Interleukin-6 and TGFβ Promote Connective Tissue Growth Factor in Chronic Cardiac Allograft Rejection

by

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Dedication

This work is dedicated to my helpmate Jessica Booth, who has endured sleep deprivation, crowded spaces, on-the-fly planning, and who regularly deals with four difficult/cranky/tired people of the y-chromosome-equipped variety on a daily basis. You made it possible for me to be a graduate student and parent. Your steadfast love, strength, selflessness, and encouragement have made it possible for me to shoulder much with joy. I love you Icca—together we are not easily broken. Ecclesiastes 4:9-12

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List of Abbreviations

6-MP 6-Mercaptopurine
AdCTGF Adenovirus Encoding Human Connective Tissue Growth Factor
AdTGFβ Adenovirus Encoding Active Human TGFβ
Adβgal Adenovirus Encoding Beta-galactosidase
ANP Atrial Natriuretic Peptide
Anti-CD4 Monoclonal Antibody that Binds CD4 Antigen
Anti-CD40L Monoclonal Antibody that Binds CD40L Antigen
Anti-CTGF Monoclonal Antibody that Binds CTGF Antigen
Anti-IL-6 Monoclonal Antibody that Binds IL-6 Antigen
APC Antigen Presenting Cell
bp Base pairs
CAV Chronic Allograft Vasculopathy
CD Cluster of Differentiation
CR Chronic Rejection
cSMA α Smooth Muscle Actin
CTGF Connective Tissue Growth Factor
ECM Extra Cellular Matrix
FS Fractional Shortening
hIgG Human Immunoglobulin
HLA Human Leukocyte Antigen
IL Interleukin
LV Left Ventricle
LVEF Left Ventricle Ejection Fraction
MHC Major Histocompatibility Complex
MMP Matrix Metalloproteinase
PWT Posterior Wall Thickness
rIgG Rat Immunoglobulin
SMAD Proteins of Family Named for *C. elegans* SMA protein and *D. melanogaster* MAD protein
STAT Signal Transducers and Activators of Transcription
TCRβ T cell receptor β constant region
TGFβ Transforming Growth Factor β
Treg T Regulatory cell(s)

Abstract

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Cardiac transplantation is an effective treatment for multiple types of heart failure refractive to therapy. Although immunosuppressive therapeutics have increased survival rates within the first year post-transplant, chronic rejection (CR) remains a significant barrier to long term graft survival. Indicators of CR include patchy interstitial fibrosis, vascular occlusion, and progressive loss of graft function. However, the functional and anatomical changes associated with CR, as well as the factors responsible for the induction and progression of the disease are not understood. These experiments utilized serial echocardiography to assess the progression of chronic rejection in vascularized mouse cardiac allografts. Cardiac allografts in mice transiently depleted of CD4+ cells

that develop chronic rejection were compared with those receiving anti-CD40L therapy that do not develop chronic rejection. Echocardiography revealed the development of hypertrophy in grafts undergoing chronic rejection which was confirmed by histologic analysis and coincided with graft fibrosis and elevated intragraft expression of IL-6. To elucidate the role of IL-6 in chronic rejection, cardiac allograft recipients depleted of CD4+ cells were treated with neutralizing anti-IL-6 mAb. IL-6 neutralization ameliorated cardiomyocyte hypertrophy, graft fibrosis, and prevented deterioration of graft contractility associated with chronic rejection. The association of IL-6 with CR prompted us to investigate the relationship between IL-6 and two other factors known to be associated with CR, namely TGFβ and connective tissue growth factor (CTGF). To this end, we utilized forced expression and neutralizing antibody approaches. Transduction of allografts with CTGF significantly increased fibrotic tissue development, though not to levels observed with TGFβ transduction. Further, intragraft CTGF expression was inhibited by IL-6 neutralization while TGFβ expression remained unchanged, indicating that IL-6 effects may potentiate TGFβ-mediated induction of CTGF. Finally, neutralizing CTGF significantly reduced graft fibrosis without reducing TGFβ and IL-6 expression levels. These findings indicate that IL-6 and TGFβ function as promoters of CR while CTGF functions as a downstream mediator of fibrosis in CR. Further, therapeutics targeting IL-6 or CTGF may hold promise in the treatment of CR of cardiac grafts.

Chapter I—The Origins of Cardiac Transplantation

The concept of human heart transplantation may have been first described in the book of Liezi around 400 AD in China. This tale describes how Bian Que exchanged the hearts of two warriors (1) with the chimerical aim of this legendary exchange being to balance the personal characteristics of both warriors. Throughout history, the human fascination with the potential of joining favorable characteristics of different bodies, much like transplantation, extended to inter-specific grafting in ancient mythology where human forms were augmented by those of other animals (merfolk, centaurs, sphinx, etc.). Those ancient amalgams are now juxtaposed with the horrific, more recent literary descriptions of vampires, werewolves, and Shelly's Frankenstein—a monster literally sewn together from the body parts of criminals. In contrast, modern transplantation uses grafting as a therapeutic to combat disease.

Beginnings of Transplant—Success and Failure

The history of modern transplantion spans over one hundred years and includes several of the most significant discoveries in the study of immunology (Figure 1). The origin of therapeutic organ transplantation traces back to the seminal work by Alexis Carrel and Charles Guthrie that overcame the first technical barrier to organ transplantation—suturing blood vessels. Carrel and Guthrie's experimental advances in anastomosis techniques paved the way for their historic 1905 report of the first successful

organ transplant in which they describe the function of a dog kidney transplanted into the neck of another dog (2). This was followed by the grafting of a puppy heart into the neck of a dog. In this experiment, the donor heart resumed beating approximately one hour after the operation. The grafted heart continued to beat for approximately two hours, at which point the experiment was interrupted due to blood coagulation in the cavities of the heart (3). These and subsequent reports by Carrel marked the beginning of the science of organ transplantation (4).
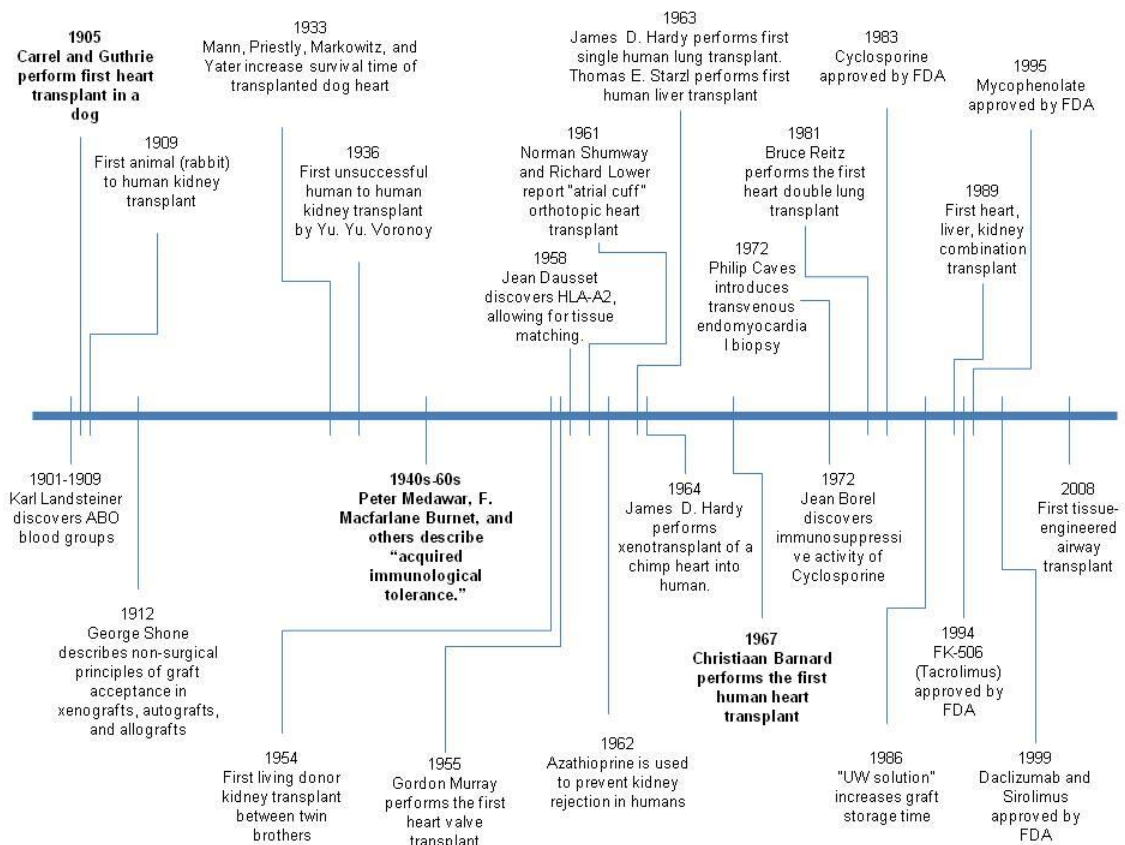
Figure 1 Timeline of Transplantation History

During the years that Carrel and Guthrie pioneered their anastomosis technique and performed the first successful orthotopic transplantations, Karl Landsteiner

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performed his seminal work that laid the foundation for understanding the "biocompatibility" of human blood and tissues. At that point in history blood transfusion was technically possible, though the results of transfusion were often deadly. During his early career, Landsteiner performed numerous autopsies on patients who had become sick as a result of a blood transfusion or injection of foreign proteins (5). The general tissue destruction and massive blood cell lysis seen in these patients intrigued Landsteiner to the point that he decided to pursue this problem rather than following his initial discovery of the polio virus in 1908 (6, 7). In studying this problem, Landsteiner conjectured that genetic differences between humans could account for serum sickness (8). The *in vitro* system that Landsteiner and colleagues developed led to the discovery of ABO blood groups and the very beginning of our understanding of biologic compatibility of transplanted tissues. However, the importance of ABO matching donor grafts to their recipients was not fully appreciated at first (9).

In 1912, George Shone summarized the growing understanding of transplant compatibility (10). In his description he summarized what was known from earliest observations—that grafts between species always failed, that autografts (self grafts) mostly succeeded, and that allografts usually failed. However, it was known that the likelihood of success for an allograft increased with closer familial relationship between donor and host. Further, the speed with which a graft failed increased when an allograft recipient received subsequent allografts. These observations of transplant failure would later become a critical building block for the understanding of immunologic tolerance.

The next significant progress in experimental heart transplantation was reported in 1933 and built upon the canine heart transplantation work begun by Carrel and Guthrie. Though the initial work described orthotopic transplant of a dog heart that resumed function, the graft functioned for less than two hours. Several important observations resulted from their findings; perhaps the most important being that a heart cut off from blood supply and moved into another recipient could resume function upon reperfusion with the recipient's blood flow. However given the short survival of the graft and that the transplant operation lasted several hours, the technique had immense room for improvement. Toward this end Mann, Priestley, Markowitz, and Yates spent considerable efforts improving the survival times of transplanted dog hearts to a mean of four hours, with one heart surviving for eight hours (11).

In 1945, R. D. Owen described red cell chimerism in dizygotic twin cattle. This observation went largely unnoticed until Frank McFarlane Burnett revisited Owens observations in his famous 1949 writing introducing the idea of a basal state of tolerance, mediated by "self molecules" (12). By 1953, experiments by Sir Peter Medawar and colleagues demonstrated that it was possible to experimentally induce tolerance to antigens by introducing them to chick embryos and fetal mice. Together, these experiments laid the foundation of our current understanding of transplant immunology, in light of "acquired immunological tolerance" (13).

Public opinion was largely against organ transplantation in the 1950s, a time when most surgeons worked throughout the night in secret and the earliest cadaveric kidney transplants in both France and the United States were performed with organs

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harvested from executed criminals, a practice that continues in some countries today (14).

Negative perceptions of organ transplantation were solidified by consistently negative results. In spite of great efforts at this time acute rejection was the rule, with organ failure within hours or days of the operation. But then in 1954 a 23 year old patient named Richard Herrick was hospitalized for kidney disease (15). As his condition deteriorated it became apparent that Richard would not survive without a functioning kidney. However, unlike the many organ recipients before him, Richard had a healthy twin brother, Ronald, who wanted to donate a kidney to save his brother. The brothers were referred to Joseph Murray at Peter Bent Brigham hospital, who was doing extensive experimental work at the time (16). After 5 ½ hours of operating, both brothers were in recovery. The transplant was successful and paved the way for many more successful identical twin transplants to come. Further, the success of identical twin transplantation demonstrated the viability of organ transplantation as a therapeutic and made clear that "non-self" factors were the culprits responsible for transplant rejection. Richard Herrick remained healthy for another 8 years, married, and had children before dying of kidney complications unrelated to the surgery while his brother Ronald was still living as of May 2008 at 77 years of age (17).

One year later in 1955 Gordon Murray performed the first heart valve transplant (18). This first transplant was followed by numerous others, each with improvement in aortic or mitral insufficiencies and showing remarkable short to mid-term durability. Though these transplants were successful, even at times crossing ABO compatibility lines, the majority of them eventually failed due to a progressive fibrosis (19). Several

questions were likely raised by the successes of such heart valve transplants, including why the transplant of a valve alone appeared to have less bioincompability compared to transplanting the whole organ.

Three years later, a major milestone in understanding the bioincompatibility of transplanted organs was achieved when Jean Dausset discovered the human leukocyte antigen (HLA)-A2, the first human major histocompatibility complex (MHC) molecule (20). Soon after Dausset discovered HLA-A2, Jon J. Van Rood and colleagues reported anti-leukocyte antibodies detected in serum taken from pregnant women (21), supporting initial observations by Paul A. Gorer that mice could produce antibodies against H-2 histocompatibility locus antigens (22). Though these reports represented only the very beginning of our understanding of tissue matching science, these discoveries were the first glimpse that it might be possible to gain molecular knowledge of the endogenous factors or "self molecules" that make some grafts more compatible with specific recipients and determine Medawar and Burnett's "acquired immunologic tolerance" (13). Advances in the understanding of tissue matching have greatly increased the potential for graft-recipient compatibility, though increased understanding of which HLA combinations are more and less immunogenic is still needed to better predict graft compatibility (23).

Transplantation Research Gains Momentum

In addition to studies that set the groundwork for defining challenges of graft acceptance by the immune system, the 1950s saw significant improvements in another major obstacle for advancing heart transplantation as a therapeutic. The vast majority of

experimental heart transplantations prior to this time had been heterotopic, or put outside of the position for the normal heart, while the native heart continued to bear the recipient's circulatory burden. However, the placement of heterotopic grafts outside the chest cavity impairs their ability to function as the primary circulatory organ. The answer to this was the development of the orthotopic transplant technique, which was made possible in part through the advent of cardiopulmonary bypass by John H. Gibbon (3, 24). Though multiple groups had approached orthotopic transplantation throughout the 50's, the technique is generally attributed to the landmark report by Shumway and Lower in 1961 (25), which first outlined the theory and execution of the atrial cuff technique. This technique, unique in that it was post operatively fully capable of supporting the recipient's circulatory system, allowed recipient dogs to resume normal exercise for weeks post transplant (3). Shumway and Lower's technique had far reaching implications for the future of cardiac transplantation, as the surgical techniques necessary for successful human heart transplantation were now in place.

It was also in the early 1960s that the first major breakthrough in treating immunologic rejection occurred. As with orthotopic transplantation, the story of the first immunosuppressant began in the 1950's, when Gertrude B. Elion and George H. Hitchins pioneered work in the use of "antimetabolites", or chemicals that antagonize the bases that comprise nucleic acids (26). By 1951, the research group had screened over 100 mercaptopurines leading to the discovery of several promising candidates, including 6-Mercaptopurine (6-MP). 6-MP demonstrated outstanding promise as a chemotherapeutic in acute childhood leukemias, but was plagued with excessive side effects, so the group

began screening variations of the drug made with chemical substitutions in hopes of finding an effective variant with diminished toxicity. By 1958, Robert Schwartz began investigating the potential of 6-MP as an immunosuppressant, supposedly in part because the lymphocyte proliferation associated with acute leukemias appeared similar to that associated with transplant rejection (26). Schwartz focused his studies on the ability of 6-MP to alter antibody production. Interestingly, Schwartz's findings were that 6-MP needed to be administered concomitantly with antigen to prevent the production of antibody (27), and that administration of antigen after immunosuppression could actually augment antibody production (28). However, the most important of Schwartz's findings was undoubtedly several years later, when they reported that tolerance to antigen could be drug induced without ablating the capacity to respond to other antigens (29).

The Beginning of Clinical Transplantation

Schwartz's findings with 6-MP sparked a series of investigations by Roy Calne, who in the early 1960's employed first 6-MP then the more efficacious azathioprine (as suggested by Gertrude B. Elion) to prevent kidney rejection in dogs (30-32). By 1962 combination treatment of azathioprine with steroids had made survival of kidney homografts in man a reality (33). Though there were still considerable side effects of the steroids administered with azathioprine, Calne's research marks the dawn of immunosuppressive drug therapy to prevent graft rejection in man. This seminal work ignited a surge of interest in organ transplantation as there was now a means to prevent immunologic rejection. 1963 saw the achievement of the first single human lung transplant by James D. Hardy (34) and the first attempts at human liver transplant by

Thomas E. Starzl (35, 36). These were certainly intrepid efforts, and though Hardy's

operation was surgically successful, these operations were performed on terminally ill

patients and the outcomes could not be considered clinical successes. However, such

early failures clearly reiterated the need for greater understanding of transplant biology

and paved the way for subsequent attempts at transplanting other organs.

In 1964, a terminally ill patient who had suffered from years of chronic

hypertension came under the care of Hardy and associates became the first human

recipient of a heart transplant (37). As no suitable human donor heart was available, they

performed the xenotransplant of a heart from a chimpanzee that Hardy had brought back

from a recent visit to another laboratory. Upon warming and reperfusion, the

transplanted heart resumed contraction for at least 90 minutes, however it was

immediately apparent that the organ had insufficient contractile capacity to support the

circulation of the large man (38). The failure of this operation prompted an outcry of

severe criticism from both the general public and the medical profession, as the United

States populace was now embroiled in the ethical, moral, and legal quandary of heart

transplantation (10). The advent of heart transplantation detonated these issues for a very

specific reason. The harvesting of the donor organ in the United States was never

performed before the donor underwent full cardiopulmonary arrest. However harvesting

organs after cardiopulmonary arrest is especially detrimental to hearts, which lose

functional capacity and the ability to be resuscitated rapidly. An additional barrier facing

Hardy and others at the time was the intense increase of restrictions on clinical research

and increased restrictions on clinical research funding (39). Hardy himself said, "The

significance of these stated or implied restrictions was attested by the fact that the second

clinical heart transplantation was performed, not in the United States, where most of the

developmental research in heart transplantation had been carried out, but in a foreign

country, where support for laboratory research would appear to be somewhat limited"

(39).

The second clinical heart transplant to which Hardy referred was performed by

the team led by Christiaan Barnard working at Groote Schuur Hospital in Cape Town,

South Africa (40). Louis Washkansky, the 54 year old recipient, received the heart of a

young woman, Denise Darvell, killed in an automobile accident. The surgical technique

was adapted from the one introduced by Shumway, and Washkansky received a barrage

of immunosuppressive treatments including intravenous hydrocortisone, oral prednisone,

nasogastric azathioprine, local irradiation of the heart with Cobalt[60], as well as

actinomycin-C (10). The graft appeared to function normally, however Washkansky

succumbed to pneumonia 18 days later, perhaps a result of the intense

immunosuppressive treatments. Barnard's group continued to perform heart transplants

and two of the group's first 10 transplant recipients lived for 13 and 23 years—long

enough to raise global optimism of the viability of cardiac transplants as therapeutics in

spite of widespread failures elsewhere (41). Thus, in Cape Town, South Africa in 1967

two streams of medical research merged; one had investigated the surgical techniques

originating from Carrel and Guthrie while the other, which sought to understand

immunologic tolerance, flowed from Medawar and Burnett.

In addition to the inception of clinical heart transplantation, the 1960s saw several significant breakthroughs in basic science related to transplant immunology. Seminal work in tissue matching by Dausset and Van Rood was bolstered by the report of the microcytotoxicity test by Paul I. Terasaki (42). The importance of this assay was not seen through immediate clinical relevance, but rather that it facilitated the classification of HLA antigens (43). It was known at the time that the main compatibility necessary for initial transplant acceptance was to avoid crossing ABO blood compatibility boundaries, as this invariably induced hyperacute rejection, irrespective of the presence of immunotherapeutic treatment (44). However, as Terasaki continued looking at host-donor compatibility, he provided in 1965 a preliminary description of hyperacute rejection in a graft that did not cross ABO compatibility lines with the donor (43). The critical observation in this case was that Terasaki was able to provide conclusive evidence that the manifestation of hyperacute rejection in this circumstance was precipitated by pre-formed anti-HLA antibodies in the recipient (45, 46). This began the use of Terasaki's crossmatch test.

Another breakthrough occurred when scientists at Sandoz in Basel, who had been screening fungal metabolite compounds for antibiotic and anti-cancer effects, decided to tune their screening process to assay immunosuppressive agents as well. This led to the discovery of Ciclosporin A (now Cyclosporine A or Sandimmune), a drug whose history is quite controversial (47-49). Early experiments comparing Cyclosporine A to other cytostatic drugs showed that Cyclosporine A was ineffective *in vitro* but was able to exert strong immunosuppression of the evolution of immune responses *in vivo* (50). However,

unlike azathioprine, Cyclosporine A was much less effective at suppressing cytoxicity of

an established immune response, indicating that known immunosuppressive drugs

exhibited a divergence in their methods of action (50). Further investigation revealed that

unlike other cytostatic and immunosuppressants, Cyclosporine A had significantly lower

myelotoxicity (51). One of the first challenges of utilizing Cyclosporine A was that the

compound is nearly insoluble in water. However, it was soon discovered that the

compound could be delivered dissolved in oil to patients (31). The first use of

Cyclosporine A in the clinic was to treat renal graft recipients, with exceptionally

promising results—improving the first year post transplant survival rate from 50% (with

azothioprine and steroids) to nearly 80% (52). The great success of Cyclosporine A

launched increased efforts to identify new immunosuppressive agents.

Advances in immunotherapeutics opened the door to increased number of

transplants by providing a means with which to combat rejection. However,

immunosuppressive drug regimens caused significant side effects. Hence, using a

minimal amount of immunosuppression would clearly be beneficial for transplant

recipients. In order to effectively combat rejection with said minimal amount of

immunosuppression, a means for both defining and monitoring graft rejection was needed

in the clinic. The answer to this problem came when Philip Caves introduced the

transvenous endomyocardial biopsy. Caves' technique utilized a modified flexible

bioptome that could be inserted into the internal jugular vein, continuing through the

superior vena cava and right atrium through the tricuspid valve into the right ventricle for

sampling (53). This technique has further benefitted from being performable as an

outpatient procedure in part because it only required local anesthesia and is repeatedly

well tolerated—even as many as 30 times through the same vessel (54). One of the

primary debates over the use of biopsy for monitoring graft rejection is the question as to

whether or not a biopsy can be assumed to represent the whole tissue. For this reason at

least 5 samples are taken from each biopsy performed to minimize the risk of

misdiagnosis (55). The use of transvenous endomyocardial biopsy had a major drawback

in its nascent years—that a uniform grading system was lacking. This problem was

eventually addressed by the International Society of Heart and Lung Transplantation first

in 1990 (56) and most recently in December of 2004 (57). These two efforts have

improved consistency in assessing rejection severity, though occurrences of endocardial

infiltrates known as "Quilty regions", humoral rejection, biopsy-negative rejection, and

the possibility of regulatory T cell infiltrates (58) still obfuscate clear analysis of clinical

samples (59, 60).

By 1983, a little over a decade after Jean Borel and colleagues discovered the

immunosuppressive activity of Cyclosporine A, the United States Food and Drug

Administration approved its use for the prevention of transplant rejection in patients and

paved the way for more transplants than ever before. The importance of Cyclosporine A

may be best illustrated by the fact that prior to its inception, transplant of hearts, lungs,

and livers were rare clinically, as these organs were much more susceptible to rejection

with previous immunosuppressive therapies. Though Cyclosporine A signaled the dawn

of effective immunotherapeutics for transplant patients, the application of transplant to

organs other than kidneys precipitated the medical research community's awareness of

another significant barrier to survival of non-kidney grafts—organ preservation. Though now of greater clinical relevance, the problem of organ preservation was understood by surgeons from the very beginning of transplant research, as one of the most significant findings of Carrel and Guthrie's first heart transplant was that a heart could resume contraction upon reperfusion. However, it was clear then, as now, that preservation techniques sufficient for intra-abdominal grafts (kidney and liver) were less effective in the preservation of intra-thoracic grafts (heart and lung) (61).

The earliest attempts at organ preservation utilized machine-operated perfusion. The machines were bulky and expensive to operate and significantly limited the procurement of donor organs. Fortunately by the late 1960s, Geoff Collins and Paul Terasaki had demonstrated that kidneys could be successfully stored by flushing with a specialized solution intended to more closely resemble intracellular solution conditions and storing on ice (62), similar to the method employed by Lower and Shumway (25). Indeed Terasaki went on to show that similar results to machine-driven perfusion could be obtained with iced storage, making the transport of donor organs faster and cheaper, as specialized equipment was no longer required (63). By the mid 1970s a majority of clinics were using Collins' technique to preserve kidneys (61). Though numerous iterations attempting to improve the preservation times of organs by both machine perfusion and cold storage, no approach emerged as better than the others throughout the 1970s (61). However, in 1986, a group working at organ preservation at University of Wisconsin at Madison introduced the UW solution. What set this preparation apart from previous perfusion solutions was the inclusion of several key factors that allowed for it to

improve the survival rates of the liver, kidney, pancreas, intestine, lung, and heart (64), though there is still room for improvement (65).

Soon after the UW solution revolutionized organ perfusion, another milestone in immunosuppressive drugs became available. A group from the Fujisawa Pharmaceutical Company isolated a soil fungus, *Streptomyces tsukubaensis*, from the Tsukuba region of northern Japan whose fermentation supernatant exhibited potent immunosuppressive capacity in their high throughput screen for molecules able to phenocopy Cyclosporine A (66, 67). The structure of the compound was determined and in August of 1986 the group presented their discovery of FK506 (Tacrolimus) for the first time (68). Subsequent investigation showed that FK506 was effective at far lower concentrations than Cyclosporine A, and could be potentially synergize with Cyclosporine A by potentiating its binding and uptake by lymphocytes with no obvious additive toxicity (69-71). Early evidence also pointed to a common intracellular target for both drugs (72). The subsequent discovery that FK-506 targets the T cell signaling intermediate calcineurin marks the first successful application of forward chemical genetics to elucidate a signaling pathway (67).

FK506 exhibited side effects, particularly in dogs, not seen with Cyclosporine A, though it appeared less toxic in initial primate studies (73, 74). Based on these findings, Thomas E. Starzl initiated clinical trials with FK-506 in liver transplant patients who were unresponsive to traditional immunotherapy (75). The ability of FK-506 to act as a "rescue agent" in transplant settings (76) was likely an important factor in the eventual FDA approval of FK506 in 1994 (77). Though it was not initially understood, one

explanation for the effectiveness of FK506 was that it is far less susceptible to removal from the cell by the multi-drug resistance gene, p-glycoprotein, than Cyclosporine A was (78). Further, p-glycoprotein mediated resistance to Cyclosporine A may be exacerbated *in vivo*, as therapeutic doses of Cyclosporine A can actually induce the expression of p-glycoprotein in certain cell types (79). The availability of multiple drugs to treat rejection and their known toxicities (70, 80-84) prompted the current use of combinatorial therapies aiming to optimize immunosuppression while minimizing side effects of the drugs.

FK-506 prompted the re-investigation of another drug, Rapamycin (Sirolimus), whose immunosuppressive function was originally discovered in 1977 (85). An important common characteristic of Rapamycin and FK-506 is the ability to reverse the multidrug resistance phenotype (78). Another key immunosuppressive drug to emerge after FK-506 was Mycophenolate mofetil, which was originally used to treat psoriasis in 1975 (86), but introduced to transplantation later with the first clinical trials aimed at determining whether or not the drug could replace azathioprine in the heart transplant "triple therapy" with Cyclosporine A and steroids (87). The effectiveness of Mycophenolate mofetil in this and other clinical trials rapidly raised interest in the drug. This interest was augmented by the discovery that it's method of action—sabotage of *de novo* purine biosynthesis—is quite specific to rapidly dividing lymphocytes as many other cells utilize the metabolic purine salvage pathway facilitated by hypoxanthine ribosyltransferase (88-90). Hence, Mycophenolate mofetil replaced azathioprine in the common "triple therapy" with one of the calcineurin inhibitors as well as steroid

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treatment. Frequently the "triple therapy" is augmented by antibody treatments (Figure 3) (91).

Drug	Cells Affected	Mode of Action	Clinical Use
Glucocorticoids	T, B, Monocytes, MΦ, and many nonimmune cells	Multiple modes of action, including Inhibition of cytokines (IL-1, 2, 3, 6, IFN-γ, TNFα, GMCSF)	Extensive
Calcineurin Inhibitors •Cyclosporine A •Tacrolimus (FK506)	Somewhat T cell specific	Inhibition of Calcineurin signaling (Cyclosporine through binding cyclophilin A, tacrolimus through binding FKBP)	Extensive
Metabolic inhibitors •Azathioprine •Mycophenolate mofetil	Lymphocyte specific	disruption of *de novo* purine synthesis used somewhat specifically by activated lymphocytes	Extensive
Lymphocyte-reactive antibodies Polyclonal Monoclonal	Ranging specificities from cell type to cell antigen	Various actions, often function through blocking or cell depleting effects	More Modest

Figure 2 Summary of Modern Immunosuppressive Approaches

Name	Effects
Anti –thymocyte globulin (ATG) Anti-lymphocyte globulin (ALG)	Polyclonal antibodies used for global depletion of thymocytes or lymphocytes
OKT3	directed against CD3ε chain, causes global depletion of CD3+ cells
Caclizumab/Basiliximab	directed against CD25α chain, inhibits IL-2 induced T cell responses
Efalizumab	directed against CD11α chain, part of leukocyte function associated antigen 1 (LFA-1), prevents LFA-1 adhesion events that mediate T cell activation, adhesion, and trafficking
LEA29Y	directed against CD80/86, blocks costimulatory signals required for T cell activation
Rituximab	directed against CD20, affects B cell function
Alemtuzumab	directed against CD52, causes T, B, Monocyte, and NK cell depletion

Figure 3 Antibody Therapeutics in Tansplantation

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Chapter II—Transplantation Today

Acute Rejection

An overview of transplant history clearly traces a progression of advances in transplant techniques and immunosuppressive therapeutics. Prior to the advances in immunosuppressive drugs, grafts were largely lost due to acute rejection characterized by intense perivascular infiltrate and parenchymal cell death (92). Acute allograft rejection remains a challenge to clinical organ transplant today and can occur through several different immunologic mechanisms. In some cases, acute graft rejection can be the result of preformed graft-reactive antibodies in the organ recipient (93-96). Indeed, antibodies can also play an important role in acute rejection even when not preformed. In addition to antibody-mediated acute rejection, acute rejection often occurs through cell-mediated immunity. It should be noted that acute rejection can occur through humoral or cellular immunity and can be associated with multiple effector lineages, including Th1 and Th2 (illustrated in Figure 6). Today, fewer transplants are lost owing to the effectiveness of modern immunosuppressive drugs (Figures 2 and 3).

Though modern therapies are excellent at limiting acute rejection and prolonging short-term graft survival, a true evaluation of the success of clinical transplantation must take long term survival into account—and therein, as Hamlet would tell us, lies the rub. Current estimates place the rate of cardiac graft failure at approximately 5% annually (97). The most significant barrier to long term graft survival is chronic rejection (CR) (98-103), which may be present to varying degrees in up to 80% of grafts by 5 years post transplant (104).

Chronic Rejection

The definition of CR remains somewhat ambiguous, and CR is often used to describe all graft loss after one year. It has been proposed that CR should specify late graft failure due to anti-graft immunity, which is then subdivided into parenchymal cell CR (associated with fibrosis of graft parenchyma) or vascular CR (associated with vascular stenosis caused by neointimal development) (105). A concise definition of CR is further complicated by the understanding that both immune and non-immune parameters contribute to CR (106). However, any rigid classification is plagued by questions as to whether or not alloantigen-dependent immunologic events are separable from alloantigen-independent responses. Therefore, CR in this manuscript will be defined by three hallmarks—progressive deterioration of graft function, interstitial fibrosis, and occlusion of luminal structures.

Though CR is the most common form of late graft failure in solid organ transplant (98-103), its etiology is not well understood. Numerous factors contribute to or have correlative associations with CR (Figure 2). These factors are generally grouped into one

of two non-exclusive categories: alloantigen-dependent or alloantigen-independent (98, 101, 103, 107). Searches for early biomarkers of CR in transplanted organs have provided limited causative results. However, factors that have been reported to correlate with late graft loss include excessive graft work load (108-110), donor related factors such as diabetes, arteriosclerosis, and cause of donor death (60), history of acute rejection (110-112), circulating donor antigen (113), infection (114, 115), duration of cold ischemic time (116), and measures of allograft immunity (111, 113). Though these and other factors may be biomarkers of CR risk, each has barriers to clinical utility. For example, with respect to previous acute rejection episodes, not all types of acute rejection episode are equivalent in elevating the risk of chronic rejection at later time points (117-119). In addition, other reports have questioned the validity of previous rejection episodes as a predictor for later development of CR (120). Diagnosis is further complicated by specific measures of graft alloimmunity, such as anti-HLA antibodies, that can be observed in both patients undergoing CR as well as those that are not experiencing rejection (121-123), though anti-idiotype antibodies are present in the non-rejecting group which may abrogate the anti-graft response. Thus, reliable prediction and diagnosis of CR remains a difficult task that must take many factors into account.
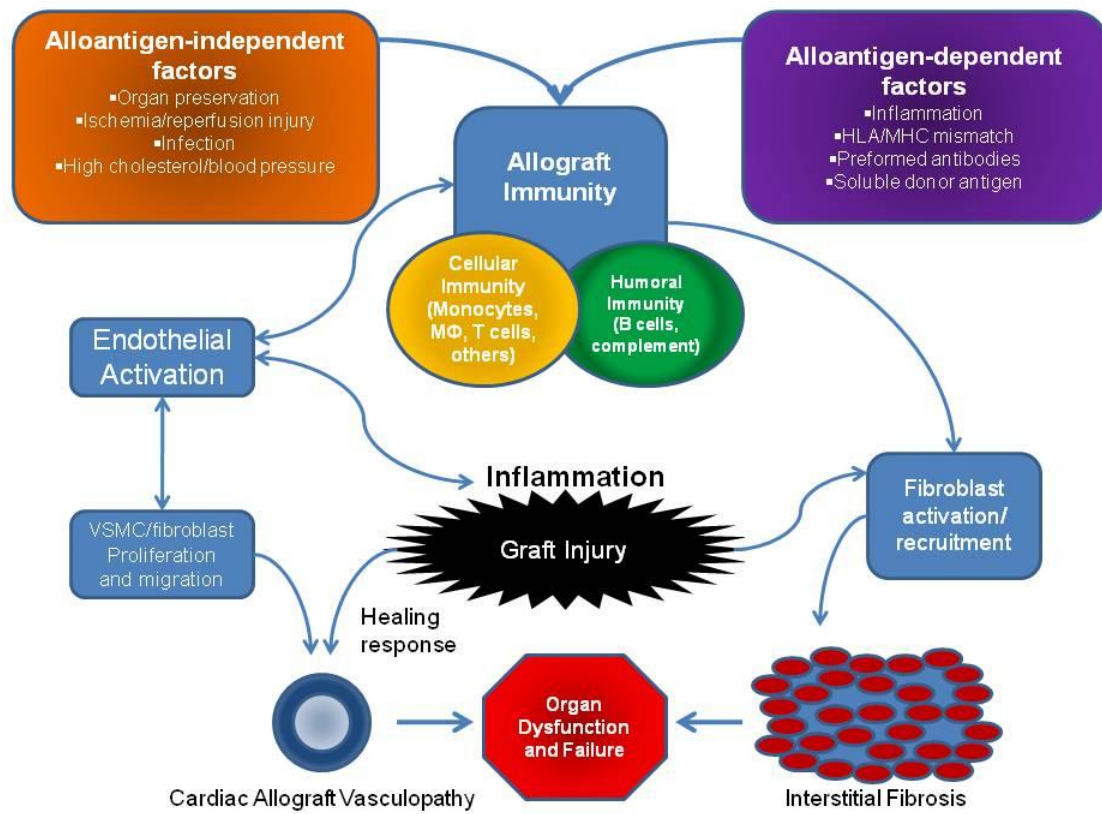
Figure 4 Factors Implicated in CR

Chronic Allograft Vasculopathy (CAV)

Though prediction of CR by risk factor and immunologic analyses is considerably

difficult, the assessment of transplant vasculature can provide additional information

about the state of transplanted organs. The occlusive vascular disease associated with CR

of cardiac grafts in humans was first reported in 1970 (124). Several reports suggest that

some level of intimal thickening can be observed in 75% of cardiac transplants within the

first year post transplant (125, 126) which may then develop CAV, estimated to occur in

up to 80% of cardiac transplants within the first 5 years post-transplant (104, 127). The

vascular disease associated with CR is now referred to by many names, including CAV,

transplant associated vasculopathy, transplant-associated coronary artery disease, transplant arteriosclerosis, graft coronary artery disease, chronic transplant dysfunction, graft coronary vascular disease, transplant coronary artery disease, and accelerated graft arteriosclerosis, and likely others. CAV can manifest as two distinct vascular pathologies (Figure 3), intimal remodeling and constrictive remodeling.
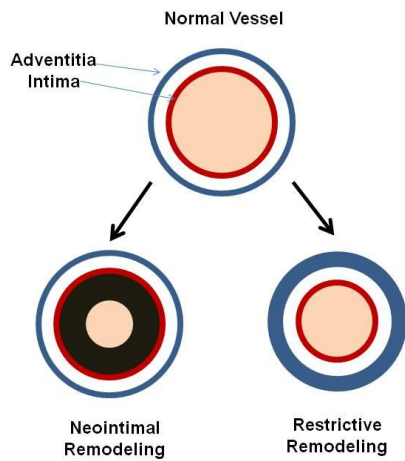
Figure 5 Vascular Remodeling in CR

For some time it was presumed that hyperplasic thickening of the vascular intima, also known as neointima, was the primary manifestation of CAV (105). However, this assumption was challenged by several studies utilizing intravascular ultrasound in which consistent constrictive remodeling was observed (128, 129). Recently, extensive investigations with intravascular ultrasound have indicated that the process of luminal occlusion in CAV may be a biphasic process with intimal thickening occurring first (130), and constrictive remodeling ultimately playing a more pathogenic role (131, 132).

Currently, no effective therapeutic exists for CAV other than retransplantation (133), a dubious therapeutic considering the constant global donor organ shortage. The current clinical approach to CAV is prevention—though this has largely been ineffective as Cyclosporine A, azathioprine, steroids, and antilymphocyte antibodies have yet to decrease the incidence of the disease (103). Recently, improvement in the incidence and severity of CAV has been observed with the newest immunosuppressive treatments, including Mycophenolate mofetil (131), Sirolimus (134), and Everolimus (103). However, there is no direct evidence that these treatments specifically target CAV associated with CR, but rather may generally provide prevention through more robust immunosuppression, as in some cases acute rejection was decreased as well (135, 136). Thus, in spite of advancements in immunotherapeutic approaches, CR remains the leading cause of death after the first year following cardiac transplantation and the greatest barrier to the effective use of available donor organs in the clinic (137).

Transplant Immunology in CAV

CAV affects arteries and veins of the graft up to, but not beyond, the suture line of the graft (103). This supports the widely held notion that graft-derived factors, likely donor alloantigens, play a critical role in the disease (98, 99, 101-103, 106). Recognition of donor alloantigens can occur through multiple pathways, in all of which T cells play a critical role (138). It should be noted that T cell recognition of foreign antigens alone renders T cells in a state of anergy (139), while allorecognition in the context of T cell costimulatory molecules can trigger a robust alloimmune response. Because T cell biology requires costimulation, there are a limited number of cellular interactions that

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may trigger alloimmune responses. These cellular interactions are typically referred to as the direct and indirect pathways (140), though recent evidence suggests the possibility of a semidirect pathway (141) and direct endothelial activation (142). In the direct pathway of allorecognition, donor MHC molecules presented by a donor antigen presenting cell (APC) are recognized by an alloreactive T cell (143) (Figure 6).
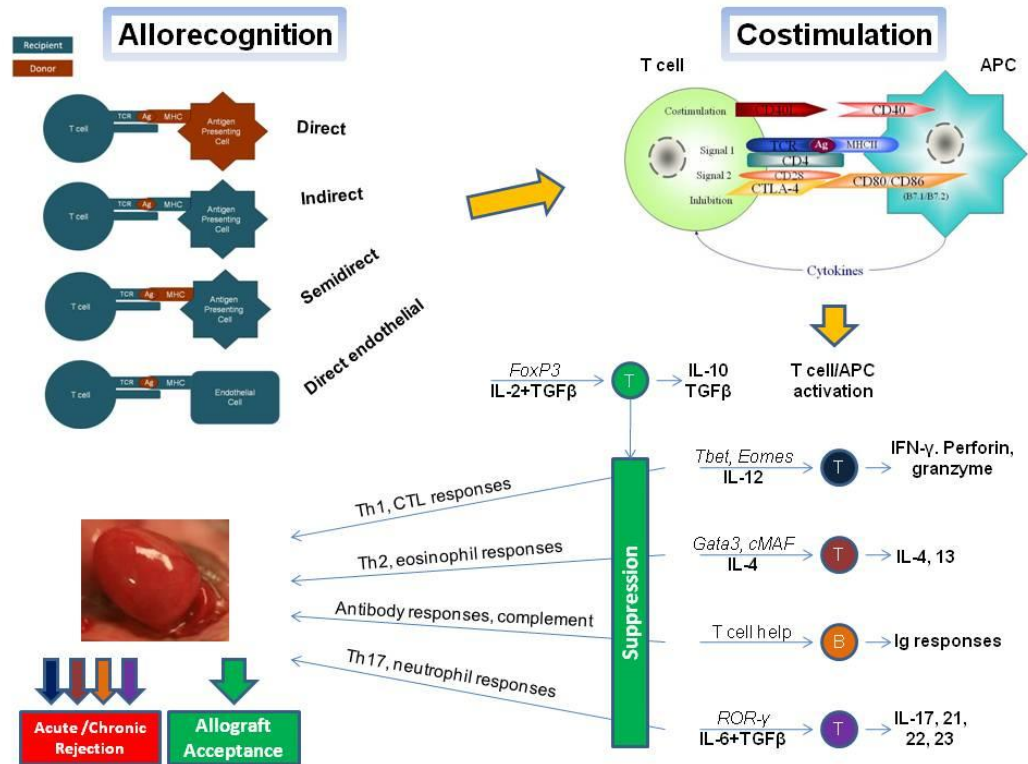
Figure 6 Immunobiology of Transplant Rejection

Allorecognition of foreign peptide in the context of self or foreign MHC molecues by the T cell receptor provides an initial signal to the T cell which then receives secondary activation through costimulation signals including CD40/CD40L interactions, then CD28 with B7-1 or B7-2 (CD80 or CD86) (144) and LFA-3/CD-2 (145, 146) (Figure 6). These costimulation signals initiate activation of the T cell which then primes

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subsequent adaptive immune responses. The indirect pathway involves the uptake of

donor antigen by recipient APCs, which then present the processed donor peptide in the

context of "self" MHC molecules (147, 148). The APC may then provide the necessary

T cell signaling and costimulation described above to prime an adaptive immune

response. In the semidirect pathway, contact between donor cells and a host APC results

in transfer of donor MHC molecule(s) to a host APC cell membrane (141, 149, 150). The

host MHC can then present the foreign MHC and peptide similarly to how a donor origin

APC would in the aforementioned direct pathway. The fourth mechanism of

allorecognition is that of direct endothelial activation (142, 151), the most controversial

mechanism of alloimmune T cell priming (152). Though human endothelial cells

generally express LFA-3 (153) and cultured primary cardiac microvascular endothelial

cells can express B7-2 following ligation of CD40 (154), the ability of endothelial cells to

provide canonical costimulatory signals sufficient for priming *in vivo* remains to be

demonstrated. Therefore, graft endothelial cell activation of T cells may likely provide

reinforcement and augmented cytokine production by cells activated through other

cellular interactions rather than priming the *de novo* differentiation of allogeneic T cells

(155, 156) (Figure 6). In summary, it remains unclear which alloreactive pathways

provide the greatest contribution to the pathology of CAV in CR of heart allografts,

though there is experimental evidence for both indirect (157-159) and suboptimal direct

(160) allorecognition being sufficient to promote CR.

　　　　Many studies have attempted to elucidate the immunologic component

responsible for CR. One hypothesis of the origin of CR is recurring insult to the graft and

vasculature through repeated immune cell attack, representing "smoldering, subacute rejection accumulating progressive subclinical (low level) damage" (105). If this hypothesis is indeed correct, it would be of great value to know which cell types are responsible for "smoldering" damage to the graft. In addition to the increased amounts of smooth muscle cells (161-163), endothelial cells (164) and fibroblasts (165) in vessels undergoing CAV, there are infiltrating immune cells including monocytes/macrophages (163, 166), dendritic cells (138), and T cells (163, 167, 168). One of the most thorough investigations into the immune cells required for CAV was performed in a model of carotid artery transplant across multiple histocompatibility barriers (169). The transplants into seven previously characterized mutant mouse strains allowed for the transplantation of coronary arteries into recipients deficient in all antigen specific responses (170), responses by CD4^{+} T cells (171, 172), B cells (173), cytotoxic T cells (174), natural killer cells (175), and macrophages (176). This investigation highlighted the importance of antigen specific responses in the development of CAV and in particular that full CAV pathology required CD4^{+} T cells, B cells, and macrophages (169). Subsequent studies have generally supported the finding that multiple types of immune responses are required for the initiation and progression of CR, particularly the necessity of CD4^{+} T cells (177) and macrophages (178), while anti-allograft antibody remains somewhat controversial (179, 180).

In summary, current understanding of CAV has been characterized as immunologic attack on endothelial cells that triggers subsequent healing responses (164), immune cell infiltration and endothelialitis (181), as well as early endothelial dysfunction

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(182). These processes may in turn produce a cytokine milieu amenable to smooth

muscle cell recruitment (183) and proliferation, progenitor cell recruitment (184, 185),

and myofibroblast transdifferentiation (186-188) culminating in CAV.

Graft Fibrosis

The changes associated with CR also include interstitial fibrosis (Figure 2),

another hallmark of CR pathology, the early extent of which correlates with long term

transplant outcome (189). Fibrosis of hearts undergoing CR is characterized by the

formation of thick web-like interstitial extracellular matrix (ECM) fibers surrounding

cardiomyocytes.   It has been suggested that graft fibrosis occurs largely in response to

CAV (103), though the relationship between these elements of graft pathology is not

known. Cardiac fibrosis is frequently divided into two types, reactive and reparative

(190-193). Reactive fibrosis generally occurs first in response to stimuli such as

cytokines and hypertension while reparative is believed to occur in response to cell death

(192, 194, 195). The increased ECM accumulation in the fibrotic heart has multiple

implications for graft function, as it provides increased tensile strength, but also stiffness,

to the myocardial wall while the sheathing of individual cardiomyocytes with ECM alters

cell to cell contacts. These changes result in disruption of the electophysiology of cardiac

myocytes (194) and likely decreases in the energy supply of cardiomyocytes, all while

the workload for graft contractility is increased (195). The intense stress of such

conditions leads to cell death, which in turn triggers additional reparative fibrosis and

may eventually lead to organ death.

Cardiac ECM

Enhanced ECM accumulation seen in tissue fibrosis is the result of two competing programs—those that promote the synthesis of ECM and those that promote ECM degradation (196, 197). The two major components of cardiac ECM are collagen types I and III (198, 199). Both types of collagens provide support and structure to the healthy heart, though type I collagen is characteristically associated with thick fibers which are thought to play a central role in determining myocardial stiffness (199). Hence, collagen type I abundance is frequently used as a surrogate index for the degree of fibrosis in heart tissues as well as those of other organs. While the accumulation of ECM in allograft fibrosis is readily observable, the cellular source of excessive ECM proteins is less clear. It was reported that cardiac myocytes can contribute to the production of collagen type I (200, 201) in addition to production of non-filamentous type IV collagen in their basement membranes (202), though it is generally accepted that the cell that regulates homeostasis of ECM within the heart is the fibroblast (202). Cardiac fibroblasts are an abundant cell type in cardiac tissue (203, 204) and are a fixture of the cardiac ECM that facilitates structural and functional connections in healthy cardiac tissue (203, 205).

Stages in Fibrosis Development

The progression of graft fibrosis can be described as occurring in three general stages (100). The first stage involves the initiation of the fibrotic response. Initiating factors for fibrotic responses can include both alloantigen-dependent (cellular and humoral immune attack) and alloantigen-independent factors (health of graft, ischemia reperfusion injury, infections), but both provide the common physiologic effect of tissue

damage. Tissue damage prompts what has been referred to as the fibrogenesis phase, characterized by the production of signaling mediators including cytokines, chemokines and growth factors (206, 207). These mediators initiate at least three cellular responses in the graft. First, cytokines and chemokines promote infiltration by non-graft immune cells, fibroblasts (208), and progenitor cells (209, 210). Infiltrating cells can further enhance production of cytokines and growth factors that drive a proliferative response in inflammatory cells, fibroblasts and epithelial cells. In addition to proliferative responses, cytokines and growth factors prompt the third phase by promoting the differentiation of cells into other cell types (188). The occurrence of endothelial to mesenchymal transformation correlates with later development of graft fibrosis (211). Though the differentiation of several cell types can be initiated in response to cytokines associated with the fibrogenesis phase, perhaps the most important cell type with respect to accumulation of ECM associated with CR may be those expressing fetal smooth muscle isoform α actin (212, 213), a marker associated with myofibroblasts (214, 215).

Myofibroblasts

Myofibroblasts are a specialized form of activated fibroblast first described in the process of cutaneous wound healing (216). Myofibroblasts are characterized by phenotypic features associated with the process of wound closure, namely the expression of α-smooth muscle actin (αSMA), intermediate filament proteins such as vimentin and desmin, and non-muscle forms of myosin (188, 202, 217, 218). The presence and persistence of myofibroblasts have been associated with alloantigen mismatch-driven scar formation in a wound healing model (219). Further, cardiac myofibroblasts have

been extensively linked to myocardial remodeling in response to cardiac cell death (220, 221). While more evidence exists to indicate the importance of myofibroblast differentiation and migration in response to myocardial infarction (222, 223), persistent myofibroblasts have been observed in interstitial infiltrate of kidney grafts undergoing CR (224, 225) and early increases in myofibroblasts may be predictive for kidney grafts that will later develop CR (226). Indeed, the lack of characterization of the role of myofibroblasts in CR of cardiac grafts may owe to the ability of myofibroblasts to acquire markers commonly associated with muscle differentiation, which obfuscates their identification in smooth muscle-rich cardiac vasculature (227). Interestingly, increases in myofibroblasts have been observed in response to acute cardiac rejection (214) as well as in response to oxygen flux that may represent conditions of ischemia/reperfusion injury (228).

Interstitial fibrosis has several effects on the myocardium of cardiac grafts that can generally be considered to inhibit function. Further, effects of cardiac myofibroblasts may extend to modulation of electrical impulses in the heart (229, 230). In addition to the effects of myofibroblasts on the production of ECM, fibroblasts can themselves function as inflammatory cells, producing cytokines, chemokines and growth factors (231). Indeed there is considerable evidence for interplay between fibroblasts regulating immune activation and the course of inflammatory responses (232). Further, activated fibroblasts can express costimulatory CD40 (233) and cardiac myofibroblasts respond to inflammatory cytokines such as tumor necrosis factor-α, IL-1, IL-6, and TGFβ (234, 235). Thus, fibroblast activation and cytokine production may provide an autocrine loop

that enhances further infiltration and dysfunction through the production of immune cytokines, chemokines and growth factors.

Factors associated with CR

TGFβ

Multiple factors have been associated with its onset and progression, especially TGFβ. TGFβ overexpression is linked with chronic rejection (236, 237), and may negatively impact graft survival through chemotactic and pro-fibrotic effects (238). TGFβ plays an important role in fibrosis of various causes in multiple organs (239). TGFβ is known to induce the differentiation of cardiac myofibroblasts (240), though this process is dependent upon osteopontin in cardiac and dermal fibroblasts (226). Further, it is likely that cardiac myofibroblast differentiation depends on cell adhesion and the extra domain A containing splice variant of fibronectin as in other myofibroblast precursors (241-243). It should be noted that fibroblasts themselves can make TGFβ (244) and myofibroblasts are rescued from apoptosis by TGFβ (245).

In addition to its deleterious fibrotic effects on the graft, TGFβ-mediates immunosuppressive and anti-proliferative functions that may be indispensable for graft and host survival (246). For example, TGFβ plays a critical role in the induction and function of T regulatory cells (Treg), which are believed to contribute to graft acceptance (247-249). Further, TGFβ inhibits T and B cell proliferation (238) and represses cancers of epithelial cell origin (250). These opposing effects make TGFβ a suboptimal target for CR treatments and have prompted investigation into the downstream mediators of TGFβ

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in CR pathology. Identifying downstream mediators of CR may facilitate the

development of therapeutics that negate the fibrosis-inducing activity of TGFβ while

sparing its anti-inflammatory and anti-proliferative effects.

Connective Tissue Growth Factor (CTGF)

CTGF is induced by TGFβ in multiple cell types (251), including cardiac

myocytes and fibroblasts (252) (Figure 4).
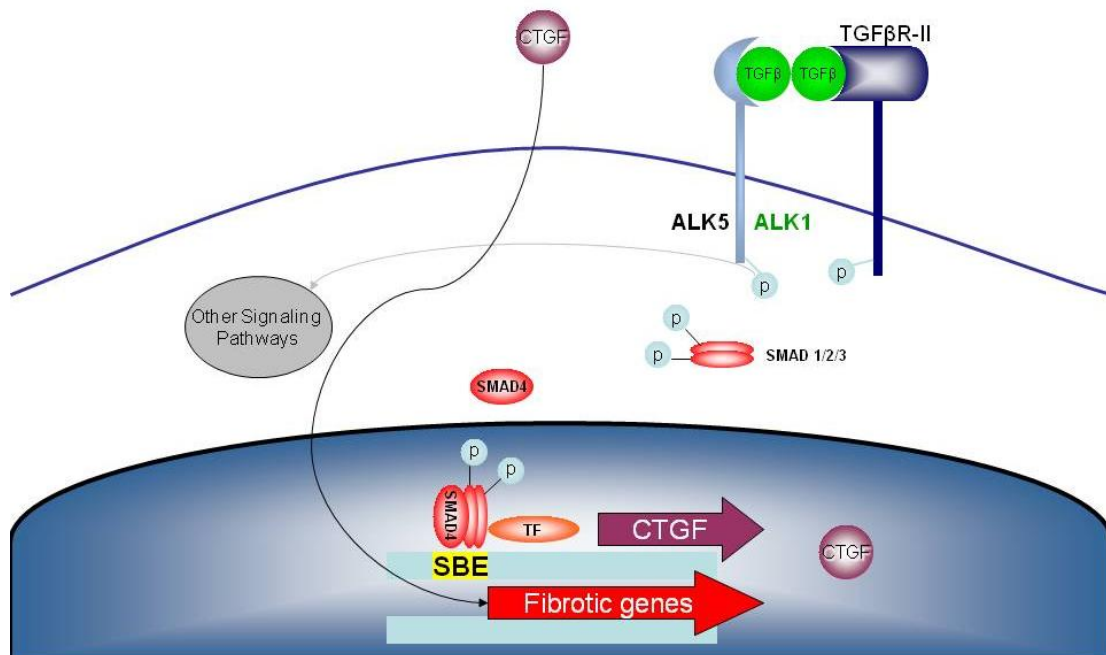
Figure 7 CTGF is induced by TGFβ

CTGF plays an important role in the development of connective tissue as well as the

formation of scar tissue (253, 254), and is upregulated in multiple fibrotic disorders,

including CR of cardiac and kidney grafts (236, 255-257). CTGF mediates multiple pro-

fibrotic effects ascribed to TGFβ including increased ECM production, fibroblast

proliferation, and enhancement of adhesive responses (257). Recently, the ability of CTGF to potently stimulate neointimal hyperplasia associated with vascular injury has been illustrated by findings that exogenous application of CTGF recapitulates the phenotype of TGFβ signaling mediator SMAD3 gene transfer (258). This suggests that CTGF may be a critical mediator of TGFβ effects in vascular injury. Thus, as CTGF is induced by TGFβ and because CTGF mediates pro-fibrotic effects and remodeling effects associated with TGFβ, CTGF has been proposed as a therapeutic target for limiting the deleterious fibrotic effects of TGFβ while sparing its immune-modulatory functions (100, 236, 259).

CTGF induction by TGFβ has been observed in settings of cardiac fibrosis (257), though transduction of syngeneic grafts with TGFβ is insufficient to induce CTGF or CR (236). Hence, TGFβ-mediated induction of CTGF *in vivo* is contextually dependent. One such contextual difference between allogeneic and syngeneic grafts is the development of alloimmune responses which may provide factors that crosstalk with TGFβ signaling (260). One such factor, investigated in this dissertation, is IL-6, a cytokine that modulates the effects of TGFβ in multiple cell types (261-263).

Dissertation Overview

The overarching aim of these studies was to better understand factors influencing the pathology of CR of cardiac grafts. The initial aims were specifically to determine if the beneficial aspects of TGFβ (immunosuppressive and anti-proliferative effects) could be separated from the deleterious aspects (proinflammatory and profibrotic effects) in the biology of CR (Figure 5).
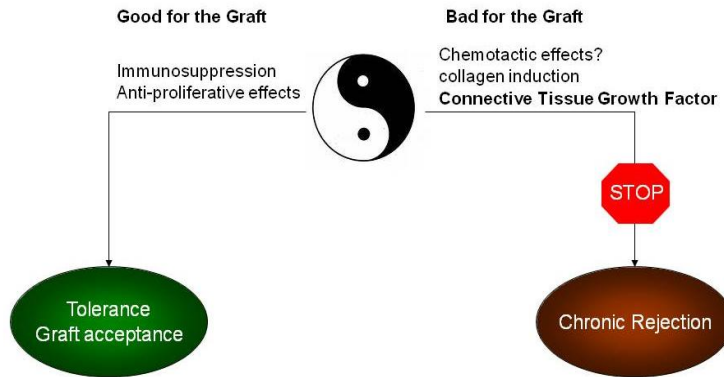
Figure 8 Potential TGFβ Effects in Cardiac Grafts

In the process of investigating the initial aim, these studies have uncovered a critical role

for IL-6 in the induction of CTGF, and CTGF is demonstrated to function as a

downstream mediator of TGFβ-mediated fibrosis. Further, these studies utilized several

neutralization approaches to suggest new therapeutic targets for the treatment of CR of

cardiac grafts.

Chapter III—Materials and Methods

Mice

WT female C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from

Charles River Laboratories (Raleigh, NC). The animals were kept under micro-isolator

conditions. The use of mice for these studies was reviewed and approved by the

University of Michigan's Committee On The Use And Care Of Animals.

Vascularized Cardiac Transplantation

Heterotopic cardiac transplantation was performed as described (264). 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.

In Vivo mAb Therapy

Anti-CD4 (hybridoma GK1.5¸ obtained from American Type Culture Collection,

Manassas, VA), anti-CD40L (hybridoma MR1, kindly provided by Dr. Randy Noelle,

Dartmouth College), and anti-IL-6 mAb (hybridoma MP5-20F3, obtained from American

Type Culture Collection, Manassas VA, with permission of DNAX) mAbs were prepared

by Bio X Cell (West Lebanon, NH). Allograft recipients were transiently depleted of CD4+ cells by i.p. injection of 1 mg of anti-CD4 mAb on days -1, 0, and 7 post transplant (236, 265). For inductive anti-CD40L therapy, allograft recipients were injected i.p. with 1 mg of anti-CD40L on days 0, 1, and 2 post transplant (236, 265). Anti-IL-6 mAb or control rat IgG (Sigma, St. Louis, MO) was administered by i.p. injection of 1 mg on days -1, 1, and 3 and weekly thereafter (265, 266). Allograft recipients treated with anti-CTGF mAb (FG-3019, kindly provided by FibroGen, Inc., San Francisco, CA (267, 268)) or control human IgG (Sigma, St. Louis, MO) received 0.5 mg i.p. twice weekly beginning on day 7 postransplant.
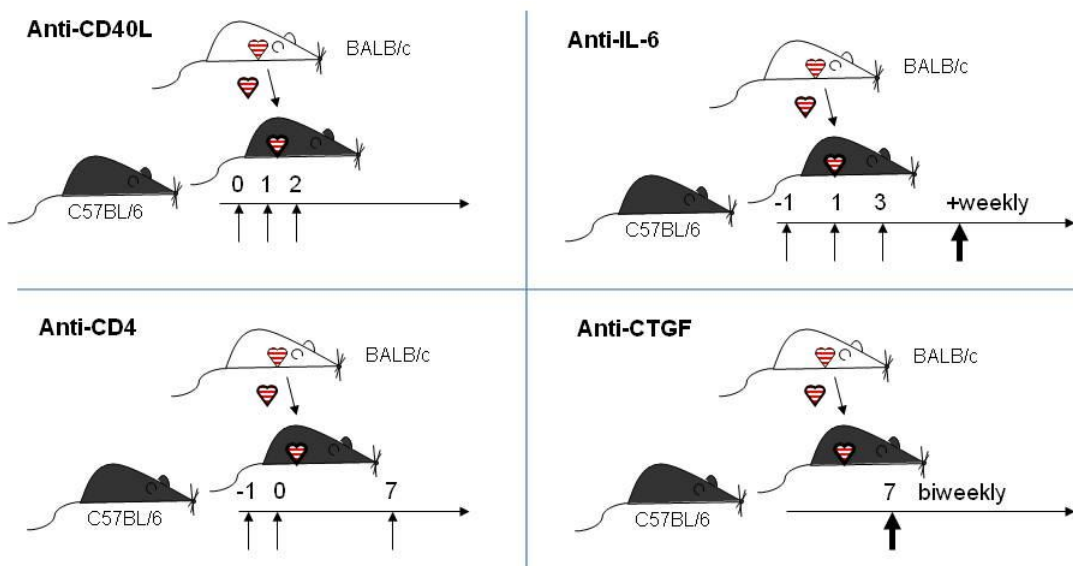
Figure 9 *In vivo* mAb Therapeutic Approaches

2D Echocardiography
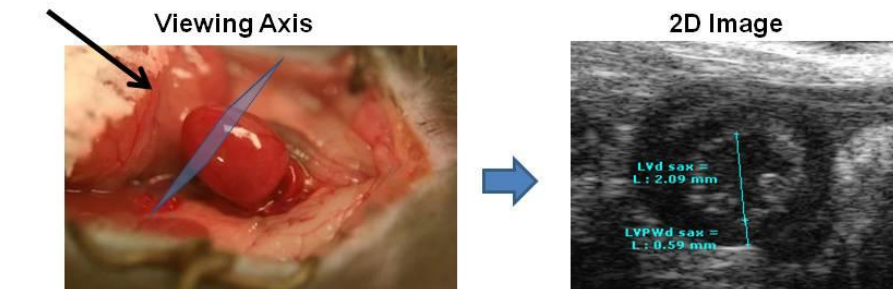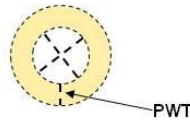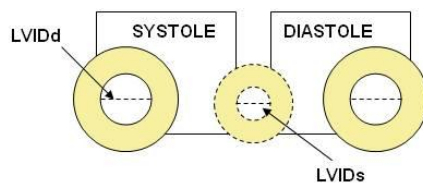
Figure 10 2D Echocardiography Technique

Serial non-invasive echocardiography was performed with a 30 MHz ultrasound probe (Vevo 770, VisualSonics Inc., Toronto, Canada). Mice were anesthetized using inhaled isofluorane. Anatomical and functional changes were assessed in the left ventricle (LV) of heterotopic cardiac grafts by obtaining short-axis views (at the mid-papillary level) of the grafts as reported by Scherrer-Crosbie *et al*. (269). Briefly, echocardiographic images were recorded in real time from both axial and longitudinal axes and saved for subsequent analysis. Measurements of posterior wall thickness (PWT) were performed in diastole and measurements for calculating fractional shortening (FS) were taken in both systole and diastole. This echocardiography

technique was validated by measuring PWT of acutely rejecting BALB/c allografts in C57BL/6 recipients, and in syngeneic C57BL/6 controls as described (269). Echocardiography was performed on allografts in recipients transiently depleted of CD4+ cells (which develop CR), allograft recipients given anti-CD40L mAb therapy (which do not develop CR), and syngeneic C57BL/6 graft controls. Serial echocardiography was performed to evaluate PWT, FS and left ventricle ejection fraction (LVEF). Measurements were obtained on day 7 and then weekly from day 21 to day 49 post transplant.

Adenoviral-mediated transduction of cardiac grafts

Transduction was performed as previously described (236, 270, 271). Briefly, cardiac grafts were perfused via the aorta with 5×10^8 pfu of E1/E3 deleted adenoviral vectors encoding the active form of human TGFβ1 (AdTGFβ) (236, 270), human CTGF (AdCTGF) (272), or beta-galactosidase (Adβgal) (236, 270, 271). Following perfusion, donor grafts were placed in iced Ringer's solution for 1 hour prior to transplantation. Previous studies with Adβgal have revealed a patchy distribution of transgene expression by both cardiac and vascular cells that persists for at least 8 weeks post transplant (271).

Morphometric analysis of cardiac graft fibrosis and hypertrophy

Graft fibrosis was quantified by morphometric analysis of Masson's trichrome stained sections using iPLab software (Scanalytics Inc., Fairfax, VA). Mean fibrotic area was calculated from 10 to 12 areas per heart section analyzed at 200X magnification (265, 273). To quantify cardiomyocyte area as a measure of hypertrophy, digital outlines were drawn around at least 80 cardiomyocytes from views of H&E stained sections at

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200X magnification. Areas within outlines were quantified using SCION IMAGE Beta

4.0.2 software (Scion Corporation, Frederick, MD) to measure cardiomyocyte cell size

(274). A minimum of 8 hearts were analyzed per group for both analysis techniques.

Quantitative real time PCR

Graft RNA was isolated by homogenizing tissues in TRIzol reagent (Invitrogen,

Carlsbad, CA) as per manufacturer's protocol. Five µg of total RNA were reverse

transcribed using Oligo dT, dNTPs, MMLV-RT (Invitrogen, Carlsbad, CA), RNAsin

(Promega, Madison, WI) in PCR Buffer (Roche, Indianapolis, IN). Resulting cDNA was

purified by a 1:1 extraction with phenol/chloroform/isoamyl (25:24:1) then precipitated

in one volume 3M NaOAc and two volumes absolute ethanol. Levels of atrial natriuretic

peptide (ANP), CTGF, IL-6, TGFβ, IL-17, and T cell receptor β constant region (TCRβ)

message were determined by quantitative real time PCR using iQ SYBR master mix

(Bio-Rad, Hercules, CA) in a Rotor-Gene 3000 thermocycler (Corbett Life Science, San

Francisco, CA). Expression levels were determined relative to GAPDH using the Rotor-

Gene Comparative Concentration utility.

Primer sequences were as follows:

ANP (*Nppa*) forward 5' GGAGGTCAACCCACCTCTG 3'

ANP (*Nppa*) reverse 5' GCTCCAATCCTGTCAATCCTAC 3'

CTGF (*Ctgf*) forward 5' GGAAAACATTAAGAAGGGCAAAA 3'

CTGF (*Ctgf*) reverse 5' CCGCAGAACTTAGCCCTGTA 3'

GAPDH (*Gapdh*) forward 5' CTGGTGCTGAGTATGTCGTG 3'

GAPDH (*Gapdh*) reverse 5' CAGTCTTCTGAGTGGCAGTG 3'

IL-6 (*Il6*) forward 5' CGTGGAAATGAGAAAAGAGTTGT 3'

IL-6 (*Il6*) reverse 5' TCCAGTTTGGTAGCATCCATC 3'

TGFβ (*Tgfb 1*) forward 5' CCTGAGTGGCTGTCTTTTGAC 3'

TGFβ (*Tgfb 1*) reverse 5' CCTGTATTCCGTCTCCTTGGT 3'

IL-17 (*Il17a*) forward 5' GGACTCTCCACCGCAATGA 3'

IL-17 (*Il17a*) reverse 5' GACCAGGATCTCTTGCTGGA 3'

TCRβ (*Tcrb-C*) forward 5' CTGCCAAGTGCAGTTCCAT 3'

TCRβ (*Tcrb-C*) reverse 5' GGCCTCTGCACTGATGTTCT 3'

Flow cytometry

Splenocytes were labeled with FITC-conjugated anti-CD3, PE-conjugated anti-CD4, and CY5-conjugated anti-CD8 (PharMingen San Jose, CA). Cell analyses were performed on lymphocytes gated using forward vs. side scatter using a Becton Dickinson FACSCalibur (San Jose, CA).

Statistical analysis

Statistical significance was calculated using an unpaired t-test with Welch's correction. *p* values ≤0.05 were considered statistically significant.

Chapter IV—Results

Experimental system

BALB/c cardiac allografts in C57BL/6 recipients receiving anti-CD40L mAb continue to function for >60 days and do not develop CR, unless transduced with TGFβ (236). In contrast, allografts in recipients transiently depleted of CD4+ cells develop CR as CD4+ cells begin to repopulate the periphery between 3 and 4 weeks following initial depletion (236, 275-277). Hence, we compared events that occurred in these two settings to identify critical elements associated with the progression of CR. Specifically, we assessed anatomical and functional echocardiographic parameters, cardiac hypertrophy, intragraft IL-6 expression, and graft fibrosis in CR. Echocardiographic and histologic analysis revealed that day 30 post transplant represents a critical point in this CR model as extensive graft hypertrophy and fibrosis are present at this time and are followed by degradation of cardiac contractility (265). Therefore, for most experiments grafts were assessed at day 30 post transplant in these studies.

Fibrosis in allografts undergoing CR

Fibrosis was most prominent among grafts transiently depleted of CD4+ cells that develop CR as assessed by morphometric trichrome analysis (p<0.0001, Figure 6). Similarly, intragraft collagen αI transcript levels were greatest in CR grafts. No

significant changes were found in either graft fibrosis (Figure 6B) or collagen αI

transcripts (Appendix 1) between day 30 and day 50 post transplant in grafts undergoing

CR, indicating a full fibrotic response by day 30 in this model.
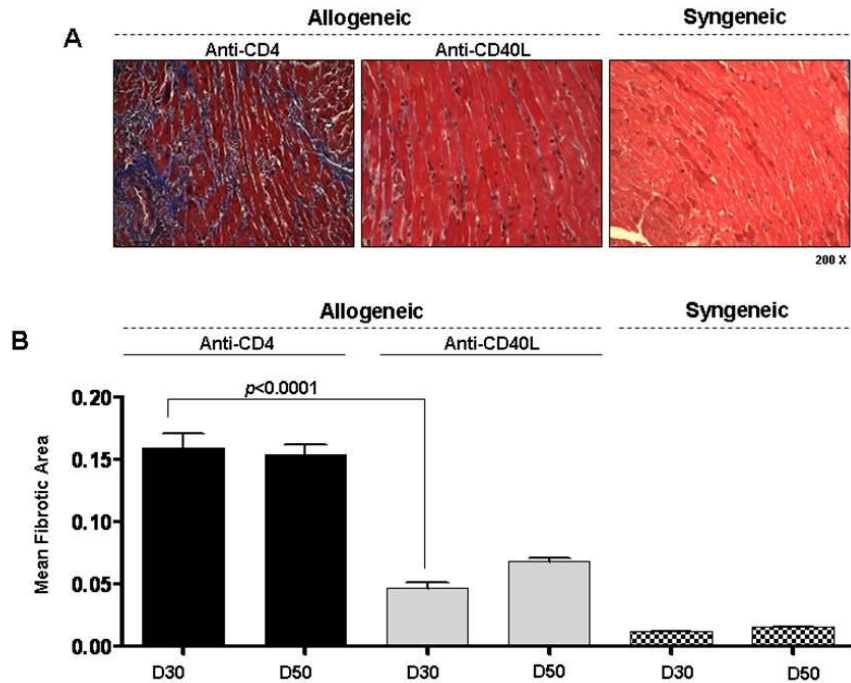
Figure 11 Fibrosis is Increased in Cardiac Grafts Undergoing CR. (A) Representative Masson's trichrome stains, in which fibrotic tissue stains blue, of heterotopic cardiac grafts at day 30 post transplant (200x magnification). Cardiac allografts from recipients transiently depleted of CD4+ cells (Anti-CD4) or allograft recipients receiving anti-CD40L mAb therapy (Anti-CD40L) are shown along with syngeneic control grafts. (B) Morphometric analysis of trichrome staining at day 30 and 50 post transplant in groups from (A). Bars represent the combined mean + S.E.M. of fibrotic (blue) area of 10–12 frames of view per heart taken from no fewer than 6 different cardiac grafts per group.

Echocardiographic assessment of the progression of CR

Serial echocardiography was performed to determine the anatomical and

functional changes associated with the progression of CR. This echocardiographic

technique was validated by assessment of unmodified syngeneic and allogeneic grafts as

previously described (269). Increases in PWT in acutely rejecting allogeneic grafts as

well as diminished thickening in control syngeneic grafts matched previously reported

results (269) in both trend and magnitude (data not shown). It should be noted that LV

intra-cavity thrombosis was observed in all groups. Following the first week post

transplant, intra-cardiac thrombus retraction (toward the apex) occurred, allowing lucid

visualization of all LV parameters as endocardial contour definition was clearly

demarcated.

Echocardiography was used to monitor allografts in recipients transiently depleted

of CD4+ cells, which undergo CR. Although it is true that heterotopic cardiac grafts

contract against a reduced load, identical surgical procedure between groups assures

similar loading conditions required for comparative assessments of graft hypertrophy and

fibrosis. Allografts undergoing CR were compared to allografts in recipients receiving

anti-CD40L therapy (that do not undergo CR) and also to syngeneic grafts (Figure 7).

PWT was greatest in grafts undergoing CR from day 7 through day 49 post transplant,

peaking at approximately day 35 (Figure 7A). The increase in PWT among CR grafts

correlated with an increased FS and LVEF (Figure 7). These parameters remained stable

in allograft recipients treated with anti-CD40L mAb, as well as in syngeneic grafts.
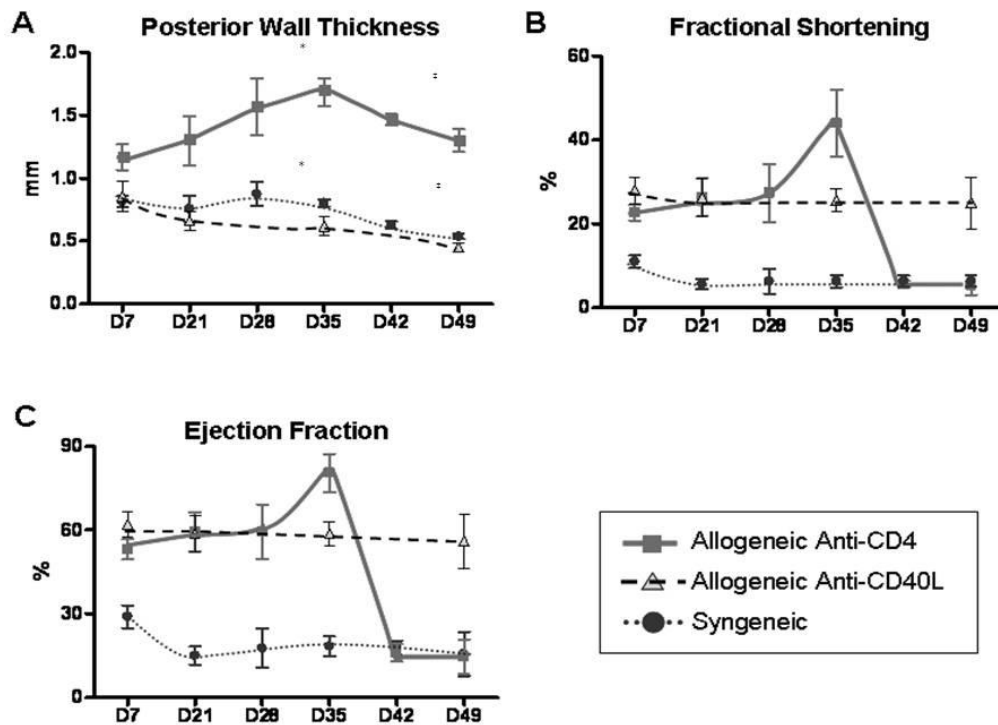
Figure 12 Echocardiographic analysis suggests hypertrophy in grafts undergoing CR. Serial echocardiography was used to monitor anatomical and functional parameters of cardiac allografts in recipients that were transiently depleted of CD4+ cells (Anti-CD4, squares), in allograft recipients receiving anti-CD40L mAb therapy (Anti-CD40L, triangles), or syngeneic graft recipients (circles). Echocardiographic parameters included posterior wall thickness (PWT) (A), fractional shortening (FS) (B), and left ventricle ejection fraction (LVEF) (C). Three transplants per experimental group were followed throughout the duration of the experiment. Individual points represent mean and bars represent ± S.E.M. at the given time points. *Day 35: Anti-CD4 vs. Anti-CD40L, p= 0.004 and Anti-CD4 vs. Syngeneic, p= 0.017. ‡ Day 49: Anti-CD4 vs. Anti-CD40L, p= 0.0126 and Anti-CD4 vs. Syngeneic, p= 0.0146.

Increases in PWT and cardiac functional parameters (FS and LVEF) by day 35 post

transplant in the anti-CD4 treated group suggested an association of cardiac hypertrophy

with CR. Following the peaks in PWT, FS and LVEF at day 35 post transplant,

deterioration of graft contractility occurred by day 42 (Figure 7B and C). Together, the

changes in graft contractile parameters between days 30 and 50 post transplant (Figure

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7B and C) and the consistency of graft fibrotic area over the same time period (Figure

7B) indicated that day 30 represents a critical time point in this CR model.

Cardiomyocyte hypertrophy and elevated IL-6 expression in grafts undergoing CR

Echocardiography revealed an anatomical increase in PWT as well as functional

increases in FS and LVEF, factors consistent with a hyperdynamic state, as is seen with

LV hypertrophy. Cardiac hypertrophy has been defined as an increase in heart mass

reflective of increased cell size rather than cell number (278). To verify the presence of

hypertrophy, cardiomyocyte areas were evaluated histologically at day 30 post transplant

(Figures 8A and B). Histologic analysis revealed that cardiomyocyte size was greatest in

CR grafts when compared to allografts in recipients receiving anti-CD40L or syngeneic

grafts ($p<0.0001$). Further, because increased levels of ANP expression have been

correlated with cardiac hypertrophy (279, 280), intragraft expression of ANP was

determined with quantitative real time PCR. Intragraft ANP expression correlated with

cardiomyocyte size in CR grafts, allograft recipients receiving anti-CD40L mAb, and

syngeneic grafts (Figure 8C).

Figure 13 Histologic confirmation of cardiac hypertrophy in CR grafts. (A) H&E stains of day 30 post transplant cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4) or recipients receiving anti-CD40L mAb therapy (Anti-CD40L) as well as syngeneic control graft recipients. Arrows highlight representative cardiac myocytes. (B) Cardiomyocyte areas were quantified using morphometric analysis. Bars represent mean + S.E.M. of area measurements taken from at least 80 cardiomyocytes per heart from 5 different hearts per group. (C) Intragraft message levels of atrial natriuretic peptide (ANP), a marker of cardiac hypertrophy, were measured with quantitative real time PCR in cardiac grafts from recipients in (B) at day 30 post transplant. Bars represent mean + S.E.M. of 3–5 grafts per group.

Since cardiac hypertrophy coincided with increased fibrosis in CR allografts, we hypothesized that there might be a common immunologic mediator of both processes. IL-6 has been linked to cardiac hypertrophy (281) and collagen production (282). Therefore, IL-6 expression was assessed in allografts undergoing CR, as well as in control grafts. IL-6 message levels were highest in CR grafts at all time points (Figure 9). While intragraft IL-6 message levels progressively decreased after day 7 among allografts treated with anti-CD40L and syngeneic grafts, CR grafts exhibited a significant resurgence of IL-6 expression (p=0.0141) on day 30 post transplant (Figure 9). Thus, a

correlation was observed between CD4+ cell return (275, 276), intragraft IL-6

expression, cardiomyocyte hypertrophy, and graft fibrosis. This prompted our

investigation into the role of IL-6 in the initiation of hypertrophy and fibrosis associated

with cardiac CR.
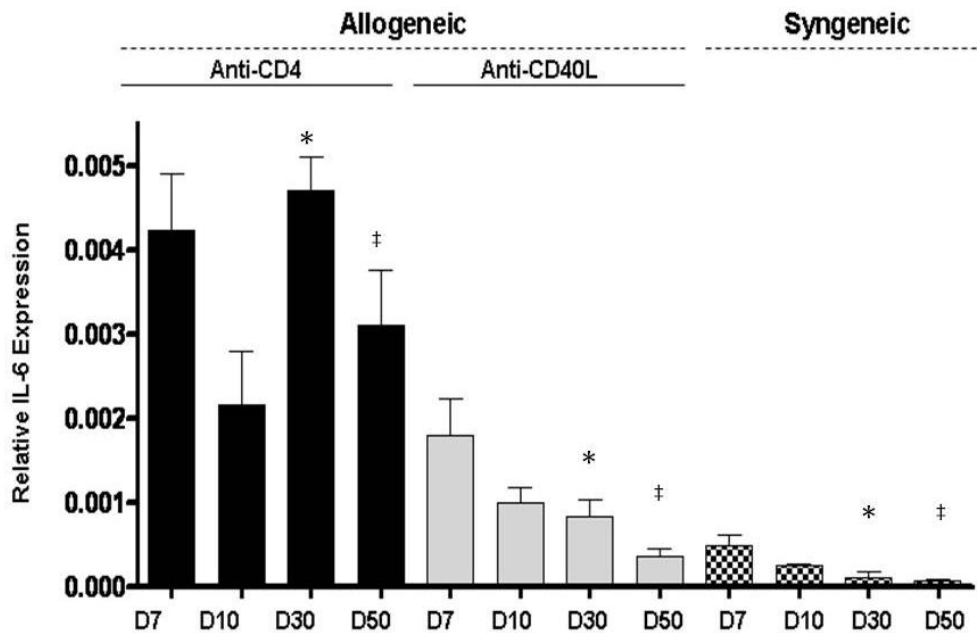
Figure 14 Increased intragraft IL-6 expression in grafts undergoing CR. IL-6 message levels in cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4), recipients receiving anti-CD40L mAb therapy (Anti-CD40L), or from syngeneic graft recipients were determined at given time points using quantitative real time PCR. Bars represent mean + S.E.M. of 3–6 grafts per group which were harvested at the given time points. *Day 30 Anti-CD4 vs. Anti-CD40L, p= 0.0141 and Anti-CD4 vs. Syngeneic, p= 0.0083. ‡ Day 49: Anti-CD4 vs. Anti-CD40L, p= 0.0150 and Anti-CD4 vs. Syngeneic, p= 0.0102.

Neutralizing IL-6 reduces cardiac hypertrophy and fibrosis

To better understand the role of IL-6 in promoting hypertrophy and fibrosis in

CR, allograft recipients transiently depleted of CD4+ cells were treated with neutralizing

anti-IL-6 mAb or control rat IgG. In recipients treated with anti-IL-6 mAb,

cardiomyocyte area ($p<0.0008$) and intragraft ANP transcript levels ($p=0.0002$) were

significantly reduced compared to recipients treated with control rat IgG (Figure 10).
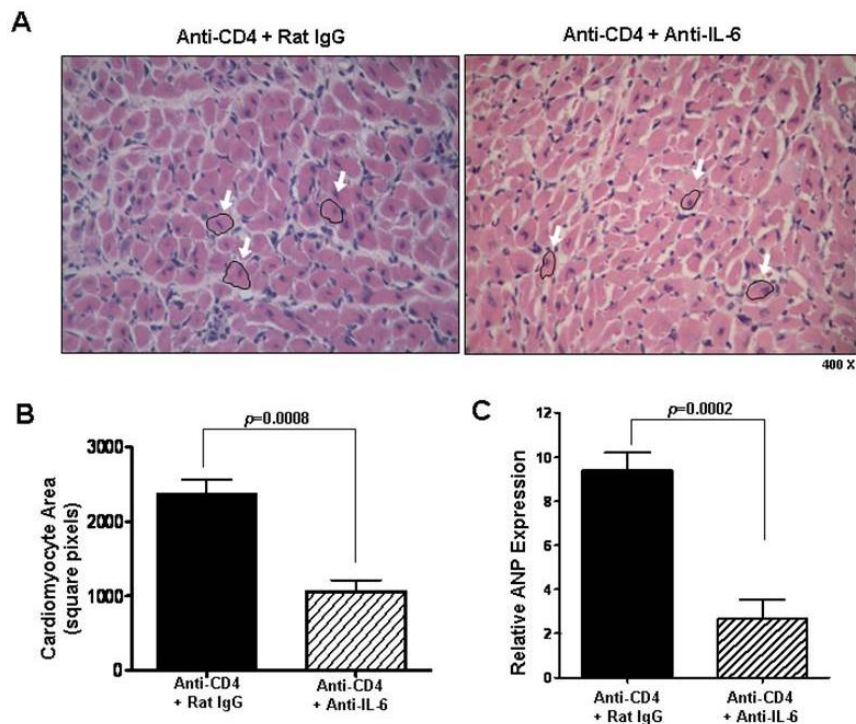
Figure 15 Neutralizing IL-6 reduces cardiomyocyte hypertrophy. (A) Representative views of H&E stained sections of day 30 post transplant cardiac allografts taken from recipients that were transiently depleted of CD4+ cells which also received either control rat IgG (Anti-CD4 + Rat IgG) or neutralizing IL-6 mAb (Anti-CD4 + Anti-IL-6). Arrows highlight representative cardiomyocytes. (B) Cardiomyocyte area quantitation of groups described in (A). Bars represent mean + S.E.M. of area measurements taken from >80 cardiomyocytes per heart in each of 5 different grafts per group. (C) Intragraft atrial natriuretic peptide (ANP) message levels in grafts described in (A) were determined using quantitative real time PCR. Bars represent mean + S.E.M. of tissue harvested from at least 5 different transplants per group.

Decreases in these parameters implicate IL-6 induces cardiac hypertrophy and

demonstrate that its neutralization can ameliorate cardiac hypertrophy associated with

CR. Furthermore, IL-6 neutralization reduced graft fibrotic tissue area ($p<0.0001$, Figure

12A, B) and intragraft expression of collagen αI ($p=0.0019$, Figure 12C) compared to

control grafts.

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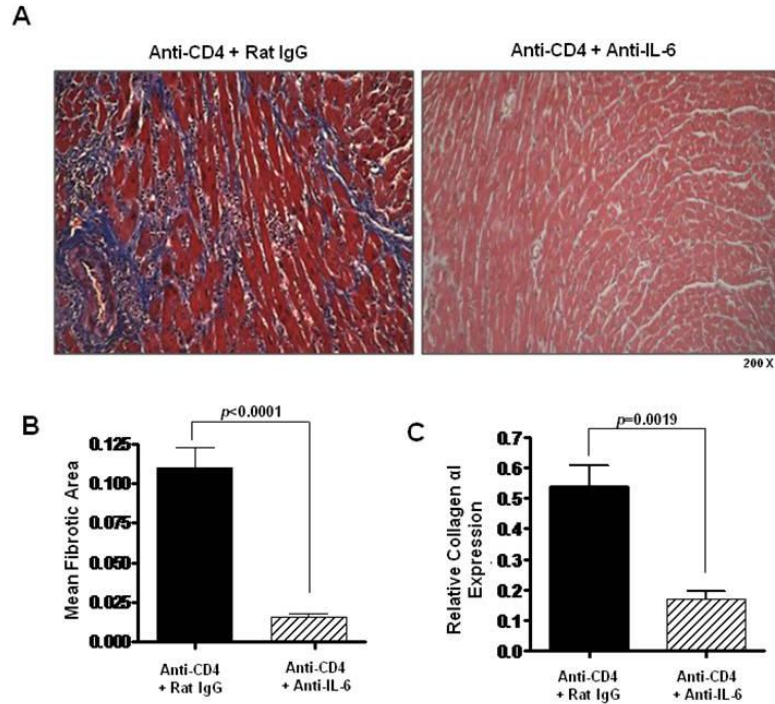
Figure 16 Neutralizing IL-6 reduces cardiac fibrosis. (A) Representative views of Masson's trichrome staining of day 30 post transplant cardiac allografts taken from recipients transiently depleted of CD4+ cells which also received either control rat IgG antibodies (Anti-CD4 + Rat IgG) or neutralizing IL-6 mAb (Anti-CD4 + Anti-IL-6). (B) Morphometric analysis of graft fibrosis in groups described in (A). Bars represent the combined mean + S.E.M. of fibrotic (blue) area from at least 10 different frames of view from each of at least 6 different cardiac grafts. (C) Intragraft collagen αI message levels in cardiac allografts taken from recipients described in (B) were determined using quantitative real time PCR. Bars represent mean + S.E.M. of tissue harvested from at least 6 different transplants per group.

Neutralizing IL-6 normalizes graft functional parameters

Since neutralizing IL-6 ameliorated both hypertrophy and fibrosis as determined by histologic and gene expression analyses, we assessed the effects of anti-IL-6 on anatomical and functional parameters of chronically rejecting hearts. Echocardiography revealed that neutralizing IL-6 prevented the increase in PWT and normalized FS and LVEF parameters in grafts which would otherwise undergo CR (Figure 13).

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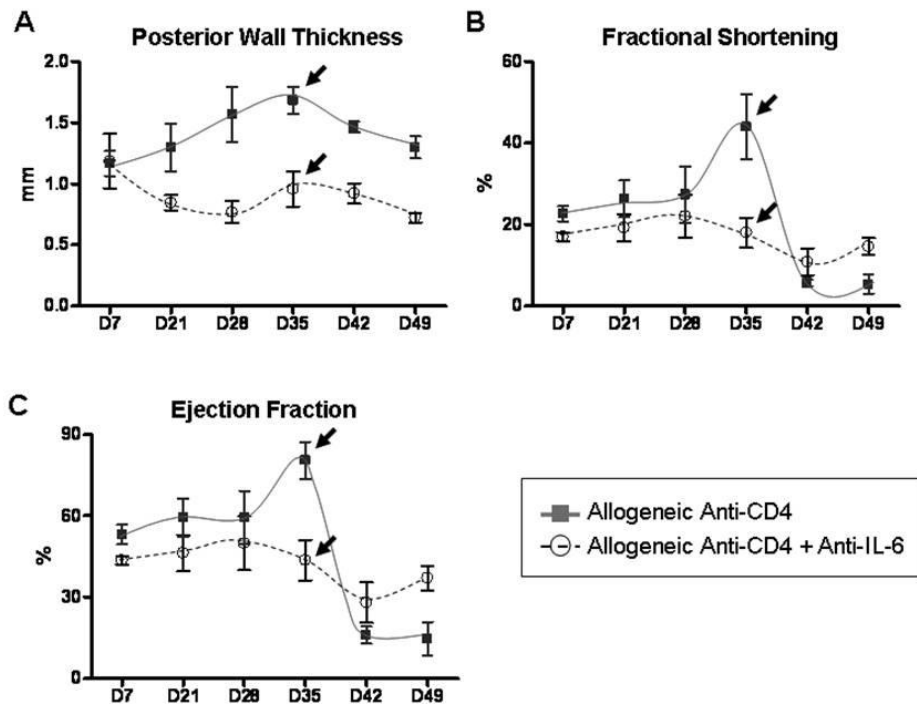
Figure 17 Neutralizing IL-6 ameliorates contractile parameter aberrations associated with CR. Serial echocardiography was used to monitor anatomical and functional parameters in cardiac allograft recipients that were transiently depleted of CD4+ cells which also received neutralizing IL-6 mAb (Anti-CD4 + Anti-IL-6, circles). Results are plotted against 3 recipients that were transiently depleted of CD4+ cells (Anti-CD4, squares) from Figure 2. Echocardiographic parameters included posterior wall thickness (PWT) (A), fractional shortening (FS) (B), and left ventricle ejection fraction (LVEF) (C). For the anti-IL-6 mAb treated group, 5 transplants were followed throughout the duration of the experiment. Individual points represent mean and bars represent ± S.E.M. of grafts analyzed at the given time point.

Thus, hypertrophy, fibrosis and subsequent deterioration of graft contractility were ameliorated in grafts whose recipients were treated with anti-IL-6 mAb. These are the first data to demonstrate that IL-6 may provide a therapeutic target for preventing CR.

IL-6 neutralization does not inhibit CD4+ cell repopulation of the periphery

In this model of CR, CD4+ cells begin to repopulate the periphery between 3 and 4 weeks post-transplant (275, 276). To ascertain the effect of IL-6 neutralization on

CD4+ cell repopulation, flow cytometry was performed on splenocytes taken from animals receiving anti-IL-6 mAb or control rat IgG. Both treatment groups had similar percentages of CD4+ cells on day 30 or day 50 post transplant (Figure 14), indicating that neutralizing IL-6 does not prevent CD4+ cell repopulation of the periphery.
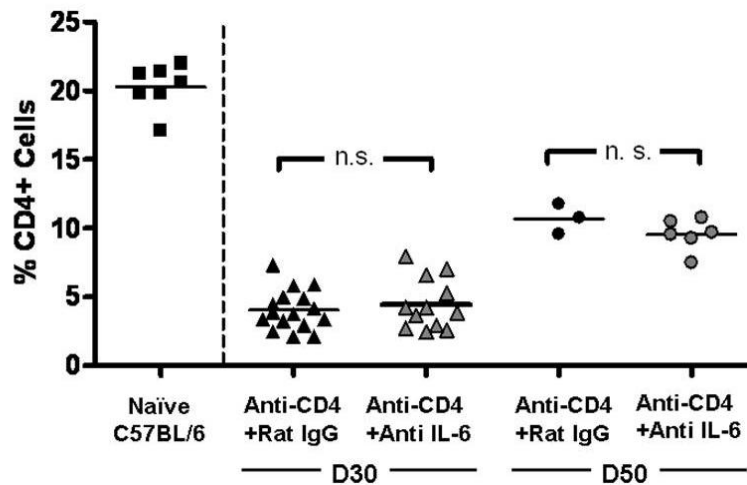
Figure 18 Neutralizing IL-6 does not prevent CD4+ cell repopulation in cardiac allograft recipients. Flow cytometric analysis was performed on splenocytes harvested from naïve mice or allograft recipients that were transiently depleted of CD4+ cells which also received either control rat IgG antibodies (Anti-CD4 + Rat IgG) or neutralizing IL-6 mAb (Anti-CD4 + Anti-IL-6). Splenocytes were harvested from cardiac allograft recipients on day 30 (triangles) or day 50 (circles) post transplant. Lines represent group mean, n.s. = not significant.

Elevated intragraft TGFβ, IL-6, and CTGF expression correlate with CR

Transduction of allografts, but not syngeneic grafts, with TGFβ is sufficient to induce CTGF and CR (236), indicating the involvement of an immune component in TGFβ-mediated fibrosis. This is further supported by the identification of IL-6 as a critical inducer of CR (Figures 10-12, 15 and 16B, (265)).
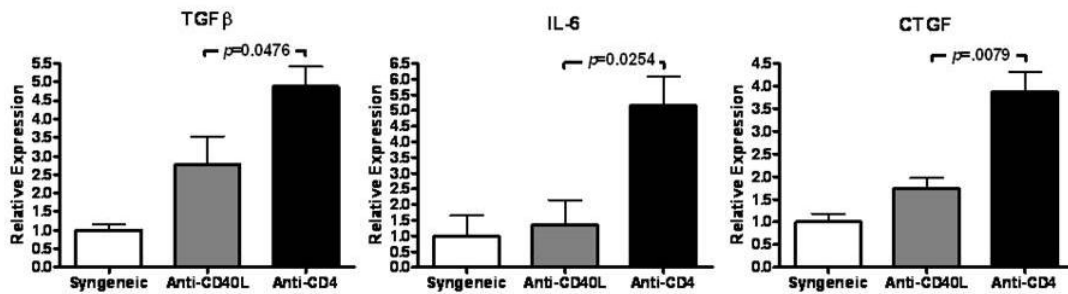
Figure 19 Elevated intragraft expression of TGFβ, IL-6, and CTGF in cardiac allografts undergoing CR. TGFβ, IL-6, and CTGF message levels were determined at day 30 post transplant using quantitative real time PCR in syngeneic cardiac grafts, cardiac allografts from recipients treated with anti-CD40L mAb therapy (Anti-CD40L), or cardiac allografts whose recipients were transiently depleted of CD4+ cells (Anti-CD4). Bars represent mean + S.E.M. of 4-9 grafts with expression relative to GAPDH normalized to the syngeneic group.

Hence, the *in vivo* interactions of TGFβ, CTGF, and IL-6 in CR were the focus of this study. TGFβ, CTGF, and IL-6 transcripts were measured in grafts whose recipients were transiently depleted of CD4+ cells, which develop CR, and compared to allografts whose recipients were treated with anti-CD40L, which do not develop CR, or untreated syngeneic grafts. Intragraft levels of TGFβ, IL-6, and CTGF were significantly increased (p=0.0476, p=0.0254, and p=0.0079 respectively) in cardiac allografts whose recipients were transiently depleted of CD4+ cells than in grafts whose recipients were treated with anti-CD40L or syngeneic controls (Figure 15). Thus, the upregulation of all three cytokines was observed in grafts undergoing CR.

Forced expression of CTGF or TGFβ promotes allograft fibrosis

To determine whether exogenous expression of CTGF promotes cardiac fibrosis, allografts and syngeneic grafts were transduced with AdCTGF. AdCTGF transduction of allografts in recipients treated with anti-CD40L caused a significant increase in fibrotic

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area by day 30 post transplant compared to allografts with control virus (Figure 15A).
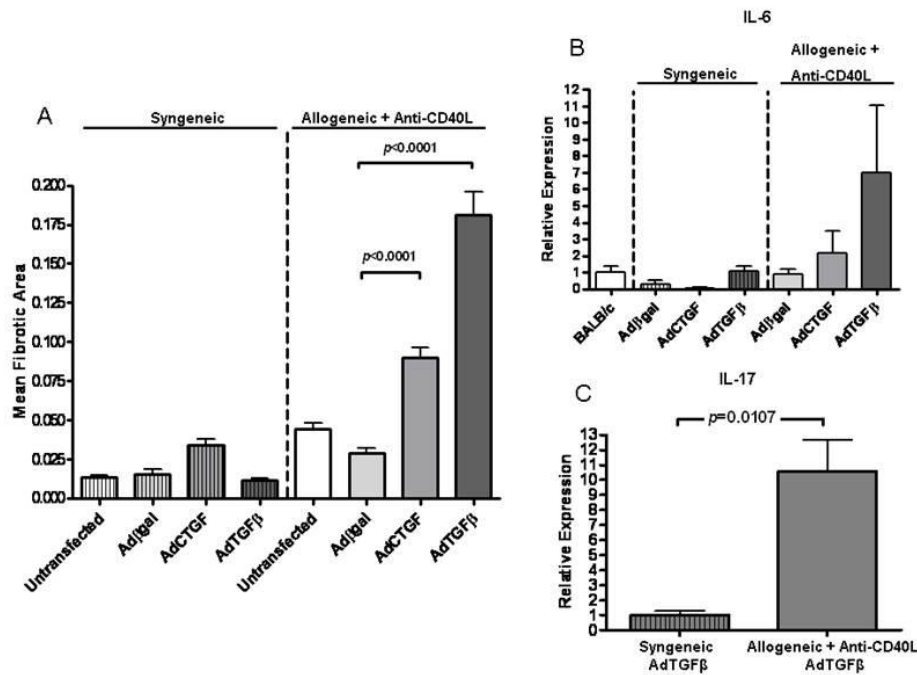
Figure 20 Forced expression of TGFβ or CTGF promotes allograft fibrosis. (A) Morphometric analysis of Masson's trichrome staining at day 30 post transplant in cardiac grafts that were left untransduced or transduced with adenoviral vectors encoding βgal (Adβgal), CTGF (AdCTGF), or TGFβ (AdTGFβ) prior to grafting into syngeneic recipients or allogeneic recipients treated with anti-CD40L. Bars represent the combined mean + S.E.M. of fibrotic (blue) area of 10-12 frames of view per heart taken from 5 to 12 different cardiac grafts per group. (B) Intragraft IL-6 message levels were determined at day 30 post transplant using quantitative real time PCR in groups from (A). Bars represent mean + S.E.M. of at least 4 hearts per group with expression relative to GAPDH normalized to naïve, untransplanted BALB/c hearts. (C) Intragraft IL-17 message levels were determined using quantitative real time PCR in syngeneic grafts transduced with AdTGFβ or allogeneic grafts transduced with AdTGFβ whose recipients received anti-CD40L treatment. Bars represent mean + S.E.M. of at least 5 independent hearts per group with expression relative to GAPDH normalized to the naïve BALB/c group.

In contrast, syngeneic grafts transduced with AdCTGF had similar levels of fibrosis to

controls. It should be noted that the mean fibrotic area for AdCTGF-transduced

allografts was less than in hearts transduced with AdTGFβ, consistent with previous

descriptions in lung transductions (283). This difference could not be accounted for by

differences in transgene expression levels, as AdTGFβ and AdCTGF expression were

comparable in these studies as determined by real time PCR (Appendix 2). Thus, while

forced expression of either TGFβ or CTGF promoted cardiac allograft fibrosis, they did

so to different extents (Figure 15) This could in part be due to TGFβ induction of

endogenous CTGF expression (236, 252, 284), thereby producing an additive effect.

It has been observed that TGFβ and CTGF are potently fibrotic in tandem while

less fibrotic individually (285, 286). Therefore, we asked whether co-transduction of

both TGFβ and CTGF vectors would induce fibrosis and CR in syngeneic grafts. No

increases in fibrosis were observed upon co-transduction of syngeneic grafts compared to

single virus transduction (Appendix 3). Thus, while injection of TGFβ and CTGF

synergize to cause fibrotic responses in the skin (286), forced expression of both was

insufficient to induce fibrosis or CR in syngeneic cardiac grafts, further supporting the

requirement of an immune component.

We next considered whether the greater fibrotic activity of AdTGFβ relative to

AdCTGF could be due to immunologic effects. TGFβ is chemotactic for multiple

immune cell types (238) that are able to produce IL-6, which we have recently reported to

play a critical role in CR (265). Therefore we asked whether differences in intragraft IL-

6 expression might account for these disparate outcomes. IL-6 transcript levels exhibited

a suggested increase in AdTGFβ, but not AdCTGF transduced allografts whose recipients

received anti-CD40L therapy. No increases in IL-6 expression were observed in

AdTGFβ or AdCTGF-transduced syngeneic grafts (Figure 15B).

TGFβ and IL-6 have been implicated in the development of Th17 responses

(261), which have recently been linked to CR (287, 288). Hence, we assessed the

expression of IL-17 in allogeneic and syngeneic grafts transduced with AdTGFβ. IL-17 expression was significantly greater (p=0.0107) in allografts than in syngeneic grafts (Figure 15C) while IL-17 expression was similar in allogeneic and syngeneic grafts transduced with AdCTGF. Thus, increased IL-17 and CTGF transcript levels may promote fibrosis associated with AdTGFβ-transduced allografts, but not AdTGFβ-transduced syngeneic grafts that do not develop fibrosis.

IL-6 neutralization reduces intragraft CTGF and IL-17 transcripts

The association between TGFβ, IL-6, and CTGF (Figure 15) may be strengthened by previous reports that IL-6 enhances TGFβ signaling by altering receptor localization in the cell membrane (263) and that IL-6 can alter the outcome of TGFβ signaling (261, 262). Indeed, we have previously reported that IL-6 neutralization prevents CR of cardiac allografts (265). We therefore asked whether IL-6 neutralization would inhibit CTGF or IL-17 expression (Figure 16).
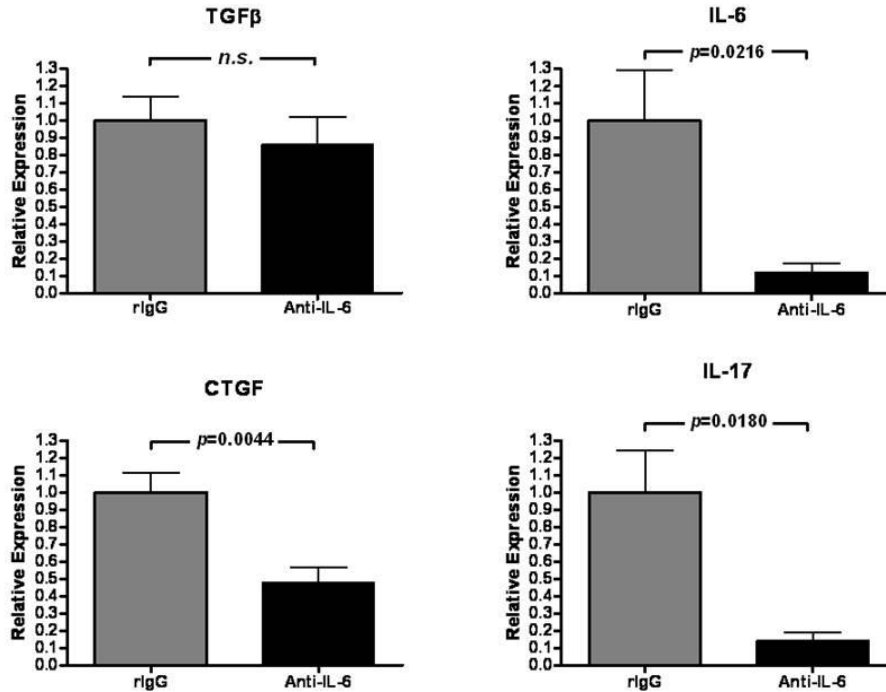
Figure 21 IL-6 neutralization reduces expression of IL-6, IL-17, and CTGF but not TGFβ in cardiac allografts undergoing CR. Intragraft IL-6, IL-17, CTGF, and TGFβ message levels were determined at day 30 post transplant using quantitative real time PCR in cardiac allograft recipients that were transiently depleted of CD4+ cells and received either neutralizing anti-IL-6 (Anti-IL-6) or control rat IgG (rIgG). Bars represent mean + S.E.M. of 6-8 grafts per group with expression relative to GAPDH normalized against rIgG-treated contols.

In allografts whose recipients were transiently depleted of CD4+ cells, treatment with

anti-IL-6 mAb significantly reduced intragraft IL-6, IL-17, and CTGF expression

(p=0.0216, p=0.0044, and p=0.0180 respectively) compared to control antibody

treatment. In contrast, TGFβ expression levels remained unchanged (Figure 16). Thus,

IL-6 promotes intragraft IL-6, IL-17, and CTGF expression.

CTGF neutralization ameliorates allograft fibrosis

To determine if CTGF neutralization would inhibit the fibrosis associated with

CR, allograft recipients that were transiently depleted of CD4+ cells were treated with

neutralizing anti-CTGF mAb or control antibody. Anti-CTGF mAb significantly reduced

fibrotic area ($p<0.0001$, Figures 17A and B), but not intragraft TGFβ, CTGF, or IL-6

transcripts (Figure 17C). These observations support a role for CTGF as a downstream

mediator of fibrosis associated with CR.
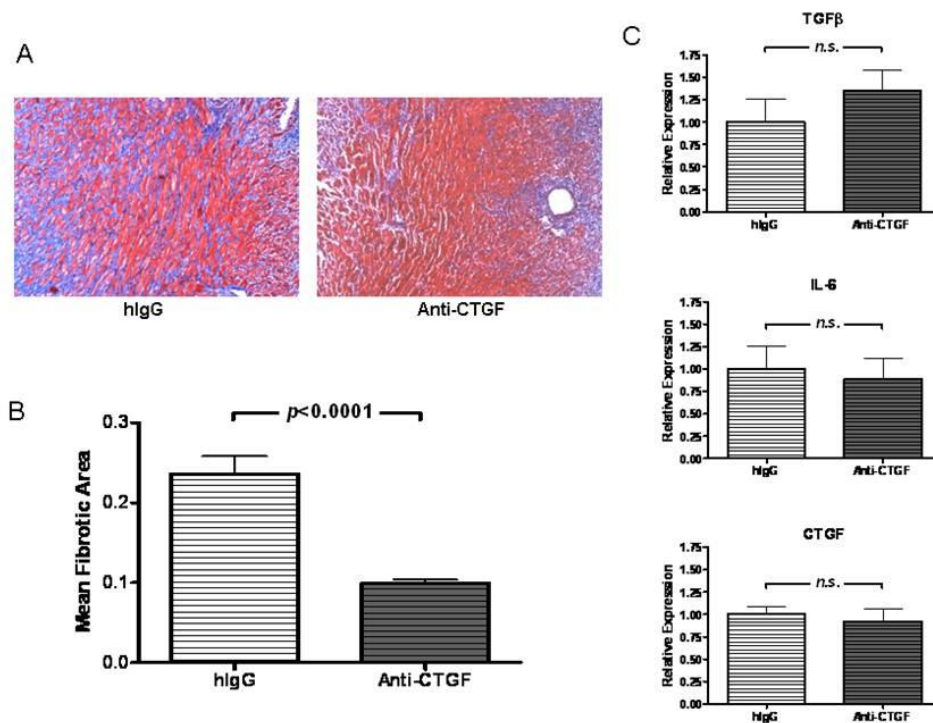
Figure 22 CTGF neutralization ameliorates fibrosis. (A) Representative sections of Masson's trichrome stains, in which fibrotic tissue stains blue, of cardiac allografts from recipients transiently depleted of CD4+ cells (Anti-CD4) at day 30 post transplant in recipients treated with control IgG or neutralizing anti-CTGF mAb (200X magnification). (B) Morphometric analysis of trichrome staining of groups in (A). Bars represent mean + S.E.M. of 10-12 frames of view from each of 6 to 9 hearts.   (C) TGFβ, IL-6, and CTGF message levels were determined at day 30 post transplant using quantitative real time PCR in cardiac allografts described in (A). Bars represent mean + S.E.M. of samples taken from 8-12 different cardiac grafts with expression relative to GAPDH normalized against hIgG-treated controls.

CTGF neutralization decreases cardiomyocyte hypertrophy associated with CR

CTGF can induce cardiomyocyte hypertrophy (289, 290), a function it shares with IL-6 (265). Since IL-6 neutralization inhibited CTGF expression (Figure 16), we assessed the effect of neutralizing CTGF on cardiomyocyte hypertrophy. Anti-CTGF treatment significantly decreased ($p<0.0001$) cardiomyocyte hypertrophy (Figure 18A) and ($p=0.0102$) intragraft expression of ANP (Figure 18B), a molecular marker of cardiac hypertrophy (279, 280). For reference, cardiomyocyte area and ANP expression levels for naïve, untransplanted BALB/c hearts and allografts transplanted into recipients receiving anti-CD40L therapy are depicted.
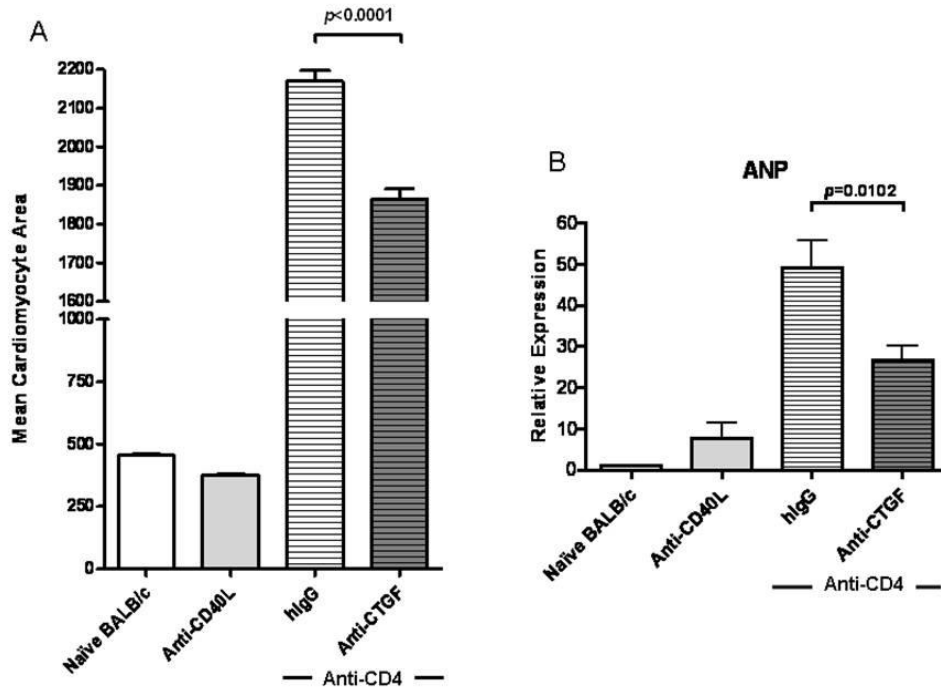
Figure 23 CTGF neutralization ameliorates cardiac hypertrophy in CR grafts. (A) Cardiomyocyte area was quantified from H&E stains of day 30 post transplant cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving CTGF neutralizing mAb (Anti-CTGF) or control antibodies (hIgG), recipients treated with Anti-CD40L mAb, or naïve, untransplanted BALB/c hearts. Bars represent mean + S.E.M. of area measurements taken from ≥100 cardiomyocytes per heart from 5 (naïve BALB/c and Anti-CD40L), 8 (Anti-CD4+hIgG), or 10 (Anti-CD4+Anti-CTGF) different hearts per group. (B) Intragraft message levels of atrial natriuretic peptide (ANP), a marker of cardiac hypertrophy, were quantified with real time PCR in cardiac grafts from groups in (A) at day 30 post transplant. Bars represent mean + S.E.M. of 8-12 grafts per experimental group (Anti-CD4+hIgG or Anti-CTGF) and 4 grafts per control group (Anti-CD40L and naïve BALB/c) with expression relative to GAPDH normalized against the naïve BALB/c hearts.

CTGF neutralization inhibits T cell infiltration of grafts

CTGF promotes integrin-mediated adhesive responses in multiple cell types (291-300) and induces the production of chemokines (301). We therefore asked whether CTGF neutralization might also alter the infiltration of immune cells into grafts undergoing CR. Histologic analysis indicated reduced cellular infiltrate in grafts

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receiving anti-CTGF (Figure 19A). Indeed, a significant decrease (p=0.0238) in TCRβ

constant region expression, a marker of graft infiltrating T cells (302), was observed

(Figure 19B).
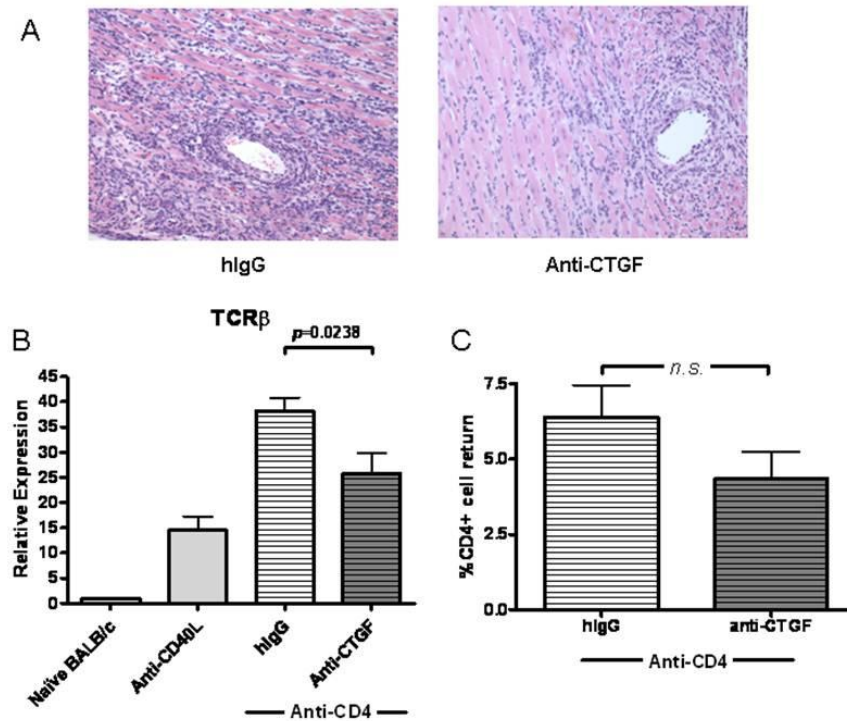
Figure 24 CTGF neutralization limits graft infiltration by T cells in CR grafts. (A) Representative H&E stains of day 30 post transplant cardiac allografts taken from recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving CTGF neutralizing mAb (Anti-CTGF) or control antibodies (hIgG). Stains suggest a reduction in perivascular infiltrate density in grafts treated with neutralizing Anti-CTGF. (B) Intragraft message levels of T cell receptor β constant region (TCRβ) were quantified at day 30 post transplant with real time PCR as a measure of T cell infiltration of allografts in recipients transiently depleted of CD4+ cells (Anti-CD4) and receiving anti-CTGF mAb or control hIgG antibodies, recipients treated with Anti-CD40L mAb, or naïve BALB/c hearts. Bars represent mean + S.E.M. of 8-12 grafts per group with expression relative to GAPDH normalized against the hIgG group. (C) Repopulation of CD4+ cells in the periphery at day 30 post transplant was determined by flow cytometric analysis of splenocytes isolated from graft recipients. Bars represent mean + S.E.M. of the percentage CD4+ cells of the gated cell population in 5 to 7 recipients tested.

To verify that this difference was not due to CTGF neutralization preventing peripheral

repopulation of CD4+ cells, we compared the percentage of CD4+ cells in anti-CTGF

and control treated graft recipients. No significant differences were observed between

these groups (Figure 19C).

Chapter V—Discussion

CR is characterized by the formation of patchy interstitial fibrosis and occlusion of vascular structures accompanied by progressive graft dysfunction (105, 303). Currently, there is no therapeutic which specifically targets CR. Although several factors have been implicated in CR, the etiology and changes associated with the progression of the disease are not fully understood. To better define these changes we monitored the progress of CR using echocardiography.

Echocardiography has been demonstrated to be effective for monitoring acute rejection (269) and long term acceptance (304) of heterotopic mouse cardiac grafts. We used echocardiography to successfully monitor the progression of CR in mice. Further, we have identified critical elements of the disease process, notably that the fibrosis of CR is associated with cardiac hypertrophy and that both processes are driven by IL-6.

Grafts undergoing CR had increased PWT, a reproducible measure of cardiac rejection in mice (269). Such increases in PWT could be caused by immunologic and inflammatory processes associated with CR such as edema, graft-infiltrating cells, cell proliferation and collagen deposition (105, 303). However, echocardiographic functional analyses (FS and LVEF) revealed increased graft contraction coincident with increased PWT by day 35 post transplant in CR grafts (Figure 7). Together, these parameters suggested the presence of cardiomyocyte hypertrophy (305) in grafts

undergoing CR. Manifestations of hypertrophy were followed by significant

deterioration of graft contractility by day 42 post transplant. Such declines in cardiac

contractility mirror events observed in human heart failure (306). These observations

implied that day 30 post transplant represents a critical time point of CR in this mouse

model as it exhibits near terminal amounts of interstitial fibrosis (Figure 6) and increases

in functional parameters indicative of hypertrophy (Figure 7).

Hypertrophy in CR grafts was confirmed by increased cardiomyocyte area (Figure

8B) and elevated levels of ANP (Figure 8C), a marker whose up-regulation has been

linked to cardiac hypertrophy in multiple models (279, 280). Although cardiomyocyte

hypertrophy has been reported in other models of cardiac failure (307-309), our data

suggest a concomitance of cardiac hypertrophy with CR. Since it is widely accepted that

immunologic factors contribute to CR, we considered that an immunologic factor might

be promoting hypertrophy (281). Indeed, it has been demonstrated that increased IL-6

signaling is sufficient to induce cardiac hypertrophy and ventricular wall thickening *in

vivo* (310). It should be noted that while sufficient to induce cardiac hypertrophy, IL-6 is

dispensable for adaptive physiologic hypertrophy responses to exercise training (311,

312). Thus, while unnecessary for physiologic hypertrophy, IL-6 has been implicated in

pathological cardiac hypertrophy in humans (313, 314) and animals (280, 315-317).

Elevated intragraft IL-6 transcript levels at day 30 post transplant coincided with

graft hypertrophy and fibrosis in grafts undergoing CR (Figures 6, 7, 9, and 14). It is of

note that significant elevation of IL-6 expression in allografts undergoing CR at days 30

and 50 post transplant occurs after a suggested (but not significant) increase at day 7

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(Figure 9). The importance of this early peak in the bimodal expression of IL-6 is unclear, though it is not likely due to ischemic/reperfusion injury in that syngeneic control grafts did not exhibit a similar early elevation of IL-6. One possibility is that the early elevation of IL-6 expression in allograft recipients treated with anti-CD4 mAb may be associated with the process of depleting CD4+ cells.

IL-6 has previously been suggested as a therapeutic target for hypertrophy (318), therefore, we neutralized IL-6 to assess its role in the hypertrophy associated with CR. Neutralizing IL-6 reduced cardiomyocyte area (Figure 10) and prevented the increases in PWT, FS and LVEF indicative of cardiac hypertrophy that were observed in CR grafts (Figure 13). These results implicate IL-6 as an inducer of hypertrophy in CR. Further, targeting IL-6 may ameliorate hypertrophy while stabilizing functional parameters in cardiac allografts undergoing CR.

Neutralizing IL-6 not only decreased cardiomyocyte hypertrophy but also lessened fibrosis of the graft (Figure 13). This finding is consistent with previous *in vitro* studies in which IL-6 increased collagen transcript levels in co-cultures of cardiac fibroblasts and cardiac myocytes (282) and treatment with IL-6 neutralizing mAb decreased cardiac fibroblast proliferation (319). Further, IL-6 may induce factors that facilitate fibroblast survival (320). Thus, decreased survival, proliferation, and collagen αI transcript levels in cardiac fibroblasts may explain the anti-fibrotic effects of neutralizing IL-6 in our study.

Beyond its roles in hypertrophy and fibrosis, IL-6 is also a potent modulator of immune responses in multiple cell types of both the innate and adaptive systems

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(reviewed in (321-323)). Hence, it is possible that IL-6 neutralization ameliorates CR in this model through immunomodulatory effects. IL-6 neutralization could prolong graft survival by impairing the transition of graft-reactive immunity from innate responses to adaptive responses, perhaps through disruption of the neutrophil to monocyte recruitment progression (321). Additionally, lymphocyte homing to the graft may be impaired, as IL-6 can induce the expression of monocyte chemotactic protein 1 in fibroblasts (324) as well as promote T cell migration on fibronectin substrate (325). In addition to promoting recruitment, IL-6 can rescue T cells from apoptosis (326). It is therefore conceivable that neutralizing IL-6 could further enhance graft survival through both failure to recruit and increased apoptosis of graft-reactive T cells.

Neutralizing IL-6 does not inhibit CD4+ cell repopulation in this system (Figure 14). Therefore, we asked whether neutralizing IL-6 had other effects on CD4+ or CD8+ cell function. To this end, ELISPOT assays (21) were performed on splenocytes harvested on day 30 post transplant from allograft recipients that were initially depleted of CD4+ cells and treated with either anti-IL-6 mAb or rat IgG. ELISPOT assays for donor-reactive IFN-γ, IL-4, and IL-17 producing cells were performed on whole splenocytes, splenocytes depleted of CD4+ cells, and splenocytes depleted of CD8+ cells. Similarly low frequencies (<50 spots/million cells) of donor-reactive cytokine producing cells were present in the spleens of rat IgG or anti-IL-6 treated recipients, indicating that IL-6 neutralization did not alter the immune response at the level of Th subset function. Hence, both CD4+ and CD8+ Th1, Th2, and Th17 remained hyporesponsive in the spleens of allograft recipients following transient depletion of CD4+ cells.

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IL-6 can augment antigen-specific antibody responses in mice (327), therefore we assessed serum levels of donor-reactive IgM or IgG. No significant difference was observed between anti-IL-6 treated recipients or controls (Appendix 4). It should be noted that these observations do not rule out other immunomodulatory effects of IL-6 neutralization in this setting. Indeed, anti-IL-6 mAb might alter lymphocyte trafficking to the graft (324, 325, 328), lymphocyte survival (326), activation and differentiation (261), as well as the immunologic events initiating fibrosis.

It has been demonstrated *in vitro* that hypertrophic stimuli such as IL-6 can induce cardiac myocyte production of factors known to promote fibrosis, including CTGF (290) and TGFβ (329). We have previously reported a strong correlation between intragraft expression of TGFβ and CTGF with CR (236). This indicated that IL-6 might induce similar pro-fibrotic gene expression *in vivo*. It should be noted that in addition to potential enhancement of TGFβ production through the induction of hypertrophy, IL-6 may also directly augment TGFβ signaling by regulating turnover and compartmentalization of its receptor (263). Furthermore, it has recently been reported that the C-terminal domain of CTGF can induce cardiomyocyte hypertrophy (289). Together, these data suggest that IL-6 may be a promoter of multiple factors able to promote both hypertrophy and fibrosis in CR.

The elucidation of a critical role for IL-6 in CR led us to evaluate of the associations of IL-6 with other factors associated with CR, especially TGFβ (237). The role of TGFβ in CR is complicated by its pleiotropic activity encompassing immunosuppressive and anti-proliferative effects in immune (238, 330-332) and non-

immune (250, 333) cells as well as the induction of Treg (334-336), which are associated with graft acceptance (247, 248, 259). Thus, TGFβ may promote graft survival and global immune tolerance while suppressing malignancy, making it ill-suited as a therapeutic target in the treatment of CR. This has prompted investigation into the downstream mediators of fibrotic TGFβ function (100, 236).

Multiple reports indicate that TGFβ requires additional factors to drive fibrosis (236, 285, 286). Indeed, syngeneic grafts do not develop fibrosis in response to TGFβ, while allografts whose recipients receive anti-CD40L mAb develop marked fibrosis in response to TGFβ (Figure 15, (236)). Hence, alloimmune responses potentiate the pro-fibrotic effects of TGFβ. We considered that IL-6 may be the immune factor that potentiates TGFβ actions in CR (265). Indeed, elevated IL-6 expression correlated with TGFβ and CTGF (Figure 15). Correlations of TGFβ with CTGF (236) and TGFβ with IL-6 (265) have previously been described. Further, we have previously observed CTGF expression associated with areas of graft-infiltrating mononuclear cells (236), whose recruitment during inflammatory responses has been linked to IL-6 (337, 338). Therefore, we considered that there may be connectivity between all three cytokines.

To ascertain the sufficiency of TGFβ and CTGF to induce CR, allogeneic and syngeneic cardiac grafts were transduced with AdTGFβ or AdCTGF and transplanted into recipients receiving anti-CD40L mAb or syngeneic recipients. AdTGFβ and AdCTGF significantly increased mean fibrotic area compared to untransduced or control vector treated allografts (Figure 15A). Consistent with a previous report of adenoviral transduction of lungs (283), the fibrotic response to TGFβ transduction in the heart was

significantly greater than the response to CTGF transduction (Figure 15A). Greater

fibrotic responses to AdTGFβ could be from synergy of TGFβ-induced immune factors

and CTGF in cardiac allografts, an effect which is not observed in syngeneic grafts (236).

Further, in cardiac allografts, TGFβ induction of endogenous CTGF may synergize with

TGFβ-mediated chemotactic effects on multiple immune lineage cells (238), which may

explain the suggested upregulation of IL-6 and significant upregulation of IL-17 (Figure

15).

Given the differences in AdTGFβ responses between allografts and syngeneic

grafts (Figure 15) and the correlation of TGFβ and CTGF with IL-6 in CR (Figure 15),

we asked whether the presence of IL-6 was required for CTGF upregulation. In cardiac

allograft recipients transiently depleted of CD4+ cells, IL-6 neutralization reduced the

expression of IL-6 and CTGF without altering TGFβ transcript levels (Figure 16). This

suggests that TGFβ transcript regulation lies upstream of IL-6 and CTGF in CR. It

should be noted that IL-6 neutralization does not prevent repopulation of CD4+ cells in

the periphery (Figure 14, (265)). This indicates that the ability of IL-6 neutralization to

prevent CR (265) could function in part through reduction of intragraft CTGF.

Another explanation of the efficacy of IL-6 neutralization could be effects in T

cell lineage decisions. It is known that the presence or absence of IL-6 may determine T

cell responses to TGFβ, as TGFβ and IL-6 result in sTh17 effector cells while TGFβ

without IL-6 can generate FoxP3+ regulatory cells (261). The possibility that this axis of

effector/regulatory T cell responses may be a key factor in CR is supported by historic

observations of IL-6, TGFβ, and T cell receptor β constant region expression in rejecting

human cardiac allografts (302). Furthermore, a role for IL-17 in the pathology of CR

seems more likely in light of observations that IL-17 alone stimulates increased collagen

production in primary mouse cardiac fibroblasts (339). This is consistent with a report

that Th17 responses to collagen type V correlate with the development of bronchiolitis

obliterans syndrome in lung transplant patients (287) and that IL-17 has recently been

associated with the development of CAV (288). In these studies IL-6 neutralization

significantly inhibited IL-17 expression (Figure 16), indicating that IL-17 might play a

role in CR possibly through CTGF induction, as IL-17 has been reported to induce

collagen production in cardiac fibroblasts (339). Another explanation for this effect

might be decreased recruitment of graft infiltrating cells which may express or induce

local cells to express CTGF (236), IL-6 and IL-17. Indeed, IL-6 induces chemotaxis and

migration of immune cells (321, 325).

Since IL-6 neutralization ameliorated CR (Figures 10-12, (265)) and decreased

intragraft CTGF expression (Figure 16), we treated cardiac allograft recipients with

neutralizing CTGF mAb. CTGF neutralization significantly reduced allograft fibrosis

(Figures 17A, B) without significantly reducing intragraft TGFβ, IL-6, or CTGF

expression (Figure 17C). These findings are consistent with CTGF being a downstream

mediator of fibrosis in CR (100, 251, 283, 340).

The significant but incomplete reduction in fibrotic area in response to CTGF

neutralization may be explained by multiple factors. Our neutralization protocol, though

effective, may not be optimal. Another possibility is the presence of CTGF-independent

pro-fibrotic effects of TGFβ and/or IL-6 (341). A further consideration is whether the

mAb FG-3019, which recognizes CTGF module 2 in humans and rodents (268), might inhibit some but not all pro-fibrotic effects of CTGF. However, this possibility seems unlikely in light of a recent report evaluating the anti-fibrotic efficacy of anti-CTGF antibodies directed against each of the four CTGF modules. In this report, only mAb directed against the von Willebrand factor type C domain (module 2) was able to inhibit TGFβ-induced fibrosis (342). Indeed, this is the same domain that the anti-CTGF mAb utilized in our study binds (267, 268).

Beyond its roles in fibrosis, CTGF can exert other effects relevant to CR. Recent studies have described a concomitance of cardiomyocyte hypertrophy with CR in humans (265, 343, 344). CTGF is produced by hypertrophic chondrocytes during development (345), and is produced by cardiac myocytes in response to hypertrophic stimuli (290). In addition, CTGF itself can induce cardiomyocyte hypertrophy (289). Treatment with neutralizing anti-CTGF mAb significantly reduced mean cardiomyocyte area (Figure 18A) and intragraft levels of ANP (Figure 18B), a marker of cardiac hypertrophy in multiple settings (265, 279, 280). However, it should be noted that anti-CTGF mAb did not inhibit cardiac hypertrophy to the extent observed with anti-IL-6 (265). This finding indicates that in addition to driving cardiac fibrosis, CTGF may augment cardiomyocyte hypertrophy associated with CR. Interestingly, hypertrophy is associated with downregulation of two recently discovered CTGF-inhibiting micro RNAs in cardiac myocytes (346). Thus, CTGF may be linked to cardiac hypertrophy on multiple levels.

Finally, as CTGF is known to play an important role in fibroblast adhesion in response to TGFβ (294, 340), we asked whether CTGF might similarly influence

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recruitment of lymphocytes to the graft. Histologic assessment of infiltrating cells was

indicative of reduced numbers of graft infiltrating lymphocytes (Figure 19A). This

observation was further supported by significant reduction of intragraft TCRβ constant

region expression (Figure 19B) in response to CTGF neutralization.

Based on these observations and others in the literature, we propose a model

representing the interactions of TGFβ, IL-6, and CTGF and their induction of

hypertrophy and fibrosis associated with CR (Figure 20A).
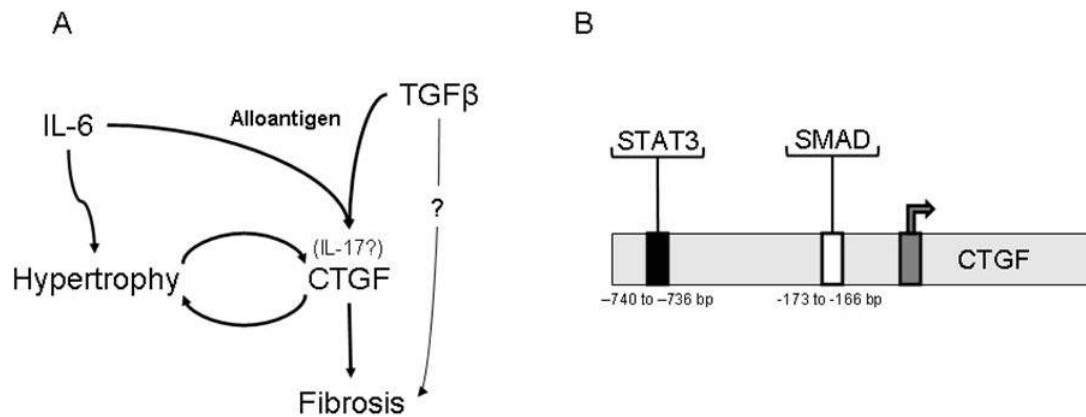
Figure 25 Proposed model of cytokine interactions in chronic rejection. (A) In cardiac allografts, TGFβ and IL-6 contribute to CTGF production. IL-6 and CTGF are both known to promote hypertrophy in cardiac myocytes, which in turn can produce CTGF. CTGF functions as a downstream mediator of fibrosis. (B) Induction of CTGF downstream of TGFβ and IL-6 could be explained by the respective presence of a consensus SMAD binding element and a STAT3 response element in 5' region upstream of the CTGF promoter. For expanded explanations, please see text.

In cardiac allografts that undergo CR, TGFβ (Figure 15, (236)) and IL-6 (Figures 9, 15,

and 16B, (265)) are induced. In syngeneic grafts, forced expression of TGFβ is

insufficient to upregulate CTGF and fibrosis (236), and IL-6 remains at basal levels

(Figure 16B). IL-6 neutralization inhibits hypertrophy and fibrosis associated with CR

(265), which may be in part through inhibition of CTGF and IL-17 expression while

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TGFβ expression remains unchanged (Figure 16). Thus, TGFβ and IL-6 appear to be upstream promoters of CTGF expression and CR. CTGF neutralization limits fibrosis (Figures 17A, B) and decreases cardiomyocyte hypertrophy (Figure 18), without altering intragraft TGFβ, IL-6, or CTGF transcripts (Figure 17C). These effects of CTGF neutralization coincide with reduction in graft infiltrating T cells (Figure 19). Together, these observations support a downstream role for CTGF in fibrosis and hypertrophy.

Contexts in which TGFβ and IL-6 are present coincide with intragraft IL-17 expression, which has been implicated in promoting cardiac remodeling (347), fibrosis (339), bronchiolitis obliterans syndrome in lung transplant patients (287), and cardiac allograft vasculopathy (288). However, the effects of IL-17 on hypertrophy and CTGF expression are unclear and merit further investigation. Our proposed model of CTGF induction downstream of IL-6 and TGFβ (and perhaps IL-17) might be explained by previous identification of both a STAT3 responsive element (−740 to −736 bp) (348) and a consensus SMAD binding element (-173 and -166 bp) (349) upstream of the CTGF promoter (Figure 20B). Hence, optimal induction of CTGF in CR may require that CTGF producing cells receive both SMAD and STAT3 signals, likely provided by TGFβ (238) and IL-6 (350) respectively.

These studies support the use of echocardiography for monitoring the *in vivo* changes occurring in CR, which helped identify a previously unrecognized immunologic axis in the development of chronic cardiac allograft rejection. These echocardiographic, histologic, and molecular findings suggest a critical role of IL-6 in the cardiac hypertrophy and fibrosis associated with CR. Together, these observations indicate that

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IL-6 neutralization may represent the first therapeutic approach to ameliorate hypertrophy and fibrosis associated with CR while stabilizing anatomical and functional parameters of the graft. These investigations also support a role for CTGF as a downstream mediator of fibrosis and highlight the essential contributions of immune elements to CR and fibrosis of cardiac grafts while elucidating relationships between TGFβ, IL-6, and CTGF. Further, these studies indicate for the first time that mAb neutralizing CTGF can ameliorate fibrosis and hypertrophy associated with CR. These findings further implicate IL-6 as a critical immune factor in CR that may potentiate TGFβ-mediated CTGF induction. Finally, TGFβ-mediated induction of fibrosis in allogeneic but not syngeneic grafts was associated with a suggested increase in intragraft IL-6 expression and a significant increase in IL-17 expression, supporting the notion that TGFβ induction of fibrosis and CR requires interaction with immune parameters.

Chapter VI—Conclusion

Summary

CR of transplanted organs remains the most significant barrier to both the long term survival of transplanted organs as well as the effective use of transplantation in the clinic. There is currently no effective treatment that targets CR, with the best hope being prevention through strong immunosuppression, which may spare the graft while it subverts long term survival of the graft recipient through increased tumorigenesis and opportunistic infections. These investigations have endeavored to better understand the physiologic and molecular etiology of CR in cardiac allografts and have tested two therapeutic approaches for CR. Through echocardiographic investigations described here and similar concurrent observations in the clinic, it has become apparent that pathologic cardiomyocyte hypertrophy is a defining physiologic feature of CR in cardiac allografts. Further, these investigations identified a critical role for IL-6 in the initiation of cardiac hypertrophy associated with CR. In addition to promoting pathologic hypertrophy associated with CR, IL-6 is an upstream promoter of CTGF and CTGF is a downstream mediator of fibrosis in CR.

These investigations in cardiac grafts have centered on hypertrophy as a signature of pathologic events occurring within the graft. However, cardiac hypertrophy is frequently observed in situations that are not pathologic, but rather physiologic. The

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factors defining physiologic versus pathologic hypertrophy has been the source of intense investigations. Some signaling elements (351) as well as the differentiation state of cardiac fibroblasts (352) were previously known to distinguish between physiologic and pathologic hypertrophy. These findings, namely the critical role of IL-6 in the induction of pathologic transplant cardiac hypertrophy, illustrate the complex and wide reaching effects of immune responses to the graft—even graft physiology.

The identification of IL-6 as a critical mediator of CR greatly informed previous experimental observations about the role of initiating immune factors in CR. Cardiac allografts administered anti-CD40L therapy and transduced to express TGFβ developed CR, whereas similarly transduced syngeneic grafts did not (Figure 16). Though it was clear that immune-mediated factors were responsible for the differences in these outcomes, the necessary immune factors were not known. These investigations indicate that IL-6 is a critical mediator of TGFβ actions in CR, potentially through the induction of anti-graft IL-17 responses (Figure 16C). These studies further indicate that the immune factors TGFβ and IL-6 induce the production of CTGF, possibly through induction of IL-17. It should be noted, however that the presence of SMAD and STAT response elements upstream of the CTGF promoter suggest that the induction of IL-17 may not be necessary for CTGF induction.

Investigations utilizing CTGF neutralization indicate that CTGF functions downstream of TGFβ and IL-6 as a true downstream mediator of fibrosis. Though CTGF neutralization did not completely abrogate fibrosis in the CR model assessed, it did provide significant reduction. The mechanisms by which CTGF reduces fibrosis are

unclear. CTGF neutralization did not significantly reduce expression of type I collagen, perhaps indicating that CTGF promotes accumulation of fibrotic tissue in CR of cardiac allografts on a post translational level.

Implication of Findings

These studies were undertaken in large part to understand the etiology of CR, a debilitating disease that will at some point affect most patients receiving a solid organ transplant. It is therefore worthwhile to consider the prospective applications of these findings clinically. The potential clinical relevance of these echocardiographic studies is highlighted by a recent report in which echocardiography defined cardiac allograft hypertrophy as a prognostic marker for the development of allograft vasculopathy and increased patient mortality (343). As pointed out in an accompanying Editorial (344), cardiac allograft hypertrophy and its association with graft vasculopathy may provide a surrogate marker for patient survival in clinical cardiac transplantation. Hence, echocardiography represents a non-invasive technique for assessment of the progression of CR. Importantly, an advantage of echocardiography for detection of CR is that it does not suffer the conundrum of judging the entire graft by a small piece, as with transvenous endomyocardial biopsy.

These findings further indicate that IL-6 may be the causative agent for transplant associated hypertrophy, recently found to correlate with poor prognosis in transplant patients. Therefore, assessment of IL-6 levels in transplant biopsies or patient serum might provide a biomarker to supplement echocardiographic assessment of cardiac hypertrophy. Further, the use of the anti-IL-6 mAb Actemra (tocilizumab) has been

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effective in treating rheumatoid arthritis in the clinic (353). Actemra was approved for the treatment of rheumatoid arthritis in the European Union and Japan in January 2009 and may soon be approved for treating patients in the United States. Thus, it appears that IL-6 neutralization could provide an alternate or supplementary immunotherapeutic that is more efficacious in preventing or treating CR than current approaches.

The potential for all immunotherapeutics, such as anti-IL-6 mAb, is hindered by the general side effects of immunosuppression. However, the side effects of global immunosuppression may be less present with CTGF neutralizing therapeutics, as no clear role for CTGF in the development or maintenance of immune responses is known. In these studies, neutralization of CTGF ameliorated the fibrosis associated with CR. Though CTGF neutralization was less potent than IL-6 neutralization in staying the onset and progression of CR, it may provide a more targeted therapeutic. In these studies CTGF appeared to decrease the presence of graft infiltrating cells. However, graft infiltrating cells were still present. Thus, targeting CTGF may provide a therapeutic for some aspects of CR pathology without significantly augmenting the immunocompromised condition of a transplant recipient. Such a therapeutic would be a seemingly excellent compliment to current immunosuppressive regimens used clinically.

Future Directions

IL-6 Future Studies

These studies have identified several key factors in the initiation and progression of CR, including a critical role for IL-6 as an inducer of processes leading to hypertrophy

as well as fibrosis, and a clear definition for CTGF as a downstream mediator of fibrosis in CR of cardiac grafts. Though these findings are important, they delineate the need for further investigations. With respect to the role of IL-6 in the induction and progression of CR a number of questions remain. First, though these investigations clearly illustrate the necessity of IL-6 in CR, the cellular and molecular mechanisms by which it contributes to CR are not known. Further investigations should probe the cellular source of IL-6 in CR. Multiple cell types are able to produce IL-6 in response to biologic stimuli. A better understanding of which cells are producing IL-6 would not only increase our understanding of the biology of IL-6 in CR, but potentially provide inroads for more targeted therapeutics.

Elucidating the cellular source of IL-6 in these models of CR is complicated by the ability of IL-6 to signal through both cis and trans mechanisms (Figure 21). In the case of cis signaling, it is expected that IL-6 signaling would only affect cells that express the cognate IL-6 receptor α. IL-6 may also signal to cells that do not express the cognate receptor, as IL-6 bound to its cognate receptor α may be proteolytically cleaved from multiple cell types, creating an IL-6/IL-6 receptor α complex that can then bind to the coreceptor for IL-6 signaling, gp-130. The gp-130 coreceptor is expressed ubiquitously throughout many tissue types and would open up the possibility that the source of IL-6 signaling in the graft may not be graft cells or graft infiltrating cells, but rather cells in the host periphery including the spleen and graft draining lymph nodes. It should be noted that these mechanisms are non-exclusive and could function simultaneously.
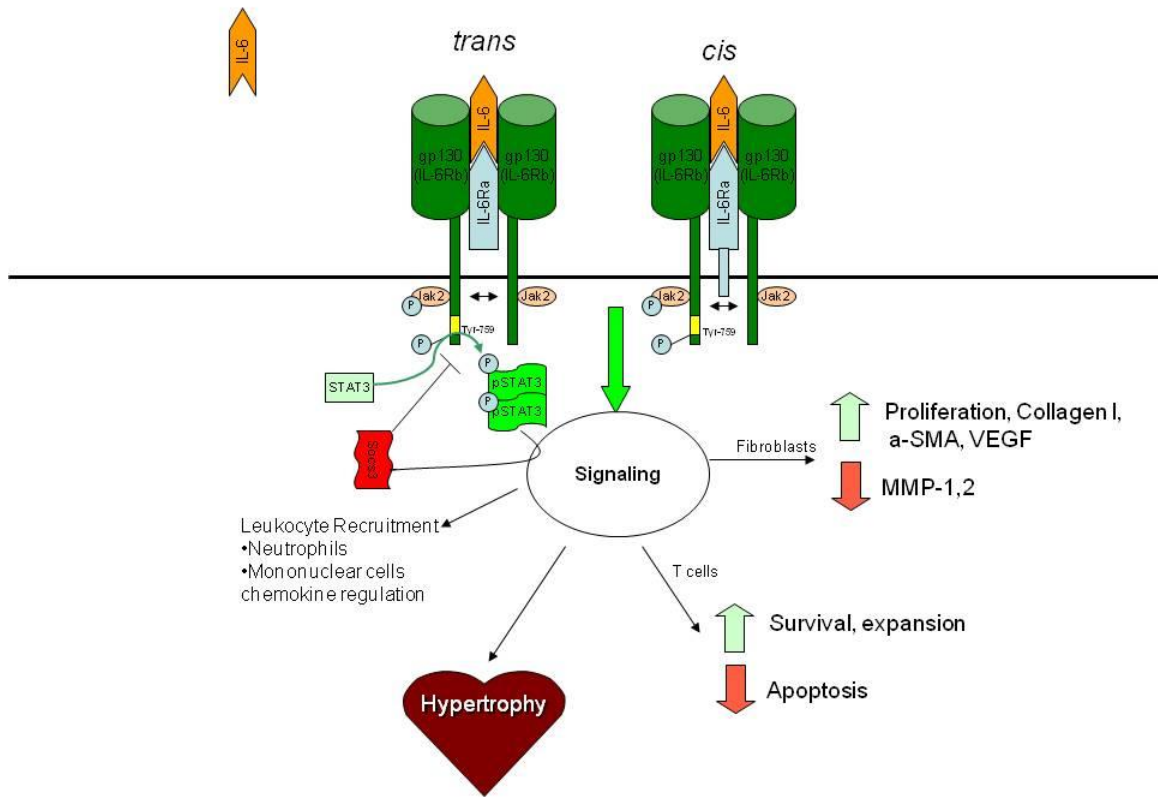
Figure 26 IL-6 Signaling and Potential Effects in CR

A primary reason for trying to understand the cellular source of IL-6 and the type of IL-6 signaling is to elucidate the biology underlying the effectiveness of IL-6 neutralization in preventing CR. Knowing the type of IL-6 signaling would facilitate further investigations into the cells that respond to IL-6 and what the ultimate effect of IL-6 upon these cells in. In particular it is unclear how IL-6 signaling culminates in CR, though several lines of investigation would be appropriate to better understand these effects. These studies indicate that IL-6 neutralization is associated with a decrease in intragraft IL-17 expression. Thus, one possibility is that the effectiveness of IL-6 neutralization largely owes to the prevention of IL-17 responses and that IL-17 is the

more critical molecule. This theory could be tested through antibody neutralization of IL-17 in these models and is currently being explored with IL-17 knockout mice.

Though there is association of IL-17 in other models of CR, these observations may point toward another putative mechanism that IL-6 neutralization works through. The pathways of T cell development into inflammatory IL-17 responses or regulatory T cell responses have been described as reciprocal. Current understanding suggests that the reciprocity between these two lineages is decided through the absence or presence of IL-6 or IL-1. Hence, another effect of IL-6 neutralization might be the developmental skewing of graft-reactive cells away from inflammatory lineages toward regulatory cell identities. This hypothesis could be tested both by antigen-specific *in vitro* suppressor assays and by adoptive transfer of sorted cells expressing the FoxP3 transcription factor from these transplant recipients into mice that would otherwise reject their grafts. The adoptive transfer approach would have the additional advantage that it would clarify whether or not any regulatory cells induced in these mice could have their regulatory cell function subverted by an IL-6 competent environment.

In addition to the potential effects of IL-6 on immune lineage cells, investigations probing the effect of IL-6 on non-immune cells including vascular endothelial cells, cardiac myocytes, and cardiac fibroblasts should be considered. IL-6 is one of several immune cytokines known to activate vascular endothelial cells, triggering multiple responses such as the induction of surface-expressed cellular adhesion molecules that are critical for sequestering immune cells to the site of inflammation. Therefore, it is possible that IL-6 signaling to endothelial cells is an important part of CR, promoting

infiltration of immune cells. Infiltrating cells may then initiate cellular damage, cytokine

production, and chronic remodeling associated with CR manifesting as CAV and

interstitial fibrosis. Infiltrating cells may also have effects upon cardiac myocytes

through cytokines, as IL-6 is known to induce hypertrophy. Further, cardiac myocytes

could be a source of factors that promote fibrotic remodeling associated with CR,

including TGFβ, CTGF, and even type I collagen. While cardiac myocytes may produce

collagen, it is more likely that cardiac fibroblasts produce most collagen in CR of the

heart.

The possibility that IL-6 has effects on all three of these cell types may be

investigated through several approaches. Since all three of these cell types are graft cells

the use of knockout mice as cardiac graft donors would be an effective approach for

determining the contributions of IL-6 signaling to graft cells. For these experiments,

mice deficient in at least four components of IL-6 signaling machinery, IL-6, IL-6

receptor α, the IL-6 coreceptor gp-130, and the intracellular STAT3 protein could be

utilized (Figure 21). Each of these may provide insights into the role of IL-6 signaling

within the graft. The outcome of transplants utilizing grafts deficient in IL-6 would

inform whether or not graft-derived IL-6, potentially produced by hypertrophic cardiac

myocytes (354, 355) or vascular endothelial cells (356), significantly contributes to CR.

If graft-derived IL-6 plays an important role in CR, it would be expected that grafts

deficient in the production of IL-6 will be protected from manifestations of CR, including

CAV, interstitial fibrosis, and cardiac hypertrophy. Though graft cells may produce

significant amounts of IL-6 that contribute to graft pathology, it is likely that graft cell responses to IL-6 are critically involved in CR as well.

To assess the importance of graft cell responses to IL-6, transplants utilizing grafts deficient in the IL-6 receptor α, the IL-6 specific element of the IL-6 receptor complex, could be used. The advantage of using IL-6 receptor α knockouts is that specific interruption of IL-6 signaling would occur in graft cells. However, the limitation of IL-6 receptor α knockout grafts is that grafts would still be able to receive signals through trans IL-6 signaling by IL-6 bound to the soluble IL-6 receptor α. If IL-6 receptor α knockout grafts were protected from CR, it would clearly implicate a role for cis IL-6 signaling to graft cells. While if the grafts were not protected it would indicate that cis IL-6 signaling to graft cells was indispensible. To determine the effects of trans IL-6 signaling on the graft, grafts deficient in the IL-6 coreceptor gp-130 or STAT3 could be utilized. In either case, the ability of graft cells to respond to cis or trans IL-6 signaling would be compromised, suggesting a role for graft responses to IL-6 signaling. However, the *caveat emptor* of interpreting experiments with these mice is that graft cell responses to all IL-6 family member cytokines (357, 358) would be abrogated. Hence, if deficiency in gp-130 or STAT3 is protective to the graft, protective effects could not be directly ascribed to diminution of IL-6 signals to graft cells, though it would be informative when interpreted alongside results with IL-6 receptor α knockout results.

In addition to elucidating the role of IL-6 signaling in the initiation and progression of CAV and interstitial fibrosis associated with CR, these studies would provide critical insights into the necessity of IL-6 for inducing pathologic hypertrophy.

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Indeed, the signals differentiating pathologic hypertrophy from physiologic hypertrophy are not fully understood (351) and likely involves cells other than myocytes (352). The studies presented here suggest that IL-6 may the initiator of pathologic hypertrophy now associated with CR of cardiac grafts. The proposed studies in which the graft is deficient in "sensing" this signal would determine if IL-6 is indeed the initiating factor for pathologic hypertrophy in CR.

CTGF Future Studies

These experiments suggest that CTGF functions as a downstream mediator of fibrosis in CR of cardiac grafts. Though CTGF neutralization was effective in reducing the deposition of fibrotic tissue, interstitial fibrosis still occurred. Though there may be multiple reasons for the incomplete inhibition of fibrosis with anti-CTGF mAb, attempts at optimizing the neutralization protocol might clarify the promise of CTGF neutralization as a fibrosis reducing therapy. In these studies, CTGF neutralization began on day 7 post transplant. This time was chosen for two reasons. First, preliminary data suggested that CTGF was upregulated as early as day 7 post transplant. Second, we were concerned that perioperative administration of anti-CTGF mAb may interfere with the healing process associated with surgery. However, early postoperative events might influence the evolution of CR. Hence, it is conceivable that perioperative and continuous neutralization of CTGF would provide greater protection against cardiac fibrosis.

In addition to its effects on fibrosis, we observed small differences in the recruitment of T cells as well as cardiomyocyte hypertrophy. Though the effect of CTGF neutralization in these areas is significant, the effect of CTGF in the onset and

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progression of CAV was not assessed and may be more important. Investigation of the role of CTGF in CAV is of the utmost importance, as CAV is a primary cause of transplant recipient death, and also because previous studies implicate CTGF as a key factor in adaptive vascular remodeling (258). Future studies should aim to determine whether CTGF neutralization reduces the frequency and/or amount of occlusion in cardiac transplants through morphometric analysis of elastin stained sections. As an alternative approach, CTGF neutralization could be attempted in recipients of vessel grafts, as the effect of CTGF neutralization could be easily assessed in this setting.

In these investigations decreased fibrotic area was not associated with decreased type I collagen transcripts. The accumulation of ECM proteins such as type I collagen is the result of two factors—the rate of synthesis and the rate of degradation. This raises the interesting possibility that CTGF neutralization inhibits graft fibrosis by maintaining ECM degrading factors rather than preventing upregulation of ECM synthesis. This interesting possibility could be examined by evaluating matrix metalloproteinase (MMP) 1 and 2 levels and activities through western blot and zymography. Indeed, suppression of ECM degradation via reduction of MMPs has already been observed in other settings of ventricular fibrosis (359).

Concluding Remarks

Together, these studies have provided significant insights into our understanding of the etiology and mechanisms of CR of cardiac grafts. First, the evolution of graft hypertrophy in response to IL-6 initiated events may provide a non-invasive biomarker for CR that cannot be detected through transvenous endomyocardial biopsy. Secondly,

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these investigations suggest that therapeutic approaches targeting IL-6 and CTGF may significantly ameliorate CR pathology in cardiac grafts. Further, CTGF neutralization may represent a therapeutic that specifically targets downstream events in CR of cardiac grafts without significantly increasing the immunosuppressed condition in which most transplant recipients live. Hopefully basic science discoveries like these will spur clinical investigations leading to improved graft survival and better quality of life for transplant recipients.

Appendix
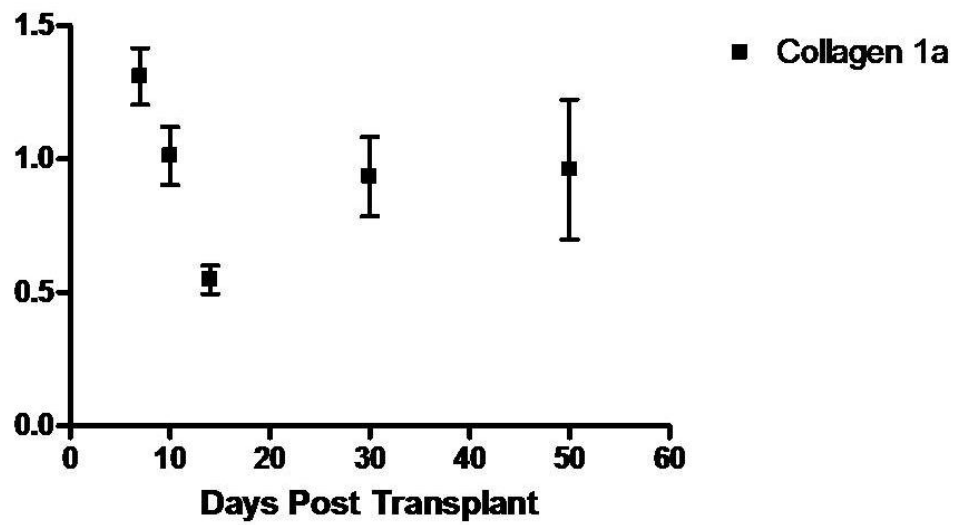
Appendix 1 Kinetic Expression of Collagen 1a in Cardiac Allografts Transiently Depleted of CD4+ cells.
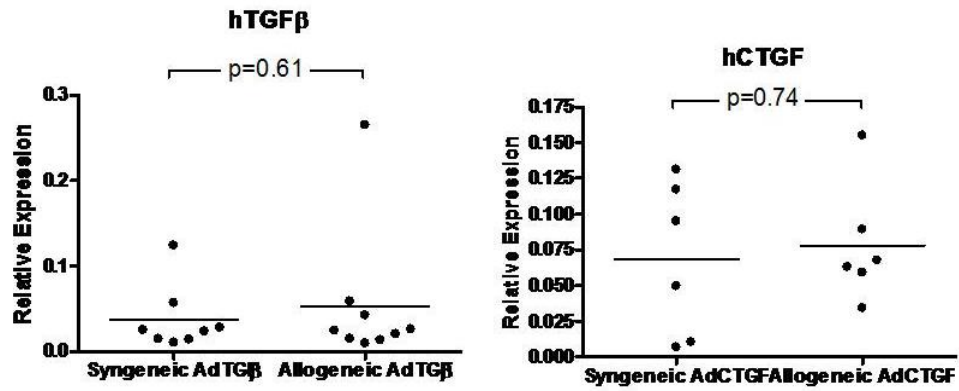
Appendix 2 Intragraft Expression of Transgenes in Cardiac Allografts Transduced with

Adenoviral TGFβ (AdTGFβ) or Adenoviral CTGF (AdCTGF).
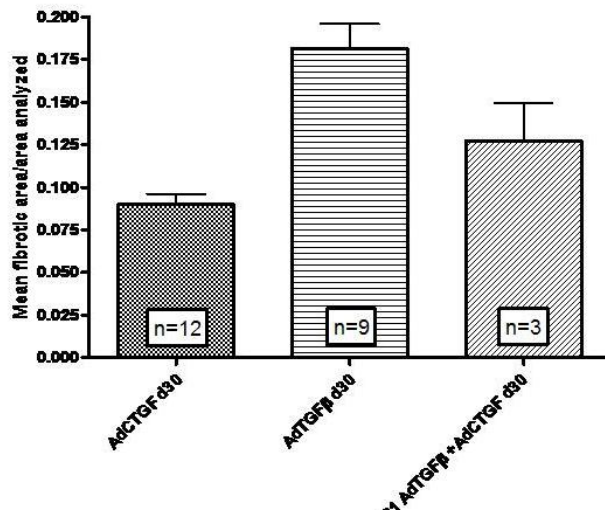
Appendix 3 Dual Transduction of AdTGFβ and AdCTGF does not produce an Additive Fibrotic

Effect in Cardiac Allografts whose recipients received anti-CD40L therapy.
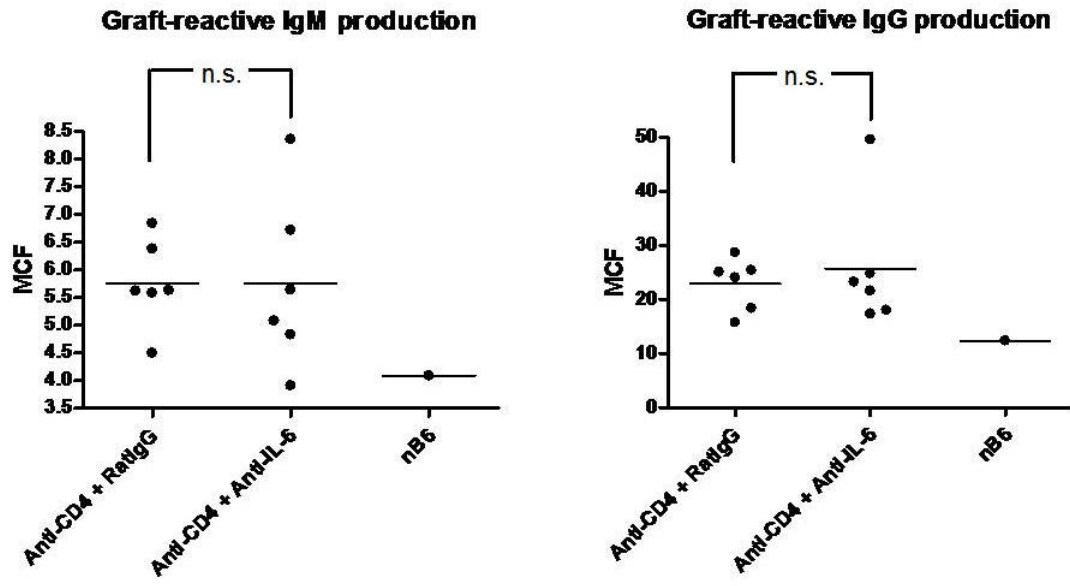
Appendix 4 **Comparison of Graft-reactive Antibody Production in Recipients of Cardiac Allografts**

Transiently Depleted of CD4+ Cells and also receiving control rat IgG or anti-IL-6 antibody therapy.

Antibody production is measured from recipient serum harvested at day 30 post transplant.

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