg play a critical role in allograft acceptance by inhibiting donor-reactive Th1 and Th2 responses and by inducing additional Treg from naïve CD4+ T cells in a TGFβ-dependent mechanism. However, recent *in vitro* studies have demonstrated that Treg do not suppress, but rather enhance IL-17 production in the course of an alloreactive response. Benghiat and colleagues demonstrate that when DCs are used as allogeneic stimulators, Tregs are critically required for driving IL-17 production in CD4+CD25- T cells. Further, Treg derived TGFβ and IL-6 generated by DCs act to synergistically induce alloreactive CD4+ T cells to produce IL-17. IL-17 may induce fibrosis through multiple mechanisms. IL-17 may act as a potent pro-inflammatory cytokine that induces endothelial cells and fibroblasts to secrete additional pro-inflammatory cytokines, such as IL-6 and TNFα. These factors may enhance the recruitment of APC and alloreactive T cells into the graft resulting in myocardial damage and ECM remodeling that favors fibrosis. IL-17 can also act directly on cardiac fibroblasts by upregulating MMP expression through NF-κB, c/EBPβ and AP-1 activation. MMPs are activated during inflammation. In addition to ECM degradation, MMP degrades the latency associated peptide and releases active TGFβ. TGFβ is then free to upregulate its downstream mediator, CTGF. CTGF in association with a mitogenic stimulus, PDGF, stimulates cardiac fibroblasts to proliferate. Further association with IGF-2 results in differentiation of fibroblasts into myofibroblasts, which upregulate collagen synthesis. Persistence of these myofibroblasts leads to fibrosis.

In addition to activating TGFβ, IL-17 induces primary CF migration in an MMP-1-dependent manner. Fibroblast migration and proliferation are two critical steps in cardiac fibrosis (233) and IL-17 may contribute to fibrosis through this mechanism. Finally, IL-17 has been reported to directly stimulate collagen and other ECM production in CF and may play a role in cardiac fibrosis and remodeling through this mechanism (92).

Only recently has IL-17 been identified as a pro-fibrotic cytokine. This Th17 cytokine is best recognized for its pro-inflammatory functions and can promote chronic tissue inflammation (91, 97, 98, 377). IL-17 upregulates NF-κB, AP-1 and c/EBP1/2 responsive pro-inflammatory cytokines, chemokines, and adhesion molecules in the heart (331). Th17 promote tissue inflammation and chronic inflammatory diseases (393). In a CR allograft, IL-17 may upregulate and synergize with the pro-inflammatory cytokines, IL-6 and TNFα, to induce inflammation and injury to the myocardium (294). Therefore, IL-17 has the potential to directly impact cardiac remodeling through its effects on TGFβ, collagen, MMPs, and tissue remodeling and/or may generate myocardial injury by inducing pro-inflammatory cytokines and chemokines that attract inflammatory cells to the allograft.

While it is clear that IL-17 plays a critical role in graft fibrosis, it remains to be elucidated whether this cytokine is both necessary and sufficient to initiate these fibroproliferative changes. In collaboration with Dr. Joseph Rabinowitz at Thomas Jefferson University, a recombinant Adeno-associated virus serotype 6 vector (AAV6) that expresses human IL-17A has been generated. AAV6 is a cardio-tropic vector that has

recently been demonstrated to provide stable, long-term expression of transgenes that are capable of reversing heart failure in a rodent model (394).

Preliminary cardiac perfusion studies with the AAV6-IL-17A vector in the mouse vascularized allograft model indicates robust transgene and protein expression as early as day 7 with increasing transgene expression over the experimental period of 50 days. In order to identify whether IL-17 is both necessary and sufficient to induce fibrotic changes associated with CR, BALB/c allografts will be perfused with the AAV6-IL17A vector prior to transplant into IL-17 deficient recipients transiently depleted of CD4+ T cells or WT recipients treated with inductive anti-CD40L mAb therapy (Figure 23). It is predicted that this strategy should rescue the CR phenotype in recipients if IL-17 is the

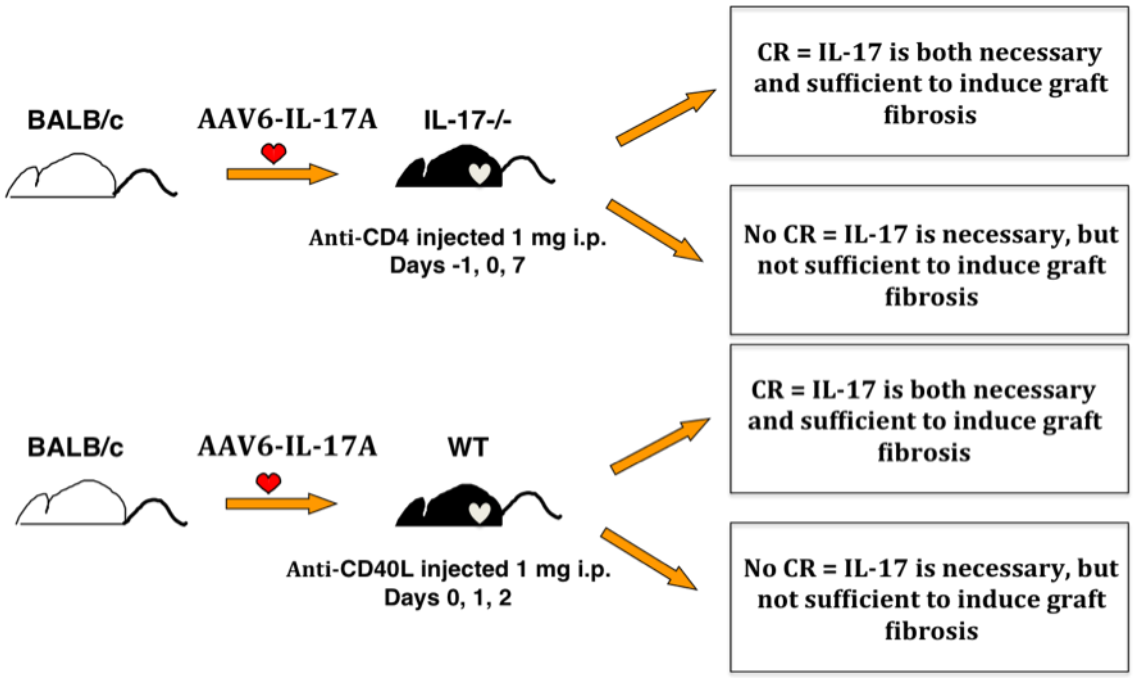


Figure 23: Forced expression of IL-17 in anti-CD4 and anti-CD40L mAb treated recipients. BALB/c allografts will be perfused with rAAV6-IL-17A prior to transplant into IL-17 deficient recipients transiently depleted of CD4+ T cells or WT recipients treated with inductive anti-CD40L mAb. Graft function will be monitored and functioning allografts will be evaluated for evidence of CR fifty days post-transplant.

causative agent of graft fibrosis. However, we may demonstrate that IL-17 is neccesary but not sufficient to sustain fibrosis. Additional IL-6 neutralization experiments will be performed with this vector to investigate the requirement for both IL-6 and IL-17 in CR, since IL-6 has been identified as a contributing cytokine in both fibrosis and cardiac hypertrophy in CR (294). These experiments will reveal the sufficiency of IL-17 in graft fibrosis and further characterize the role IL-17 plays in CR.

5.3 Concluding remarks

Organ transplantation is one of the most significant therapeutic advancements made in the second half of the 20th century. Research in the transplant field has revealed the most basic tenets of immunology: the discovery and characterization of the MHC, immunological self-tolerance, the requirement for TCR activation and co-stimulation, inflammatory 'danger' signals, Treg, effector and memory cell development and function. Knowledge gained in studying the immune response toward the transplanted organ has deepened our understanding of the immune system in health and in disease and has given us a model to harness immunological self-tolerance. The data herein has provided two therapeutic targets that can potentially be neutralized locally within the allograft to suppress the progression of CR: TGFβ and IL-17. Additionally, it is clear that there are TGFβ-dependent and -independent mechanisms of allograft acceptance. Hence, it is possible that therapies that rely on the immunosuppressive activities of TGFβ to control alloreactive responses may have the undesired effect of generating T cell dependent graft fibrosis. In conclusion, a vast quantity of experimental data exists that transplant tolerance can be achieved. Perhaps one of the most significant therapeutic accomplishments of this next century will be attaining the 'Holy Grail'.

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