Role of Effector $CD8^+$ T Cells in Allograft Vasculopathy

By

Anton Ivan Skaro

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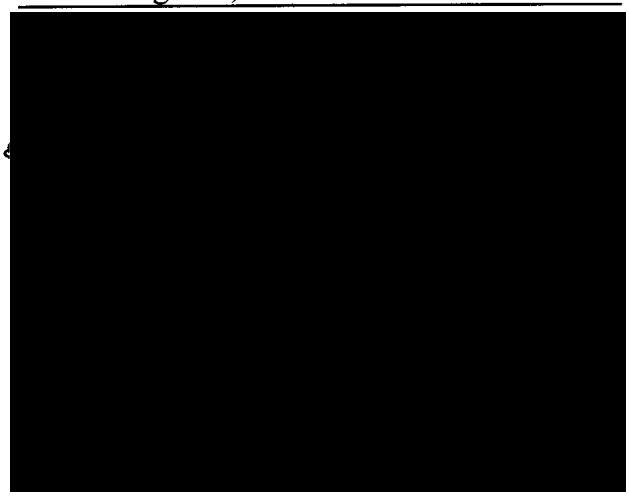

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Author: Anton Ivan Skaro

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For Mary Ann:

"Your support and encouragement have made this thesis a reality"

Table of Contents

List of Figures and Tables

Chapter 1 – General Introduction

Chapter 4 – Impairment of Recipient Cytolytic Activity Inhibits Medial Smooth Muscle Cell Apoptosis and Attenuates Allograft Vasculopathy

Chapter 5 – $CD8^+$ T Lymphocytes Mediate Allograft Vasculopathy by Direct Cytolysis and by an Interferon-γ-Dependent Indirect Effector Pathway

Abstract

Allograft vasculopathy (AV) has emerged as the major obstacle to long-term survival in clinical heart transplantation. Immune events are implicated in the development of AV, but the cellular and molecular mechanisms involved remain unclear.

In this thesis, we examined the role of T cell subsets ($CD4^+$ and $CD8^+$) and the effector mechanisms involved using knockout mice and adoptive cell transfer in a fully MHC-mismatched murine aortic allograft model of AV.

The inhibition of $CD8^+$ T cells or cytotoxic T lymphocyte (CTL) effector mechanisms led to preservation of medial smooth muscle cells and attenuation of AV. In contrast, CD4 deficient mice exhibited striking medial smooth muscle loss and intimal hyperplasia which was similar in extent to wildtype recipients. Moreover, $CD8^+$ T cells are essential to the development of AV in the presence of cyclosporine A-based immunosuppression.

The transfer of allo-primed $CD8^+$ T lymphocytes into immunodeficient recombinase activating gene knockout ($RAG^{-/-}$) mouse recipients of aortic allografts resulted in the development of AV, confirming that $CD8^+$ effector T cells are sufficient to induce AV. In addition, we established that $CD8^+$ T cell-mediated AV occurs by contact-dependent direct cytolysis and bya distinct interferon -γ-dependent indirect effector pathway. Furthermore, AV mediated by$CD8^+$ T cells, but not $CD4^+$ T cells, is refractory to cyclosporine A treatment. Given the resistance of this cell type to conventional immunosuppression these results may have important therapeutic implications. The development of novel immunosuppressive agents capable of inhibiting $CD8^+$ T cells might prevent AV in the clinic.

Abbreviations

AV	Allograft vasculopathy
AICD	Activation induced cell death
APAF1	Apoptosis activating factor 1
APC	Antigen presenting cell
BID	BCL-2 interacting domain
B6	C57BL/6
CHF	Congestive heart failure
CTL	Cytotoxic T lymphocyte
CyA	Cyclosporine A
DAG	Diacylglycerol
DISC	Death inducing signalling complex
DTH	Delayed type hypersensitivity
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FasL	Fas Ligand
FACS	Fluorescence activated cell sorting
FADD	Fas associated death domain
Gld	Fas ligand-deficient
ICOS	Inducible co-stimulator
IFN-γ	Interferon-gamma
H & E	Hematoxylin and eosin
IGF	Insulin-like growth factor
iNKR	Inhibitory natural killer cell receptors
IP_3	inositol 1,4,5-triphosphate
I-TAC	Interferon-inducible T cell α chemoattractant
JNK	Jun kinase
LAD	Left anterior descending
Lpr	Fas-deficient
MHC	Major histocompatibility complex

MIG	Monokine induced by interferon-γ
MLR	Mixed lymphocyte reaction
NK	Natural killer cell
NF-κB	Nuclear factor-kappa B
NFAT	Nuclear factor of activated T lymphocytes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PECAM	Platelet-endothelial cell adhesion molecule
$Pfp^{-/-}$	Perforin-deficient
PKC	Protein Kinase C
$PLC\gamma_1$	Phospholipase C
PTK	Protein tyrosine kinases
RAG	Recombinase activating gene
SCID	Severe combined immunodeficient
SEM	Scanning electron microscopy
SMC	Smooth muscle cell
Tc1	type 1 cytotoxic T cell
TCR	T cell receptor
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
TUNEL	Terminal deoxytransferase mediated dUTP nick end labeling

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Chapter 1

1.0 General Introduction

Congestive heart failure (CHF) is the leading cause of hospitalization and death in the industrialized world, affecting up to 2% of the adult general population (Sutton, 1990). The incidence of CHF has increased during the past 3 decades, and it will continue to increase. This increase is related to the development of effective palliative therapies, which have extended the lives of CHF patients. Our aging population contributes further to this increase. Heart failure affects more than 400,000 Canadians, with over 50,000 new cases diagnosed annually (Johansen *et al*, 2003). The treatment of CHF represents a tremendous economic burden on both public and private systems of financing health care worldwide. In the United States, more than $34 billion is spent annually for the care of CHF patients (O'Connell and Bristow, 1994). Similarly, medical costs related to CHF represent 1% of total health expenditures in Western Europe (Eriksson, 1995). The prognosis for patients with CHF is poor and is comparable to even the worst malignancies. As many as 50% of patients with CHF die within 4 years, whereas 50% of patients with end-stage heart disease die within 1 year (Schocken *et al*, 1992).

Over the last two decades heart transplantation has evolved as the most effective therapy for end-stage heart disease (Légaré *et al*, 1999). Despite being a well-established treatment for end-stage organ failure, solid organ transplantation continues to be limited by rejection (Häyry *et al*, 1993, Azuma *et al*, 1997, Hosenpud *et al*., 2001). Transplant rejection has been clinically classified on the basis of histopathologic features into acute and chronic allograft rejection. This classification scheme implies a temporal sequence of events resulting from discrete pathologic mechanisms (Shirwan, 1999). However, this

may not be the case in the strictest sense given that similar immune mechanisms have been implicated in acute and chronic rejection and that these pathologic features often co-exist in the same graft (Bolton *et al*, 1989, Hall, 1991, Shirwan, 1999).

There is considerable evidence that acute allograft rejection is a T cell mediated phenomenon (Lubaroff, 1973, Hall *et al*, 1978, Bolton *et al*, 1989) directed against allo-antigens, primarily those of the major histocompatibility complex (MHC), expressed by the graft (Pescovitz *et al*, 1984, Shoskes and Wood, 1994, Gould and Auchincloss, 1999). The anti-graft immune response is initiated upon T cell recognition of allo-antigen by direct and indirect antigen presentation pathways leading to the activation and proliferation of allo-reactive T cell clones (Shoskes and Wood, 1994, Gould and Auchincloss, 1999). It is thought that $CD8^+$ cytotoxic T lymphocytes (CTL) and $CD4^+$ T helper cells, once activated, exert discrete injurious effector mechanisms capable of allograft destruction leading to organ failure (Turvey and Wood, 1999, Libby and Pober, 2001). T helper cells elaborate pro-inflammatory cytokines and chemokines, which are involved in macrophage recruitment to the graft, and mediate delayed type hypersensitivity (DTH)-like intragraft responses (Shirwan, 1999, Libby and Pober, 2001). CTL and macrophages mediate graft-directed cytotoxicity through the engagement of death receptors expressed by graft parenchymal and vascular cells or alternatively by the release of potent effector molecules (Shirwan, 1999, Libby and Pober, 2001).

At the molecular level, graft cell damage by CTL is mediated by two major pathways of direct cytotoxicity (Kagi *et al*, 1994, Berke, 1995, Nagata and Golstein, 1995). The granule-exocytosis pathway involves the release of macromolecules contained within membrane-bound vesicles of mature effector CTL. These macromolecules include

the pore-forming protein perforin and cytotoxic proteases (granzymes) capable of initiating programmed cell death or apoptosis (Kagi *et al*, 1994, Berke, 1995). Alternatively, Fas ligand (FasL)-bearing activated CTL engage Fas death receptor-expressing target cells and this ligand-receptor interaction leads to the initiation of an intracellular signalling cascade resulting in target cell apoptosis (Kagi *et al*, 1994, Berke, 1995, Nagata and Golstein, 1995).

The development of novel immunosuppressive agents capable of effectively inhibiting acute rejection has facilitated the widespread acceptance of organ transplantation as a viable treatment modality for organ failure (Calne *et al*, 1978). Although modern immunosuppression has dramatically improved early transplant survival, failure of cardiac allografts due to chronic rejection has emerged as a major clinical problem (Hosenpud *et al*., 2001). Chronic rejection is the leading cause of patient mortality and late allograft failure beyond the first post-transplant year (Uretsky *et al*, 1992, Schnetzler *et al*, 1998, Hosenpud *et al*., 2001). More than 50% of transplanted hearts display significant pathologic features of chronic rejection within 5 years of transplantation (Olivari *et al*, 1989).

Chronic rejection poses a diagnostic dilemma since most patients do not develop angina pectoris. The first clinical presentation is often progressive cardiac failure, ventricular arrhythmia or sudden death (von Scheidt W, 2000). The most common and intractable pathologic presentation of chronic rejection is a diffuse, concentric intimal proliferative lesion within the epicardial coronary arteries of the transplanted heart, termed allograft vasculopathy (AV) (Figure 1, Libby and Pober, 2001). Of the three most commonly transplanted solid organs (kidney, liver and heart), the relationship of AV to

late graft failure seems most clear for heart allografts, however AV has been described in all solid organ transplants (Häyry *et al.*, 1993). Indeed, AV has been suggested to contribute to irreversible organ damage in non-cardiac transplants (Azuma *et al.*, 1997).

Similar to acute rejection, AV develops as a result of T cell-driven immune responses directed against the graft vasculature (Hall, 1991, Ensminger *et al*, 2002). It has been suggested that $CD4^+$ T helper cells play the dominant role (Shi *et al*, 1996, Ensminger *et al*, 2002, Yamada *et al*, 2003); however recent evidence suggests that AV may be less dependent than acute rejection on T helper cell function (Han *et al*, 2000). Moreover, there is anecdotal evidence suggesting that effector CTL are resistant to cyclosporine A (Horsburgh *et al*, 1980, Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001), implicating these cells in the failure of current calcineurin inhibitor-based pharmacotherapy.

This thesis will evaluate the role of $CD8^+$ CTL in the development of AV using a murine aortic allograft model (Mennander *et al*, 1991, Koulack *et al*, 1995). It will address a variety of aspects of this topic including the effector mechanisms employed by $CD8^+$ T cells to injure the graft leading to the repair and remodelling process characteristic of AV. The central hypothesis is that $CD8^+$ T cells are capable of mediating robust AV and that under conditions of clinical immunosuppression this $CD8^+$-mediated pathway results in the observed vascular disease.

Figure 1. ***Characteristic histopathologic features of AV.***

Representative photomicrographs of human proximal left anterior descending (LAD) coronary artery from a normal heart (**a**) and from a cardiac allograft explanted 8 years post-transplantation due to severe AV (**b**). These photomicrographs are constructed from composite photomicrographs captured at 40x magnification and stained with hematoxylin and eosin.

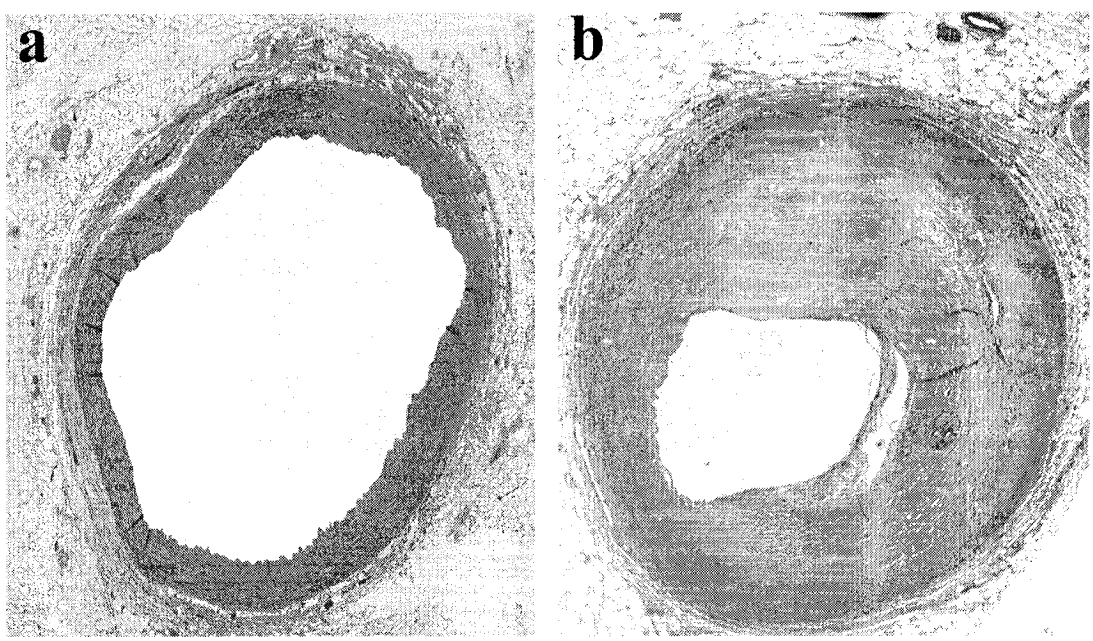


FIGURE 1

Chapter 2

2.0 Background

2.1 T cell Activation

Over 3 decades ago, Bretscher and Cohn proposed the two signal hypothesis in the activation of B lymphocytes to produce antibody to explain Ehrlich's observation of "horror autotoxicus" (Bretscher and Cohn, 1970). In this model, the recognition of foreign antigen by B lymphocytes (signal 1) without signal 2 results in the unresponsiveness of the antibody producing cell. T helper cell-derived cytokines and signalling molecules provide the necessary signal 2 following recognition of an antigenic epitope processed by the B cell.

Lafferty and Cunningham adapted the two signal hypothesis to explain the activation of T lymphocytes (Lafferty and Cunningham, 1975). In their model, the delivery of signal 1 was achieved following interaction between antigen and the T cell receptor. They further postulated that a stimulator cell-derived product with co-stimulatory activity provides the necessary signal 2 required for conversion of the T cell from a resting to an activated state. This model was later refined to state that activation results from T cell recognition of antigen in the context of an MHC molecule providing signal 1, while signal 2 results from the interaction of co-stimulatory molecules, expressed by an antigen presenting cell (APC), with its cognate receptor on the T cell (Carreno and Collins, 2002, Schwartz, 2003).

2.1.1 Signal 1

The T cell receptor is a heterodimer of α and β, or γ and δ chains linked by disulphide bridges (Davis and Bjorkman, 1988, Van Der Merwe and Davis, 2003). The mature T cell receptor (TCR), after gene rearrangement, confers antigen specificity to each individual T cell clone, and interacts with the peptide-MHC complexes presented on the surface of the APC (Schwartz, 1985, Van Der Merwe and Davis, 2003). The intracytoplasmic domain of the TCR is insufficient in length for signal transduction by itself and for this purpose it associates with the CD3 complex (Clevers *et al*, 1988). CD4 and CD8 co-receptors are expressed with the TCR/CD3 complex on T helper cells and CTL, respectively (Janeway, 1992). These proteins bind to the non-polymorphic regions of MHC molecules and act to strengthen or prolong the TCR-MHC/peptide interaction, thus facilitating optimal transduction of signal 1 (Norment *et al*, 1988, Garcia *et al*, 1996).

Upon TCR-MHC binding, signal transduction is initiated by CD45-mediated dephosphorylation (Hermiston *et al*, 2003) of two members of the src family of protein tyrosine kinases (PTK), p56Lck and p59Fyn (Samelson *et al*, 1990, Abraham *et al*, 1991, Samelson, 2002). Activated p56Lck and p59Fyn then phosphorylate tyrosine residues present in the intracytoplasmic domains of ε and ζ chains of the CD3 complex (Latour *et al*, 2001). This allows for their association with another PTK known as ZAP-70 which is subsequently involved in the phosphorylation and activation of phospholipase C ($PLC\gamma_1$) (Chan *et al*, 1994, Latour *et al*, 2001). $PLC\gamma_1$ is then involved in the cleavage of plasma

membrane phospholipids to form the second messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Samelson, 2002).

In the first signalling pathway, DAG mediates the activation of protein kinase C (PKC) which in turn phosphorylates the inhibitory protein IκB causing it to dissociate from nuclear factor-kappa B (NF-κB) (Karin and Ben-Neriah, 2000, Isakov and Altman, 2002). This allows for the nuclear translocation of NF-κB where it binds to a specific region of the IL-2 gene promoter sequence (Baeuerle and Henkel, 1994, Baldwin, 1996).

In the second pathway, IP_3 triggers the release of Ca^{2+} from intracellular stores thereby facilitating the calcium-dependent activation of calmodulin (Lewis, 2001). Together, Ca^{2+} and calmodulin mediate the activation of calcineurin which possesses potent phosphatase activity capable of dephosphorylating the cytoplasmic component of the nuclear factor of activated T lymphocytes (NFAT) (Rao *et al*, 1997). It is at this point in the intracellular signalling cascade where the calcineurin inhibitors cyclosporine A (CyA) and FK-506 (tacrolimus) act to interrupt T cell activation through signal 1 blockade (Shevach, 1985, Sigal and Dumont, 1992). The active form of NFAT then translocates into the nucleus where it associates with the AP-1 complex composed of c-Jun and c-Fos and binds a distinct site within the promoter region of the IL-2 gene (Rao *et al*, 1997).

Together, NF-κB and NFAT initiate IL-2 gene transcription which is an important growth factor involved in the induction of T cell proliferation (Minami *et al*, 1993). However, this requirement for IL-2 as a T cell growth factor may not be absolute (Sepulveda *et al*, 1999). Specifically, $CD8^+$ T cells have been suggested to posses less stringent IL-2 requirements for antigen-driven activation (Sepulveda *et al*, 1999, Dai *et*

al, 2000, van Stipdonk *et al*, 2001, Li *et al*, 2001, Jones *et al*, 2002, Weng *et al*, 2002). Alternatively, $CD8^+$ T cells may produce sufficient amounts of IL-2 which can function in an autocrine fashion leading to activation and subsequent proliferation (van Stipdonk *et al*, 2001). Given this data, it is not surprising that a subset of CTL have been identified as being resistant to signal 1 blockade by treatment with the calcineurin inhibitor CyA (Horsburgh *et al*, 1980).

There is clear evidence that robust T cell activation still occurs despite blockade of IL-2 signalling through treatment with calcineurin inhibitors (Li *et al*, 2001) or when more completely interrupted using IL-2 knockout mice (Steiger *et al*, 1995). These data suggest that the proliferative signal conferred by IL-2 ligation of the IL-2 receptor on the T cell is dispensable for T cell activation. Indeed, other cytokines including IL-4, IL-7, IL-12, and IL-15, among others, have been implicated as surrogate growth factors capable of promoting the activation and proliferation of $CD8^+$ T cells (Kanegane and Tosato, 1996, Huang *et al*, 2000, Li *et al*, 2001, Ferrari-Lacraz *et al*, 2001, Schluns *et al*, 2002, Kieper *et al*, 2002, Schmidt and Mescher, 2002, Weng *et al*, 2002). Thus, the availability of cognate antigen as well as growth factors provided by APC and T helper cells act to regulate the activation of naïve $CD8^+$ T cells into mature effector CTL. In addition, a subset of $CD4^+$ $CD25^+$ T cells function as a negative regulator of $CD8^+$ T cells, inhibiting their activation and effector function providing a further level of control over CTL immunity (Piccirillo and Shevach, 2001, van Maurik *et al*, 2002). However, the impact of immunosuppressive agents on the regulatory function of $CD4^+$ $CD25^+$ T cells remains unclear and may have profound implications for the development of transplantation tolerance through allo-immune regulation (Wood and Sakaguchi, 2003).

2.1.2 Signal 2

The activation of second messenger pathways that occurs upon interaction of the TCR with the peptide-MHC complex is not sufficient for optimal T cell activation. Quill and Schwartz demonstrated this when T cells exposed to purified MHC molecules inserted into liposomes which were pulsed with processed peptide failed to proliferate (Quill and Schwartz, 1987). Similarly, T cell clones stimulated with the mitogen concanavalin A in the absence of antigen presenting cells fail to proliferate despite the generation of second messengers resulting from an intact signal 1 (Mueller *et al*, 1989). As a result, modulation of signal 2 is an attractive therapeutic option because the manipulation of co-stimulatory signals might provide a means either to enhance or to terminate immune responses.

The second signal, so-called co-stimulation, is provided through various molecules utilizing distinct mechanisms that physically reorganize the TCR complex, lower the threshold for TCR signalling, amplify T cell activation and modify the outcome of antigenic activation, preventing anergy or death (Carreno and Collins, 2002, Sharpe and Freeman, 2002). Several cell surface receptors including LFA-1, ICAM-1 and VLA-4, initially investigated for their role in cell adhesion, have been implicated in T cell co-stimulation (Mondino and Jenkins, 1994, Chirathaworn *et al.* 2002, Kohlmeier *et al*, 2003). However, the interaction of the CD28 superfamily of receptors on T cells with their ligands, B7-1 (CD80), and B7-2 (CD86) on the APC have garnered the most interest as co-stimulatory molecules for T cell activation (Carreno and Collins, 2002, Sharpe and Freeman, 2002). CD80 and CD86 have a dual specificity for two CD28 superfamily

members, the stimulatory receptor CD28 and the inhibitory receptor CTLA-4 (CD152) (Carreno and Collins, 2002, Sharpe and Freeman, 2002). CTLA-4 is the higher-affinity receptor for CD80 and CD86 (Linsley *et al*, 1994). CD28 is constitutively expressed on the surface of T cells, whereas CTLA-4 expression is rapidly up-regulated following T-cell activation (Carreno and Collins, 2002). On most APC populations, CD86 is expressed constitutively at low levels and is rapidly up-regulated, whereas CD80 is induced much later after activation has occurred (Hathcock *et al*, 1994). CD80 and CD86 have largely overlapping functions, yet they are differentially expressed by different cell types or at different times during an immune response (Sharpe and Freeman, 2002).

A critical role for CD28 as a co-stimulatory molecule in the activation of T cells was established when Su and colleagues found that concurrent stimulation through the TCR and CD28 resulted in a 30-fold increase in Jun kinase (JNK) activity, a necessary protein tyrosine kinase (PTK) for the phosphorylation of c-Jun and activation of the AP-1 complex (Su *et al*, 1994). However, stimulation through the TCR or CD28 alone resulted in minimal JNK activity confirming the importance of CD28 in T cell co-stimulation (Su *et al*, 1994). Further evidence supporting a pivotal role for CD28-mediated co-stimulation in the activation of T cells was obtained using a potent inhibitory fusion protein construct of CTLA-4 and an immunoglobulin constant chain, referred to as CTLA-4Ig (Linsley *et al*, 1991). In transplantation models, CTLA-4Ig treatment is able to abolish T cell-mediated acute rejection responses *in vivo* (Turka *et al*, 1992, Lenschow *et al*, 1992). However, transplantation experiments with CD28-deficient mice later demonstrated that T cell-mediated acute rejection of skin allografts remains intact in the absence of co-stimulation through CD28 (Kawai *et al*, 1996). Szot and colleagues later demonstrated

that CD28 blockade-resistant $CD8^+$ T cells are involved in the rejection of cardiac allografts by CD28-deficient recipients, implicating alternate co-stimulatory pathways in the activation of this subset of $CD8^+$ T cells (Szot *et al*, 2001).

The CD40-CD40 ligand (CD154) receptor-ligand pair has also been suggested to contribute to the co-stimulation of T cells. CD40 is expressed by dendritic cells, activated macrophages and B cells, whereas CD154 is found almost exclusively on T cells (Banchereau *et al*, 1994). Interestingly, CD154-deficient animals exhibited profound defects in T cell activation *in vitro* and *in vivo* indicating that this receptor –ligand pair might be directly involved in T cell co-stimulation (Grewal *et al*, 1995, Grewal *et al*, 1996). However, it has been hypothesized that T cells from CD154-deficient mice are incapable of properly conditioning APCs such that the CD40-CD154 interaction was involved in the up-regulation of CD80 and CD86 on the APC and CD28 on the T cell compromising CD28-mediated co-stimulation (Yang and Wilson, 1996). Nonetheless, there is strong evidence that $CD4^+$ T cells (Blazar *et al*, 1997, Blazar *et al*, 1998) and $CD8^+$ T cells (Borrow *et al*, 1996, Schoenberger *et al*, 1998, Clarke, 2000, Bourgeois *et al*, 2002) require an intact CD40-CD154 interaction for optimal activation and effector function. In contrast, other groups have identified a subset of $CD8^+$ T cells which under certain circumstances can be activated independently of the CD40-CD154 interaction (Whitmire *et al*, 1996, Whitmire *et al*, 1999, Gao *et al*, 2000, Guo *et al*, 2001). Recently, Jones and colleagues have demonstrated that $CD4^+$ T cell-mediated cardiac allograft rejection is sensitive to CD40-CD154 blockade, whereas a subset of $CD8^+$ T cells remained fully functional, capable of mediating acute rejection (Jones *et al*, 2000).

In a transplantation model, Larsen and colleagues demonstrated that simultaneous blockade of B7/CD28 and CD40/CD154 is effective in abrogating acute and chronic rejection of murine cardiac allografts (Larsen *et al*, 1998). Similarly, Li and colleagues found that combined co-stimulatory blockade successfully abrogated acute rejection of murine cardiac allografts (Li *et al*, 1999). Interestingly, rapamycin was synergistic in preventing rejection, whereas when CyA was added to co-stimulatory blockade, rejection promptly re-appeared (Li *et al*, 1999). Treatment with rapamycin results in a G1 arrest of many cell types including T cells, largely through inhibition of down-stream IL-2 signalling. However, interruption of IL-2 signalling occurs at a functionally distinct location of the pathway from calcineurin inhibitors such that the permissive signal for activation induced cell death (AICD) is maintained despite rapamycin treatment (Li *et al*, 1999). Thus, from the point of view of the establishment of peripheral tolerance through co-stimulatory blockade, rapamycin does not break tolerance by maintaining the necessary proximal IL-2 signal which promotes AICD (Li *et al*, 1999). However, other groups, using high responder strain mice, have shown that co-stimulation resistant cells remain functionally intact and are capable of rejecting allografts despite a similar treatment regimen (Newell *et al*, 1999, Williams *et al*, 2000, Ha *et al*, 2001). Larsen's group later showed that this co-stimulatory blockade resistant rejection is mediated by asialo $GM1^+$ $CD8^+$ T cells (Trambley *et al*, 1999). Antigen-specific $CD8^+$ T cells have been found to rapidly up-regulate surface expression of natural killer (NK) cell associated markers upon activation, particularly in high responder strain C57BL/6 mice (Oughton and Kerkvliet, 1999, Slifka *et al*, 2000, Assarsson *et al*, 2000). Furthermore, there is evidence from these high responder strain mice (Trambley *et al*, 1999) and in humans

(Inaba *et al*, 1987, Young and Steinman, 1990) that CD4 "help" is not always required to generate $CD8^+$ CTL.

The persistence of rejection and apparent T cell activation despite blockade of CD28-B7 and CD40-CD154 signalling implies the activity of other non-conventional co-stimulatory molecules. Recently, inducible co-stimulator (ICOS) a distinct, yet functionally related molecule to CD28, capable of providing the necessary signal 2 for T cell activation, has been discovered (Hutloff *et al*, 1999). It was initially thought that ICOS played a more prominent role within the co-stimulation of $CD4^+$ T cells (Lohning *et al*, 2003). However, ICOS-B7H signalling has been shown to be important for $CD8^+$ T cell mediated cytotoxicity against tumour cells *in vivo* (Liu *et al*, 2001, Wallin *et al*, 2001).

Ozkaynak and colleagues examined the impact of ICOS-B7H signalling in a murine model of acute and chronic cardiac allograft rejection using blocking anti-ICOS monoclonal anitbodies or ICOS-deficient mice bred onto the C57BL/6 background (Ozkaynak *et al*, 2001). They demonstrated that animals treated with CD154 blockade alone exhibited a profound up-regulation of ICOS by T lymphocytes indicating that co-stimulation provided through ICOS-B7H signalling might rescue T cell activation capable of mediating transplant rejection (Ozkaynak *et al*, 2001). Contrary to initial reports that ICOS may be expressed mostly by $CD4^+$ T cells, the authors found that both $CD4^+$ and $CD8^+$ T cell subsets were inhibited by anti-ICOS therapy (Ozkaynak *et al*, 2001). Interestingly, when ICOS blockade was added to anti-CD154 treatment, acute and chronic rejection were inhibited within fully mismatched BALB/c cardiac allografts transplanted into high responder CD57BL/6 recipients (Ozkaynak *et al*, 2001).

The co-stimulatory requirements of $CD4^+$ and $CD8^+$ T cells can be further distinguished by the importance of the 4-1BB–4-1BB ligand interaction during T cell activation (Laderach *et al*, 2002). For instance, the expansion of LCMV virus-specific $CD8^+$ or $CD4^+$ T cells is differentially affected in mice respectively lacking 4-1BB ligand or CD154 (Tan *et al*, 1999). Shuford *et al.*demonstrated that signalling through 4 -1BB preferentially induces the IL-2-independent proliferation of $CD8^+$ T cells while also enhancing CTL activity and the production of interferon-γ (Shuford *et al*, 1997). Later, Takahashi *et al.* demonstrated that 4-1BB stimulation significantly increases the survival of superantigen-activated $CD8^+$ T cells *in vivo* (Takahashi *et al*, 1999). The promotion of $CD8^+$ T cell proliferation and survival by 4-1BB stimulation appears to be mediated, at least in part, through the enhanced expression of Bcl-xL, an anti-apoptotic BCL-2 family member (Lee *et al*, 2002). As described above, the blockade of traditional co-stimulatory molecules fails to inhibit rejection in high responder strain mice where $CD8^+$ T cells are sufficient to mediate rejection (Trambley *et al*, 1999). In an intestinal allograft model utilizing high responder recipients, blocking the 4-1BB pathway significantly inhibited rejection mediated by $CD8^+$ but not $CD4^+$ T cells (Wang *et al*, 2003). This correlated with a disruption of priming of allo-specific $CD8^+$ T cells, whereas $CD4^+$ T cell activation remained largely intact (Wang *et al*, 2003). Taken together, these results indicate that 4-1BB has a crucial role in the generation of effector cytotoxic T lymphocytes by enhancing their proliferative capacity and survival while supporting a type 1 cytotoxic T cell (Tc1) phenotype capable of mediating graft rejection (Shuford *et al*, 1997).

2.2 Cytotoxic T Lymphocyte Effector Mechanisms

Killing activity exerted by cytotoxic T lymphocytes (CTL) against offending pathogens is integral to the survival of humans and animals alike. Initially it was believed that membrane damage was sufficient to cause cell death. However, it is now apparent that the destruction of target cells by CTL is a multifaceted process composed of complex pathways leading to apoptotic or necrotic cell death (Barry and Bleackley, 2002). This was first appreciated in experiments where antibody and complement damage was observed to be restricted to the plasma membrane, while lymphocytes produced a more general internal disintegration of the target cell, including the nucleus (Russell *et al*, 1980). Apoptosis or programmed cell death results from activation of an enzymatic cascade intrinsic to the cell. It is characterized by preservation of the plasma membrane, membrane blebbing, condensation of chromatin, and fragmentation of nuclear DNA. This leads to a 'silent' form of cell loss, distinguishing it from the intense inflammatory response incited by necrotic cell death (Barry and Bleackley, 2002).

It has been established that the two dominant mechanisms of contact-dependent, lymphocyte-mediated cytotoxicity are the granule-exocytosis and the Fas-Fas ligand (FasL) pathways (Kagi *et al*, 1994). Although this interpretation is true in that measurable CTL activity is mediated almost exclusively by the granule exocytosis and Fas pathways (Kagi *et al*, 1994), emerging experimentation has uncovered other cytotoxic effector molecules. As such, it may be more useful to consider the two mechanisms as those initiated by Fas-associated Death Domain (FADD) via a target cell death receptor and those that require the pore forming protein perforin (Russell and Ley, 2002).

Experiments using Fas-deficient (*lpr*) or FasL-deficient *(gld)* mice have demonstrated that Fas (CD95) is the most physiologically important receptor initiating death through the recruitment of FADD and caspase 8 (Nagata and Golstein, 1995, Krammer, 2000). However, other members of the tumour necrosis factor (TNF) receptor pathway, including tumour necrosis factor-related apoptosis-inducing ligand(TRAIL) receptor, converge on FADD as well (Kuang *et al*, 2000). Moreover, the granule-exocytosis pathway represents a series of parallel pathways that involve the delivery, into the target cell cytoplasm, of several cytotoxic granules including various granzymes and the more recently described granulysin (Trambas and Griffiths, 2003). Several modes of entry have been described, but each is dependent on perforin for their eventual liberation from membrane-bound vesicles, which is an absolute requirement for cytotoxicity to occur (Trambas and Griffiths, 2003).

Naïve or precursor $CD8^+$ CTL do not possess a readily available cytotoxic armamentarium rendering them incapable of target cell killing (Russell and Ley, 2002). Upon antigen-driven activation, a rapid induction of cytotoxic effector molecules including granule components and TNF-related ligands confers maximal CTL activity after several days of stimulation (Russell and Ley, 2002). CTL-target conjugate formation, based on an antigen-specific TCR-MHC class I and peptide interaction (signal 1), initiates the development of an immunological synapse (Stinchcombe *et al*, 2001). This synapse formation is bolstered by adhesion molecules such that the interaction between LFA-1 and target-derived ICAM-1 provides signal 2 (Lancki *et al*, 1987). These two signals lead to triggering, whereby the CTL reorients its cytotoxic effector molecules contained in vesicles to the region of receptor activation and releases the granule

components into the intercellular cleft between the killer and the target (Trambas and Griffiths, 2003).

In the granule-exocytosis pathway of killing, granzymes or granulysin gain entry into the target cell through poly-perforin pores in the plasma membrane of the target (Barry and Bleackley, 2002). Alternatively, cytotoxic granules can be internalized by the cation-independent mannose-6-P receptor (CI-MPR) and subsequently released from endocytic vesicles by perforin (Russell and Ley, 2002). Once in the cytoplasm, there are numerous substrates for granzymes to act upon including caspase 3 and the pro-apoptotic BCL-2 family member BID (BCL-2 interacting domain; Barry and Bleackley, 2002). In particular, granzyme B is capable of activating the mitochondrial as well as caspase-dependent pathways of apoptosis (Barry and Bleackley, 2002). More recently, granzyme B has been shown to cause DNA fragmentation by directly activating apoptotic nucleases (Thomas *et al*, 2000). Nonetheless, a crucial role for perforin in this pathway is clear since CTL from perforin-deficient mice are unable to induce membrane damage or apoptosis via granule-exocytosis, rendering them susceptible to infection and tumorigenesis (Kagi *et al*, 1994, van den Broek *et al*, 1996). However, CTL activity against Fas-expressing targets remains fully intact in these mice (Kagi *et al*, 1994).

Similarly, the expression of the TNF family of ligands including FasL and TRAIL requires antigen-driven activation (Russell and Ley, 2002). However, maximal induction occurs up to two hours after TCR stimulation and the long half-life of ligand on the surface allows effector cells to continue to display cytotoxic activity in the absence of TCR stimulation leading to the phenomenon of bystander killing (Wang *et al*, 1996). This pathway appears to be active in all CTL but it is most commonly utilized by $CD4^+$ Th1

cells (Ju *et al*, 1994). Thus, $CD4^+$ CTL are capable of the promiscuous killing of non-MHC class II-bearing targets after initial TCR stimulation (Wang *et al*, 1996). This could have important implications in transplant rejection, as relatively few cells express MHC class II, but once stimulated these $CD4^+$ CTL are capable of killing all death receptor-expressing cells in the vicinity.

In the Fas-FasL pathway, CTL-derived FasL engages the Fas death receptor of the target leading to a cascade of events involving FADD and caspase 8, depending on the target cell type (Scaffidi *et al*, 1998, Barry and Bleackley, 2002). The cytoplasmic region of Fas contains a death domain that, upon ligand-induced clustering, forms a binding site for the adaptor protein FADD. FADD, through its death effector domain, can recruit pro-caspase 8 to the complex. Receptor-associated procaspase 8 undergoes autocatalytic activation to caspase 8 (also known as FLICE), which can then cleave and activate effector caspases 3, 6, and 7 at aspartic acid residues (Barry and Bleackley, 2002). Activated effector caspases cleave a variety of cellular protein substrates while initiating endonucleases, leading to DNA fragmentation and apoptotic cell death. Activated caspase 8 can also cleave a small cytosolic protein called BID, enabling BID to bind to mitochondria and to release cytochrome *c* into the cytoplasm. Released cytochrome *c* binds to apoptosis-activating factor 1 (APAF-1), forming a complex (apoptosome) that in the presence of ATP can bind and promote autocatalytic activation of procaspase 9 (Barry and Bleackley, 2002). Caspase 9, like caspase 8, can cleave and activate effector caspases. In some cell types (type 1) sufficient amounts of caspase 8 are activated at the death-inducing signaling complex (DISC) so as to efficiently initiate apoptosis, whereas

others (type 2) form less caspase 8 and must use the mitochondrial pathway to amplify the Fas signal (Scaffidi *et al*, 1998).

$CD8^+$ CTL also produce several cytokines, such as TNF and IFN-γ that have cytotoxic activity when secreted in the vicinity of target cells (Lattime and Stutman, 1992, Ruegg *et al*, 1998). IFN-γ produced by CTL up-regulates the expression of MHC class I (Gobin *et al*, 1999) and Fas (Mullbacher *et al*, 2002) by target cells and may thereby potentiate direct recognition and contact-dependent killing. For example, IFN-γ has recently been implicated as an indirect effector molecule in the rejection of skin grafts by $CD8^+$ T cells (Valujskikh *et al*, 2002). However, in these experiments IFN-γ may be exerting its effect by the potentiation of direct recognition and killing of recipient-derived neo-vasculature, necessary for survival of the skin graft (Valujskikh *et al*, 2002). These data implicate cross-primed CTL-derived IFN-γ as an important effector involved in the destruction of tumour neo-vasculature necessary for tumour growth and subsequent metastasis (Waldmann, 2002). In addition, $CD8^+$ T cell-derived IFN-γ is capable of mediating delayed type hypersensitivity (DTH)-like reactions, whereby macrophages are specifically recruited and activated to exert killing activity (Muller *et al*, 1994).

There is emerging evidence of an important regulatory role for IFN-γ in CTL-mediated contact-dependent killing of tumour targets (Lukacher, 2002). Malmberg and colleagues pre-treated ovarian tumour targets with IFN-γ to enhance MHC class I expression to boost CTL recognition and killing (Malmberg *et al*, 2002). Interestingly, they observed reduced killing activity in these experiments (Malmberg *et al*, 2002). It was previously described that $CD8^+$ CTL express inhibitory natural killer cell receptors

(iNKR) such as CD94/NKG2A, which are up-regulated in response to IFN-γ (McMahon and Raulet, 2001). This led to the hypothesis that ligation of iNKR on the CTL, up-regulated by IFN-γ was responsible for the diminished killing activity. Indeed, when antibodies to CD94 were added to the culture, killing activity was restored (Malmberg *et al*, 2002). Moreover, ligation of iNKR, by non-classical MHC class I molecules such as Qa-1, potently inhibits CTL killing while inducing IFN-γ production, further promoting iNKR expression (Lukacher, 2002). Consistent with this hypothesis is the observation of enhanced killing activity by $CD8^+$ CTL-derived from IFN-γ-deficient mice *in vitro* (Saleem *et al*, 1996) and robust acute rejection of cardiac allografts despite co-stimulatory blockade *in vivo* (Bishop *et al*, 2001).

2.3 Acute Rejection

Upon transplantation of a vascularized cardiac allograft, donor dendritic cells migrate from the graft through the circulation to the spleen (Larsen *et* al, 1990). These professional APCs persist in the spleen for several days and associate primarily with $CD4^+$, but also with $CD8^+$ T cells of the host (Larsen *et al*, 1990). Host T cell recognition of foreign class I and class II MHC molecules, expressed on donor dendritic cells, leads to T cell activation and is thought to initiate acute allograft rejection (Larsen *et al*, 1990, Lechler *et al*, 2001). Both $CD4^+$ Th cells and $CD8^+$ CTL are thought to be involved in acute allograft rejection by mechanisms involving DTH-like responses and T cell-mediated cytotoxicity, respectively (Lowry *et al*, 1983, Mason *et al*, 1984, Dengler and Pober, 2000). The relative importance of $CD4^+$ and $CD8^+$ T cells in allograft rejection

remains a contentious issue. Most adoptive transfer and cell depletion experiments indicate that $CD4^+$ Th cells play a dominant role in this process (Hall, 1991). Under most circumstances, $CD4^+$ T cells reject allogeneic tissues irrespective of the presence of $CD8^+$ T cells (Hall, 1991, Shelton *et al*, 1992, Bishop *et al*, 1993, Morton *et al*, 1993), while the reverse is not always true (Hall 1991, Bishop *et al.* 1992, Bishop *et al.* 1993).

Bishop and colleagues have demonstrated that at early stages following heart transplantation, $CD4^+$ T cells are the dominant T cell subset in the graft (Bishop *et al*, 1992). However, at later stages $CD8^+$ T cells constitute the majority of graft infiltrating cells. Although graft destruction strongly correlated with $CD8^+$ CTL infiltration, this appears to $CD4^+$ T cell-dependent, as depletion of $CD4^+$ T cells from recipient mice prior to transplantation resulted in abrogation of the $CD8^+$ infiltrate and prolonged allograft survival (Bishop *et al*, 1992). Krieger observed similar results while using a different mouse strain combination for their transplants (Krieger *et al*, 1996).

In a later study, Bishop and colleagues showed that treatment with rIL-2 of transplant recipients depleted of $CD4^+$ T cells restored $CD8^+$ infiltration, but did not result in the generation of CTL activity and graft rejection (Bishop *et al*, 1993). In addition, depletion of $CD8^+$ T cells in this system did not inhibit acute rejection demonstrating that $CD4^+$ T cells were both necessary and sufficient to initiate graft destruction (Bishop *et al*, 1993). The authors argued that the development of CTL activity was dependent on $CD4^+$ T cells and that other factors in addition to IL-2 were important in activation of CTL (Bishop *et al*, 1993).

In a complimentary line of experimentation, Krieger and colleagues observed indefinite cardiac allograft survival in CD4-deficient ($CD4^{-/-}$) recipients (Krieger *et al*,

1996). Moreover, the adoptive transfer of $CD4^+$ T cells into $CD4^{-/-}$ mice restored the ability of these mice to acutely reject hearts (Krieger *et al*, 1996). Taken together, these results indicate that $CD8^+$ CTL are dispensable for acute rejection and that they require $CD4^+$ T cell help in order to contribute to the rejection process.

One explanation of these findings is that CTL activity is not essential in the development of acute allograft rejection. Another is that, unconventional pathways of CTL induction are active in the absence of $CD8^+$ T cells, and that these take part in the rejection process. The significant redundancy of immune effector mechanisms would suggest that the latter is a serious possibility. For example, transplantation of CD8-deficient ($CD8^{-/-}$) mice with MHC class I-disparate skin grafts leads to prompt rejection of these grafts (Dalloul *et al*, 1996). Interestingly, in this model rejection was associated with the development of a highly lytic population of $CD4^-$ $CD8^-$ TCR $\alpha\beta^{\text{intermediate}}$ (DN) T cells (Dalloul *et al*, 1996). Moreover, *in vitro* studies demonstrated that activation of $CD4^+$ T cells in the absence of $CD8^+$ T cells leads to development of $CD4^+$ CTL capable of killing allogeneic targets by the granule-exocytosis pathway (Williams and Engelhard, 1997), normally utilized by $CD8^+$ CTL for target cell killing (Kagi *et al*, 1994, Russell and Ley, 2002). Recent studies in a rat small intestinal transplantation model have confirmed *in vivo* that depletion of $CD8^+$ T cells does not reduce intragraft expression of granzyme B and perforin (Krams *et al*, 1998). In this *in vivo* model apoptosis of graft parenchymal cells was not reduced and graft rejection was unimpeded (Krams *et al*, 1998). These data suggest that in the absence of $CD8^+$ T cells other pathways of cytotoxic activity are induced, which are capable of mediating acute allograft rejection.

The studies above show that $CD8^+$ T cells are not required or even sufficient to reject grafts. However, this evidence does not rule out the possibility that $CD8^+$ T cells significantly contribute to the acute rejection of solid organ allografts under normal conditions. Studies employing the adoptive transfer of previously allo-sensitized $CD8^+$ T cells have demonstrated a significant role for $CD8^+$ T cells in acute allograft rejection (Prowse *et al*, 1983, Hall *et al*, 1985). In addition, $CD8^+$ T cells have been shown to mediate rejection of MHC class I mismatched skin grafts independently of $CD4^+$ T cells (Rosenberg *et al*, 1986). Rosenberg and colleagues utilized high responder strain C57BL/6 mice as recipients, which contain a population of helper-independent $CD8^+$ T cells capable of providing sufficient IL-2 production for autocrine stimulation leading to development of CTL in the absence of $CD4^+$ T cells (Roopenian *et al*, 1983, Andrus *et al*, 1984).

It has been appreciated that $CD8^+$ CTL, through recognition of donor MHC class I, are the major effector cell implicated in the destruction of cardiac myocytes in transplanted hearts (Libby and Zhao, 2003). Indeed, intense apoptosis of cardiac myocytes has been described in heart allografts undergoing acute rejection (Shaddy, 1997). Until recently, it was felt that the activation of $CD8^+$ CTL is dependent on $CD4^+$ T helper function. Kreisel and colleagues have demonstrated that IFN-γ-stimulated mouse vascular endothelial cells are capable of activating naïve $CD8^+$ T cells in a B7-dependent manner *in vitro* (Kreisel *et al*, 2002). Moreover, they demonstrated that these CTL had the ability to produce IFN-γ and IL-2 and exhibited an effector CTL phenotype. In a separate study, these authors demonstrated that vascular endothelial cells are indeed capable of activating $CD8^+$ CTL *in vivo* leading to the acute rejection of cardiac allografts

(Kreisel *et al*, 2002). Similarly, Halamayand colleagues have shown that 2C tr ansgenic (H-2L^d-specific clone) CD8$^+$ T cells are suffient to initiate acute cardiac allograft rejection in the absence of CD4$^+$ T cells (Halamay *et al*, 2002). Again, these 2C mice are bred onto a C57BL/6 background and exhibit helper-independent proliferative and CTL responses to the 2C allo-antigen (Halamay *et al*, 2002). These data clearly implicate a role for CD8$^+$ T cells in the acute rejection process, particularly since CD8$^+$ CTL from humans might exhibit similar helper-independent function (Inaba *et al*, 1987, Young and Steinman, 1990).

In humans, the appearance of transcripts for granzyme B and perforin in kidney (Lipman *et al*, 1994, Strehlau *et al*, 1997) or heart (Legros-Maida *et al*, 1994) biopsies strongly correlates with acute rejection episodes. Expression of these markers is reduced following immunosuppressive therapy (Legros-Maida *et al*, 1994). Similarly, a correlation between acute rejection and granzyme A expression was found in biopsies of human cardiac allografts (Alpert *et al*, 1995). Wever and colleagues developed infiltrating cell lines from human renal biopsies, and noted that these CTL use the granule-exocytosis pathway to kill proximal tubular epithelial cell targets derived from the corresponding biopsies (Wever *et al*, 1998). Similar results correlating the expression of CTL-specific cytotoxic molecules and acute allograft rejection have been obtained in murine models of transplantation (Chen *et al*, 1993, McDiarmid *et al*, 1995, Chen *et al*, 1996, Krams *et al*, 1998). Although acute allograft rejection can occur in the absence of CTL, identification of CTL effector molecules appears to be a good predictor of allograft rejection.

Allograft rejection is thought to be primarily mediated by DTH and cytotoxic T-lymphocyte activity both of which are dependent on type 1 cytokines (Hall, 1991). Indeed, type 1 cytokines are often found in grafts undergoing acute rejection (Dallman, 1993). Various pharmacologic interventions such as treatment with cyclosporine A (Takeuchi *et al*, 1992), rapamycin (Ferraresso *et al.* 1994), anti-CD4 monoclonal antibody (Takeuchi *et al*, 1992, Siegling *et al*, 1994, Mottram*et al*, 1995 and Binder *et al*, 1996), and CTLA-4Ig (Sayegh *et al*, 1995) result in down-regulation of type 1 immunity yielding prolongation of graft survival.

IFN-γ transcription has been consistently detected in cardiac and renal grafts undergoing acute rejection, and because in some cases long term allograft survival was associated with decreased IFN-γ expression, researchers have proposed that this cytokine mediates acute allograft rejection (Dallman, 1993). However, some data suggests that the role of IFN-γ in acute rejection may be more complex than was previously thought. Lakkis and co-workers have shown that IFN-γ transcripts persist in long surviving cardiac allografts in mice treated with CTLA4-Ig (Lakkis *et al*, 1997). In addition it has been demonstrated that both IFN-γ-deficient (Saleem *et al*, 1996) and IFN-γR-deficient (Steiger *et al*, 1998) mice are capable of acutely rejecting cardiac and islet allografts, respectively, suggesting that this cytokine is not essential for acute rejection.

Perhaps most surprising were the results obtained by Konieczny and co-workers, demonstrating that IFN-γ-deficient mice fail to accept cardiac and skin allografts following combined co-stimulatory blockade with CTLA4-Ig and anti-CD154 (Konieczny *et al*, 1998). Moreover, injection of anti-IFN-γ monoclonal antibody into wildtype allograft recipients undergoing treatment with co-stimulatory blockade led to

prompt acute allograft rejection, suggesting that IFN-γ may be required for tolerance induction rather than rejection (Konieczny *et al*, 1998). The mechanisms by which IFN-γ may mediate tolerance are not clear, however it has been suggested that IFN-γ could be involved in the negative regulation allo-reactive T cell expansion (Konieczny *et al.* 1998) and CTL activity (Lukacher, 2002). In support of this are studies demonstrating that the presence of IFN-γ leads to decreased allo-specific T cell proliferation and CTL activity in response to challenge *in vitro* (Dalton *et al*, 1993, Konieczny *et al*, 1998, Lukacher, 2002).

2.4 T Lymphocytes in Native Atherosclerosis

The localization of immune cells including macrophages and lymphocytes in human atherosclerotic lesions has implicated the involvement of innate and adaptive immunity in the pathogenesis of atherosclerosis (Hansson, 2001). This has prompted significant interest in the field of inflammation and immunity as they pertain to atherosclerosis (Binder *et al*, 2002, Libby, 2002). Consequently, the prevailing view has become that atherosclerosis is a chronic inflammatory disease induced by a lipid disturbance at sites of hemodynamic shear stress within the arterial wall, leading to the induction of an over-exuberant fibroproliferative repair process (Hansson *et al*, 2002, Libby, 2002).

T lymphocytes are among the earliest cell types to infiltrate the early fatty streak lesion of human atherosclerosis (Hansson *et al*, 2002). T cell clonal expansion has been demonstrated in lesions from animal models and humans (Ross, 1999). The exact nature

of the inciting pathogen(s) in atherosclerosis remain(s) uncertain. Numerous candidate pathogens have been identified in animal models or as risk factors by epidemiologic studies (Ross, 1999). The most likely culprit is oxidized forms of LDL (oxLDL) (Kearney, 2000), but emerging evidence implicating microbial antigens derived from infectious agents such as *Chlamydia pneumoniae* have garnered attention recently (Libby, 2002).

Nonetheless, the majority of vessel wall infiltrating T cells express an effector/memory phenotype and specifically recognize oxLDL (Kearney, 2000, Binder *et al*, 2002, Libby, 2002). Moreover, detection of anti-oxLDL IgG antibodies, distinct from natural low-avidity germline-encoded IgM antibodies, in atherosclerotic patients and hypercholesterolemic mice support a role for ox-LDL specific lymphocytes in atherogenesis (Kearney, 2000). The presence of IgG antibodies in sera derived from humans and animals with atherosclerosis suggests the activity of T cells in providing the necessary signals for B cell antibody class switch.

The inhibition of lesion formation in atherogenic mice treated with co-stimulatory blockade, by interruption of the CD40-CD154 interaction, provides further evidence of an important role for T cells (Mach *et al*, 1998). Similarly, Lutgens *et al* showed a profound reduction in atherosclerosis in apolipoprotein e-deficient ($Apoe^{-/-}$) CD154-deficient mice (Lutgens *et al*, 1999). Moreover, two independent reports showed that delayed treatment with anti-CD154 blockade was effective in preventing the progression of atherosclerosis by stabilizing the plaque and potentially preventing clinically significant events related to rupture (Schönbeck *et al*, 2000, Lutgens *et al*, 2000). These data support an important role for activated T lymphocytes in atherogenesis.

Most T cells identified in lesions from humans and animal models are $CD4^+$ $\alpha\beta TCR^+$ and resemble the Th1 lineage through secretion of IFN-γ, and IL-2 (Hansson, 2001). Moderate numbers of $CD8^+$ Tc1 cells are also present which exhibit cytolytic activity and antigen-specific secretion of IFN-γ (Binder *et al*, 2002, Gupta *et al*, 1997). The elaboration of IFN-γ leads to complex cross-talk between T lymphocytes and macrophages which are ultimately pro-atherogenic. Th2 lymphocytes are found less frequently in early lesions but become more prominent in advanced lesions and function to counteract the pro-atherogenic effects of the Th1 lineage (Pinderski-Oslund *et al*, 1999). Th2-derived IL-10 acts to stabilize plaques by decreasing lipid content, and enhancing the smooth muscle cellular and collagen components of the atheroma (Mallat *et al*, 1999). These effects can be primarily attributed to antagonizing the pro-atherogenic and plaque destabilizing effects of Th1-secreted IFN-γ (Gupta *et al*, 1997).

Zhou and colleagues more directly assessed the impact of T cells in atherosclerosis using adoptive cell transfer into immunodeficient chow-fed $Apoe^{-/-}$ SCID mice under physiologic levels of lipid stress (Zhou *et al*, 2000). They observed a marked reduction in fatty streak lesions in unreconstituted $Apoe^{-/-}$ SCID mice compared to controls indicating that T lymphocytes, while not obligatory, do play a major role in forming the early fatty streak lesion (Zhou *et al*, 2000). Moreover, the transfer of $CD4^+$ T cells into $Apoe^{-/-}$ SCID hosts resulted in a substantial increase in lesion size which was accompanied by marked T cell infiltration, IFN-γ production and enhanced MHC class II expression (Zhou *et al*, 2000). In contrast, other groups performed similar experiments using immunodeficient mice under conditions of severe lipid stress and observed no attenuation of disease (Daugherty *et al*, 1997, Dansky *et al*, 1997). However, in the

setting of severe hypercholesterolemia significant activation of innate immunity, by the engagement of highly conserved pattern recognition receptors by oxidized LDL species, is sufficient to trigger atherogenesis (Hansson, 2001). Thus, in the presence of severe lipid stress, far beyond what is observed in humans, atherogenesis appears to be lymphocyte-independent. Taken together, these data are consistent with the hypothesis that under physiologically relevant levels of hypercholesterolemia T cell immunity plays a major role in atherogenesis.

In addition, T lymphocytes play a major role in the development of plaque instability and subsequent rupture leading to clinically significant sequelae characterized by the acute coronary syndrome (Libby *et al*, 2002). Emerging evidence supports a role for T lymphocytes and intense pro-inflammatory responses at sites of plaque instability capable of precipitating myocardial infarction (Van der Wal *et al*, 1994). T lymphocytes are but one cell type capable of igniting such intense inflammation by elaboration of the cytokine IFN-γ resulting in recruitment of activated macrophages and other T lymphocytes (Mach *et al*, 1999). T-lymphocyte-derived IFN-γ incites inflammation, inhibits smooth muscle cell proliferation while promoting apoptosis, prevents collagen deposition and enhances the activity of matrix metalloproteinases which ultimately lead to rupture and tissue factor-mediated thrombosis (Hansson, 2001). A physical disruption of the atheromatous plaque precipitates thrombus formation in the vast majority of cases (Davies, 1995). Smooth muscle cells are the primary producer of interstitial collagen that provides strength to the fibrous cap. Inhibition of smooth muscle cell proliferation and synthetic capacity renders the fibrous cap vulnerable to rupture and exposes tissue factor precipitating thrombosis (Libby, 2002). Further, T lymphocyte-derived IFN-γ results in

potentiation of smooth muscle cell apoptosis leading to the formation of a necrotic core resulting in further instability (Hansson, 2001). These integral players have been found in abundance in both human plaques and advanced lesions of atherosclerosis prone mice. Interruption of T cell-derived IFN-γ results in greater plaque stability with less frequent complications associated with plaque rupture (Libby, 2002). Moreover, expression of IL-10 by Th2 lymphocytes directly opposes the pro-atherogenic effects of IFN-γ and further promotes stabilization of atherosclerotic lesions (Pinderski *et al*, 2002). Thus, while not necessary for plaque initiation under conditions of severe lipid stress, activated T lymphocytes play a major role in the development of clinically significant atherosclerosis characterized by myocardial infarction and sudden cardiac death.

2.5 Allograft Vasculopathy

The median survival of heart transplants performed in Canada is 8 to 9 years (Légaré *et al*, 1999). Heart transplant recipients are treated with combination immunosuppressive therapy which ablates acute rejection thereby prolonging graft survival. However, late graft failure primarily due to chronic allograft rejection has emerged as a major problem in clinical heart transplantation (Hosenpud *et al*, 2001). The most common and intractable pathological presentation of chronic rejection in cardiac transplants is a concentric intimal proliferative response within the arteries of transplanted organs termed allograft vasculopathy (AV). AV bears many similarities to native atherosclerosis in terms of its pathophysiology; however patients with AV have no symptoms of angina pectoris (Uretsky *et al*, 1992). When present, AV often progresses

rapidly leading to ventricular arrhythmias, silent myocardial infarction, congestive heart failure and sudden death (Uretsky *et al*, 1992). Currently, there is no effective therapy capable of limiting the mortality and morbidity associated with AV. Conventional revascularization strategies are ineffective because of the diffuse nature of the disease and re-transplantation is marred by poor results while also compounding the already critical problem of organ shortage (von Scheidt W, 2000).

The two most widely used and well characterized animal models of AV are the murine heterotopic heart transplant (Corry *et al*, 1974) and aortic interposition allograft (Koulack *et al*, 1995). In the first model, a donor heart is transplanted heterotopically into the abdomen of the recipient such that the heart receives modified perfusion from the abdominal aorta and venous drainage via the vena cava. Anastamosis of the donor and recipient aortae provides coronary perfusion to the graft myocardium, while venous outflow is achieved by anastamosis of the donor pulmonary artery to the recipient inferior vena cava. In this regard, the left ventricle is rendered non-functional and remains empty throughout the cardiac cycle. This represents a substantial deviation from the normal functional characteristics of human cardiac allografts and might have profound implications on the pathophysiology of AV observed in this model. Similar to human transplantation, the murine cardiac allograft requires some form of immunosuppressive therapy to prevent acute rejection prior to the development of AV within the coronary arteries which forms many weeks later.

In the second model, a short segment of abdominal aorta is interposed orthotopically into the abdominal aorta of the recipient, where it develops AV in histoincompatible strain combinations (Koulack *et al*, 1995). The aortic interposition

graft model lacks the parenchyma of a heart allograft and as such does not require immunosuppression in order to prolong graft survival sufficiently to form AV. For this reason, this model has come under intense scrutiny because some feel it does not adequately represent the pathophysiology of AV occurring within whole organ transplants (Pober, 2002). However, this model has several advantages including its consistency and the ease of quantifying lesion formation. Moreover, Ensminger *et al* have shown that the degree of intimal hyperplasia is highly concordant between aortic allografts and whole organ transplants in a combined heterotopic heart and aortic allograft model (Ensminger *et al*, 2000). A similar correlation was found in allograft aortas and coronary arteries of human transplants undergoing AV (Mehra *et al*, 1997). Furthermore, we (Légaré *et* al, 2001, Johnson *et al*, 2002) and others (Hillebrands *et al*, 2001) have found that immunosuppression using cyclosporine A, which ameliorates acute rejection in this model, does not affect the generation of AV. In fact, data from both immunosuppressed (Hillebrands *et al*, 2001, Johnson *et al*, 2002) and non-immunosuppressed (Shimizu *et al*, 2001, Johnson *et al*, 2002) animal models including aortic allografts (Shimizu *et al*, 2001, Hillebrands *et al*, 2001, Johnson *et al*, 2002) and whole organ transplants (Shimizu *et al*, 2001, Hillebrands *et al*, 2001, Saiura *et al*, 2001) have conclusively demonstrated that recipient-derived cells form the neointimal lesion (Skaro *et al*, 2002). These observations using the aortic allograft model have been pivotal in revising the previously accepted hypothesis regarding the etiology of AV.

In arterial allografts, AV is characterized by a chronic perivascular infiltrate present in the adventitia of the vessel wall (Plissonnier *et al*, 1995). Immunocytochemical analysis of these allografts reveals that these cells are predominantly monocytes as well

as $CD4^+$ and $CD8^+$ T lymphocytes (Plissonnier *et al*, 1995). Another striking feature of AV is the gradual disappearance of vascular smooth muscle cells from the medial compartment (Mennander *et al*, 1991, Adams *et al*, 1992, Hirsch *et al*, 1998) with a concurrent $CD8^+$ T cell infiltration (Plissonnier *et al*, 1995). Perhaps the most clinically relevant pathologic feature of AV is a progressive intimal hyperplasia which becomes occlusive over time eventually leading to ischemic graft failure (Libby and Pober, 2001). While the intimal lesion is a hallmark of AV, inward vessel remodelling may also play a role in the hemodynamic compromise of AV (Pethig *et al*, 1998, Pasterkamp *et al*, 2000). However, the clinical significance of inward vessel remodelling remains to be formally evaluated in human transplants.

The pathogenesis of AV combines immune and non-immune driving forces activating a complex cascade of injurious events in the graft arterial tree leading to uncontrolled vascular remodelling characterized by progressive lumenal narrowing. Concomitant parenchymal fibrosis occurs in response to ischemia due to lumen loss and restricted perfusion (Libby and Pober, 2001). Immunological factors implicated in AV include the degree of histoincompatibility and the number of acute rejection episodes (Libby and Pober, 2001). Non-immunogenic risk factors for the development of AV include ischemia-reperfusion, serum cholesterol and age of the donor and recipient (Johnson, 1992). Ischemic injury during organ procurement and transplantation has been identified as a particularly important factor (Gaudin *et al*, 1994). Gaudin *et al*, for example, showed that perioperative ischemic injury predicts the development of AV in humans (Gaudin *et al*, 1994). Since prolonged ischemic time enhances the development

of AV in allografts but not syngeneic grafts, ischemic injury by itself is not sufficient to induce AV (Knight *et al*, 1997).

AV is clearly an immunological phenomenon in that inbred animals receiving syngeneic transplants exhibit neither intimal hyperplasia nor medial smooth muscle cell loss (Mennander *et al*, 1993). Moreover, allo-sensitization by the administration of donor type splenocytes prior to allo-transplantation profoundly accelerates the development of AV (Kolb *et al*, 1996). Studies involving the interruption of T cell function including gene knockout strategies, antibody depletion, and inhibition of T cell activation by co-stimulatory blockade have all demonstrated the importance of cell-mediated immune responses in the development of AV (Shi *et al*, 1996, Larsen *et al*, 1996, Allan *et al*, 1997, Ensminger *et al*, 2000, Ensminger *et al*, 2002, Szeto *et al*, 2002, Yamada A *et al*, 2003). The role of humoral responses remains unclear with the evidence to date suggesting that allo-antibody is not required (Chow *et al*, 1996), but nonetheless capable of inducing AV (Russell *et al*, 1994, Russell *et al*, 1997, Hancock *et al*, 1998).

AV, like acute rejection, is a T lymphocyte-dependent process where allo-reactive T cells target the graft vasculature triggering abnormal repair and remodelling responses within the vessel wall (Hall, 1991, Ensminger *et al*, 2002). The relative contribution of the T cell subsets in eliciting AV remains a controversial issue. The majority of evidence supports a dominant role for $CD4^+$ T cells in the generation of AV (Shi *et al*, 1996, Ensminger *et al*, 2002, Yamada *et al*, 2003). Shi and colleagues demonstrated that $CD4^{-/-}$ recipients of carotid allografts do not develop AV, whereas intimal lesion formation occurs unhindered in $CD8^{-/-}$ recipients (Shi *et al*, 1996). Similarly, Ensminger and colleagues showed that reconstitution of T cell-deficient athymic nude or recombinase-

activating gene knockout mice, which lack T cells and B cells, with $CD4^+$ but not $CD8^+$ T cells led to the generation of AV in MHC class I-mismatched aortic allografts (Ensminger *et al*, 2002). AV generated by these $CD4^+$ T cells must be occurring through the indirect allo-recognition pathway (Ensminger *et al*, 2002). Similarly, Yamada and colleagues demonstrated that long surviving MHC class II-deficient cardiac allografts from recipients depleted of $CD8^+$ T cells developed AV also via cross-primed $CD4^+$ T cells (Yamada *et al*, 2003). Taken together, these data suggest that $CD4^+$ T cells are necessary for the development of AV while also implicating $CD4^+$ T cells primed by the indirect allo-recognition pathway in the rejection process.

More recently, it has been suggested that AV may be less dependent than acute rejection on T helper function (Han *et al*, 2000). Han and colleagues, using anti-CD4 antibody transgenic mice as recipients, which are completely devoid of $CD4^+$ T cells by endogenous depletion, showed that fully mismatched cardiac allografts developed AV mediated by infiltrating $CD8^+$ T cells (Han *et al*, 2000). The $CD8^+$ T cells from these high responder strain C57BL/6 background mice have been previously shown to be helper-independent (Roopenian *et al*, 1983, Andrus *et al*, 1984), presumably allowing their activation and effector function capable of generating AV (Han *et al*, 2000). This fully disparate model of AV more closely approximates human AV, since human cardiac transplantation is performed across a complete MHC mismatch. Other models of AV employing variable degrees of histoincompatibility are artificial and preferentially elicit the allo-reactivity of a particular T cell subset.

Allan and co-workers, using an MHC class I mismatched inbred miniature swine cardiac allograft model demonstrated that anti-CD8 monoclonal antibody therapy in

addition to CyA was effective in ameliorating AV (Allan *et al*, 1997). Animals treated with CyA alone developed florid AV in association with an intense $CD8^+$ T cell infiltration of the graft (Allan *et al*, 1997). Moreover, there is anecdotal evidence suggesting that effector CTL are resistant to cyclosporine A (Horsburgh *et al*, 1980, Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001), potentially implicating these cells in the failure of current calcineurin inhibitor-based pharmacotherapy.

2.5.1 Immunosuppression and AV

Given that acute (Hall, 1991, Yamada *et al*, 2003) and chronic rejection (Hall, 1991, Ensminger *et al*, 2002) are both dependent on T cell function and that the prevailing belief is that the $CD4^+$ T cell subset plays the dominant role (Hall, 1991, Ensminger *et al*, 2002, Yamada A *et al*, 2003), current immunosuppressive drugs have been tailored to address the activation, cytokine production, and proliferation of these cells. Most of these drugs are small organic compounds originally isolated from microorganisms and later shown to possess immunosuppressant properties. The remainder were specifically engineered and organically synthesized to exploit different targets within the pathways of T cell activation and effector function (Denton *et al*, 1999, Libby and Pober, 2001). Each of these drugs has discrete modes of action which serve to inhibit the immune response to the transplanted organ. However, they also produce toxic side-effects, not the least of which is non-specific immunosuppression, which limits their effective use in clinical transplantation (Denton *et al*, 1999).

Multi-drug regimens were developed to minimize toxicity while maintaining a sufficient level of immunosuppression to prevent acute rejection (Denton *et al*, 1999, von Scheidt W, 2000). Most transplant centers currently use combination immunopharmacotherapy which includes the use of a calcineurin inhibitor (cyclosporine A or tacrolimus), anti-proliferative agent (azathioprine or mycophenolate mofetil) and corticosteroids. The overall level of immunosuppression is closely monitored to minimize the complications of infection and post-transplant lymphoproliferative disease. Despite the use of multiple drugs with complementary modes of action (Nair *et al*, 1995), sparing of certain resistant effector limbs of the immune system capable of generating AV has become apparent.

The calcineurin inhibitors cyclosporine A (CyA) and tacrolimus (FK-506) are the foundation of current immunosuppressive regimens in clinical transplantation. After binding their respective immunophilin receptors (cyclophilin and FK-binding protein 12), the resulting complex inhibits the enzyme calcineurin which is necessary for IL-2 gene transcription. Interruption of this positive signal for T cell activation potently suppresses the cell-mediated immunity responsible for acute rejection (Jamieson *et al*, 1979, Légaré *et al*, 2001). Despite this beneficial effect, CyA in particular has been implicated in augmenting AV in a rat aortic allograft model (Mennander *et al*, 1991). It has been postulated that this occurs through cyclosporine A-induced up-regulation of the pro-fibrogenic cytokine transforming growth factor (TGF)-β (Shin *et al*, 1998) Parenchymal fibrosis within renal allografts receiving long-term CyA therapy has also been described and might occur through a similar pathway (Bennett *et al*, 1996). However, no such mechanism has been implicated with the use of Tacrolimus (van Riemsdijk *et al*, 2002)

and despite further improvement in the rates of acute rejection, AV still occurs largely unimpeded (Crespo-Leiro *et al*, 2002).

More recently, another report has refuted the claim that CyA exacerbates AV using a minor mismatched (Lewis to Fisher 344) rat heart allograft model (Richter *et al*, 2002). In their study, treatment with 12 mg/kg/d CyA resulted in mild attenuation of AV, while no difference from control animals was observed in the 3 mg/kg/d CyA-treated animals (Richter *et al*, 2002). Interestingly, the 12 mg/kg/d CyA treated animals exhibited drug levels in excess of 2200 ng/ml which is 4-fold greater than those permitted in humans and is certain to result in nephrotoxicity in the clinical setting (Richter *et al*, 2002). Despite this, neither group of CyA-treated animals experienced an exacerbation in the degree of AV indicating that CyA, even at doses toxic to humans, does not promote the development of AV (Richter *et al*, 2002).

Another important implication in the failure of calcineurin inhibitor-based immunosuppression is the emerging sentiment that certain effector limbs of the immune response are resistant and thus spared from immunosuppression (Libby and Pober, 2001). The importance of these subsets of T cells might be overlooked in animal models of allograft vasculopathy where immunosuppression is not commonly employed (Ensminger *et al*, 2002, Yamada *et al*, 2003). Indeed, CD8-dependent mechanisms may play a dominant role in clinical transplantation where $CD4^+$ T cell function is substantially inhibited by current calcineurin-inhibitor based immunosuppressive regimens (Horsburgh *et al*, 1980, Hruban *et al*, 1990, Bujan *et al*, 2001, Bishop *et al*, 1992).

Horsburgh and co-workers first identified a CyA-resistant population of CTL over twenty years ago (Horsburgh *et al*, 1980). Later, Hruban and colleagues observed in

human cardiac allografts undergoing AV that predominantly $CD8^+$ T cells infiltrated these grafts despite the calcineurin inhibitor-based immunosuppression their patients received (Hruban *et al*, 1990). Those allografts with no AV specifically lacked this $CD8^+$ T cell-associated endothelialitis leading the authors to speculate that AV is the result of CTL immune responses directed against the endothelium despite immunosuppression (Hruban *et al*, 1990). Bujan *et al* observed in the Lewis to Fisher 344 heart allograft model that AV was associated with the infiltration of $ED1^+$ macrophages and $CD8^+$ T lymphocytes despite daily treatment of 5 mg/kg of CyA (Bujan *et al*, 2001). This is in striking contrast to non-immunosuppressed animals where allografts undergoing AV show $CD4^+$ and $CD8^+$ T cell infiltration (Plissonnier *et al*, 1995, Ensminger *et al*, 2002, and our unpublished observations). Furthermore, when Bishop and colleagues examined the draining lymph nodes of sponge allograft recipients treated with calcineurin inhibitors, particularly those animals treated with CyA, they found that most if not all primed T cells were of the $CD8^+$ subset (Bishop *et al*, 1992). This anecdotal evidence supports the hypothesis that $CD4^+$ and $CD8^+$ T cells exhibit differential susceptibility to calcineurin inhibitors with $CD8^+$ T effector function being relatively resistant to this commonly used immunosuppressive agent.

Interestingly, there are many recent reports of a subset of $CD8^+$ T cells from high responder strain mice and humans (Inaba *et al*, 1987, Young and Steinman, 1990) which are IL-2 independent (Li *et al*, 2001, Jones *et al*, 2002, Weng *et al*, 2002). IL-15 may act as a rescue cytokine which promotes attainment and proliferation of the memory phenotype; whereas in certain circumstances IL-2 acts to limit the proliferation of memory $CD8^+$ T cells (Ku *et al*, 2000). Responsiveness to these T cell growth factors by

$CD8^+$ T cells still relies upon strong TCR signalling, which is undoubtedly provided by the robust stimulation encountered in response to allo-antigen (Gett *et al*, 2003). This lack of dependence on IL-2 signalling for activation and proliferation of $CD8^+$ T cells in lieu of other growth promoting cytokines such as IL-15 would predict that $CD8^+$ T cells are resistant to calcineurin inhibitors. However, this question has not been directly addressed in a transplant model of AV.

Rapamycin is produced by *Streptomyces hygroscopicus* and was first isolated from soil samples retrieved from Easter Island. It is a macrolide antibiotic with potent anti-fungal activity and later found to be structurally very similar to FK-506 (Sigal and Dumont, 1992, Ghosh *et al*, 2002). This similarity prompted its evaluation as an immunosuppressant. Although both FK-506 and rapamycin bind to the same intracellular immunophilin receptor FKBP12, their mechanisms of action significantly diverge as the rapamycin-FKBP12 complex inhibits the serine-threonine kinase mammalian target of rapamycin (mTOR) (Brown *et al*, 1994). This results in a G1 arrest of many cell types including T cells, largely through inhibition of down-stream IL-2 signalling. However, interruption of IL-2 signalling occurs down-stream of IL-2 receptor signal transduction termed signal 3, such that the permissive signal for AICD is maintained despite rapamycin treatment (Li *et al*, 1999). Thus, from the point of view of the establishment of peripheral tolerance through co-stimulatory blockade, rapamycin does not break tolerance by maintaining the necessary proximal IL-2 signal which promotes AICD (Li *et al*, 1999).

Another advantage to rapamycin is the avoidance of significant nephrotoxicity as a result of calcineurin inhibitor-mediated inhibition of nitric oxide synthase (Attur *et al*,

2000) which is essential for autoregulatory functions governing renal perfusion (Baylis and Qiu, 1996). Most importantly, the well described antiproliferative effects of rapamycin on various cell types including endothelium and vascular smooth muscle cells would intuitively be favourable in the prevention of AV (Marx *et al*, 1995, Mohacsi *et al*, 1997). Many immunosuppressant drugs including CyA, FK506, leflunomide and mycophenolate mofetil exhibit varying degrees of inhibitory effects on the proliferation of rat and human endothelium and smooth muscle cells, but this occurs mostly at or near toxic levels of these drugs (Mohacsi *et al*, 1997). In contrast, rapamycin exhibits the most profound antiproliferative effects well within the therapeutic range of the drug effectively avoiding major side-effects and toxicity (Mohacsi *et al*, 1997).

Morris and Billingham first showed the efficacy of rapamycin in preventing AV within a rat cardiac allograft model (Meiser *et al*, 1991). Since then, rapamycin has been shown to prevent chronic rejection of heart, renal, and small bowel allografts in various small animal (Schmid *et al*, 1995, Murphy *et al*, 2003) and nonhuman primate models (Ikonen *et al*, 2000). Moreover, rapamycin has been shown to not only inhibit but also reverse the development of AV in rat (Poston *et al*, 1999, Gregory *et al*, 1993) and non-human primate models (Ikonen *et al*, 2000) of AV.

Interestingly, Slavik *et al* have identified a subset of $CD8^+$ T cells from human individuals which are resistant to the antiproliferative effects of rapamycin (Slavik *et al*, 2001). They showed that when rapamycin is co-cultured with either freshly purified human $CD8^+$ T cells or $CD8^+$ T cell clones that proliferation still occurs. This proliferative response was highly dependent on the strength of the TCR signal and the presence of co-stimulation, as they could inhibit the proliferation of this specific subset of

rapamycin-resistant $CD8^+$ T cells by adding suboptimal concentrations of antigen or anti-CD3 and anti-CD28 (Slavik *et al*, 2001). Moreover, they could similarly inhibit this proliferative response through the addition of anti-IL2 monoclonal antibody indicating that this rapamycin-resistant $CD8^+$ T cell subset was dependent on IL-2 and not IL-15. Another group performed similar experiments using human $CD8^+$ T cells and found that neither CyA nor rapamycin were able to completely inhibit the proliferative responses of these cells (Combates *et al*, 1995). When rapamycin and CyA were used in combination there was a synergistic inhibition of $CD8^+$ T cell proliferation indicating that together these two drugs might more adequately address the expansion of allo-reactive $CD8^+$ T cells *in vitro* (Combates *et al*, 1995). However, it is important to point out that in their experiments, Combates and colleagues used a drug concentration of rapamycin which was well within the level that would be expected *in vivo* without causing toxicity. In contrast, the CyA used in these experiments was well above acceptable levels observed in human cardiac transplantation (Combates *et al*, 1995). This level is more than 2-fold what is clinically permissible again bringing into question the ability of calcineurin inhibitors to block $CD8^+$ T cell activity within its non-toxic range. It would have been interesting to note whether the activity of these $CD8^+$ T cells could have been rescued by the addition of the exogenous T cell growth factors IL-2, IL4, or IL-15. Sources of these cytokines are plentiful in the context of allo-transplantation *in vivo* and would most certainly determine the failure of immunosuppressive drug regimens using rapamycin. Nonetheless, rapamycin seems to more adequately address the function and activity of $CD8^+$ T cells and is likely to be more ideally suited for the prevention of AV. The functional

significance of rapamycin-resistant $CD8^+$ T cells will have to be further elucidated as we await the results of long-term studies using rapamycin in clinical transplantation.

2.5.2 Type 1 Cytokines in Allograft Vasculopathy

Upon activation, T cells differentiate into multiple lineages of T helper cells (Th1, Th2, Th3 and maybe others) based on the cytokine milieu in which activation occurs resulting in distinct cytokine secretion profiles (Mosmann *et al.*, 1986). In the presence of IL-12 and IFN-γ, Th1 cells develop and secrete IL-2, TNF-β, and IFN-γ, whereas IL-4 yields Th2 cells which produce IL4, IL6, IL-10 and IL-13 (Mosmann *et al.*, 1986). These distinct subsets of $CD4^+$ T helper cells lead to dissimilar immune responses. Th1 cells are generally involved in protective immunity against bacterial and viral pathogens as well as autoimmunity, whereas Th2 responses are implicated in allergy and atopic disease (Heinzel *et al.*, 1989). $CD8^+$ T cells were later demonstrated to exhibit similar cytokine secretion profiles as differentiating T helper cells allowing there classification into type 1 and 2 cytotoxic T cells (Tc1 and Tc2) (Li *et al*, 1997).

Type 1 immune responses play a major role in the development of various acute and chronic inflammatory diseases including multiple sclerosis (MS; Horwitz *et al*, 1997). In a murine model of MS (experimental allergic encephalomyelitis), treatment with agents which inhibit IFN-γ release, such as the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor atorvastatin, led to significant inhibition of IFN-γ production by both Th1 and Tc1 cells and remission of neurologic disease (Youssef *et al.*, 2002). As mentioned above, the type 1 cytokine interferon-γ has been

strongly implicated in the development of native atherosclerosis, particularly in the potentiation of plaque instability and the genesis of acute coronary syndromes (Gupta *et al*, 1997). Similarly, IFN-γ is involved in the development of AV (Russell *et al*, 1994, Raisanen-Sokolowski *et al*, 1997, Nagano *et al*, 1997, Raisanen-Sokolowski *et al*, 1998, Nagano *et al*, 1998). In wildtype mouse heart transplant recipients, AV was markedly attenuated after the administration of neutralizing anti-IFN-γ antibodies (Russell *et al*, 1994). Using an IFN-γ gene knockout strategy, two independent groups demonstrated that IFN-γ-deficient recipients develop little or no intimal hyperplasia, emphasizing the importance of IFN-γ in the development of AV (Raisanen-Sokolowski *et al*, 1997, Nagano *et al*, 1997, Raisanen-Sokolowski *et al*, 1998, Nagano *et al*, 1998). Furthermore, therapies such as HMG-CoA reductase inhibitors and angiotensin II receptor 1 (AT-1) blockers which cause immune deviation to type 2 responses, thereby limiting IFN-γ production, exhibit some efficacy in attenuating AV (Kobashigawa *et al*, 1995, Richter *et al*, 2000). These data from animal models and humans support an important role for IFN-γ in the development of AV.

Interestingly, Nagano and colleagues observed significant parenchymal rejection, presumably due to residual acute responses, in hearts from IFN-γ-deficient recipients which exhibited no AV (Nagano *et al*, 1997). Similarly, others have demonstrated that acute cardiac allograft rejection proceeds normally in IFN-γ-deficient mice (Saleem *et al*, 1996, Zand *et al*, 2000). In fact, Halloran and colleagues have shown that IFN-γ may have an early protective effect on murine cardiac and renal allografts by preventing immune-mediated collapse of the graft microcirculation leading to early necrosis (Halloran *et al*, 2001). Thus IFN-γ has diverse and potentially contradictory effects on

organ allograft survival, acting both on the immune system and on the graft itself, the net effect depending on the time post-transplant (Konieczny *et al*, 1998, Halloran *et al*, 2001). These data also suggest IFN-γ-dependent effectors are obligatory for the generation of AV, yet non-essential in the development of acute parenchymal rejection and imply distinct mechanisms for these pathologies.

Tellides and colleagues later showed that IFN-γ treatment of immunodeficient SCID-beige mouse recipients of human or pig vessel allografts leads to the development of AV (Tellides *et al*, 2000). The authors claimed that IFN-γ is species-specific in its ability to elicit responses such that only pig cells will respond to pig IFN γ and human IFN-γ only stimulates human cells (Tellides *et al*, 2000). This would predict donor origin of the lesion cells in their experiments, as only the graft itself is able to respond to the IFN-γ (Tellides *et al*, 2000). However, this is in direct conflict with emerging evidence from our lab (Johnson *et al*, 2002, reviewed in Skaro *et al*, 2002) and others (Hillebrands *et al*, 2001, Saiura *et al*, 2001, Shimizu *et al*, 2001) indicating that the lesion cells of AV are recipient-derived.

Moreover, Tellides and colleagues further theorized that IFN-γ led to the up-regulation of platelet derived growth factor (PDGF)-B receptors and thus potentiated smooth muscle cell mitogenesis exerted by PDGF (Tellides *et al*, 2000). However, other groups have demonstrated that IFN-γ exerts a negative effect on smooth muscle cell proliferation (Nunokawa and Tanaka, 1992, Shimokado *et al*, 1994). Nonetheless, these data imply a pivotal role for IFN-γ in the generation of AV. It remains to be elucidated whether IFN-γ is involved in the cytolytic cell-induced apoptosis of vascular wall

components or the recruitment of leukocytes and potentially lesion cells through the induction of IFN-γ-inducible chemokines.

2.5.3 Type 2 Cytokines in AV

As mentioned above, immune deviation from type 1 to type 2 mediated responses by drugs such as HMG-CoA reductase inhibitors and AT-1 receptor antagonists results in attenuation of AV. Several groups have evaluated the role of type 2 cytokines in AV foreseeing a potentially protective role for the anti-inflammatory cytokines IL-10 and TGF-β (Koglin *et al*, 1998, Furukawa *et al*, 1999, de Groot-Kruseman *et al*, 1999, Koglin *et al*, 2000). Furukawa and colleagues examined the role of IL-10 in the development of AV using a MHC class II-mismatch murine cardiac allograft model (Furukawa *et al*, 1999). Interestingly, they observed exaggerated AV mediated by increased numbers of graft infiltrating $CD8^+$ CTL in animals treated with exogenous IL-10 (Furukawa *et al*, 1999). This is counterintuitive given that AV is mediated preferentially by a type 1 cytokine response (Raisanen-Sokolowski A *et al*, 1997, Koglin *et al*, 2000) and that IL-10 would naturally antagonize this effect. Moreover, in this MHC II disparate model activation of $CD8^+$ T cells would require indirect presentation (cross-priming) in order for effector CTL to develop. However, IL-10 has been previously described to exert potent chemoattractant activity for human $CD8^+$ T cells *in vitro* (Jinquan *et al*, 1993). In addition, IL-10 promotes the differentiation of $CD8^+$ T cells into effector CTL (Chen and Zlotnik, 1991) capable of direct cytolysis as well as mediating DTH (Li *et al*, 1997). Thus, it is conceivable that $CD8^+$ T cells recruited and stimulated in response to IL-10 are

mediating AV in this model (Furukawa *et al.*, 1999). Not surprisingly, neutralizing IL-10 antibody had no impact on the development of AV in the class II disparate model, which appeared to be mediated in large part by infiltrating Th1 cells (Furukawa *et al*, 1999). Therefore, while AV seems to be Th1 dominated, immune deviation through administration of IL-10 is not beneficial and might in fact be detrimental to the allograft.

Koglin and colleagues evaluated the role of TGF-β in the development of AV (Koglin *et al*, 1998). They found that C57BL/6 TGF-β_1 knockout recipients of fully mismatched CBA cardiac allografts developed more severe AV than wildtype control mice (Koglin *et al*, 1998). They also found up-regulation of STAT4, IFN-γ and IL-2 expressed by graft infiltrating T lymphocytes, whereas type 2 cytokine levels were unchanged (Koglin *et al*, 1998). These data are more in keeping with the anti-inflammatory properties of TGF-β. This suggests that TGF-β_1, when present, antagonizes type 1 responses and acts to attenuate AV. However, TGF-β has also been shown to have pro-fibrogenic effects under certain circumstances (Workalemahu *et al*, 2003). Indeed, TGF-β production in response to calcineurin inhibitor-induced nephrotoxicity has been observed in chronic allograft nephropathy (Khanna *et al*, 2002). Alternatively, one might foresee the beneficial effects of TGF-β on the induction of T regulatory cells which would serve to dampen allo-reactive T cells and inhibit responses leading to AV (Cobbold *et al*, 2003).

Koglin and colleagues studied the impact of immune deviation on the development of AV using STAT4 and STAT6 deficient mice (Koglin *et al*, 2000). $STAT6^{-/-}$ mice showed no change in the magnitude of AV indicating that type 2 immunity is dispensable for lesion development (Koglin *et al*, 2000). However, $STAT4^{-/-}$

recipients exhibited marked mitigation of AV indicating that type 1 responses are dominant in the pathogenesis of AV (Koglin *et al*, 2000). However, in the absence of STAT4 signalling AV still develops (Koglin *et al*, 2000). This data would suggest that residual type 1 immunity and/or type 2 responses are capable of mediating AV in this situation. It is possible that type 2 cytokines lead to the recruitment of effector cells such as eosinophils which are capable of damaging the allograft leading to the abnormal repair and remodelling processes implicated in the development of AV (Nolan *et al*, 1995).

2.5.4 Interferon-γ-inducible Chemokines in AV

The importance of chemokines and chemokines receptors in the recruitment and infiltration of allografts by leukocytes leading to rejection has been a focus of intense research (Hancock, 2002). An attractive strategy in the prevention of acute and chronic rejection of organ allografts has been to interrupt the signals mediated by chemokines derived from inflamed tissues with their cognate chemokine receptors on infiltrating leukocytes. There are a myriad of chemokine-chemokine receptor pairs each with a distinct role in various types of immune responses (Hancock, 2002). Some of these chemokine receptors are quite promiscuous and bind multiple chemokines further complicating the issue (Hancock, 2002). However, amid the vast array of these molecules the CXCR3 chemokine receptor has been shown to be important in the infiltration of Th1 and Tc1 cells into inflamed tissues (Nakajima *et al*, 2002). Interestingly, CXCR3 recognizes a family of three interferon-γ inducible chemokines which include IFN-γ-inducible protein of 10 kD (IP-10), monokine induced by IFN-γ (MIG) and interferon-

inducible T cell α chemoattractant (I-TAC) (Nelson and Krensky, 2001, Hancock, 2002). Given the established importance of IFN-γ in the development of AV, the involvement of the CXCR3-binding chemokines has become an attractive area of study.

It has been theorized that the important role of IFN-γ is exerted through the release of this family of chemokines resulting in the recruitment of leukocytes which act to damage the graft (Nelson and Krensky, 2001, Hancock, 2002). Alternatively, these chemokines might be involved in the recruitment of recipient-derived lesion forming cells (α-actin$^+$ smooth muscle-like cells) from the circulation to the subendothelial space. In support of this, Wang and colleagues demonstrated that IP-10 is a potent mitogenic and chemotactic factor for vascular smooth muscle cells *in vitro* (Wang *et al*, 1996). Moreover, IP-10 mRNA was up-regulated when smooth muscle cells were challenged *in vitro* with either IFN-γ or IL-1β (Wang *et al*, 1996). Similarly, enhanced expression of IP-10 mRNA is observed in rat carotid artery after injury mediated by balloon angioplasty (Wang *et al*, 1996). However, this latter hypothesis remains to be directly evaluated in humans or animal models of transplantation.

Hancock's group first examined the role of CXCR3 in the development of acute and chronic rejection within cardiac allografts using CXCR3-deficient mice (Hancock *et al*, 2000). They found that CyA-treated (10 mg/kg/d) CXC3$^{-/-}$ mice exhibited permanent engraftment of fully mismatched cardiac allografts with no histologic evidence of AV (Hancock *et al*, 2000). It is important to note that this dose of CyA was only able to prolong graft survival by 3 d in wildtype recipients (Hancock *et al*, 2000). They found a significantly reduced number of infiltrating T lymphocytes (CD4$^+$ and CD8$^+$) and macrophages in allografts from CXCR3$^{-/-}$ mice (Hancock *et al*, 2000). Similarly, they

found that anti-CXCR3 monoclonal antibody was able to indefinitely prolong allograft survival in CyA-treated wildtype mice even if started after the onset of rejection (Hancock *et al*, 2000). Rejection in wildtype mice was associated with a profound increase in the expression of IFN-γ, IP-10, I-TAC and MIG (Hancock *et al*, 2000). These data would indicate that CXCR3 and its family of chemokines are important in the recruitment and activation of allo-reactive T lymphocytes capable of mediating acute and chronic allograft rejection.

Yun and colleagues examined the role of MIG in the development of AV using a murine cardiac allograft model with an isolated MHC II mismatch (Yun *et al*, 2002). They found that macrophage derived MIG was important in the recruitment of $CD4^+$ T lymphocytes during the development of AV (Yun *et al*, 2002). When MIG was neutralized using a monoclonal antibody there was a significant reduction in intimal thickening (Yun *et al*, 2002). Moreover, the presence of $CD4^+$ T cells was necessary for sustained macrophage production of MIG (Yun *et al*, 2002). This would imply some sort of positive feedback loop, whereby $CD4^+$ T cells provide the source of IFN-γ leading to macrophage production of MIG and further recruitment of $CD4^+$ T lymphocytes. It is not surprising that $CD8^+$ T lymphocytes play a less profound role under the experimental conditions of an isolated MHC II mismatch. However, $CD8^+$ T lymphocytes can also mediate DTH-like immune responses and under certain circumstances are a plentiful source of IFN-γ (Stinn *et al*, 1998).

Recently, Zhao and colleagues evaluated a subset of their cardiac transplant patients and found a positive correlation between the expression of CXCR3-binding chemokines and AV (Zhao *et al*, 2002). Only allograft hearts contained mRNA for

CXCR3-binding chemokines while normal hearts did not (Zhao *et al*, 2002). Furthermore, they observed a persistent elevation of IP-10 and ITAC, whereas macrophage derived MIG was only elevated during acute rejection episodes (Zhao *et al*, 2002).

In another recent report Hancock *et al* showed that donor-derived IP-10 was important in early leukocyte trafficking to the allograft (Hancock *et al*, 2001). Moreover, once leukocyte infiltration, in response to IP-10, had occurred, the up-regulation of MIG and I-TAC closely followed (Hancock *et al*, 2001). Transplantation of cardiac allografts from IP-10-deficient mice into wildtype recipients led to prolonged graft survival in the absence of immunosuppression and correlated with significantly reduced leukocyte infiltration (Hancock *et al*, 2001). Unfortunately they did not add low-dose immunosuppression and evaluate these grafts for the presence of AV at later time points. Nonetheless, modulation of IFN-γ and its associated chemokines through novel drug therapies might be successful in preventing AV. These data provide important new therapeutic targets for exploitation by scientists and industry alike.

2.6 Objectives

The specific objectives of the research performed in this thesis are:

1) To assess the T cell subsets ($CD4^+$ and $CD8^+$) and effector mechanisms involved in the generation of medial smooth muscle cell apoptosis and its relationship to the development of the intimal occlusive lesion characteristic of AV.

2) Assess the role of $CD8^+$ T cells in mediating AV in immunosuppressed animals.

3) Assess the involvement of contact-dependent, direct cytolytic and indirect effector mechanisms employed by $CD8^+$ T cells in the allo-immune mediated injury of aortic allografts leading to the development of AV.

Chapter 3

3.0 Materials and Methods

3.1.0 Chapter 4

3.1.1 *Animals*

Male 6-8 wk old C3H/HeJ (C3H; H-2^k), and C3.MRL-*Tnfrsf6*^{lpr} (Fas$^{-/-}$; *lpr*; H-2^k) mice were used as donors. Male 6-8 wk old C57BL/6J (B6; H-2^b), B6.129S2-*Cd4*^{tm1Mak} (CD4$^{-/-}$; H-2^b), B6.129S2-*Cd8a*^{tm1Mak} (CD8$^{-/-}$; H-2^b), B6.129P2-*B2m*^{tm1Unc} (β2m$^{-/-}$; H-2^b), C57BL6/J-*Pfp*^{tm1Sdz} (*pfp*$^{-/-}$; H-2^b), and B6Smn.C3H-*FasL*^{gld} (FasL$^{-/-}$; *gld*; H-2^b), mice were used as recipients. Syngrafts from B6 donors transplanted into B6 recipients served as negative controls. Allografts from C3H donors transplanted into wildtype B6 recipients served as positive controls. Experimental groups consisted of C3H donors and CD4$^{-/-}$, CD8$^{-/-}$, β2m$^{-/-}$, *pfp*$^{-/-}$, or FasL$^{-/-}$ recipients. In addition, Fas$^{-/-}$ donor aortas were transplanted into *pfp*$^{-/-}$ recipients to completely inhibit CTL activity.

All mice were >25 g and were purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in the Carleton animal care facility with food and water *ad libitum*. Sentinel mice were serologically negative for all viral pathogens. All animal experimentation was undertaken in compliance with the guidelines of the Canadian Council on Animal Care.

3.1.2 *Aortic Transplantation*

Infrarenal abdominal aortic interposition transplantation was performed as we have previously described (Koulack *et al*, 1995). Briefly, donor C3H strain mice were anaesthetized with intraperitoneal sodium pentobarbital (5 mg/kg) and the abdomen was entered through a midline laparotomy incision. The infrarenal abdominal aorta was exposed and carefully dissected free from the adjacent inferior vena cava. A 10 mm aortic segment was excised and flushed with heparinized saline prior to allo-transplantation. Recipient B6 mice were anaesthetized and the abdominal aorta was similarly exposed and a microvascular clamp was applied to obtain proximal and distal control prior to division with microsurgical scissors. The donor aorta was implanted orthotopically by an end-to-end microsurgical anastomotic technique using sterile interrupted 11-O nylon sutures (Pike Surgical).

Grafts were harvested at 14 and 60 d post-transplant, with 5-10 animals per group per time point. Harvested grafts were flushed with heparinized saline before fixing in 4% buffered formalin at 4°C overnight and then transferred to phosphate buffered saline (PBS) until processing. Aortic tissues were embedded in paraffin and 5μm sections were prepared. Eight cross sections per aortic graft were stained with hematoxylin and eosin (H&E) for general histology and digital-assisted morphometric analysis. Sections were also stained for apoptotic nuclei and α-smooth muscle actin immunocytochemistry.

3.1.3 *Immunosuppression*

Wildtype and $CD8^{-/-}$ B6 recipients of aortic allografts were injected subcutaneously with aqueous cyclosporine A (CyA; Sandimmune™, Novartis, Canada) 50 and 70 mg/kg/d until harvest. This daily dose of CyA has been shown in our laboratory to prevent the acute rejection of cardiac allografts in the C3H to C57BL/6 strain combination.

3.1.4 *In Situ Detection of Apoptosis by TUNEL*

Nine sections each from control and experimental aortic grafts harvested at 14 d post-transplant were subjected to the terminal deoxytransferase-mediated dUTP nick end labeling (TUNEL) assay for the *in situ* detection of apoptotic nuclei as we have previously described (Légaré *et al*, 2000). Briefly, sections were de-paraffinized in xylene and hydrated in a graded series of ethanol into 0.1 mol/L PBS followed by digestion with RNase A (100μg/mL in PBS; Boehringer Mannheim, Laval PQ) in a humidified chamber for 40 min at 37°C. Slides were washed three times in 2x standard saline citrate buffer. Endogenous peroxidase activity was quenched in 0.15% hydrogen peroxide in 0.1 mol/L PBS for 25 minutes at room temperature, rinsed in 0.1 mol/L PBS, and digested with proteinase K (20μg/ml in Tris-EDTA buffer; Boehringer Mannheim, Laval, PQ) at 37°C for 1 min. Slides were then incubated in cold 2mg/ml glycine (Sigma, St Louis, MO) before incubation with ethanol: acetic acid (2:1) at -20°C for 10 min. Slides were rinsed with 2x standard saline citrate buffer and treated with Equilibration

Buffer (Apoptag™ Kit, Oncor, Gaithersburg, MD) for 20 min. Sections were then incubated with TdT (Apoptag™ Kit, Oncor) in Reaction Buffer (Apoptag™ Kit, Oncor) at 37°C for 2 hr and the reaction was terminated by washing with 2x standard saline citrate buffer. Digoxigenin labeled nucleotides were detected by incubation with an anti-digoxigenin peroxidase-conjugated antibody in 0.1% Tween 20 (Apoptag™ Kit, Oncor) following the manufacturer's instructions. Staining was visualized with diaminobenzidine in 0.01% H_2O_2 and methyl green (Sigma) was used as a counterstain. Normal mouse intestine was used as a positive control tissue. For negative controls, distilled water was substituted for the TdT enzyme. Apoptotic nuclei were quantified by counting the number of TUNEL-positive nuclei within the media of each aortic graft cross section using a microscope at 400X magnification.

3.1.5 *Digitized Image Analysis of Tissue Sections*

Formalin-fixed and paraffin-embedded tissues were used for conventional histology as previously described (Légaré *et al*, 2000). Eight sections from each graft were analyzed by light microscopy using a Zeiss Axiovert 200 microscope (Karl Zeiss, Thornwood, NY) and images were captured using a Spot Cooled AxioCam color digital camera (Carl Zeiss). Digital image analysis was performed in a blinded fashion and intimal and medial areas were measured using Scion Image software (Scion Corp., Frederick, MD) as previously described (Légaré *et al*, 2000). The intima to media ratio was then calculated to control for donor variation and section obliquity and concurrently

reflects both the medial and intimal events characteristic of AV. Area calculations were performed for control and experimental groups at 60 d post-transplantation.

3.1.6 *Immunocytochemistry*

Sections were de-paraffinized in xylene, hydrated, washed in PBS, and digested with 20μg/mL proteinase K (Boehringer Mannheim, Laval, PQ) in Tris-EDTA. Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Avidin/biotin blocking was performed using the Vector® Avidin/biotin blocking kit following the manufacturer's instructions (Vector Labs Inc. Burlingame, CA). The primary antibody was a monoclonal anti-α smooth muscle actin antibody (1A4; 1:100; Sigma, St Louis, Missouri). Secondary antibody was a polyclonal biotinylated anti-mouse IgG reagent (1:50; Vector Labs Inc.). Sections were washed in PBS and incubated with a peroxidase avidin/biotin complex (Vector Labs, Inc.). Staining was visualized with diaminobenzidine in 0.01% H_2O_2 and sections were counterstained using methyl green (Sigma).

3.1.7 *Isolation and Primary Culture of Vascular Smooth Muscle Cells*

Segments of abdominal and thoracic aorta were aseptically recovered from 4 to 6 wk old C3H wildtype and $Fas^{-/-}$ *lpr* mice within a laminar flow hood. The adventitia was excised under magnification using a dissecting microscope. The lumen of the vessel was flushed with 5mM ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Burlington, ON)

to denude the endothelium. The remaining medial layer of the aorta was then cut longitudinally and plated in uncoated 6 well culture plates (Corning, NY). The aortic explants were covered with a sterile stainless steal mesh screen and 1 mL of RPMI 1640 (ICN, Aurora, OH) supplemented with 100 units/ml of penicillin, 100 μg/ml streptomycin (Invitrogen,), 2.5 μg/mL fungizone (Invitrogen), 20% fetal bovine serum (FBS) (Invitrogen), 20 mM HEPES (Invitrogen) and 50 μM 2-ME (Sigma) was added to allow the cells to grow beyond the mesh. After the cells had reached confluence, these passage 1 cells were incubated in trypsin-EDTA from(Invitrogen) at 37°C for 4 min and were split into T75 flasks from (Corning).

3.1.8 *Vascular Smooth Muscle Cell Phenotypic Analysis*

Smooth muscle cell phenotype was confirmed by the presence of immunoreactivity for α-actin (anti-α-smooth muscle actin mAb clone 1A4; Sigma; 1:100) in more than 95% of the cells and was performed as described above. All experiments were performed using smooth muscle cells from passages 3 to 5.

3.1.9 *CTL Mediated Killing of Vascular Smooth Muscle Cells In Vitro*

To identify the contribution of cytolytic effector mechanisms utilized by CTL in the killing of vascular smooth muscle cells *in vitro*, CTL were cultured with smooth muscle cell (SMC) targets in an *in vitro* CTL assay. Briefly, primed, purified CTL from B6 wildtype and perforin-deficient mice, expanded *in vitro* for 72 h, were prepared as

effector cells. [^{3}H]-Thymidine (100 μCi for 24 h at 37°C)-labeled allogeneic C3H wildtype and *lpr* smooth muscle cells were used as targets. Radiolabeling of SMC targets was facilitated by exposure to 5 ng/ml PDGF-BB (Sigma, St. Louis, MO) in the culture medium. The effector and target cells were plated in flat-bottom 96-well microtiter plates ($1x10^4$ target cells/well) at effector to target cell ratios ranging from 25:1 to 3:1 in RPMI 1640 medium. After incubation at 37°C for 4 h, the plates were harvested using an automatic cell harvester (Skatron, Sterling, VA) as previously described (Liwski and Lee, 1999, Liwski *et al*, 2000). Specific apoptosis was calculated using the following formula: % specific apoptosis = ([^{3}H]TdR in targets at T_{4h} - [^{3}H]TdR in effector/targets at T_{4h}) / ([^{3}H]TdR in targets at T_{4h}) x 100%.

3.2.0 Chapter 5

3.2.1 *Animals*

Male 6 wk old BALB/cJ (BALB/c; H-2^d), C3H/HeJ (C3H; H-2^k), C3.MRL-Tnfrsf6^{lpr} (*lpr*; H-2^k), C57BL/6 (B6; H-2^b), C57BL/6J-*B2m*^{tm1Unc} (B6 β2m$^{-/-}$; H-2^b), MRL/MpJ (MRL; H-2^k) and MRL.129P2(B6)-*B2m*^{tm1Unc} (MRL β2m$^{-/-}$; H-2^k) mice were used as donors. Male 8 wk old B6.129S7-RAG-1^{tm1Mom} (B6 RAG-1$^{-/-}$; H-2^b), and C3H.129S6(B6)-*Rag2*^{tm1} N12 (C3H RAG-$2^{-/-}$; H-2^k) mice were used as recipients. Male 6 wk old B6, C3H, C57BL/6-Pfp^{tm1Sdz} (*pfp*; H-2^b), B6Smn.C3-Tnfsf6^{gld} (*gld*; H-2^b), and B6.129S7-*Ifng*^{tm1Ts} (IFN-$\gamma^{-/-}$; H-2^b) were used as cell donors for adoptive transfer and *in vitro* culture.

3.2.2 *Aortic Transplantation*

Abdominal aortas were transplanted as described above (Koulack *et al*, 1995). In these experiments aortic graft and spleen harvest were performed at 60 d following transplantation.

3.2.3 *Allo-immunization*

Stimulator BALB/c splenocytes were isolated as previously described (Liwski *et al*, 2000), for *in vivo* allo-immunization of B6 cell donors. Briefly, BALB/c stimulator cells were isolated and inactivated by adding mitomycin C 25 μg/ml (Sigma, St. Louis, MO) for 30 min. Groups of 6 wk old male B6 mice (4 mice per group) were immunized intraperitoneally with 5 x 10^7 mitomycin C-treated BALB/c splenocytes in 200 μl of PBS. On day 7 after immunization all mice were sacrificed by cervical dislocation and splenocytes were isolated and either whole T cells or $CD8^+$ T cells were purified as described below. We have previously shown this immunization protocol to be effective in generating robust CTL and proliferative responses from T cell populations (Liwski *et al*, 2000).

3.2.4 *Whole T cell and $CD8^+$ T cell isolation*

Seven days following allo-immunization, spleens were harvested and splenocyte suspensions were purged of erythrocytes as previously described (Liwski *et al*, 2000). Whole T cells were enriched by nylon wool (Polysciences, Warrington, PA) followed by passage through CD3 immunocolumns (R&D Systems, Minneapolis, MN). For $CD8^+$ T cell isolation, whole T cell suspensions were purified by negative selection with CD8 immunocolumns (R&D Systems) (13). CD8 purity was > 93% with no $CD4^+$ or $B220^+$ contaminants. To control for null cell effects in the $CD8^+$ transfer population, prior to nylon wool enrichment and passage through CD8 immunocolumns splenocytes were

treated with anti-CD8 mAb (0.2μg/10^6 cells; clone YTS169.4; Cedarlane, Mississauga, ON) and low-tox-M rabbit complement (Cedarlane).

For $CD4^+$ and $CD8^+$ T cell isolation by positive selection, enriched T cell populations were incubated with anti- L3T4 (anti-CD4) and anti-Ly-2 (CD8α) magnetic beads (Miltenyi Biotec, Sunnyvale, CA). The cell suspension was pelleted and adjusted to 1×10^7 cells per 100μl of 2% FBS (Invitrogen) in PBS on ice. Ten microlitres of antibody-labeled beads; either anti-L3T4 (anti-CD4) or anti-Ly-2 (CD8α) was added to the 100μl cell suspension at a concentration of 10 μl antibody per 10^7 cells, and the solution incubated in the dark at 4°C for 15 min. After incubation with antibody, the solution was pelleted, washed twice in cold 2% PBS, resuspended in 500μl of the 2% PBS solution and placed on a pre-washed (with 2% PBS) column that was suspended within a magnetic field (Miltenyi Biotec). The effluent was discarded. The column removed from the magnet and the cells adherent to the magnetic beads were freed by washing them free of the column. The cells were then pelleted, resuspended in serum-free RPMI, and cells counted. Isolated cells were typically >99% pure as determined by flow cytometric analysis and appeared viable by exclusion of trypan blue.

3.2.5 *FACS Analysis*

Cells (1 x 10^6) were washed twice in PBS containing 1% FBS. Cells were incubated in the dark with 2 μg/ml of anti-CD4 mAb (rat $IgG2_b$ anti- mouse; clone YTS 191.1; Cedarlane), anti- CD8α mAb (rat $IgG2_b$ anti-mouse; clone YTS 169.4; Cedarlane) anti-B220 FITC mAb (mouse $IgG2_b$ anti-mouse; clone 5a-8; Cedarlane), or isotype

control (rat $IgG2_b$; Cedarlane) at 4°C for 30 min. Cells were then washed three times, fixed in serum-free RPMI containing 0.1% paraformaldehyde and stored at 4°C overnight. FACS analysis was performed on a FACSCalibur using CellQuest software (Becton Dickinson, Mountain View, CA).

3.2.6 *Direct Cytotoxic T lymphocyte (dCTL) activity*

Ex vivo: allo specific dCTL activity was assessed to confirm allo-activation. Briefly, freshly purified, primed B6 whole T and $CD8^+$ T cells were cultured in the presence of [^{3}H]-Thymidine ([^{3}H]-TdR)-labeled allogeneic P815 (H-2^d; ATCC) or third party BW5147.3 (H-2^k; ATCC) tumor cell targets at effector to target cell ratios ranging from 50:1 to 6:1. The plates were incubated for 18h at 37°C and after harvesting apoptosis was calculated based on the loss of [^{3}H] due to DNA fragmentation as described above.

In vitro: To assess direct killing in the absence of the granule-exocytosis and Fas-Fas ligand pathways, primed, purified $CD8^+$ T cells from B6 perforin-deficient mice, expanded *in vitro* for 72 h, were prepared as effector cells and [^{3}H]-Thymidine-labeled allogeneic C3H *lpr* Concanavalin A lymphoblasts were used as target cells. Cells were plated at effector to target cell ratios ranging from 25:1 to 3:1. After incubation at 37°C for 4 h, the plates were harvested and specific apoptosis assessed.

3.2.7 *Adoptive cell transfer*

Twenty-four hours after transplantation, B6 RAG-$1^{-/-}$ recipients were injected intraperitoneally with $1x10^7$ primed whole T cells, or primed purified $CD8^+$ T cells.

3.2.8 *Histology and Digital Assisted Morphometry*

Formalin-fixed and paraffin-embedded tissues were used. Eight sections from each graft were stained with Verhoeff elastin stain. Digitized images were captured using a Zeiss Axiovert 200 & AxioCam camera (Carl Zeiss, Thornwood, NY) and the intimal and medial areas were measured using Scion Image software (Scion Corp., Frederick, MD) as described above.

3.2.9 *Antibodies and Reagents*

Primary monoclonal antibodies used for immunocytochemistry were anti-CD3e (145-2C11), anti-CD4 (RM4-5), anti-CD8a (53-6.7), anti-CD11b (M1/70), and anti-CD19 (1D3) purchased from PharMingen (SanDiego, CA). Secondary antibodies included biotinylated polyclonal anti-rat (PharMingen) and biotinylated anti-hamster IgG cocktail (G70-204, G94-56, PharMingen).

3.2.10 *Immunocytochemistry*

Six micron frozen sections were stained with the following primary antibodies: anti-CD3e (145-2C11; 1:50), anti-CD4 (RM4-5; 1:25), anti-CD8 (53-6.7;1:50), anti-CD11b (M1/70; 1:50), and anti-CD19 (1D3; 1:25) purchased from PharMingen (SanDiego, CA). Primary antibody was detected with biotinylated anti-hamster IgG cocktail (G70-204, G94-56; 1:50; PharMingen) for CD3e and polyclonal anti-rat IgG antibody (1:50; PharMingen) for CD4, CD8, CD11b, and CD19 followed by treatment with peroxidase avidin/biotin complex (Vector, Burlingame, CA), and 3, 3'-diaminobenzidine as the chromogen. As a negative control, species and IgG isotype matched antibodies were used in place of primary antibody (PharMingen).

3.2.11 *Statistics*

Data are presented as mean ± standard error of the mean (SEM) for each experimental group. One-way ANOVA and the Tukey-Kramer multiple comparisons test or Student's *t* test were used. Values of $p < 0.05$ were considered significant.

Chapter 4

4.0 Impairment of Recipient Cytolytic Activity Inhibits Medial Smooth Muscle Cell Apoptosis and Attenuates Allograft Vasculopathy

4.1 Introduction

Orthotopic cardiac transplantation is an established therapy in the treatment of end-stage heart failure in selected patients. The discovery of immunosuppressive agents which effectively ablate acute rejection has facilitated the widespread use of this therapeutic modality. Despite these advances, chronic rejection has emerged as the leading cause of death beyond the first post-transplant year and the most frequent cause of late graft failure (Hosenpud *et al*, 2001). Current immunosuppressive therapies do not inhibit the development of AV (Tilney *et al*, 1991, Kuo and Monaco, 1993, MacDonald *et al*, 1994, Koskinen *et al*, 1995) and re-transplantation is marred by both poor results and a critical shortage of organs (Srivastava *et al*, 2000).

Due to the absence of angina in most patients, the first clinical presentation is often progressive cardiac failure, ventricular arrhythmia or sudden death (von Scheidt W, 2000). The most common and clinically significant pathological feature of chronic rejection in heart transplants is allograft vasculopathy (AV). While there are organ specific changes associated with chronic rejection, AV has been described in all solid organ transplants (Häyry *et al*, 1993). AV is central to late graft failure following heart transplantation, and has been suggested to contribute to irreversible organ damage in non-cardiac transplants (Azuma *et al*, 1997). Of the three most commonly transplanted solid organs (kidney, liver and heart), the relationship of AV to late graft failure seems most clear for heart allografts.

The pathogenesis of AV combines immune and non-immune driving forces activating a complex cascade of injurious events in the graft arterial tree leading to

uncontrolled vascular remodeling characterized by progressive lumenal narrowing. Concomitant parenchymal fibrosis occurs in response to ischemia due to lumen loss and restricted perfusion (Libby and Pober, 2001). The failure of current management strategies is the result of an incomplete understanding of this rejection process.

There is strong evidence from T cell-deficient (athymic nude) animals, which do not develop AV, implicating T cell-derived immune responses in the disease process (Hall, 1991, Ensminger *et al*, 2002). However, the relative contribution of T cell subsets ($CD4^+$ and $CD8^+$) to the development of AV remains controversial. Moreover, the effector mechanisms utilized by $CD4^+$ and $CD8^+$ T cells to damage the allograft, triggering the over-exuberant repair and vascular remodeling process characteristic of AV, are poorly understood.

The majority of evidence suggests that $CD4^+$ T helper cells play the major role in the generation of AV (Shi *et al*, 1996, Ensminger *et al*, 2002, Yamada *et al*, 2003). Shi *et al* claimed that $CD4^+$ T helper cells, macrophages and allo-antibody are required for intimal lesion development in a murine loop carotid allograft model, while $CD8^+$ T cells and NK cells are not necessary (Shi *et al*, 1996). Similarly, Ensminger and colleagues suggested that $CD8^+$ T cell effectors and allo-antibody production are dependent on indirectly primed T helper cells (Ensminger *et al*, 2002).

However, there is accumulating evidence that the development of AV is less dependent than acute rejection on $CD4^+$ T cell helper function (Han *et al*, 2000). Han and colleagues, using anti-CD4 antibody transgenic mice that are devoid of $CD4^+$ T cells by endogenous depletion, showed that AV still occurs despite the absence of T helper cells (Han *et al*, 2000). This lack of dependence on $CD4^+$ T cells suggests an important role for

$CD8^+$ T lymphocytes. Allan *et al* demonstrated the impact of $CD8^+$ T cells by inhibiting the development of AV in MHC class I mismatched cardiac allografts in miniature swine using anti-CD8 monoclonal antibody therapy (Allan *et al*, 1997). Indeed, CD8-dependent mechanisms may play a dominant role in clinical transplantation where $CD4^+$ T cell function is substantially inhibited by current calcineurin-inhibitor based immunosuppressive regimens (Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001).

In both human cardiac transplants and murine aortic allografts, AV is characterized by the formation of a progressive neointimal lesion within the epicardial coronary arteries that becomes occlusive over time. Ensminger *et al* have shown that the degree of intimal hyperplasia is highly concordant between aortic allografts and whole organ transplants in a combined heterotopic heart and aortic allograft model (Ensminger *et al*, 2000). A similar correlation was found in allograft aortas and coronary arteries of human transplants undergoing AV (Mehra *et al*, 1997).

The occlusive lesion consists of α-$actin^+$ smooth muscle-like cells and various infiltrating leukocytes. In animal models the underlying media has been closely examined and smooth muscle cell (SMC) loss in this compartment is evident (Hirsch *et al.*, 1998). In human cardiac transplants less attention has been paid to vascular SMC and conflicting reports exist regarding medial events during AV (Häyry *et al*, 1992, Billingham, 1994, Geraghty *et al*, 1996). A close examination of human coronary arteries undergoing AV, however, reveals significant medial SMC loss associated with areas of intimal lesion formation (Figure 1).

Until very recently, it was generally accepted that the neointimal lesion characteristic of AV was populated by donor SMC transmigration from the graft media with subsequent proliferation within the subendothelial space (Akyurek *et al*, 1996, Bojakowski *et al*, 2000). The stimulus for this differentiation and migration was suggested to be growth factors (e.g. basic fibroblast growth factor, bFGF; platelet-derived growth factor, PDGF) released by platelets and endothelial cells subjected to ischemic and immune-mediated damage (Billingham, 1994). Ischemic damage contributes to the extent of lesion formation (Ewel *et al*, 1993, Gaudin *et al*, 1994, Andersen *et al*, 1995, Knight *et al*, 1997), but is not itself sufficient to cause intimal hyperplasia since syngeneic transplants do not show signs of AV (Hirsch *et al*, 1998).

The hypothesis based on SMC migration has been refuted by recent evidence that the neointimal cells are of recipient, not donor origin (Shimizu *et al*, 2001, Saiura *et al*, 2001, Li *et al*., 2001, Hillebrands *et al*, 2001, Johnson *et al*, 2002, Hillebrands *et al*, 2003). For example, Shimizu and colleagues, using a transgenic mouse in which all somatic cells express the LacZ gene and gene product (β-gal), have demonstrated that the neointimal lesion of AV in cardiac and aortic allografts is entirely composed of cells of recipient origin (Shimizu *et al*, 2001). We have recently confirmed and extended this work in a rat aortic allograft model using primers for specific Class I MHC genes and polymerase chain reaction (PCR; Johnson *et al*, 2002). In this model, we also demonstrated that immunosuppression, to ablate acute rejection, did not change the recipient origin of the cells within the neointima (Johnson *et al*, 2002). Similarly, recipient origin of neointimal cells has recently been demonstrated in human renal allografts (Grimm *et al*, 2001).

Given the above, depletion of SMC from the vascular media of grafts undergoing AV can no longer be convincingly argued to be due to transmigration of SMC to the subendothelial space. Evidence from animal models suggests that medial SMC are being destroyed by allo-immune responses (Hirsch *et al*, 1998). We have shown that apoptosis occurs within the medial SMC compartment during the early stages of AV (Légaré *et al*, 2000). Concurrently, we have demonstrated increased expression of cytotoxic molecules associated with killer T lymphocytes in grafts undergoing AV (Hirsch *et al*, 1998). We therefore hypothesized that cytolytic $CD8^+$ T cells induce apoptosis of vascular SMC and that medial damage contributes to the generation of AV.

In this study we examined the contribution of $CD4^+$ and $CD8^+$ T cell subsets to the generation of early medial SMC destruction and the induction of vascular remodeling, which are hallmark pathological features of AV. Moreover, we studied the impact of impairment of recipient cytotoxic T cell (CTL) function on the progression of medial smooth muscle cell loss and intimal lesion formation. Our data supports the hypothesis that $CD8^+$ T cells are responsible for the induction of apoptosis within medial SMC of allografts undergoing AV. Moreover, $CD8^+$ T cells were capable of generating a robust allo-immune response which triggers vascular remodeling in the absence of $CD4^+$ T cell helper function. This study also demonstrates that CTL activity plays a critical role in the destruction of medial SMC leading to the development of AV. Given that $CD8^+$ T cells have been shown to be relatively resistant to standard, clinical immunosuppressive agents (Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001), these data might explain the limited efficacy of current transplant pharmacotherapy (Hosenpud *et al*, 2001).

4.2 Results

4.2.1 *Early medial apoptosis is mediated by CD8$^+$ T cells through pathways of direct cytolysis*

To assess the role of CD8$^+$ T cells in the development of early medial destruction, a hallmark feature of AV, C3H aortic allografts were transplanted into either C57BL/6 (B6) CD8 alpha chain knockout (CD8$^{-/-}$) or $\beta_2$microglobulin knockout (β2m$^{-/-}$) mice. Since preliminary experimentation in wildtype mice showed peak medial smooth muscle cell (SMC) apoptosis is evident at 14 d post-transplantation, grafts were harvested from knockout mice at this time point. We found that wildtype controls receiving syngeneic aortic grafts showed virtually no medial apoptosis (Figures 2 and 3), while allografts into wildtype B6 recipients showed a marked, and highly significant ($p \leq 0.0001$) increase in medial apoptotic activity (Figures 2 and 3). Allografts from a CD8-depleted environment (CD8$^{-/-}$ and β2m$^{-/-}$) exhibited a significant ($p < 0.001$) reduction in medial SMC apoptosis when compared to allografts from wildtype B6 mice (Figure 3).

Given that depletion of CD8$^+$ T cells resulted in a marked reduction in early medial SMC apoptosis and that CD8$^+$ T cells are known to lyse target cells through direct cytolysis, we sought to determine the role of CTL killing pathways in this process. To assess the contribution of the two major arms of the cytolytic response, mice deficient in either of the main pathways of cytolysis were used as allograft recipients. C3H aortic grafts were transplanted into perforin-deficient (*pfp*$^{-/-}$) mice, which are incapable of mediating cytotoxicity through the granule-exocytosis pathway, and into *gld* mice which

lack Fas Ligand rendering them incapable of killing by the Fas/FasL interaction. Recipient mice with impaired pathways of cytolysis, either perforin- or Fas ligand-deficient, also showed significantly ($p< 0.01$; $p< 0.001$, respectively) reduced levels of medial apoptosis similar to CD8-deficient animals (Figure 3). Therefore, the lack of either $CD8^+$ T cells or inhibition of cytotoxic mediators, expressed mainly but not exclusively by $CD8^+$ T cells, significantly reduces, but does not eliminate, early medial SMC apoptosis.

4.2.2 *The role of $CD4^+$ and $CD8^+$ T cell subsets and pathways of cytolysis in late medial SMC loss*

The demonstration of reduced medial cell apoptosis at the 14 d time point suggests a potential long-term preservation of medial cells in animals lacking $CD8^+$ CTL activity. To quantify the extent of medial destruction late in the course of AV, medial area measurements were performed by digital morphometry at 8 wk post-transplantation and expressed as a percentage of the medial area of an undamaged syngeneic transplant. Allogeneic aortic grafts into wildtype mice showed a marked (43.1% ± 6.901) decline in medial area relative to syngeneic grafts into wildtype mice (Figure 4). Grafts transplanted into $CD4^{-/-}$ mice showed a similar reduction in medial area (37.2% ± 2.624; Figure 4), whereas the CD8-depleted environment of $CD8^{-/-}$ and $\beta2m^{-/-}$ mice promoted medial area preservation (both $p<0.001$; Figure 4).

Aortic grafts from recipient environments with impaired pathways of direct cytolysis, by interruption of perforin or FasL were also subjected to medial area

measurements to assess the contribution of the major pathways of cytolysis to the generation of late medial SMC destruction. Aortic grafts from *pfp*$^{-/-}$ mice with disruption of the granule-exocytosis pathway exhibited a significant preservation in medial area compared to wildtype allografts ($p<0.001$; Figure 4). Similarly, when the Fas/FasL pathway was inhibited in FasL$^{-/-}$ mice and *pfp*$^{-/-}$ recipients of Fas death receptor-deficient (*lpr*) aortic allografts, a significant preservation (both $p<0.001$) of medial area was observed (Figure 4).

To confirm that medial area measurements accurately represented the degree of SMC loss, α-smooth muscle actin immunocytochemistry was performed on control and experimental grafts. Numerous α-actin^{+} cells were visualized within the medial compartment of control syngeneic aortic grafts transplanted into wildtype mice (Figure 5). In control allogeneic grafts, SMC loss in the media was evident and α-actin^{+} smooth muscle-like cells could be seen to populate the neointima (Figure 5). However, some residual α-actin^{+} medial smooth muscle cells could be visualized in allografts derived from a CD8-deficient environment (Figure 5) and those where the Fas/FasL pathway of cytolysis were interrupted (FasL$^{-/-}$ recipients and *lpr* grafts into *pfp*$^{-/-}$ recipients; Figure 5) indicating that the medial area measurements are consistent with the degree of medial SMC damage observed at 8 wk post-transplantation..

4.2.3 *CD8$^+$ T cell-mediated direct cytolysis of vascular smooth muscle cells in vitro*

Having implicated CD8$^+$ T cells and pathways of cytolysis in the destruction of medial smooth muscle cells at early and late time points during the generation of AV, we assessed the relative contribution of the major pathways of direct cytolysis used by CD8$^+$ T cells in an *in vitro* CTL assay with smooth muscle cells as targets. Mouse vascular smooth muscle cells derived from aortic explants of wildtype C3H (H-2^k) and Fas-deficient C3H *lpr* (H-2^k) mice morphologically resembled mature SMC with expression of α-actin (Figure 6) and a low level of surface MHC class I (data not shown).

As expected, primed wildtype CD8$^+$ T cells induced substantial killing of allogeneic wildtype smooth muscle cell targets *in vitro* (Figure 7). When the granule-exocytosis pathway was interrupted by using perforin-deficient (*pfp*$^{-/-}$) effector cells killing activity against smooth muscle cell targets was preserved (Figure 7). Disruption of the Fas-FasL pathway by using *lpr* smooth muscle cell targets resulted in a significant reduction in CTL activity (Figure 7). CTL activity was completely ablated when both pathways were interrupted simultaneously by using *pfp*$^{-/-}$ effector cells against *lpr* SMC targets (Figure 7). These data indicate that CTL lyse vascular SMC targets *in vitro* primarily through the Fas-FasL interaction.

4.2.4 *$CD8^+$ T cell-mediated direct cytolysis contributes to the generation of AV*

The data above show that early SMC apoptosis and late medial damage are partly ameliorated in animals lacking $CD8^+$ T cells or with genetically impaired cytolytic activity. The data also demonstrate the eventual loss, although incomplete in some instances, of SMC in all groups except syngeneic controls. In order to determine the impact of functional impairment of $CD8^+$ CTL on the generation of clinically significant AV lesions, we assessed the degree of intimal hyperplasia at 8 wk post-transplantation using digital morphometry.

Intimal and medial areas were defined by internal and external elastic laminae respectively, and intimal thickening was expressed as the intima to media ratio. Syngeneic transplants, like native aortic segments, showed no intimal lesion and exhibited a normal single cell thick intimal layer (Figure 8 and 9). Allografts in fully immunocompetent wildtype mice, however, showed substantial AV lesions characterized by medial smooth muscle cell loss and intimal hyperplasia (Figures 8 and 9). Similarly, aortic allografts into $CD4^{-/-}$ mice exhibited marked AV, which was similar in extent and nature to allografts from a wildtype environment (Figures 8 and 9). However, allografts in CD8-depleted ($CD8^{-/-}$ and $\beta2m^{-/-}$) recipients showed significantly reduced intimal thickening compared to WT ($p<0.001$ and $p<0.05$, respectively) and $CD4^{-/-}$ ($p<0.001$ and $p<0.01$, respectively) animals (Figures 8 and 9). This indicates that $CD8^+$ T cells, while not necessary, are sufficient and perhaps play a more significant role in the generation of intimal hyperplasia in this fully MHC disparate murine aortic allograft model of AV.

Having established that $CD8^+$ T cells are important in the induction of medial damage and the subsequent generation of AV, we then examined the role of direct pathways of cytolysis commonly employed by $CD8^+$ T cells in target cell killing. Recipient mice with impaired pathways of cytolysis through interruption of perforin or Fas ligand or both pathways were assessed for the generation of intimal thickening. Aortic allografts from *pfp*$^{-/-}$ recipients, in which the granule-exocytosis pathway was interrupted, showed no significant change in intimal thickening (Figure 8 and 9). Interruption of the Fas/FasL pathway in *gld* mice showed a significant reduction ($p<0.05$; Figure 8 and 9) in intimal hyperplasia implicating an important role for this interaction in the generation of AV. However, when both the granule-exocytosis and Fas/FasL pathways were interrupted simultaneously, as seen in *pfp*$^{-/-}$ recipients of *lpr* aortic allografts, there was a marked (almost 70 percent) mitigation in intimal thickening, indicating that both pathways of classical CTL activity, mainly employed by but not exclusive to $CD8^+$ T cells, play an important role in the generation of AV ($p<0.001$; Figure 8 and 9).

4.2.5 *AV in cyclosporine A-treated mice occurs by a $CD8^+$ T cell-dependent process*

The generation of AV within aortic allografts in the absence of immunosuppression could potentially be due to $CD8^+$ T cell-mediated acute rejection responses. To demonstrate an important role for $CD8^+$ T cells in the generation of AV in the clinically relevant setting of cyclosporine A (CyA)-based immunosuppression, fully mismatched C3H aortic allografts were transplanted into wildtype and $CD8^{-/-}$ recipients

treated with CyA. Prior to these experiments we transplanted C3H cardiac allografts into wildtype B6 recipients treated with a similar dose of CyA to establish that acute rejection was eliminated. As expected, untreated cardiac allografts were promptly rejected within 12 d of transplantation, whereas CyA-treated (50 mg/kg/d) cardiac allografts remained functionally intact and survived to greater than 60 d.

Having established that CyA (50 mg/kg/d) is capable of inhibiting acute rejection through prolongation of cardiac allograft survival, we transplanted C3H aortic allografts into wildtype and $CD8^{-/-}$ mice treated with CyA. Allografts from wildtype mice treated with CyA (50 mg/kg/d) exhibited marked intimal hyperplasia and medial smooth muscle cell loss (Figure 10). However, allografts from $CD8^{-/-}$ recipients treated with CyA 50 mg/kg/d exhibited normal vessel morphology with no evidence of intimal hyperplasia or medial smooth muscle cell loss. These data indicate that in the presence of CyA, at doses capable of inhibiting acute rejection, AV develops through a $CD8^{+}$ T cell-dependent process.

4.3 Discussion

AV is thought to result from a low-grade allo-immune response directed against the graft endothelium. This has been suggested to lead to cytokine and growth factor cascades which result in SMC de-differentiation, migration from the media, and proliferation to form an occlusive neointima which eventually leads to ischemic graft failure (Häyry *et al*, 1993, Häyry *et al*, 1995). The evidence to support this hypothesis is circumstantial and recently we (Johnson *et al*, 2002) and others (Shimizu *et al*, 2001, Saiura *et al*, 2001, Li *et al*, 2001, Hillebrands *et al*, 2001, Hillebrands *et al*, 2003) have provided substantial data which contradicts this hypothesis. For example, we have previously demonstrated that the loss of SMC in the media of grafts undergoing AV is due to apoptosis, and not migration (Hirsch *et al*, 1998). Others (Koglin and Russell, 1999) have also demonstrated apoptosis in AV.

We have provided further direct evidence, in a rat aortic allograft model (by PCR analysis of MHC Class I haplotype), that the α-actin^{+} smooth muscle-like cells in the expanding neointima are of recipient, not donor origin (Johnson *et al*, 2002). This confirms that graft (donor) SMC migration is not responsible for the accumulation of smooth muscle-like cells in the subendothelial space forming the occlusive neointima. Recently, other groups have provided further compelling evidence, in other rodent models (Shimizu *et al*, 2001, Saiura *et al*, 2001, Li *et al*, 2001, Hillebrands *et al*, 2001, Hillebrands *et al*, 2003) and humans (Grimm *et al*, 2001), of a recipient-derived lesion. These data have necessitated a re-evaluation of the previously accepted mechanism of AV. This new hypothesis must explain the loss of SMC by a process which does not include de-differentiation and migration out of the medial compartment. Loss of SMC

from the media is undoubtedly immune mediated since it does not occur in syngeneic transplants or immune-incompetent animals (Koulack *et al*, 1995, Koulack *et al*, 1996, Shi *et al*, 1996, Russell *et al*, 1997) but what remains unanswered is the mechanism by which it occurs and its pathological significance.

In acute rejection, there is convincing evidence that $CD4^+$ T cells play a dominant role. For example, long term graft acceptance occurs in CD4 knockout recipient mice but CD8 knockout mice exhibit normal acute rejection (Krieger *et al*, 1996, Krieger *et al*, 1997). However, in adoptive transfer experiments graft rejection occurs faster if $CD4^+$ and $CD8^+$ cells are transferred together (Hall *et al*, 1990), suggesting that $CD8^+$ T cells play a role under normal conditions. Such a role appears to be linked to the activities of $CD4^+$ T cells, but that link may not be essential in all cases (Jones *et al*, 2000). Recently, Kreisel and colleagues have demonstrated that $CD8^+$ T cells activated by non-hematopoietic antigen presenting cells are capable of mediating acute rejection of cardiac allografts in the absence of $CD4^+$ T cells (Kreisel *et al*, 2002).

Allograft vasculopathy is also a T cell dependent pathology (Hall, 1991, Ensminger *et al*, 2002) but the contribution of T cell subsets is less clear. Although the effector mechanisms employed by allo-specific T cells have been well characterized, little is known regarding their role in the generation of AV. The development of AV seems less dependent than acute rejection on $CD4^+$ T cell helper function. Han *et al* found that AV occurs in anti-CD4 antibody transgenic mice, which are devoid of $CD4^+$ T cells by endogenous depletion (Han *et al*., 2000), suggesting an important role for $CD8^+$ T cells. Allan *et al* have directly implicated $CD8^+$ T cells by inhibiting the development

of AV within cardiac allografts using depleting anti-CD8 monoclonal antibody therapy in their MHC class I disparate miniature swine model (Allan *et al.*, 1997).

There is accumulating evidence to suggest that $CD8^+$ T cell effector mechanisms play a significant role in AV by inducing apoptotic events in the transplant vasculature (Hirsch *et al*, 1998, Koglin and Russell, 1999, White *et al*, 1997). In aortic grafts, anti-CD8 antibody therapy significantly reduces medial apoptosis (Légaré *et al*, 2000). Further, we have demonstrated abundant granzyme B, perforin and FasL expression in grafts undergoing AV (Hirsch *et al*, 1998). This data implicates $CD8^+$ CTL but is not incontrovertible since non T cell sources of these factors have been associated with apoptosis (Ogura *et al*, 2001).

To assess the role of $CD8^+$ T cells and the major CTL killing pathways in the induction of medial cell damage, aortic interposition transplants were performed using CD4 and CD8 knockout recipient mice. Allografts placed into an immune-deficient environment lacking $CD8^+$ T cells by CD8-α and $\beta_2$microglobulin gene deletion, showed a marked reduction in apoptosis within the medial cell compartment at 2 wk post-transplant. Allografts transplanted into immune environments with impaired CTL pathways (either by perforin or Fas ligand gene deletion), commonly used by, but not exclusive to, $CD8^+$ T cells, also showed a similar reduction in SMC cell apoptosis at this time point. These data provide strong evidence to support the hypothesis that $CD8^+$ CTL play an important role in medial cell destruction by inducing apoptosis of SMC in transplants undergoing AV. Since, medial apoptotic activity was not completely ablated by depletion of $CD8^+$ T cells or cytolytic impairment, significant redundancy in the early anti-graft response exists. Other potential mediators of this early apoptosis include

residual CTL activity mediated by $CD4^+$ T cells, innate immune effectors and allo-antibody induced apoptosis.

Assessment of medial apoptosis at 2 wk post-transplant is a static representation of a very dynamic rejection process. Medial damage at later stages (8 wk post-transplant) was assessed by medial area measurements and expressed as a percentage of the medial area of an undamaged syngeneic aortic graft. To confirm that a reduction in the observed medial area measurements reflected a loss of vascular smooth muscle cells, α-smooth muscle actin immunostaining was used to differentiate medial SMC. As expected, allografts from wildtype mice showed substantial medial SMC loss when compared to syngeneic controls. Impairment of $CD8^+$ CTL effector cells by deletion or by inhibiting cytolysis resulted in significant medial area preservation and partial sparing of α-$actin^+$ medial smooth muscle cells indicating that the area measurements are reliable. Despite the residual medial damage observed in experimental allografts, where $CD8^+$ T cells or CTL pathways were inhibited, a potential role for $CD8^+$ T cell-mediated smooth muscle cell apoptosis in the generation of AV can be postulated. Although the current accepted hypothesis regarding the etiology of AV suggests that endothelial disruption is a necessary step in the development of AV, these data, which are consistent with arterial injury models, implicate smooth muscle cell damage as a possible inciting stimulus for the vascular remodelling process observed in AV.

Graft loss from AV is primarily due to the arteriosclerotic lesion which impedes blood flow within the transplanted heart eventually leading to ischemic organ failure necessitating re-transplantation. It was therefore of interest to examine the role of $CD8^+$ CTL activity on the generation of this lesion, especially given the data that early medial

apoptotic events are compromised in CD8-deficient animals. Others have also examined the development of the neointimal lesion in the absence of $CD8^+$ cells with conflicting results. Depletion of $CD8^+$ T cells by antibody treatment in a rat heterotopic heart model showed $CD8^+$ cells to be unnecessary for lesion formation (Forbes *et al*, 1994). Similarly, Szeto and colleagues found that $CD8^+$ T cells were neither necessary nor sufficient to induce cardiac allograft vasculopathy in rat chimeric hearts (Szeto *et al*, 2002). Ensminger *et al* using adoptive transfer of T cell subsets into nude or $RAG^{-/-}$ mice showed that cross-primed $CD4^+$ T cells were sufficient to induce AV in MHC class I disparate aortic allografts, while $CD8^+$ T cells were not (Ensminger *et al*, 2002).

In contrast, heterotopic heart transplants carried out in $CD8^+$ T cell depleted (by anti-CD8 monoclonal antibody treatment) inbred miniature swine showed no evidence of lesion formation (Allan *et al*, 1997). These results would indicate that $CD8^+$ T cells are essential for the development of intimal hyperplasia in this model. Studies using cardiac grafts transplanted into mice depleted of $CD4^+$ T cells showed evidence of lesion formation coinciding with the presence of $CD8^+$ T cells (Han *et al*, 2000). Moreover, experiments using anti-CD40L (CD154) co-stimulatory blockade have suggested an important role for $CD8^+$ T cells in the development of graft arteriosclerosis (Ensminger *et al*, 2000).

In the present study, using the fully MHC disparate mouse aortic allograft model, the lack of $CD8^+$ T cells in $CD8^{-/-}$ and $\beta 2m^{-/-}$ mice resulted in significant attenuation of AV compared to allografts from wildtype and $CD4^{-/-}$ mice. This finding supports an important role for $CD8^+$ T cells in the murine aortic allograft model of AV. The AV observed in non-immunosuppressed aortic allografts seems to be mediated mostly but not

exclusively by $CD8^+$ T cells. Moreover, when allo-transplantation was performed under CyA-based immunosuppression, animals which lack $CD8^+$ T cells showed no evidence of pathology, while wildtype mice with normal $CD8^+$ T cell effector function developed significant AV. Moreover, a similar dose of CyA was successful in eliminating the acute rejection response to a cardiac allograft in the identical strain combination indicating that acute rejection responses are not necessarily implicated in the generation of vasculopathy. The differential susceptibility of $CD4^+$ and $CD8^+$ T cell subsets to CyA-based immunosuppression (Horsburgh *et al*, 1980, Hruban *et al*, 1990, Bujan *et al*, 2001, Bishop *et al*, 1992) might explain the limited efficacy of current transplant pharmacotherapy in ameliorating AV.

Effector $CD8^+$ T cells commonly, but not exclusively utilize direct pathways of cytolysis to kill allogeneic target cells. Subbotin and colleagues have suggested a critical role for Fas ligand (FasL) mediated killing in the development of AV by demonstrating the complete abrogation of lesion formation in recipient *gld* mice which lack FasL (Subbotin *et al*, 1999). However, the interpretation of their data is complicated by the age dependent immune hyporesponsiveness observed in *gld* mice and its subsequent effects on the kinetics of graft rejection. Djamali *et al* showed that 6wk old *gld* mice manifest normal acute rejection of cardiac allografts, whereas 13 wk old *gld* mice exhibit significantly prolonged graft survival (Djamali*et al* , 1998). Since the animals used by Subbotin *et al* were up to 12 wk old at the time of transplant and nearly 18 wk old at harvest, immune dysregulation and not Fas/FasL interruption is implicated in the abrogation of AV in their study.

Recently, Krupnick and colleagues have suggested that the granule-exocytosis pathway is more important than the Fas/FasL pathway in the killing of allogeneic endothelial cells (Krupnick *et al*, 2002). However, susceptibility to killing in their 4 h *in vitro* CTL assay does not necessarily reflect *in vivo* killing, which occurs in an inflammatory milieu. For example, Li *et al* showed that interferon-γ, commonly present in an allograft environment, induces sensitivity of human endothelium to Fas/FasL mediated apoptosis *in vitro* (Li *et al*, 2002). Here we demonstrate that the Fas/FasL interaction is important in the induction of medial smooth muscle cell apoptosis both *in vitro* and *in vivo* during the generation of AV. However, the most marked reduction in clinically significant intimal hyperplasia was when both pathways of direct cytolysis were inhibited simultaneously indicating the involvement of both pathways in the pathogenesis of AV. It is entirely possible that CTL-mediated allo-responses against both the graft endothelium and vascular smooth muscle cells occur during the initiation of the AV process. Moreover, Fas-sensitive smooth muscle cells would be predictably injured via CTL-derived FasL, while damage to the Fas-resistant endothelium may be largely mediated by granule-exocytosis.

Thus, the evidence presented here suggests that $CD8^+$ CTL induce medial SMC damage primarily via an apoptotic mechanism employing Fas ligand and perforin/granzymes as effectors. This study provides strong *in vivo* evidence that $CD8^+$ CTL are inducing medial smooth muscle cell loss via apoptosis. The 8 wk data confirm that in the absence of $CD8^+$ CTL activity, medial SMC killing is partially ameliorated. However, residual medial SMC destruction is clearly apparent and likely occurs through redundant immune effector pathways. Despite the widely accepted theory that allo-

immune destruction of the endothelium leads to the development of AV (Mennander *et al*, 1993, Libby and Pober, 2001), it is possible that a relationship between medial SMC damage and intimal proliferation exists. In non-transplant vascular injury models there is evidence that at least partial medial damage is essential for neointimal lesion formation (Reidy, 1985). The evidence suggests that a threshold of medial damage, as opposed to endothelial damage, predicts the extent of intimal thickening during vessel remodeling (Bjorkerud *et al*, 1969, Clowes *et al*, 1983, Reidy, 1985). Complete destruction of the medial SMC population is not required for the formation of the intimal lesion in vascular injury models (Bjorkerud *et al*, 1969, Clowes*et al*, 1983, Reidy, 1985, Linder *et al*, 1993). Thus, even in the absence of $CD8^+$ CTL, the early medial damage caused by redundant pathways could be sufficient to cause the residual neointimal lesion formation observed during cytolytic impairment.

If the redundant pathways are mediated by $CD4^+$ T cells they may play much less of a role in clinical transplantation where CyA-based immunosuppression is common. This is because $CD4^+$ T cells are very sensitive to the effects of CyA whereas $CD8^+$ T cells are relatively resistant (Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001). For example, $CD4^+$ T cell mediated allo-DTH responses are down regulated by CyA (Bretscher and Havele, 1992), whereas $CD8^+$ T cell mediated allo-CTL responses are unaffected (Kroczek *et al*, 1987). Further evidence that $CD4^+$ T cell (but not $CD8^+$ T cell) thymic maturation events are affected by cyclosporine A suggests a potential global downregulation of $CD4^+$ T cell activity upstream to peripheral T cell activation (Takeuchi *et al*, 1989). Thus, in the clinical situation, $CD8^+$ T cell dependent mechanisms may play a dominant role in the development of AV. Therapies

which specifically address the activation, proliferation and effector function of $CD8^+$ CTL might be more effective in the treatment and prevention of AV within human heart transplants.

Figure 1. ***Human cardiac allografts explanted due to severe AV exhibit medial smooth muscle cell loss in addition to intimal hyperplasia.***

Representative photomicrographs of human left anterior descending (LAD) coronary artery from a cardiac allograft explanted 4 years post-transplantation due to severe AV. The patient suffered from diffuse allograft vasculopathy resulting in ischemic cardiac failure necessitating re-transplantation. (**a**) There is a marked intimal occlusive lesion as well as a paucity of vascular smooth muscle cells in the contracted media. (H&E; 200x magnification). (b) Same section at 400x magnification. Arrows indicate internal and external elastic laminae; L indicates the intimal lesion.

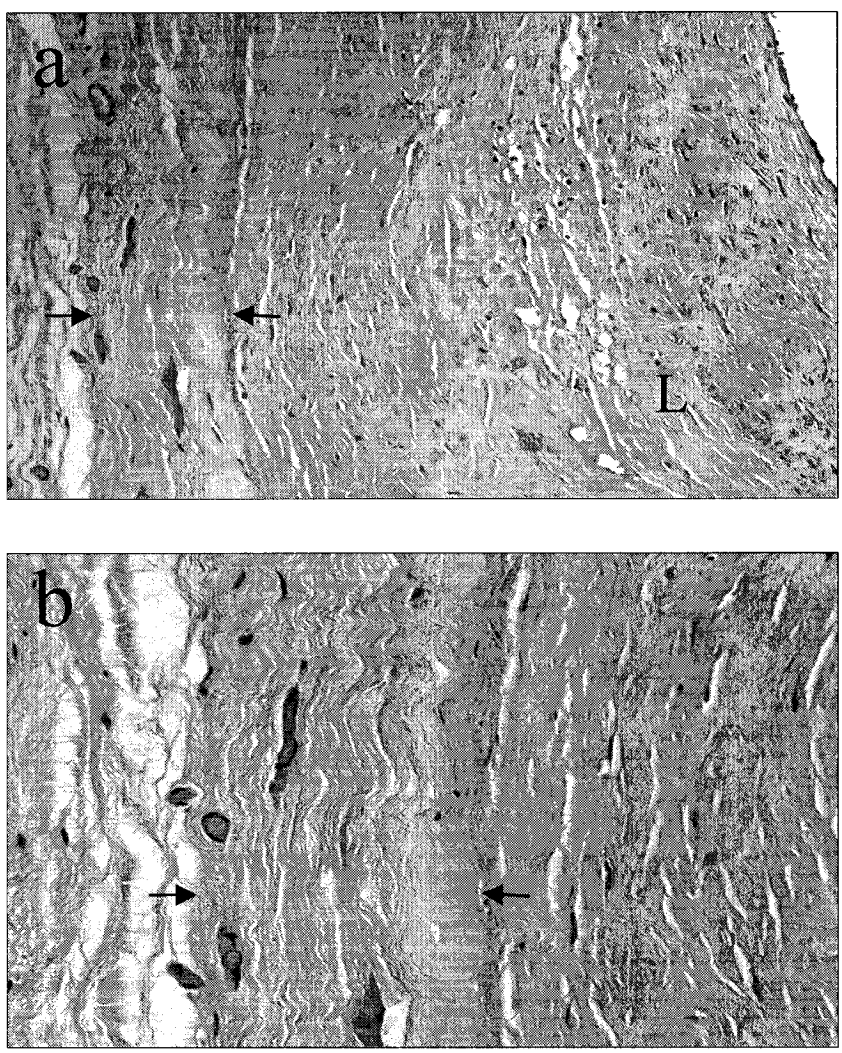


FIGURE 1

Figure 2. ***Aortic allografts undergoing AV exhibit early medial cell apoptosis.*** Representative photomicrographs from (**a**) syngraft and (**b**) wildtype allograft serial cross sections at 14 d post-transplant. Sections stained using TUNEL technique to detect apoptotic nuclei. Arrows indicate internal and external elastic laminae. 200x magnification.

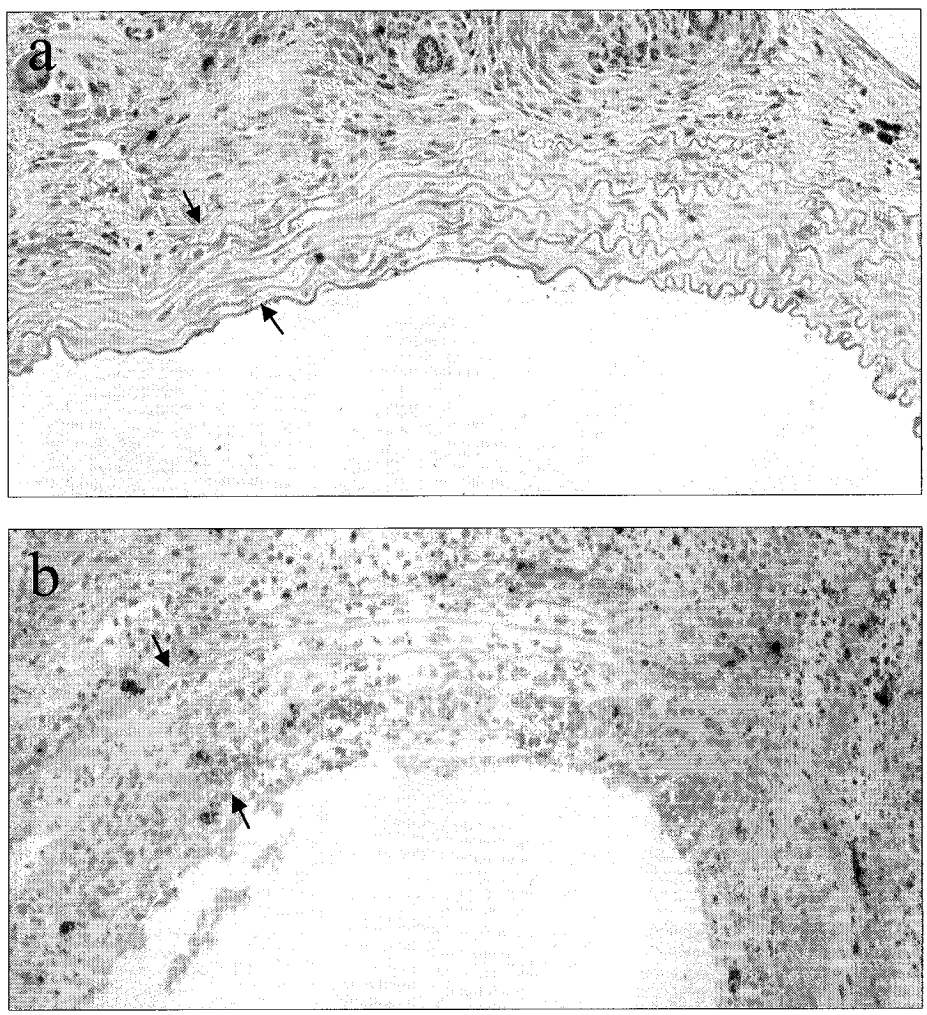


FIGURE 2

Figure 3. ***Deletion of CD8$^+$ T cells or inhibition of CTL effector molecules inhibits early medial cell apoptosis.***

Apoptotic (TUNEL$^+$) nuclei per cross section of syngeneic grafts in wildtype recipients, allogeneic grafts in wildtype recipients and allogeneic grafts in environments depleted of CD8$^+$ T cells (CD8$^{-/-}$ and β2m$^{-/-}$) and allografts in environments deficient in CTL pathways of cytolysis (perforin- and FasL-deficient) was determined at 2 weeks post-transplant. CD8$^{-/-}$ (n=5; $p<0.001$), β2m$^{-/-}$ (n=6; $p<0.001$), perforin-deficient (*Pfp*$^{-/-}$; n=5; $p<0.01$), and FasL-deficient (n=4; $p<0.001$) groups are significantly different from wildtype allograft (n=5) controls. Data are expressed as mean ± SEM.

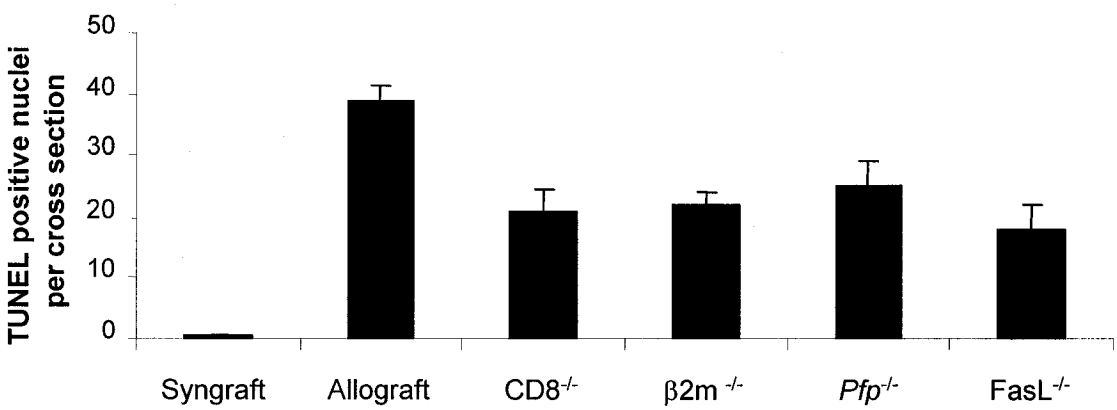

TUNEL positive nuclei per cross section
50
40
30
20
10
0
Syngraft
Allograft
CD8-/-
β2m -/-
Pfp-/-
FasL-/-

FIGURE 3

Figure 4. ***Deletion of CD8$^+$ T cells or CTL effector molecules promotes late medial area preservation.***

The degree of medial preservation is expressed as a percentage reduction from the medial area of undamaged syngeneic aortic grafts for allografts in wildtype recipients (Allograft) and allogeneic grafts in environments depleted of CD8$^+$ T cells (CD8$^{-/-}$ and β2m$^{-/-}$) and allografts in environments deficient in CD8$^+$ CTL pathways of cytolysis (perforin- and FasL-deficient) was determined at 8 wk post-transplant. CD8$^{-/-}$ (n=6), β2m$^{-/-}$ (n=6), perforin-deficient (*Pfp*$^{-/-}$; n=5;), and FasL-deficient (FasL$^{-/-}$; n=5;) groups are significantly different from allograft controls (n=6; $p<0.001$) and CD4$^{-/-}$ recipients (n=10; $p<0.001$). Data are expressed as mean ± SEM.

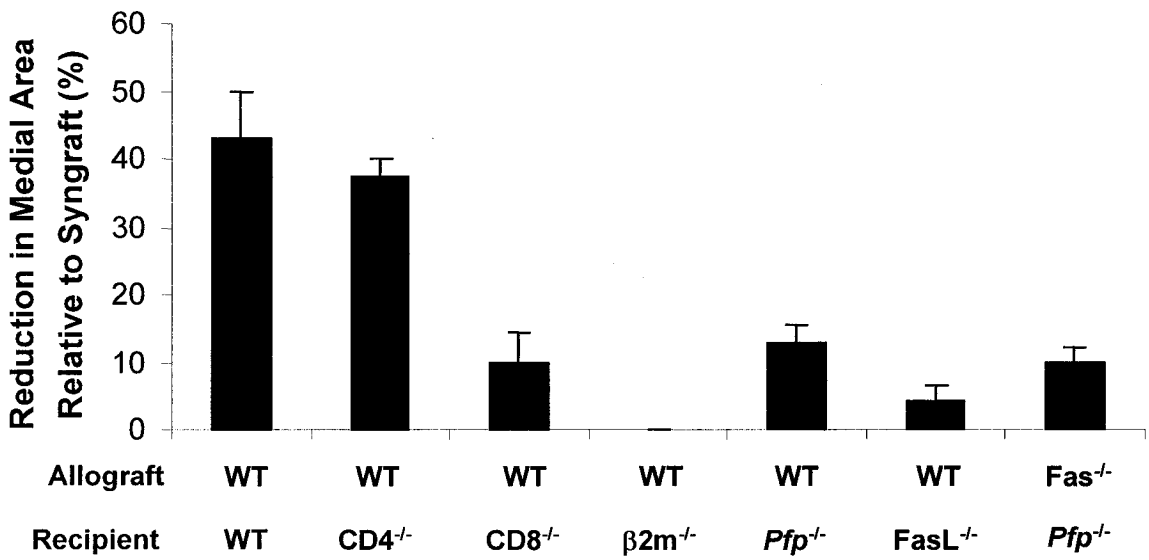

Reduction in Medial Area Relative to Syngraft (%)
60
50
40
30
20
10
0
Allograft WT WT WT WT WT WT Fas-/-
Recipient WT CD4-/- CD8-/- β2m-/- Pfp-/- FasL-/- Pfp-/-

FIGURE 4

Figure 5. ***Deletion of CD8$^+$ T cells or CTL effector molecules leads to preservation of α-actin$^+$ medial smooth muscle cells.***

Representative photomicrographs at 8 wk post-transplant showing immunocytochemistry detecting α-actin (clone 1A4) positive smooth muscle cells from wildtype grafts into (**a**) syngeneic control, (**b**) allogeneic wildtype control, (**c**) CD4$^{-/-}$ recipient, (**d**) CD8$^{-/-}$ recipient, (**e**) FasL$^{-/-}$ recipient, and (**f**) Fas-deficient *lpr* allograft into a perforin-deficient recipient. Arrows indicate internal and external elastic laminae. Photographed at 200x magnification.

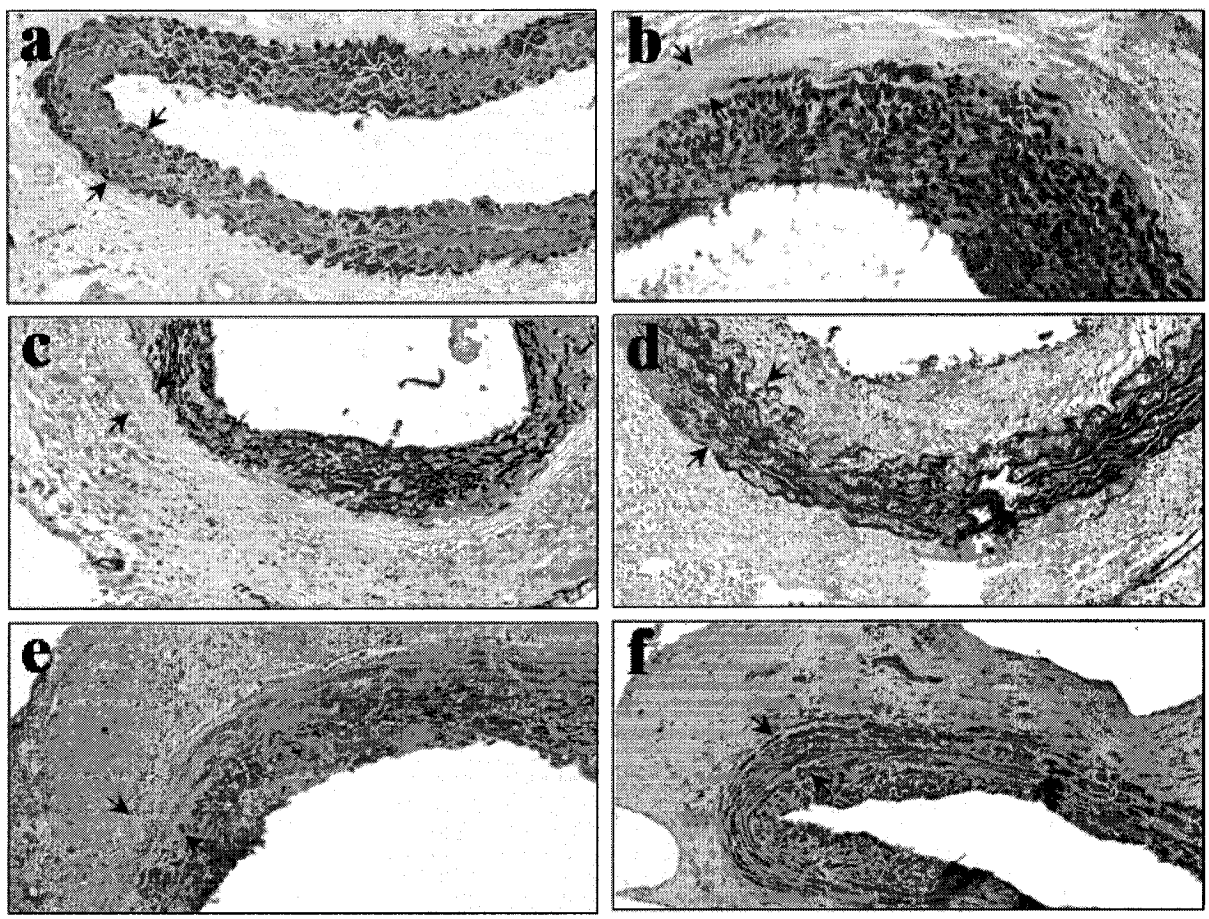


FIGURE 5

Figure 6. ***Vascular smooth muscle cells from aortic explants express α-actin during in vitro culture.***

Representative photomicrographs of cultured vascular smooth muscle cells from aortic explants derived from (**a**) wildtype C3H/HeJ and (**b**) Fas-deficient C3H *lpr* mice immunostained with α-actin (clone 1A4). Photoghraphed at 400x magnification.

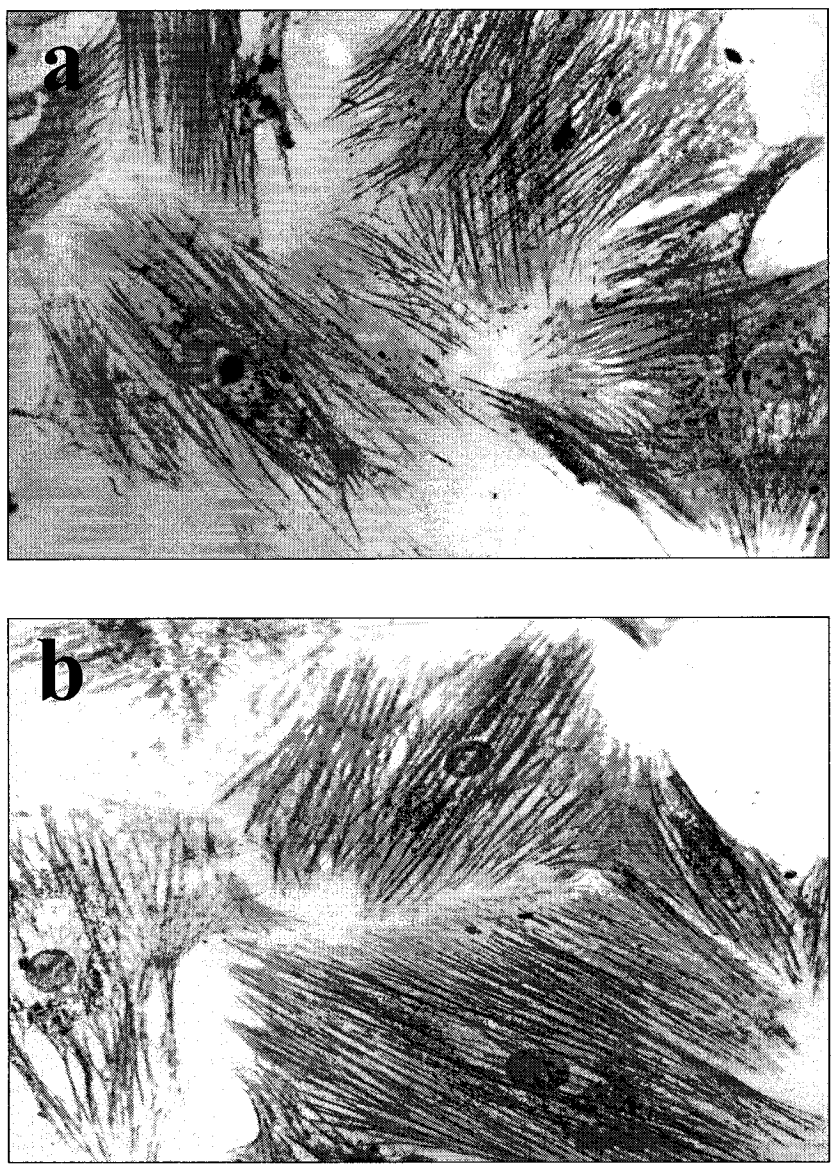


FIGURE 6

Figure 7. ***CD8$^+$ T cells lyse vascular smooth muscle cell targets in vitro primarily through the Fas/FasL interaction.***

$CD8^+$ T cells, freshly purified from allo-primed animals were tested for killing activity in an in vitro CTL assay. Allogeneic C3H $H2^k$ vascular smooth muscletargets from wildtype (■, ●) and Fas-deficient *lpr* (□, ○) mice were incubated with primed $CD8^+$ T cells from wildtype (■, □) or perforin-deficient (●, ○) C57BL/6 mice. No killing of third party targets was observed. Both wildtype and *lpr* targets were susceptible to killing mediated by anti-H2-D^k antibody and complement. Non-specific lysis was <10%.

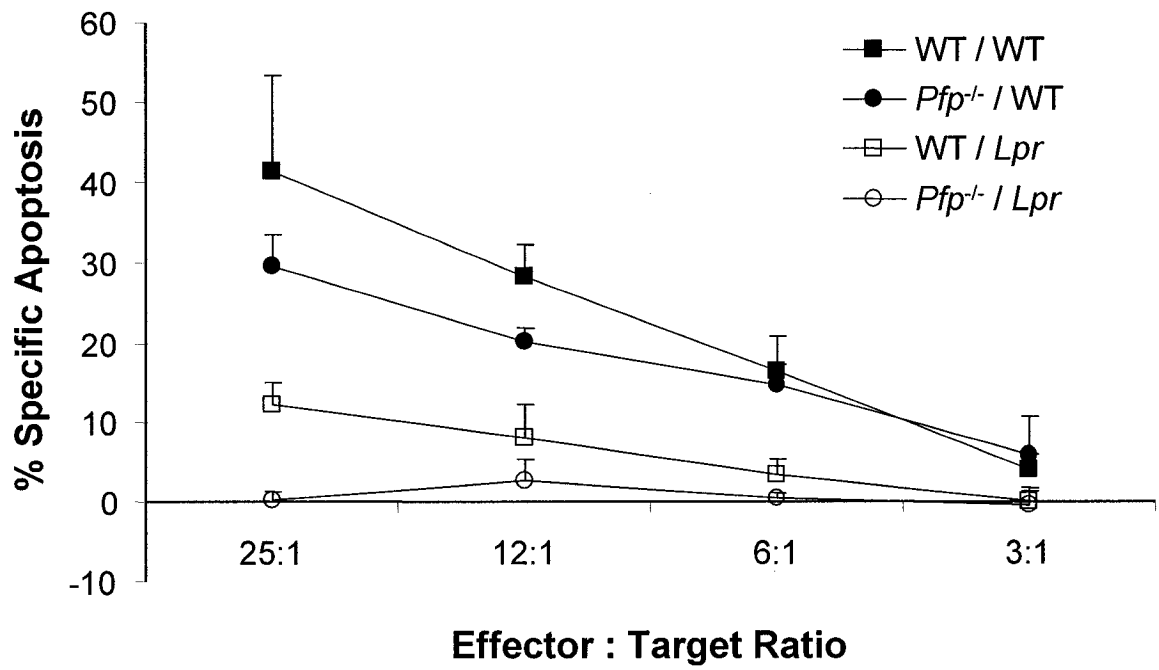

60
50
40
30
20
10
0
-10
% Specific Apoptosis
25:1
12:1
6:1
3:1
Effector : Target Ratio
WT / WT
Pfp-/- / WT
WT / Lpr
Pfp-/- / Lpr

FIGURE 7

Figure 8. ***Role of T cell subsets and CTL effector molecules in the generation of AV.*** Representative photomicrographs of (**a**) control syngraft, (**b**) wildtype control allograft, (**c**) allograft into $CD4^{-/-}$ recipient, (d) allograft into a $CD8^{-/-}$ recipient, (e) allograft into $\beta 2m^{-/-}$ recipient, (f) allograft into perforin-deficient recipient, (g) allograft into Fas ligand-deficient recipient, and (h) Fas-deficient *lpr* allograft into perforin-deficient recipient. Photoghraphed at 200x magnification.

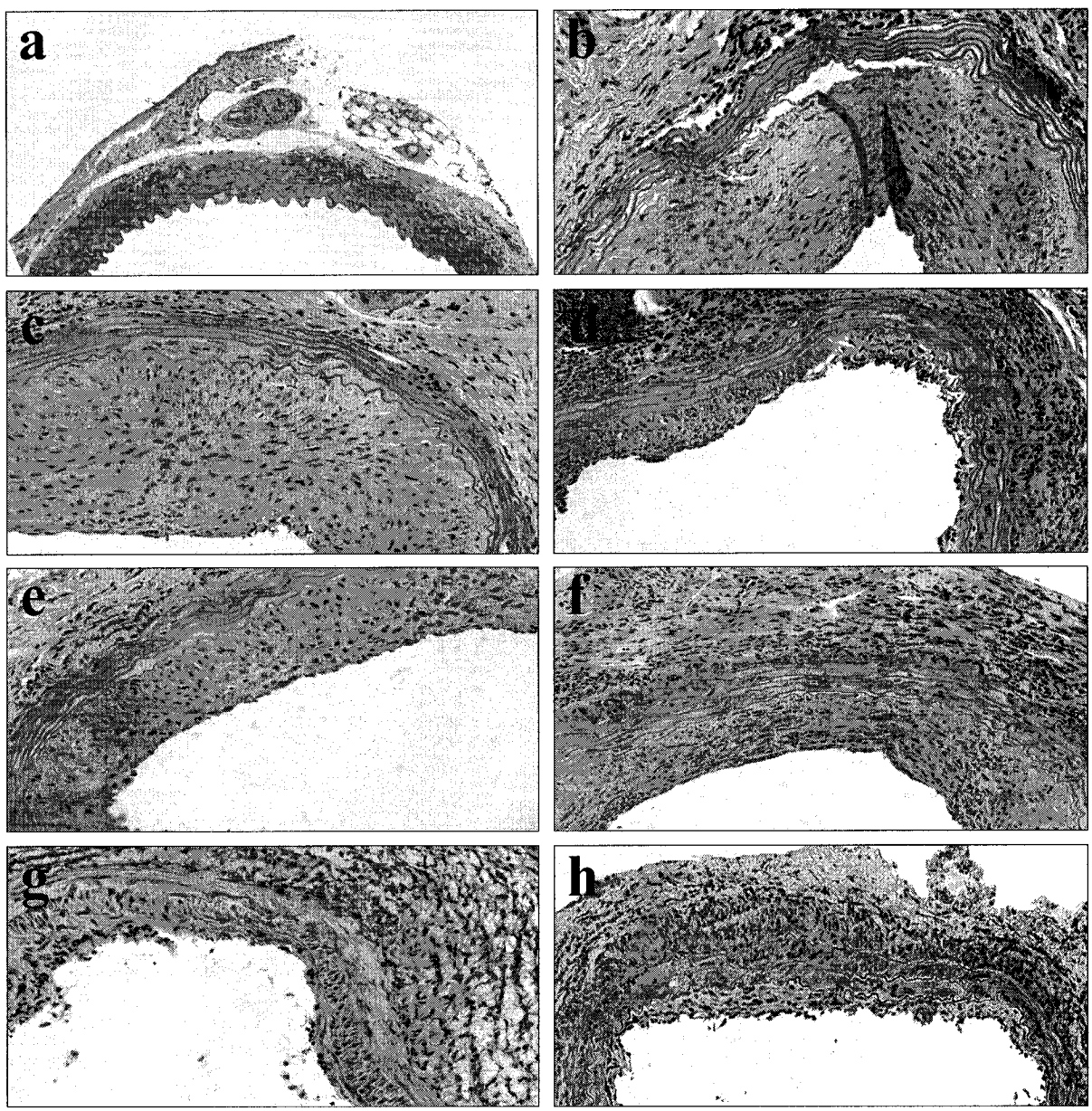


FIGURE 8

Figure 9. ***Inhibition of CD8$^+$ T cells or CTL effector molecules attenuates AV.***

Intima to media ratios for syngeneic grafts in wildtype recipients, allogeneic grafts in wildtype recipients, allogeneic grafts in environments depleted of $CD8^+$ T cells ($CD8^{-/-}$ and $\beta2m^{-/-}$), allogeneic grafts in environments deficient in pathways of cytolysis (perforin- and FasL-deficient), and Fas-deficient *lpr* allografts in perforin-deficient recipients were determined at 8 wk post-transplant. Wildtype allografts in $CD8^{-/-}$ (n=5; $p<0.001$), $\beta2m^{-/-}$ (n=5; $p<0.05$; $p<0.01$), $FasL^{-/-}$ (n=5; $p<0.05$; $p<0.01$) and $Fas^{-/-}$ allografts in perforin-deficient ($Pfp^{-/-}$; n=10; $p<0.001$) are significantly different from wildtype allograft controls (n=6) and $CD4^{-/-}$ recipients (n=10). Data are expressed as mean ± SEM.

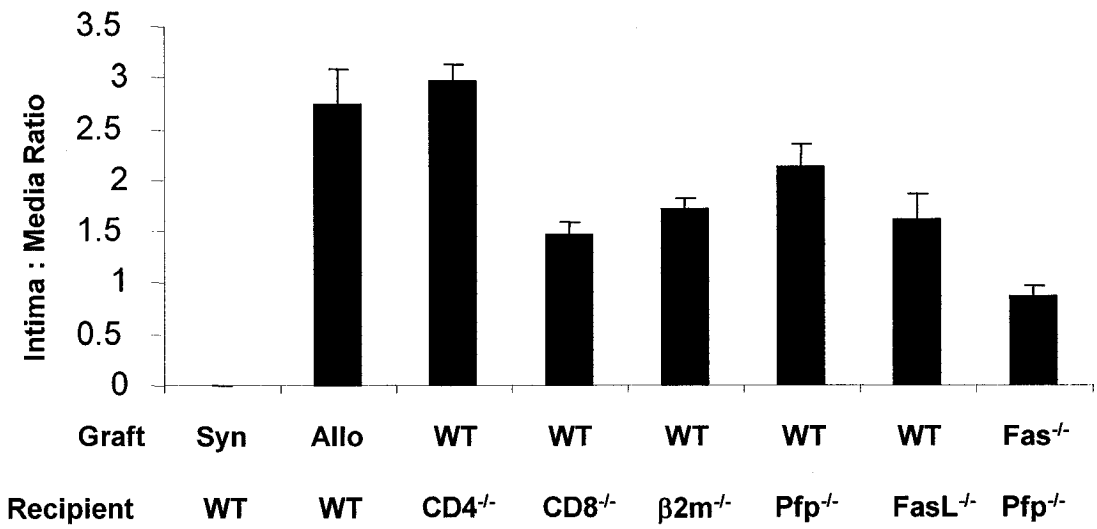

Intima : Media Ratio
3.5
3
2.5
2
1.5
1
0.5
0
Graft
Syn
Allo
WT
WT
WT
WT
WT
Fas-/-
Recipient
WT
WT
CD4-/-
CD8-/-
β2m-/-
Pfp-/-
FasL-/-
Pfp-/-

FIGURE 9

Figure 10. ***AV in immunosuppressed animals is a CD8$^+$ T cell-dependent process.*** Representative photomicrographs of allografts from wildtype B6 recipients (**a** and **c**) and B6 CD8$^{-/-}$ recipients (**b** and **d**) treated with cyclosporine A 50 mg/kg/d or 70 mg/kg/d, respectively. CD8$^{-/-}$ recipients did not develop AV at either dose. The AV observed in wildtype B6 recipients treated with cyclosporine A is dependent on the presence and functionality of CD8$^+$ T cells; n=4-5 animals per group; scale bars = 200 μm.

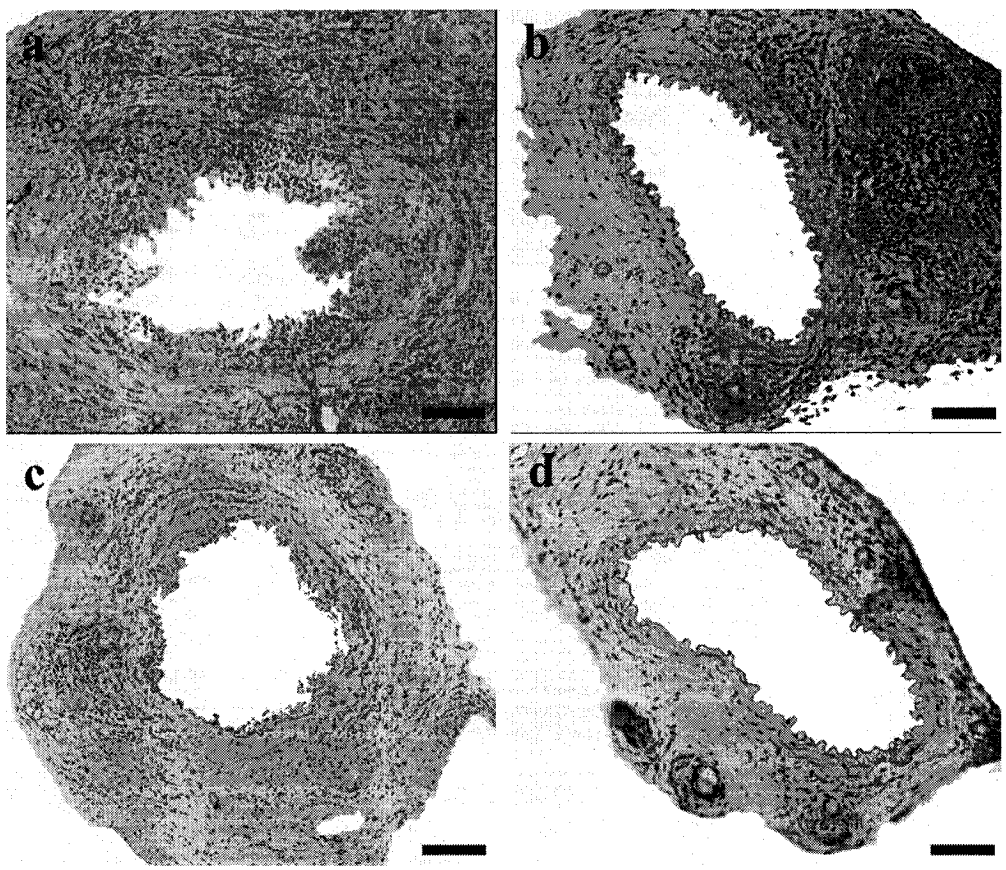


FIGURE 10

Chapter 5

5.0 $CD8^+$ T Lymphocytes Mediate Allograft Vasculopathy by Direct Cytolysis and by an Interferon-γ-Dependent Indirect Effector Pathway

5.1 Introduction

Heart transplantation is the only treatment option for many patients suffering from end-stage heart failure. Although modern immunosuppression has dramatically improved early survival by ameliorating acute rejection, loss of cardiac grafts due to AV has emerged as the major limitation in heart transplantation (Hosenpud *et al.*, 2001). More than 50% of transplanted hearts will fail within 10 years, primarily from the pathologic effects of AV (Hosenpud *et al.*, 2001). AV consists of a concentric thickening of the intimal layer of the epicardial coronary arteries, leading to lumenal occlusion, thrombosis and eventually ischemic organ failure (Billingham, 1994). It is also characterized by a leukocytic infiltrate in the adventitia and loss of smooth muscle cells from the media (Hirsch *et al.*, 1998). Current immunosuppressive regimens do not inhibit AV (Hosenpud *et al.*, 2001) and conventional revascularization strategies are ineffective because of the diffuse nature of the disease (Billingham, 1994).

The exact cellular and molecular events responsible for AV are not known. It is clearly T cell mediated, in that T cell-deficient athymic nude animals do not exhibit AV (Hall, 1991, Ensminger *et al.*, 2002), although recent evidence suggests that innate immunity may also contribute to lesion formation (Russell *et al.*, 2001). While some have suggested that $CD4^+$ T cells are required for the generation of AV (Ensminger *et al.*, 2002, Yamada *et al.*, 2003), others (Han *et al.*, 2000) have shown that AV occurs in anti-CD4 antibody transgenic mice that are devoid of $CD4^+$ T cells by endogenous depletion (Han *et al.*, 2000). We have shown that marked apoptosis occurs within the medial SMC compartment during the early stages of AV with a concomitant upregulation of mRNA

for cytotoxic T lymphocyte derived mediators of target cell apoptosis (Hirsch *et al.*, 1998). These data suggest an important role for $CD8^+$ T cells in AV.

$CD8^+$ T lymphocyte effects on AV could be mediated through either direct cytolysis (Hirsch *et al.*, 1998, Légaré *et* al., 2000) or through indirect effector pathways (Nagano *et al.*, 1998, Delfs *et al.*, 2001). The available evidence suggests that direct cytolytic activity leading to apoptosis in the transplant vasculature could contribute significantly to AV (Hirsch *et al.*, 1998, Légaré *et* al., 2000). Alternatively, indirect effector mechanisms mediated by type 1 cytokines could also contribute to AV (Nagano *et al.*, 1998, Delfs *et al.*, 2001). These direct and indirect events have been suggested as inciting stimuli leading to the vascular remodeling and neointimal hyperplasia that are characteristic of AV (Delfs *et al.*, 2001).

Our data in this study demonstrate that $CD8^+$ T cells can induce AV in the absence of $CD4^+$ T cells or antibody, and that CTL-mediated direct cytolysis and interferon-γ-dependent non-CTL effector pathways are both involved. Given that $CD8^+$ T cells have been suggested to be less sensitive than $CD4^+$ T cells to calcineurin inhibition (Bishop *et al.*, 1992, Bujan *et al.*, 2001, Hruban *et al.*, 1990, Libby and Pober, 2001), these data might explain the limited efficacy of current immunosuppressive therapy in preventing AV.

5.2 Results

The experimental system used in this study entailed the adoptive transfer of effector $CD8^+$ T cells into immunodeficient recombinase activating gene-1 knockout ($RAG\text{-}1^{-/-}$) mice that lack functional B cell and T cell compartments. The C57BL/6 (B6) $RAG\text{-}1^{-/-}$ recipient mice were transplanted with fully mismatched BALB/c aortic allografts prior to reconstitution with primed B6 effector T cell subsets. In this study we restricted our observations to the effector limb of AV, by allowing primary antigen activation to occur in the wildtype host and subsequently transferring specific populations of effector T cells into immunodeficient mice.

5.2.1 *Generation of primed effector $CD8^+$ T cells*

Primed whole T cell populations were derived by immunizing wildtype B6 animals with BALB/c splenocytes. Primed $CD8^+$ T cells were purified from the enriched T cell populations (Figure 1, Table 1) by negative selection using immunocolumns as described above and contained no detectable $CD4^+$ or $B220^+$ cells (Figure 2, Table 1). To confirm that immunization had led to allo-activation, we tested freshly purified, primed $CD8^+$ T cells for direct CTL activity *ex vivo*. The allo-primed $CD8^+$ T cells killed (up to 70% specific apoptosis) allogeneic ($H\text{-}2^d$) targets, but not third party ($H\text{-}2^k$) targets (Figure 3). In contrast, $CD8^+$ T cells isolated from naïve B6 mice did not kill (Figure 3). Deletion of $CD8^+$ cells or asialo $GM1^+$ cells from allo-primed whole T cell populations eliminated *ex vivo* killing (Figure 3).

Similarly, when either primed whole T cell or primed, purified $CD8^+$ T cell populations were cultured in a secondary mixed lymphocyte reaction (MLR) with mitomycin C-inactivated allogeneic stimulators, significant proliferation of responder cells was detected (Figure 4). In contrast, whole T cells and purified $CD8^+$ T cells derived from naïve B6 mice exhibited reduced proliferation in a primary MLR (Figure 4). Antigen specificity was demonstrated by the diminished proliferation of allo-primed $CD8^+$ T cells in response to third-party ($H\text{-}2^k$) stimulators (Figure 4). Taken together, these data would suggest that allo-immunization is effective in generating effector whole T cell and $CD8^+$ T cell populations exhibiting both proliferative capacity and killing activity of allogeneic stimulators and targets, respectively.

Having demonstrated successful priming of whole T cell and $CD8^+$ T cell populations, we then confirmed the survival of these cells *in vivo* after transfer into immunodeficient RAG-$1^{-/-}$ mice that did not receive transplants. Two weeks after the transfer of $1x10^7$ primed B6 whole T cells, or primed, purified B6 $CD8^+$ T cells into the B6 RAG-$1^{-/-}$ mice, spleen samples were analyzed by fluorescence activated cell sorting (FACS) analysis or immunocytochemistry. Despite the lack of further allo-antigen exposure *in vivo*, immunodeficient mice injected with whole T cells exhibited successful reconstitution of both the $CD4^+$ and $CD8^+$ T cell compartments (Figure 5), while mice injected with primed, purified $CD8^+$ T cells showed successful reconstitution of only the $CD8^+$ T cell compartment (Figure 5). This confirms that the transferred $CD8^+$ T cell population is viable, able to reconstitute the immunodeficient host and uncontaminated with $CD4^+$ T cells (Figure 5). Neither whole T cell injection nor $CD8^+$ T cell injection reconstituted the B cell compartment (Figure 6).

5.2.2 *Role of CD8$^+$ effector T cells in the generation of AV*

After demonstrating that priming led to the generation of allo-specific B6 CD8$^+$ T cells capable of direct CTL activity *ex vivo*, we assessed their ability to generate AV in B6 RAG-1$^{-/-}$ recipients of fully mismatched BALB/c aortic allografts. We observed no AV in allografts transplanted into unreconstituted RAG-1$^{-/-}$ mice whereas reconstitution with allo-primed whole T cells or with purified, allo-primed CD8$^+$ T cells led to the generation of robust AV with a marked intimal lesion, virtually complete loss of medial SMC, and a profound adventitial mononuclear cell infiltrate (Figure 7). Wildtype recipients, RAG-1$^{-/-}$ recipients reconstituted with primed whole T cells, and RAG-1$^{-/-}$ recipients reconstituted with primed CD8$^+$ T cells all developed intimal hyperplasia at similar levels (Figure 8).

In order to confirm that the AV observed in RAG-1$^{-/-}$ recipients reconstituted with primed, purified CD8$^+$ T cells was not due to the small (5-7%) population of null (non-T and non-B) cells within the transfer inoculum, we deleted CD8$^+$ cells from the transfer population using anti-CD8 monoclonal antibody and complement-mediated lysis prior to nylon-wool enrichment and passage through CD8 immunocolumns. A similar number of null cells (1 x 10^7) derived from allo-immunized B6 mice were then transferred into B6 RAG-1$^{-/-}$ recipients of BALB/c allografts and assessed for the development of AV. Deletion of CD8$^+$ cells from the purified CD8$^+$ T cell transfer population abrogated AV (Figure 7 and 8). Moreover, highly purified, primed CD8$^+$ T cells, with a purity in excess of 99% (Figure 9), isolated using a positive selection technique for subsequent adoptive transfer into immunodeficient recipients yielded similar results to negative selection

purification (Figure 10 and 11). These two observations confirm that the small population of null cells in the negatively selected primed $CD8^+$ T cell preparation are not responsible for the development of AV.

To eliminate the contribution of concurrent ischemic vessel injury we performed adoptive transfer 4 and 7 d after transplantation where the initial non-specific inflammatory response to ischemia would be attenuated. Our results were unchanged indicating that robust $CD8^+$ T cell allo-responses mediate AV irrespective of early ischemic injury (Figure 12).

The infiltration of transferred effector $CD8^+$ T cells into the grafts undergoing AV was confirmed by immunocytochemistry. As expected, allografts harvested from unreconstituted RAG-$1^{-/-}$ recipients did not contain $CD4^+$ or $CD8^+$ cells (Figure 13). Allografts harvested from RAG-$1^{-/-}$ mice reconstituted with whole T cells exhibited infiltration by both T cell ($CD4^+$ and $CD8^+$) subsets (Figure 13). Allografts harvested from $CD8^+$ T cell-reconstituted RAG-$1^{-/-}$ mice showed $CD8^+$ T cells localized predominantly within the media with no $CD4^+$ T cells in the grafts (Figure 13).

Serum samples from $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ allograft recipients were taken and subsequently analyzed for the presence of allo-specific immunoglobulin G (IgG) by FACS analysis. There was no detectable allo-specific IgG in the serum derived from $CD8^+$ T cell injected immunodeficient mice (Figure 14). In contrast, serum harvested from wildtype B6 mice, which exhibited marked rejection of aortic allografts, contained significant levels of allo-specific immunoglobulin (Figure 14). To confirm that other classes of immunoglobulin such as IgM, which might not have been detected by FACS analysis, were indeed not present, we assayed serum from $CD8^+$ T cell

reconstituted RAG-$1^{-/-}$ for killing activity against allogeneic splenocytes in a complement-dependent cytotoxicity assay. Serum from wildtype B6 mice showed dose-dependent cytotoxicity (ID_{50} 1:1024), while serum from $CD8^+$ T cell reconstituted immunodeficient mice did not (Figure 15). These data confirm that transferred effector $CD8^+$ T cells migrate to the graft and mediate AV in the absence of $CD4^+$ T cells or B cell activity.

5.2.3 *Disruption of cytotoxic mediators eliminates in vitro CTL killing*

We examined the role of direct cytolytic and non-CTL effector mechanisms in $CD8^+$ T cell mediated AV. We first assessed direct killing *in vitro* by using primed $CD8^+$ T cells with targeted deletions of effector molecules necessary for the granule-exocytosis and Fas-Fas ligand pathways of cytotoxicity.

When the granule-exocytosis pathway was interrupted, by using perforin deficient (*pfp*) effector cells, killing of allo targets was not reduced (Figure 16). Likewise, interruption of the Fas-FasL pathway, by using Fas receptor deficient (*lpr*) target cells, did not reduce killing (Figure 16). However, when both pathways were interrupted, by using *pfp* effector cells against *lpr* targets, complete abrogation of killing activity was observed (Figure 16). These data confirm that, at least *in vitro,* minor pathways of cytotoxicity are not relevant in this system.

5.2.4 *CD8$^+$T cell-mediated direct cytolysis contributes to the generation of AV*

When primed CD8$^+$ T cells from B6 FasL-deficient (*gld*) mice or B6 perforin deficient (*pfp*) mice were used to reconstitute B6 RAG-1$^{-/-}$ allograft recipients we observed robust AV (Figure 17), confirming that neither CTL pathway is essential for the generation of AV. When we blocked both of the major pathways of cytotoxicity simultaneously by transferring primed CD8$^+$ T cells derived from *pfp* animals into RAG-1$^{-/-}$ recipients transplanted with *lpr* aortic allografts, the mice exhibited attenuated AV lesions. The AV lesions were characterized by both intimal hyperplasia and medial SMC loss (Figure 17), but there was an almost 50% reduction in intimal hyperplasia compared to wildtype controls (Figure 18; $p<0.01$) suggesting an important, but not essential, role for direct killing in the etiology of AV, as well as the activity of some alternate non-CTL CD8$^+$ T cell effector pathway.

5.2.5 *A distinct CD8$^+$ T cell non-CTL effector pathway is capable of generating AV and is interferon-γ-dependent*

To examine the contribution of non-CTL effector mechanisms we transferred purified, primed C3H CD8$^+$ T cells into immunodeficient C3H RAG-2$^{-/-}$ recipients of aortic allografts from B6 β2microglobulin-deficient (B6 β2m$^{-/-}$) donors. β2m$^{-/-}$ mice are profoundly deficient in surface Class I MHC (Grusby *et al.*, 1993) and are referred to as MHC I-deficient mice. We (Figure 19) and others (Zijlstra *et al.*, 1990, Apasov *et al.*, 1993, Glas *et al.*, 1992) have shown that cells from such mice are not susceptible to direct

killing. The cells do, however, produce Class I peptides that can be presented by recipient dendritic cells and used to engage the transferred effector $CD8^+$ T cells. Such $CD8^+$ T cells may then mediate killing by indirect non-CTL means. Indeed, MHC I-deficient allografts transplanted into RAG-$2^{-/-}$ recipients reconstituted with primed, purified $CD8^+$ T cells from wildtype C3H mice exhibited substantial AV (Figure 20), but the extent of intimal hyperplasia was reduced by approximately one third when compared to wildtype control allografts (Figure 21; $p=0.0406$). These data support the involvement of non-cytolytic effector pathways in $CD8^+$ T cell-mediated AV.

Having implicated non-cytolytic $CD8^+$ T cell effectors in the generation of AV, we postulated that interferon-γ (IFN-γ) would be a prime mediator of this pathway. To confirm both the indirect non-CTL effect and a role for IFN-γ in this pathway, we used primed, purified $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mice to reconstitute RAG-$1^{-/-}$ recipients of MHC I-deficient allografts. Primed $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mice were unable to generate AV in these allografts despite a significant $CD8^+$ T cell infiltrate (Figure 22). Interestingly, primed $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mice triggered AV in wildtype allografts (Figure 22). These data confirm that an indirect non-CTL pathway is involved in the observed AV in MHC I-deficient allografts and that IFN-γ is the essential mediator of this process.

5.2.6 *$CD8^+$ T cell-mediated AV is refractory to cyclosporine A treatment.*

Primed, highly purified $CD4^+$ and $CD8^+$ T cell subsets are sufficient to induce AV in non-immunosuppressed $RAG^{-/-}$ allograft recipients (Figure 23). In contrast, only primed, highly purified $CD8^+$ T cells remained capable of inducing AV in the presence of cyclosporine A (50 mg/kg/d) treatment, whereas $CD4^+$ T cells did not (Figure 23). $CD4^+$ T cell function is potently inhibited by the addition of immunosuppression, whereas $CD8^+$ T cell effectors are relatively resistant to cyclosporine A (Figure 24).

5.3 Discussion

It is widely accepted that various non-specific inflammatory events contribute to the generation of AV (Schmid *et al.*, 1997). Ischemic injury during organ procurement and transplantation has been identified as a particularly important factor. Gaudin *et al*, for example, showed that perioperative ischemic injury predicts the development of AV in humans (Gaudin *et al.*, 1994). Although many immune and non-immune factors contribute to the generation of AV (Schmid *et al.*, 1997), it is well established that in the absence of effective T cell activity (by using athymic nude, severe combined immunodeficient (SCID), RAG-deficient animals or very high dose immunosuppression) AV does not occur (Hall, 1991, Ensminger *et al.*, 2002, Russell *et al.*, 1994, Delfs *et al.*, 2001, Hillebrands *et al.*, 2001). In this study T and B cell deficient RAG-$1^{-/-}$ mice did not exhibit AV.

Some have suggested that $CD4^+$ T helper cells are necessary for the generation of AV (Ensminger *et al.*, 2002, Yamada *et al.*, 2003). However, we (using CD4 knockout mice; see chapter 2) and others (using endogenous antibody depletion; Han *et al.*, 2000) have found that AV is not dependent on the activities of $CD4^+$ T cells. This is of considerable importance given the fact that $CD8^+$ T cells appear to be relatively resistant to the actions of current calcineurin pathway based immunosuppressive therapies (Bishop *et al.*, 1992, Bujan *et al.*, 2001, Hruban *et al.*, 1990, Libby and Pober, 2001) and the fact that such therapies do not ablate AV (Hosenpud *et al.*, 2001).

In this study we utilize adoptive transfer into immunodeficient RAG-$1^{-/-}$ recipients to examine the contribution of $CD8^+$ effector T cells to the generation of AV. One caveat

of cell transfer systems into immunodeficient hosts is the potential for homeostasis-driven proliferation of elements in the transferred population (Goldrath *et al.*, 2000). Such homeostatic proliferation results in cells with a functional state similar to that of the memory $CD8^+$ T cell phenotype capable of cytokine production and effector function (Schluns *et al.*, 2000, Ge *et al.*, 2002). For this reason we restricted our observations to the effector limb, by allowing primary antigen activation to occur in the wildtype host and subsequently transferring effector T cell populations; thus avoiding the issue of homeostasis-driven proliferation.

We demonstrate here that primed $CD8^+$ T cells are sufficient to generate AV. Transferred purified effector $CD8^+$ T cells induced robust AV in RAG-$1^{-/-}$ mice which lack both T cells and B cells. When $CD8^+$ cells were deleted from this transfer population AV induction was abrogated, confirming that the AV lesions seen are completely dependent on effector $CD8^+$ T cells. Moreover, when highly purified $CD8^+$ T cells isolated by positive selection were used in the adoptive transfer AV was again observed. While the intimal lesion is a hallmark of AV (Billingham, 1994), we recognize that inward vessel remodeling may also play a role in the hemodynamic compromise of AV (Pethig *et al.*, 1998, Pasterkamp *et al.*, 2000). However, the relative contribution of these two processes remains unclear.

$CD8^+$ effector T cells are capable of direct cytolysis (Hirsch *et al.*, 1998, Légaré *et* al., 2000), but they also produce cytokines which mediate DTH-like immune responses (Nagano *et al.*, 1998, Delfs *et al.*, 2001). We first examined the contribution of direct CTL-mediated killing. When either the granule-exocytosis or the FasL pathway was blocked independently, AV lesion formation was unimpeded. These data are in contrast

to the observations of Subbotin and colleagues (Subbotin *et al.*, 1999), who claimed that Fas-FasL interactions were critical for AV. Complicating the interpretation of their data, however, is the age dependent expression of immunological hyporesponsiveness in the *gld* mice they used (Djamali *et al.*, 1998). For example, Djamali *et al* showed that 6wk old *gld* mice exhibit normal acute rejection but 13wk old mice exhibit long term graft survival (Djamali *et al.*, 1998). While the $CD8^+$ T cells used in our experiments were derived from 6 wk old *gld* mice, Subbotin *et al* used 10-12 wk old *gld* mice at the start of their experimentation (18 wk old at harvest) at which time the immunological competency of these animals is in question (Subbotin *et al.*, 1999).

When both pathways were blocked concurrently we observed a significant attenuation of $CD8^+$ T cell-mediated AV indicating that direct killing plays a major role in the induction of AV. However, since complete inhibition of AV was not seen, other pathways must be active. Since simultaneous blockade of both cytolytic pathways eliminated direct killing *in vitro*, minor pathways of cytotoxicity are unlikely to be active within this system. Given that unreconstituted recipients do not develop lesions, NK cells of the host are not implicated. Although, indirect $CD8^+$ T cell effectors might recruit and activate RAG-$1^{-/-}$-derived macrophages or NK cells in a DTH-like manner capable of damaging the allograft. Taken together this data indicates that non-CTL effector mechanisms contribute to $CD8^+$ T cell mediated AV.

We investigated this non-CTL effector pathway by evaluating the ability of allo-primed $CD8^+$ T cells to reject MHC I-deficient grafts. $CD8^+$ T cells are unable to directly interact with such grafts (Zijlstra *et al.*, 1990, Apasov *et al.*, 1993, Glas *et al.*, 1992, Qian *et al.*, 1996). In our study, primed $CD8^+$ T cells generated AV in these allografts despite

the mitigation of direct killing. Moreover, primed $CD8^+$ T cells generated by immunization with allogeneic wildtype cells do not kill these MHC I-deficient targets *in vitro* (Figure 19, Glas *et al.*, 1992) confirming our *in vivo* observations. This data strongly supports the hypothesis that non-CTL effectors are implicated in the generation of AV in MHC I-deficient allografts, as well as in *lpr* grafts of *pfp* reconstituted RAG-$1^{-/-}$ mice in which pathways of direct cytolysis are attenuated.

Interferon-γ (IFN-γ) would be a likely mediator of such non-CTL effector mechanisms (Nagano *et al.*, 1998, Delfs *et al.*, 2001). Graft infiltrating $CD8^+$ T lymphocytes produce significant amounts of IFN-γ (Stinn *et al.*, 1998, Valujskikh *et al.*, 2001) and IFN-γ could potentiate PDGF-induced effects on the cells forming the neointimal lesion (Tellides *et al.*, 2000) as well as inducing DTH-like cell mediated responses (Muller *et al*, 1994). Other groups have previously demonstrated an important role for host-derived IFN-γ in the development of AV as IFN-$\gamma^{-/-}$ recipients exhibit markedly, although not completely, attenuated AV (Nagano *et al.*, 1997, Raisanen-Sokolowski *et al.*, 1997, Raisanen-Sokolowski *et al.*, 1998). Similarly, in this study primed $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mie transferred into immunodeficient recipients of wildtype allografts generated robust AV, albeit significantly reduced when compared to wildtype $CD8^+$ T cells. However, transfer of allo-primed $CD8^+$ T cells from IFN-$\gamma^{-/-}$ mice into RAG-$1^{-/-}$ recipients where the direct CTL effector pathways are blocked did not induce AV, thus demonstrating that the observed non-CTL effector pathway is IFN-γ dependent.

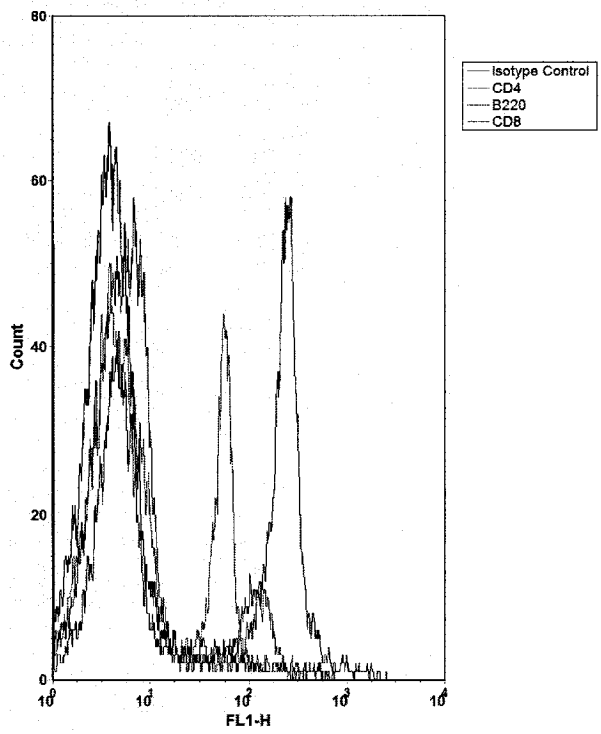
A role for $CD8^+$ effector T cells in AV may explain its resistance to calcineurin inhibitor based immunosuppressive treatments which primarily target $CD4^+$ T cell

activation (Bishop *et al.*, 1992, Bujan *et al.*, 2001, Hruban *et al.*, 1990, Libby and Pober, 2001). This correlates with our evidence (Johnson *et al.*, 2002, Légaré *et al*, 2001) and the evidence of others (Hillebrands *et al.*, 2001) that in animal models moderate calcineurin inhibition ablates $CD4^+$ T cell mediated acute rejection but has little to no effect on AV. Indeed, we have found that B6 $CD8^+$ T cells transferred into RAG-$1^{-/-}$ allograft recipients treated with cyclosporine A (50 mg/kg/d) developed AV, whereas similarly treated RAG-$1^{-/-}$ recipients reconstituted with $CD4^+$ T cells did not (Figure 23 and 24). This demonstrates a relationship between $CD8^+$ T cell effector function and AV in the context of effective $CD4^+$ T cell immunosuppression. Moreover, the activity of non-CTL effector $CD8^+$ T cells might be particularly implicated in the failure of modern transplant pharmacotherapy, since indirect pathways, in general, are thought to be most resistant to calcineurin inhibition (Lee *et al.*, 2001, Sawyer *et al.*, 1993, Hornick *et al.*, 2000).

Figure 1. ***Characterization of splenocyte populations prior to and following T cell enrichment.***

Splenocytes **(a)** isolated from allo-immunized C57BL/6 mice were subjected to nylon wool T cell enrichment **(b)**. Splenocytes and enriched T cells were then labeled with FITC-anti- CD4 (red), FITC-anti-B220 (blue), and FITC-anti- CD8 (purple) monoclonal antibodies and fluorescence activated cell sorting (FACS) analysis was performed to assess the purity of the isolated cell populations. Background fluorescence was determined using FITC-rat $IgG2_b$ (isotype control; black). These enriched T cells were subsequently subjected to either whole T cell or $CD8^+$ T cell isolation using either negative or positive selection techniques. Data are representative of 10 experiments.

a) Splenocytes



b) Enriched T cells

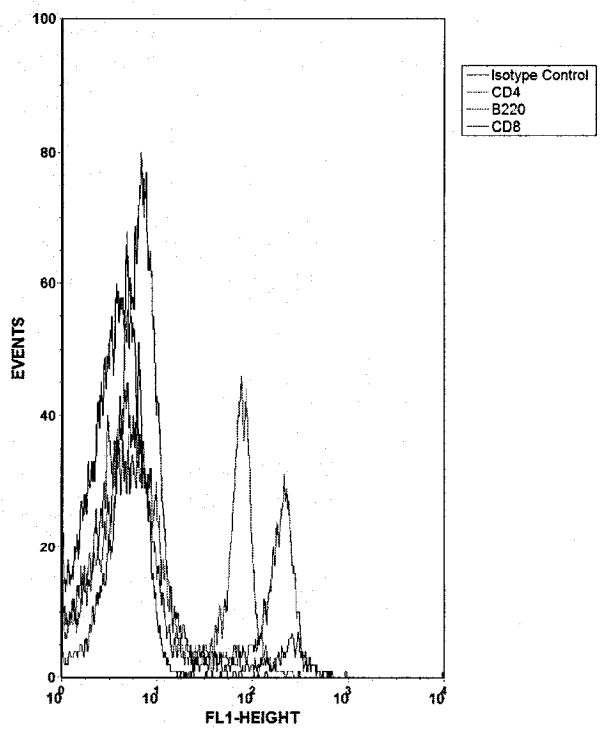
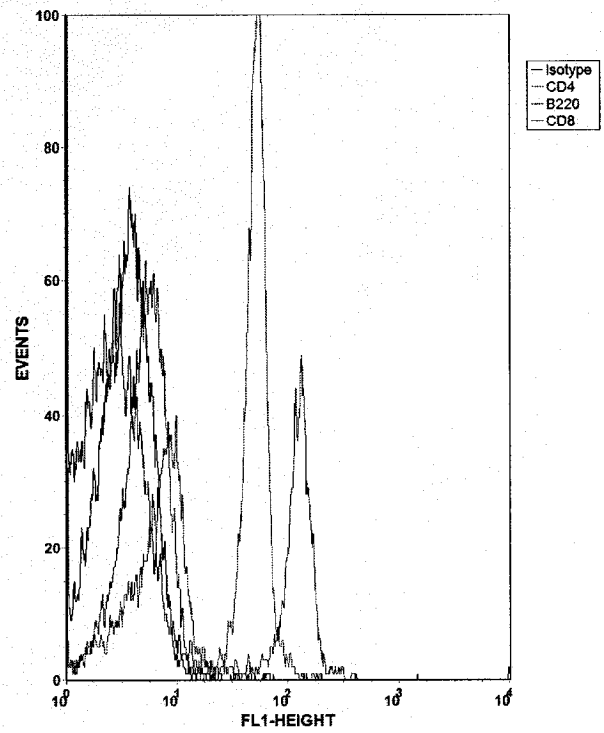


FIGURE 1

Figure 2. ***Purity of whole T cells and CD8$^+$ T cells using negative selection isolation technique.***

Spleen T cells from allo-immunized C57BL/6 mice were isolated by nylon wool enrichment. For whole T cell isolation by negative selection, enriched T cells were subsequently passed through CD3 immunocolumns to eliminate B cells and other non-T cells **(a)**. Similarly, CD8$^+$ T cells were isolated by negative selection from enriched T cell populations by passage through CD8 immunocolumns to eliminate CD4$^+$ T cells and B cells **(b)**. Whole T cells and CD8$^+$ T cells were then labelled with FITC-anti-CD4 (red), FITC-anti- B220 (blue), FITC-anti- CD8 (purple), and PE-anti- αβTCR (not shown) monoclonal antibodies and FACS analysis was performed to assess the purity of the isolated cell populations. Background fluorescence was determined by using FITC-rat $IgG2_b$ (isotype control; black). Whole T cell and CD8$^+$ T cells populations were subsequently utilized for *in vitro* assays and *in vivo* adoptive transfer experimentation. Data are representative of 10 experiments.

a) CD3 (Negative Selection)



b) CD8 (Negative Selection)

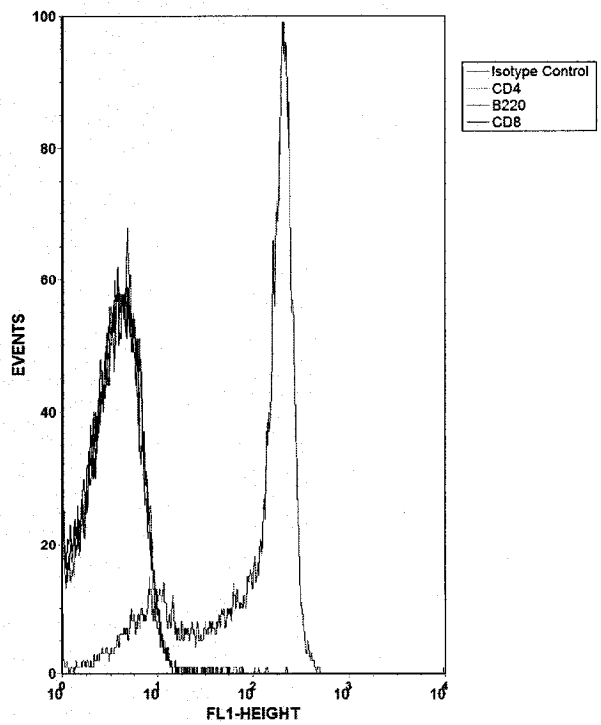


FIGURE 2

	CD4	B220	CD8	αβTCR
Splenocytes	28.3±1.5	51.0±3.2	13.4±2.1	—
Enriched T cells	49.6±4.6	6.4±2.4	36.7±4.5	—
T cells (- Selection)	56.6±3.8	0.5±0.2	36.0±3.7	93.4±2.4
$CD8^+$ T cells (- Selection)	0.6±0.2	0.6±0.2	93.6±2.6	91.2±1.5
T cells (+ Selection)	56.5±3.6	0.4±0.3	37.3±2.3	98.4±1.1
$CD8^+$ T cells (+ Selection)	0.5±0.2	0.4±0.1	99.0±0.9	97.6±1.3

TABLE 1. ***Purity of adoptive cell transfer populations.***

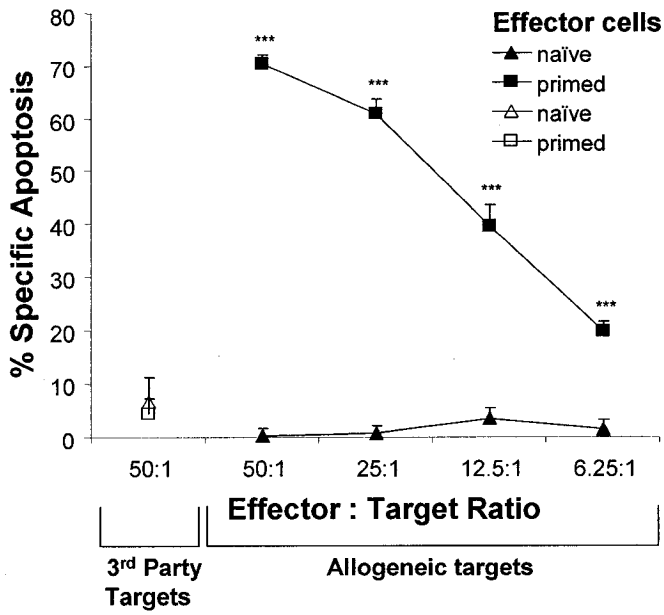
Splenocytes, nylon wool enriched T cells, whole T cells and purified $CD8^+$ T cells isolated by either positive or negative selection techniques were assessed for CD4, B220, CD8 and αβTCR expression by FACS analysis. Data shown are expressed as mean ± SEM of positively stained cells and are representative of n=5-10 experiments.

Figure 3. ***Generation of primed allo-specific CD8$^+$ CTL.***

(**a**) CD8$^+$ T cells, freshly purified from allo-primed animals were tested for killing activity in an *ex vivo* direct CTL assay. Allogeneic targets were incubated with naïve CD8$^+$ T cells (▲), or primed CD8$^+$ T cells (■). Third party targets were also used (Δ, □).

(**b**) Whole T cells from naïve (▲), or primed (■) animals were tested for CTL activity against allogeneic targets. Primed whole T cells treated with anti-CD8 (Δ) or anti-asialoGM1 (□) monoclonal antibodies and complement were also tested. Data shown are expressed as mean ± SEM and are representative of seven separate experiments (*** $p<0.001$, ANOVA).

a) CD8+ T cells



b) Whole T cells

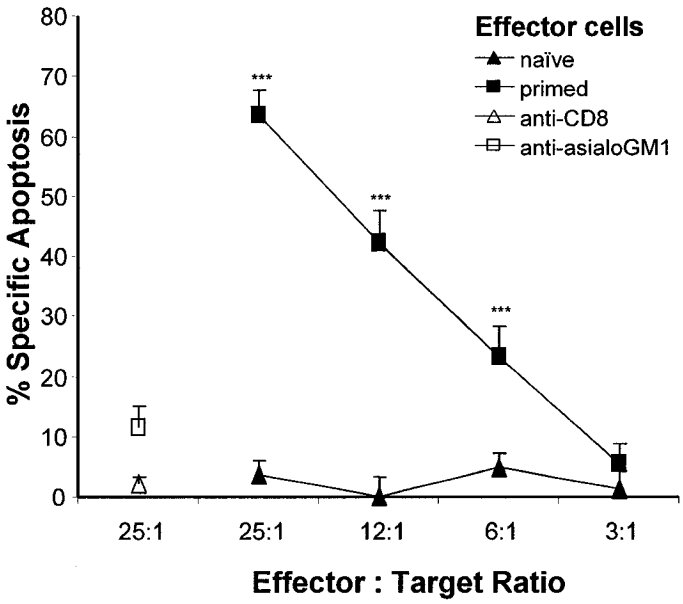
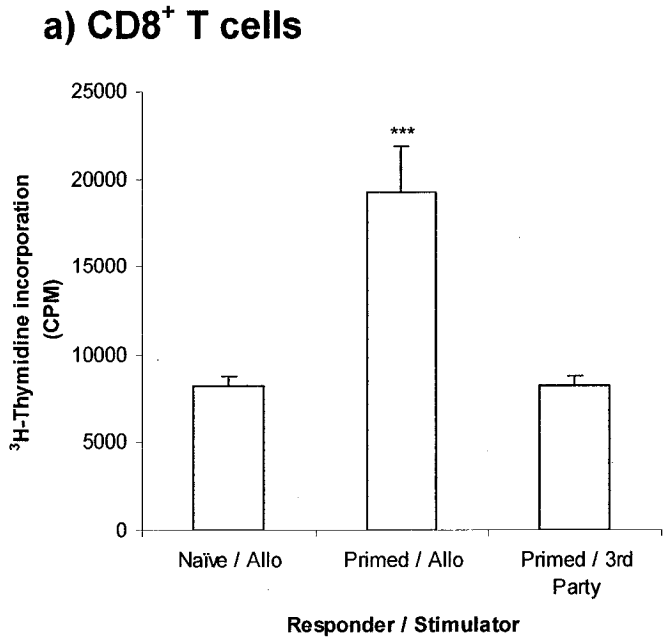


FIGURE 3

Figure 4. ***Allo-immunization leads to generation of effector CD8$^+$ T cells with proliferative capacity.***

Allo-specific proliferation in a secondary mixed lymphocyte reaction (MLR) of primed CD8$^+$ T cells (**a**) and whole T cells (**b**) was assessed by ^{3}H-Thymidine incorporation. CD8$^+$ T cells (**a**) and whole T cells (**b**) from unimmunized (naïve) C57BL/6 mice served as a primary MLR control. Purified CD8$^+$ T cells and whole T cells were activated in the presence of mitomycin C-treated, T cell-depleted, BALB/c (allogeneic) or C3H (third party) stimulator splenocytes, at a responder to stimulator ratio of 4:1. Data shown are expressed as mean ± SEM of 3 experiments (*** $p<0.001$, ANOVA).


a) CD8+ T cells
25000
20000
15000
10000
5000
0
3H-Thymidine incorporation (CPM)
***
Naïve / Allo
Primed / Allo
Primed / 3rd Party
Responder / Stimulator

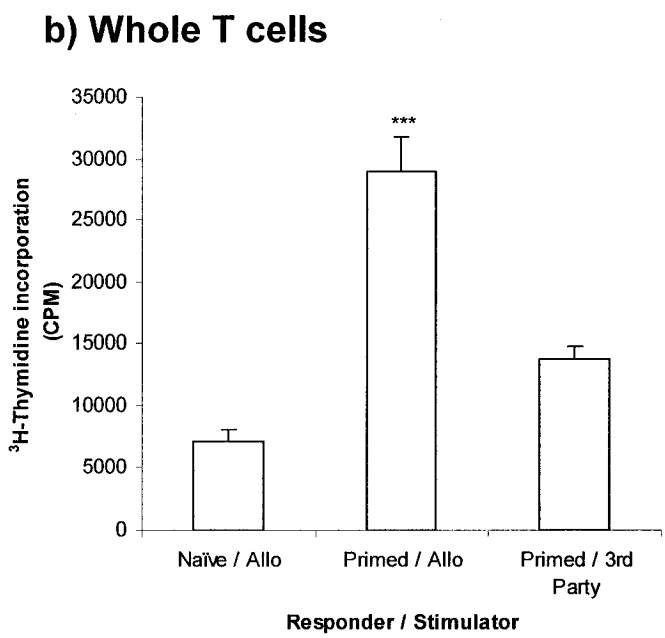

b) Whole T cells
35000
30000
25000
20000
15000
10000
5000
0
3H-Thymidine incorporation (CPM)
***
Naïve / Allo
Primed / Allo
Primed / 3rd Party
Responder / Stimulator

FIGURE 4

Figure 5. ***Reconstitution of RAG-1$^{-/-}$ mice with syngeneic primed allo-reactive cells.*** Two weeks after reconstitution, splenocytes from RAG-1$^{-/-}$ mice were probed with anti-CD4 (blue), anti-CD8 (red), and isotype control (black) monoclonal antibodies to confirm survival of transferred cells. Unreconstituted RAG-1$^{-/-}$ mice (**a**); whole T cell reconstituted mice (**b**); and CD8^{+} T cell reconstituted mice (**c**) were assayed. Experiment shown is representative of three separate experiments.

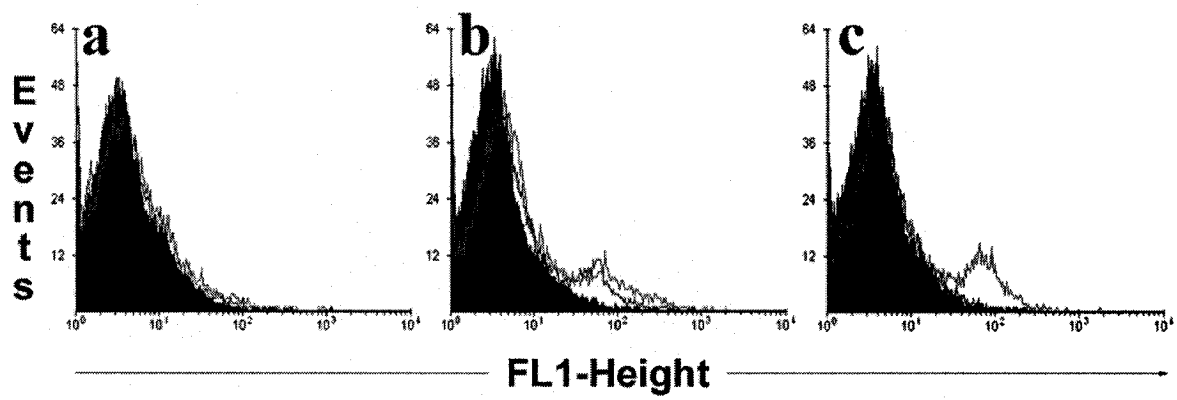


FIGURE 5

Figure 6. ***Adoptive transfer of whole T cells or CD8$^+$ T cells did not reconstitute the B cell compartment of immunodeficient mice.***

Two weeks after reconstitution, 5 μm frozen sections of spleen tissue were probed with anti-CD19 monoclonal antibody immunocytochemistry to detect the presence of B cells. Spleen tissue from wildtype C57BL/6 **(a)**; from whole T cell injected RAG-1$^{-/-}$ **(b)**; and from CD8$^+$ T cell injected RAG-1$^{-/-}$ **(c)** mice was immunostained. Photomicrographs are representative of n=3 per group; scale bars = 200 μm.

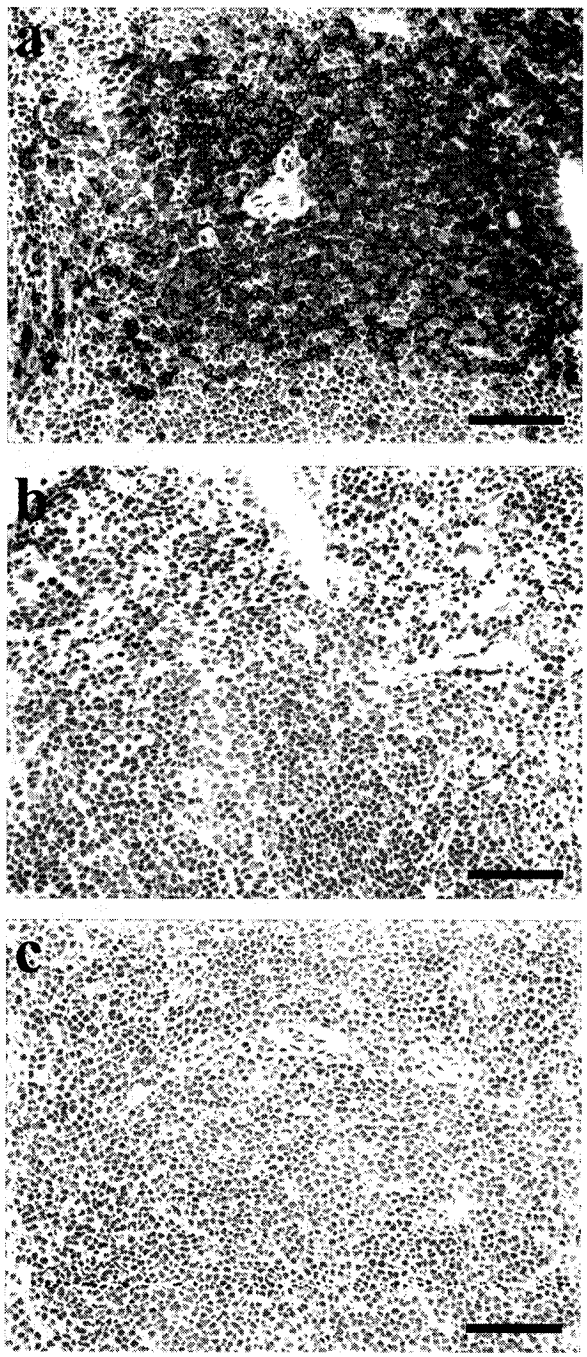


FIGURE 6

Figure 7. ***Primed CD8$^+$ T lymphocytes induce AV.***

Representative photomicrographs of aortic allografts from an unreconstituted RAG-1$^{-/-}$ mouse **(a)**; from a RAG-1$^{-/-}$ mouse reconstituted with allo-primed whole T cells **(b)**; or allo-primed, purified CD8$^+$ T cells **(c)**; or allo-primed null cells (non-CD8$^+$) **(d)**; n=5-9 animals per group; scale bars = 200 μm.

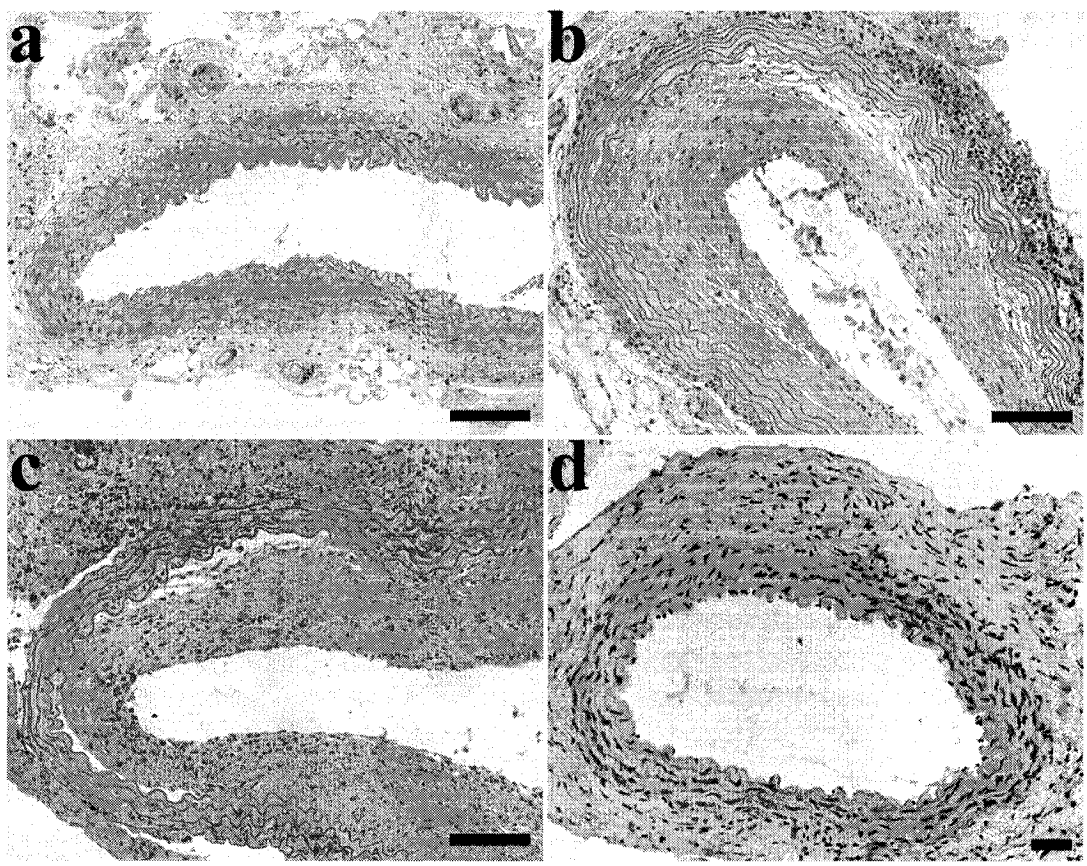


FIGURE 7

Figure 8. ***$CD8^+$ T cell-mediated intimal hyperplasia is similar in extent to AV observed in wildtype recipients.***

Digital image analysis of histological sections from aortic allografts shows intimal area calculations for the five groups: wildtype recipients, unreconstituted RAG-$1^{-/-}$ recipients, primed whole T cell reconstituted RAG-$1^{-/-}$ recipients, primed $CD8^+$ T cell reconstituted recipients, and primed non-$CD8^+$ cell reconstituted RAG-$1^{-/-}$ recipients; n=5-9 for the groups (***, $p<0.001$, ANOVA).

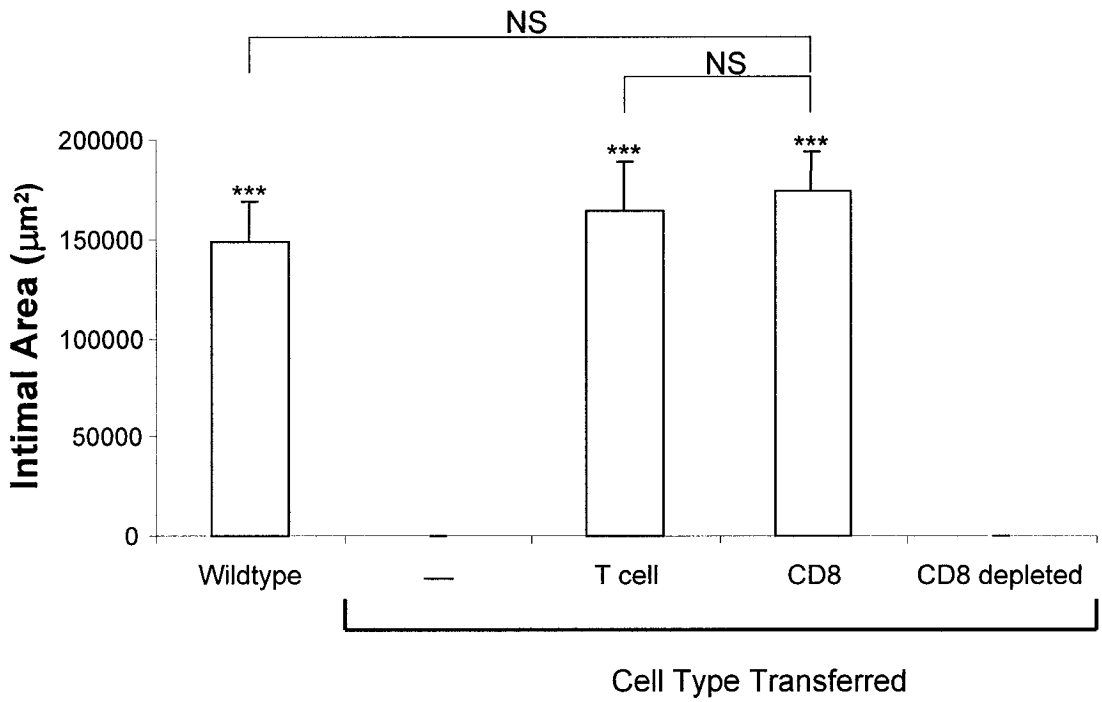
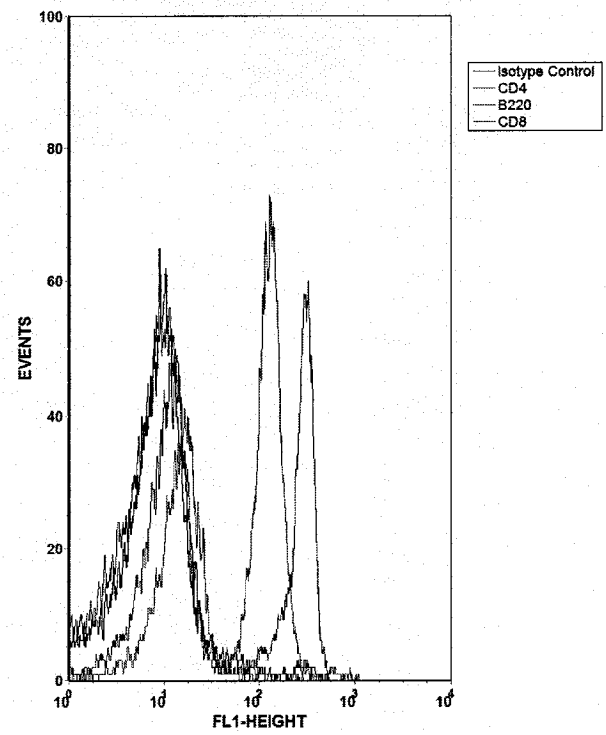

NS
NS
***
***
***
200000
150000
100000
50000
0
Intimal Area (μm²)
Wildtype
—
T cell
CD8
CD8 depleted
Cell Type Transferred

FIGURE 8

Figure 9. ***Purity of whole T cells and CD8$^+$ T cells using positive selection isolation technique.***

Spleen T cells from allo-immunized C57BL/6 mice were isolated by nylon wool enrichment. Whole T cells were subsequently positively selected from enriched T cell populations using anti-CD90 (Thy 1.2) microbeads followed by magnetic column separation **(a)**. Similarly, CD8$^+$ T cells were positively selected from enriched T cell populations using anti-CD8 microbeads followed by magnetic column separation **(b)**. Purified whole T cells and CD8$^+$ T cells were then labelled with FITC-anti- CD4 (red), FITC-anti- B220 (blue), FITC-anti- CD8 (purple), and PE-anti- αβTCR (not shown) monoclonal antibodies and FACS analysis was performed to assess the purity of the isolated cell populations. Background fluorescence was determined by using FITC-rat $IgG2_b$ (isotype control; black). Whole T cell and CD8$^+$ T cells populations were subsequently utilized for *in vitro* assays and *in vivo* adoptive transfer experimentation. Data are representative of 10 experiments.

a) CD90/Thy 1.2 (Positive Selection)



b) CD8 (Positive Selection)

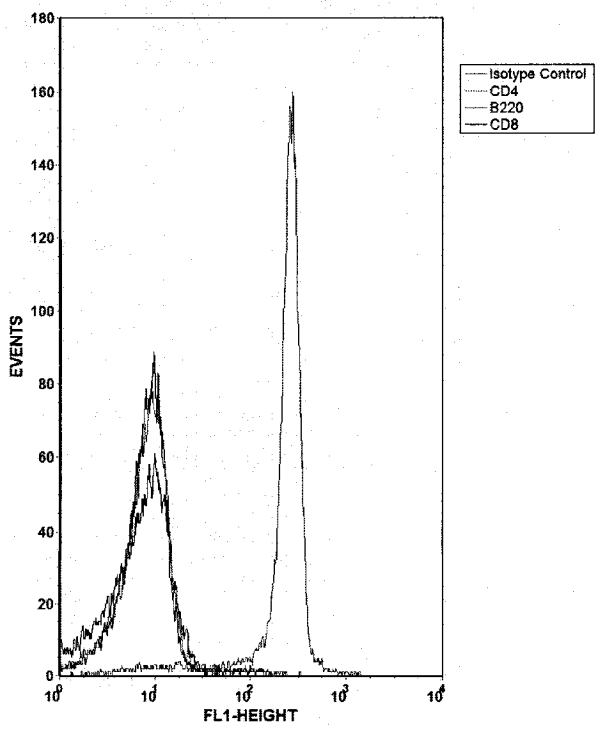


FIGURE 9

Figure 10. ***Highly purified CD8$^+$ T cells isolated by positive selection induce AV.*** CD8$^+$ T cells isolated to >99% purity by positive selection were assessed for their ability to induce AV in aortic allografts transplanted into RAG-1$^{-/-}$ recipients. Representative photomicrographs of aortic allografts from RAG-1$^{-/-}$ mice reconstituted with allo-primed, highly purified whole T cells (**a**); or allo-primed, highly purified CD8$^+$ T cells (**b**); n=6 per group; scale bars = 200 μm.

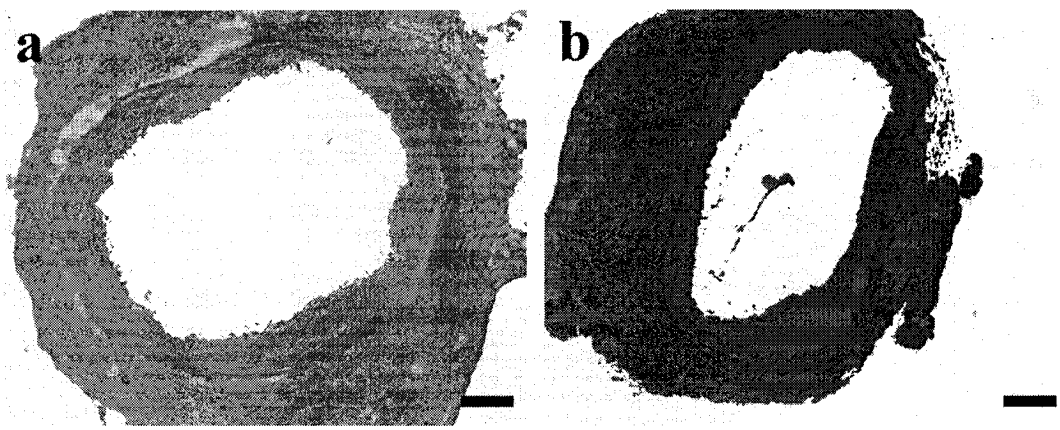


FIGURE 10

Figure 11. ***Intimal hyperplasia mediated by highly purified CD8$^+$ T cells is similar in extent to AV observed in whole T cell reconstituted immunodeficient recipients.***

Digital image analysis of histological sections from aortic allografts shows intimal area calculations for the two groups: allo-primed, highly purified whole T cell reconstituted RAG-1$^{-/-}$ recipients, and allo-primed, highly purified CD8$^+$ T cell reconstituted RAG-1$^{-/-}$ recipients; n=6 for the groups.

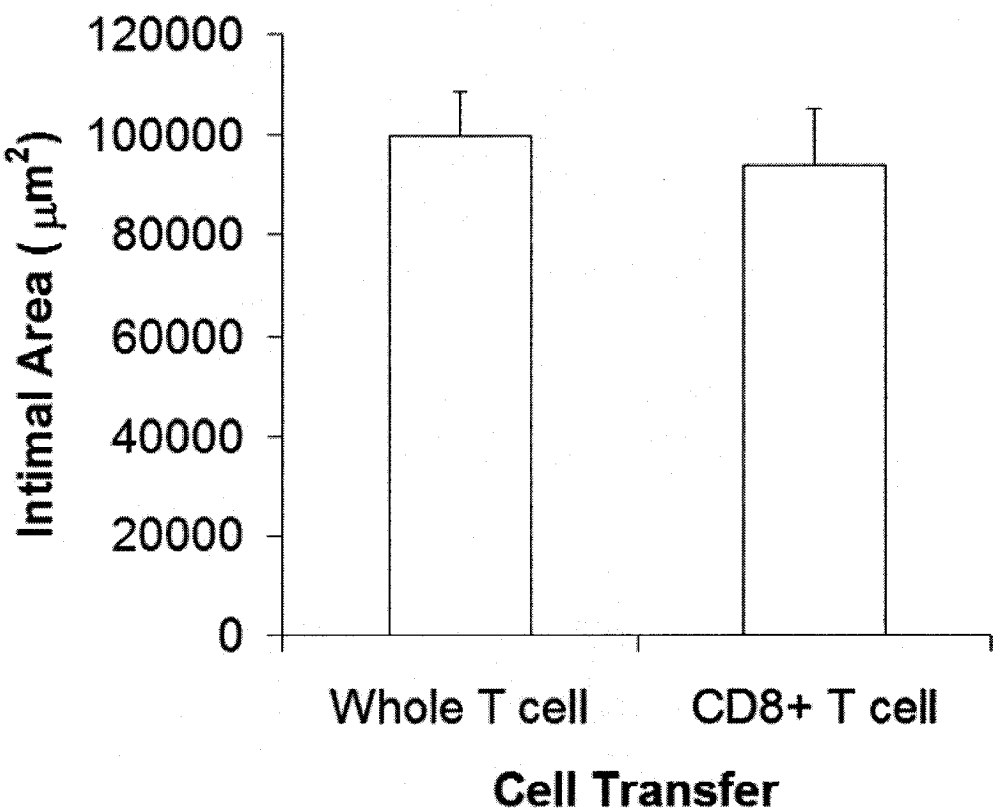

120000
100000
80000
60000
40000
20000
0
Intimal Area (μm²)
Whole T cell
CD8+ T cell
Cell Transfer

FIGURE 11

Figure 12. ***AV in CD8$^+$ T cell reconstituted RAG-1$^{-/-}$ mice occurs irrespective of concurrent ischemic injury.***

To control for concurrent ischemic graft injury, RAG-1$^{-/-}$ allograft recipients were reconstituted with primed, purified CD8$^+$ T cells 24, 96, and 168 h after transplantation. Digital image analysis of histological sections from aortic allografts shows intimal area calculations for the three groups; n=4-9 for the groups.

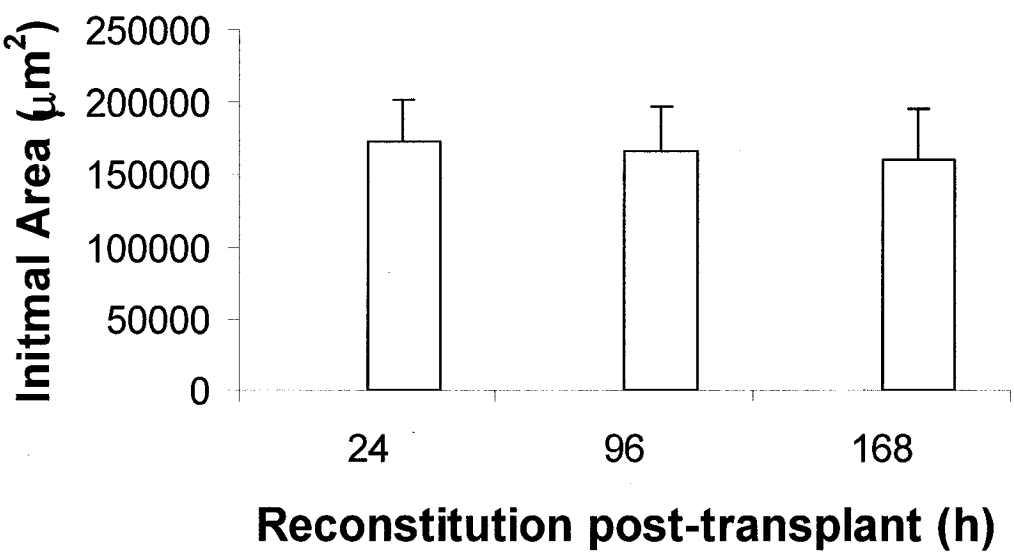

Initmal Area (μm²)
250000
200000
150000
100000
50000
0
24
96
168
Reconstitution post-transplant (h)

FIGURE 12

Figure 13. ***Graft infiltration by lymphocyte subsets after reconstitution of RAG-1$^{-/-}$ recipients.***

Panels **a**, **c** and **e** are probed with anti-CD4. Tissue represents allografts from unreconstituted RAG-1$^{-/-}$ mice (**a**); RAG-1$^{-/-}$ mice reconstituted with primed whole T cells (**c**); or RAG-1$^{-/-}$ mice reconstituted with primed CD8^{+} T cells (**e**). Panels **b**, **d** and **f** are probed with anti-CD8 and represent similar reconstitution as noted for **a**, **c** and **e** above. Micrographs representative of n=5-9 per group; scale bars = 200 μm.

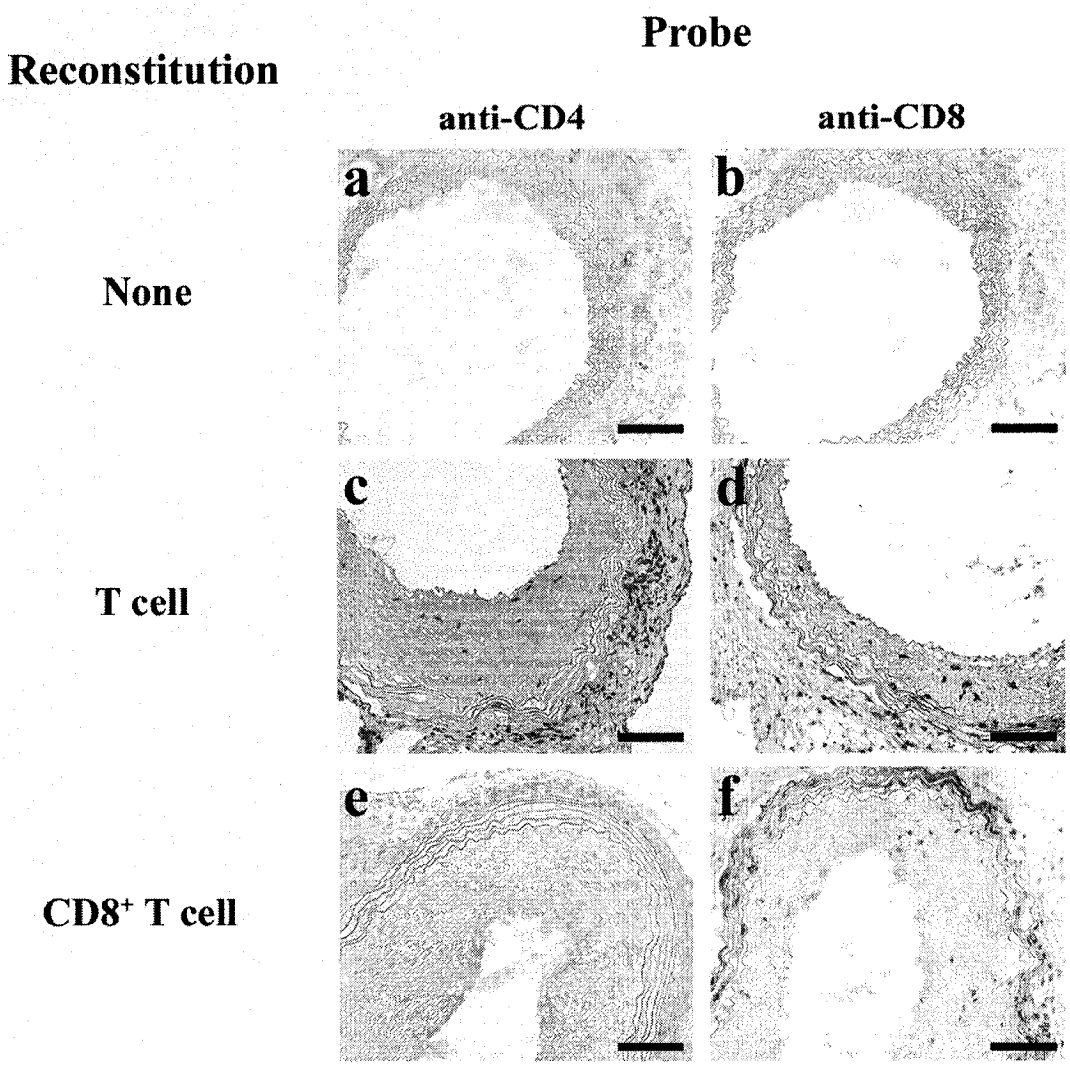

Probe
Reconstitution
anti-CD4
anti-CD8
None
T cell
CD8+ T cell
a
b
c
d
e
f

FIGURE 13

Figure 14. ***CD8$^+$ T cell reconstituted RAG-1$^{-/-}$ mice do not produce allo-specific IgG.*** Serum from CD8$^+$ T cell reconstituted RAG-1$^{-/-}$ (red) and wildtype (purple; antiserum) recipients with rejected allografts was incubated with allogeneic T cells. No serum incubated with allogeneic T cells (media; black) and antiserum incubated with syngeneic T cells (blue) served as negative controls. These allogeneic and syngeneic T cells were subsequently labeled with FITC-anti- mouse IgG and FACS analysis was performed to detect the presence of allo-specific IgG.

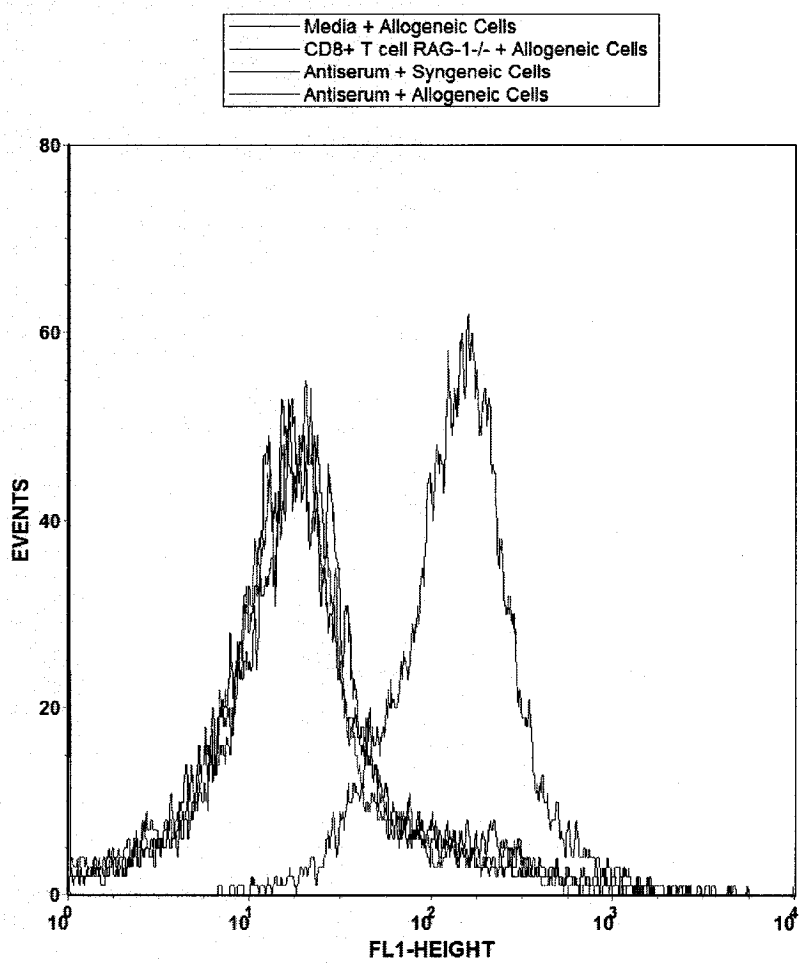

Media + Allogeneic Cells
CD8+ T cell RAG-1-/- + Allogeneic Cells
Antiserum + Syngeneic Cells
Antiserum + Allogeneic Cells
80
60
40
20
0
EVENTS
10^0
10^1
10^2
10^3
10^4
FL1-HEIGHT

Figure 14

Figure 15. ***$CD8^+$ T cell reconstituted RAG-$1^{-/-}$ mice do not produce antibodies capable of complement-dependent cytotoxicity.***

To confirm that the AV observed in $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ mice was independent of cytotoxic antibody, serial dilutions of serum collected from these animals was incubated with allogeneic splenocytes and complement. Dead cells were stained with trypan blue and quantified by cell counting using a hemocytometer. Antiserum from wildtype recipients and naïve mice left untransplanted served as positive and negative controls respectively. Data are shown as mean ± SEM of three separate experiments.

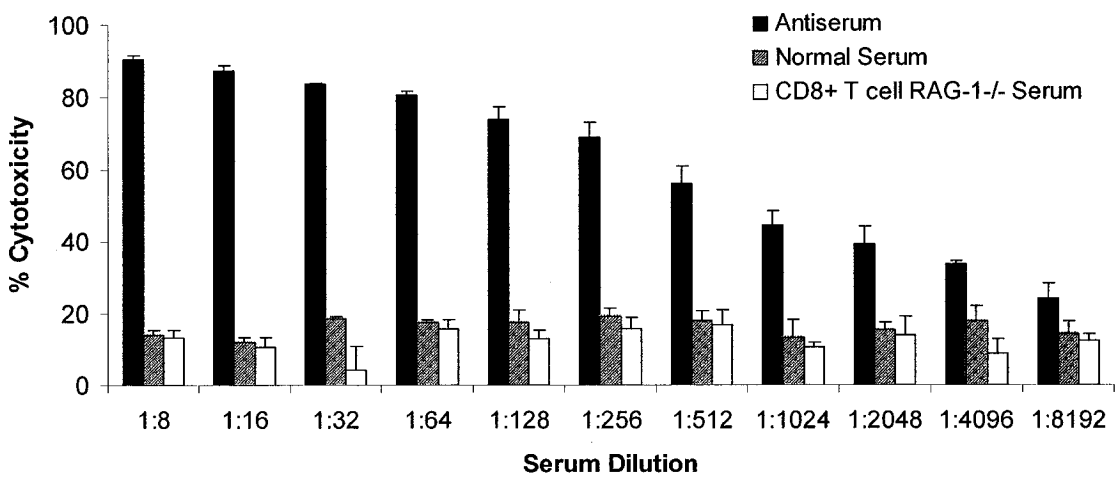

Antiserum
Normal Serum
CD8+ T cell RAG-1-/- Serum
% Cytotoxicity
100
80
60
40
20
0
1:8
1:16
1:32
1:64
1:128
1:256
1:512
1:1024
1:2048
1:4096
1:8192
Serum Dilution

FIGURE 15

Figure 16. ***Ablation of CTL activity by interruption of cytotoxic mediators.***

Wildtype allogeneic lymphoblast targets were incubated in an *in vitro* CTL assay with wildtype CTL (■), or with CTL from perforin-deficient mice (□). Fas-deficient targets were incubated with wildtype CTL (●), or CTL from perforin-deficient mice (○). Representative of four separate experiments (*** $p<0.001$, ANOVA). Non-specific lysis was <5%.

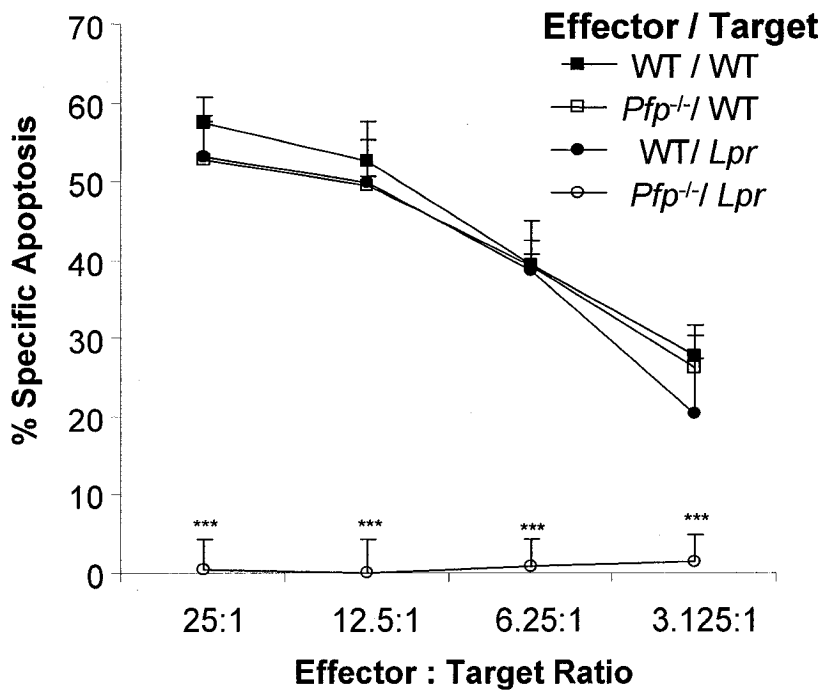

Effector / Target
WT / WT
Pfp-/-/ WT
WT/ Lpr
Pfp-/-/ Lpr
% Specific Apoptosis
70
60
50
40
30
20
10
0
***
***
***
***
25:1
12.5:1
6.25:1
3.125:1
Effector : Target Ratio

FIGURE 16

Figure 17. ***Contribution of CD8$^+$ T cell-mediated direct cytolysis to AV.***

Wildtype allografts were transplanted into RAG-1$^{-/-}$ mice which received primed CD8$^+$ T cells from either wildtype mice (**a**), FasL$^{-/-}$ mice (**b**), or from perforin-deficient mice (**c**). These combinations block either the Fas-FasL pathway (**b**) or the granule-exocytosis pathway (**c**) of killing. Also examined were allografts from Fas$^{-/-}$ mice transplanted into RAG-1$^{-/-}$ mice reconstituted with primed CD8$^+$ T cells from perforin-deficient mice (**d**) (interrupts both major pathways of cytotoxicity). Micrographs are representative of n=5-6 in all groups; scale bars = 200 μm.

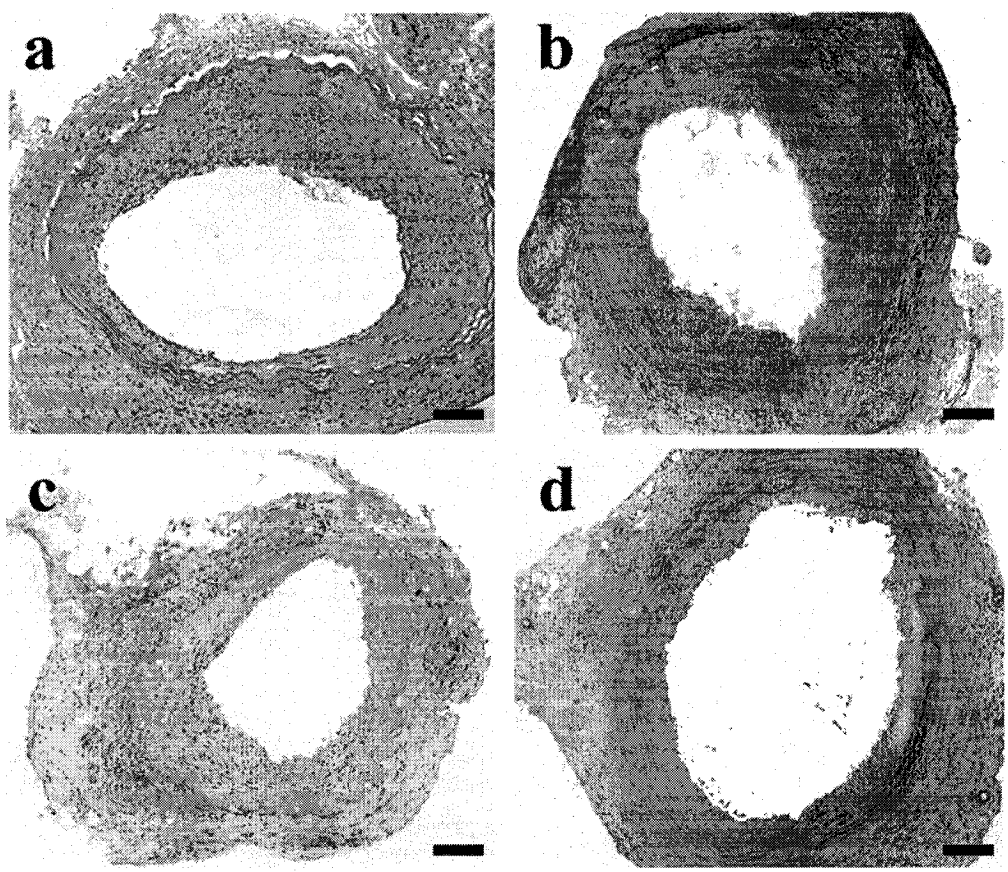


FIGURE 17

Figure 18. ***Ablation of mediators of direct cytotoxicity attenuates AV.***

Digital image analysis of sections yielded quantitative data of the extent of intimal hyperplasia in the five groups: unreconstituted RAG-$1^{-/-}$ recipients of a wildtype allograft; primed wildtype $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ recipients of a wildtype allograft; primed FasL-deficient $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ recipients of a wildtype allograft; primed perforin-deficient $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ recipients of a wildtype allograft; and primed perforin-deficient $CD8^+$ T cell reconstituted RAG-$1^{-/-}$ recipients of a Fas-deficient allograft; n=5-6 in all groups (**, $p<0.01$, ANOVA).

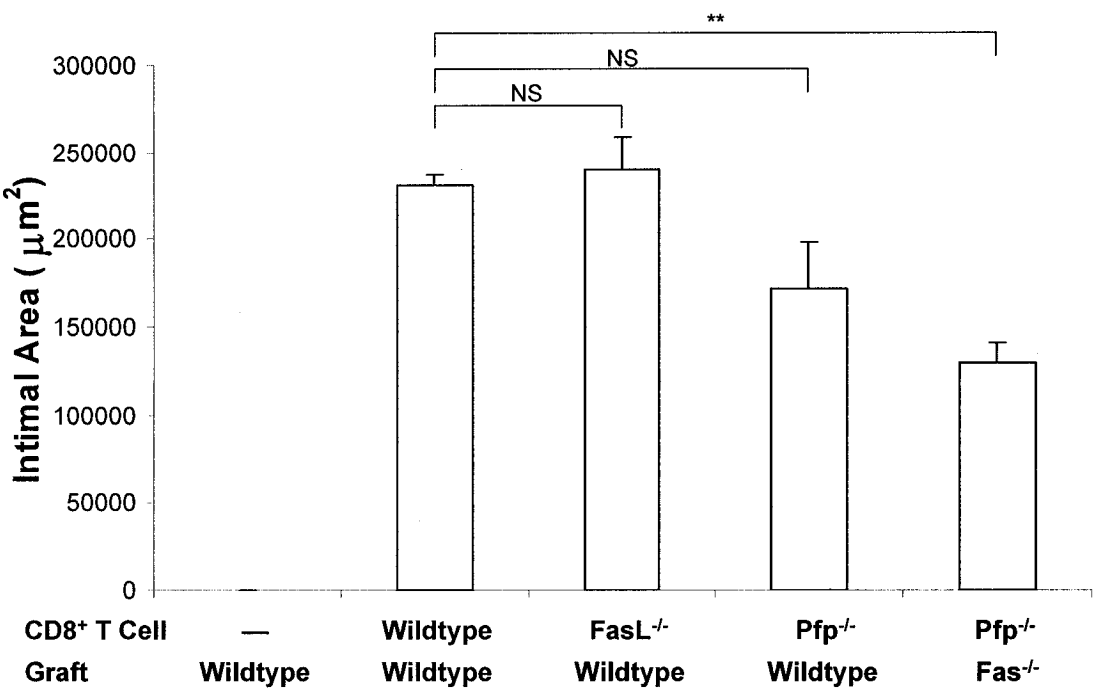

**
NS
NS
300000
250000
200000
150000
100000
50000
0
Intimal Area (μm²)
CD8⁺ T Cell
Graft
—
Wildtype
Wildtype
Wildtype
FasL-/-
Wildtype
Pfp-/-
Wildtype
Pfp-/-
Fas-/-

FIGURE 18

Figure 19. ***Ablation of CTL activity by interruption of target derived MHC Class I.***

Wildtype CTL were incubated with wildtype (■) and β2microglobulin-deficient (○; MHC-$I^{-/-}$) allogeneic targets in an *in vitro* CTL assay. Third party control targets were also tested and no killing was observed. Representative of 3 separate experiments (*** $p<0.001$, ANOVA). Non-specific lysis was <5%.

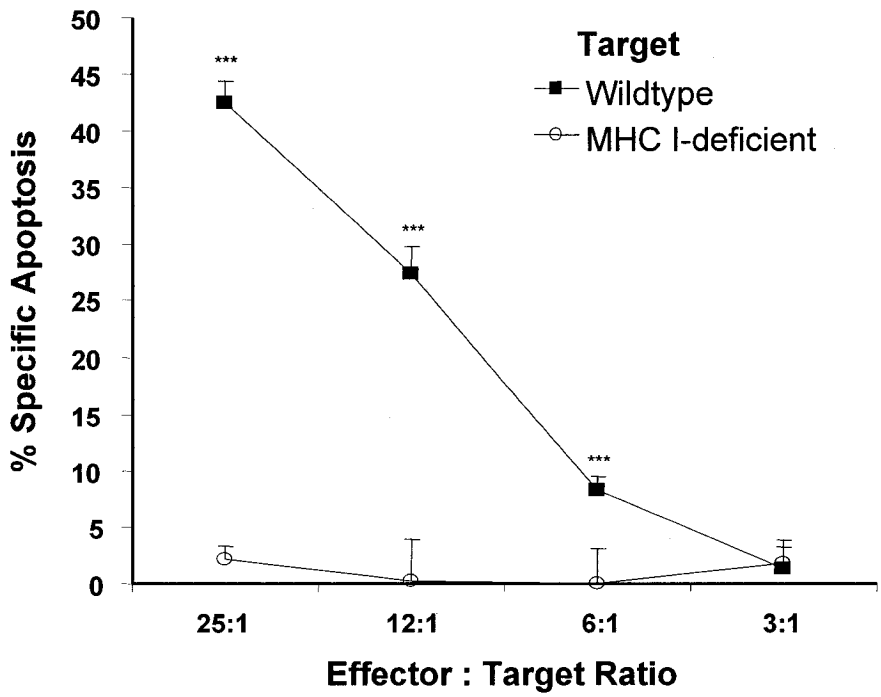

50
45
40
35
30
25
20
15
10
5
0
% Specific Apoptosis
***
***
***
Target
Wildtype
MHC I-deficient
25:1
12:1
6:1
3:1
Effector : Target Ratio

FIGURE 19

Figure 20. ***Contribution of indirect effectors to CD8$^+$ T cell-mediated AV.***

Representative photomicrographs of aortic allografts from wildtype B6 **(a)**, or B6 β2m$^{-/-}$ **(b)** mice were transplanted into C3H RAG-2$^{-/-}$ recipients receiving primed, purified CD8$^+$ T cells from wildtype C3H mice. C3H RAG-2$^{-/-}$ recipient of B6 β2m$^{-/-}$ allograft left unreconstituted **(c)**. AV in β2m$^{-/-}$ grafts is due to indirect CD8$^+$ T cell mediated effects. Scale bars = 200 μm.

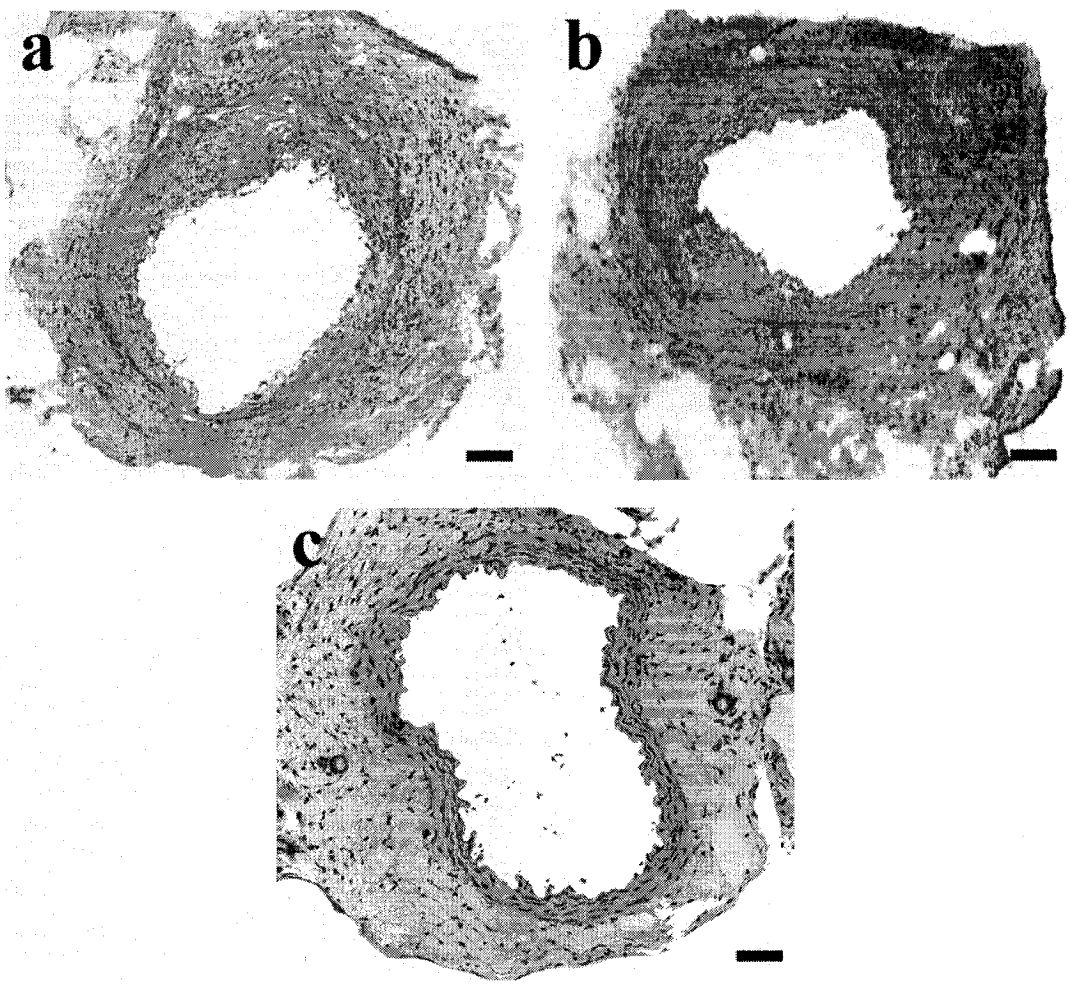


FIGURE 20

Figure 21. ***Direct CTL activity and a non-CTL indirect effector pathway are involved in CD8$^+$ T cell-mediated AV.***

Allografts from wildtype B6 or B6 β2m$^{-/-}$ mice were transplanted into C3H RAG-2$^{-/-}$ recipients receiving primed, purified CD8$^+$ T cells from wildtype C3H mice. Digital image analysis of histologic sections quantified the intimal lesion area in these groups; n=4 for the groups (*, p=0.0406, Unpaired T-test).

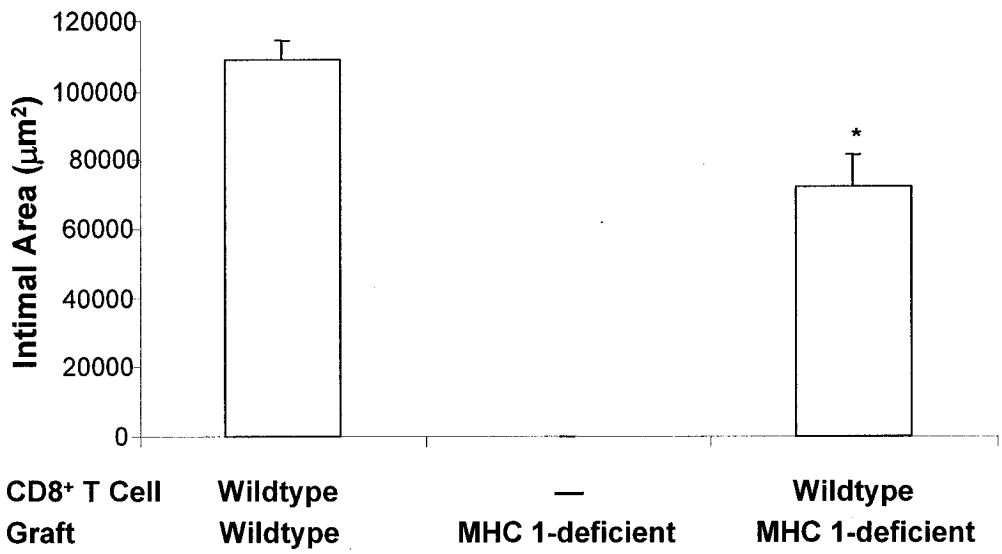

120000
100000
80000
60000
40000
20000
0
Intimal Area (μm²)
*
CD8+ T Cell
Graft
Wildtype
Wildtype
—
MHC 1-deficient
Wildtype
MHC 1-deficient

FIGURE 21

Figure 22. ***AV mediated by indirect CD8$^+$ T effector cells is interferon-γ dependent.*** Representative photomicrographs of allografts from wildtype MRL mice transplanted into B6 RAG-1$^{-/-}$ mice reconstituted with CD8$^+$ T cells from interferon-γ$^{-/-}$ mice (**a**); allografts from MRL β2m$^{-/-}$ mice transplanted into B6 RAG-1$^{-/-}$ mice reconstituted with primed, purified CD8$^+$ T cells from wildtype B6 mice (**b**); or reconstituted with primed, purified CD8$^+$ T cells from B6 interferon-γ$^{-/-}$ mice (**c**) and probed with anti-CD8 immunocytochemistry (**d**); n=5 for the groups; scale bars = 100 μm.

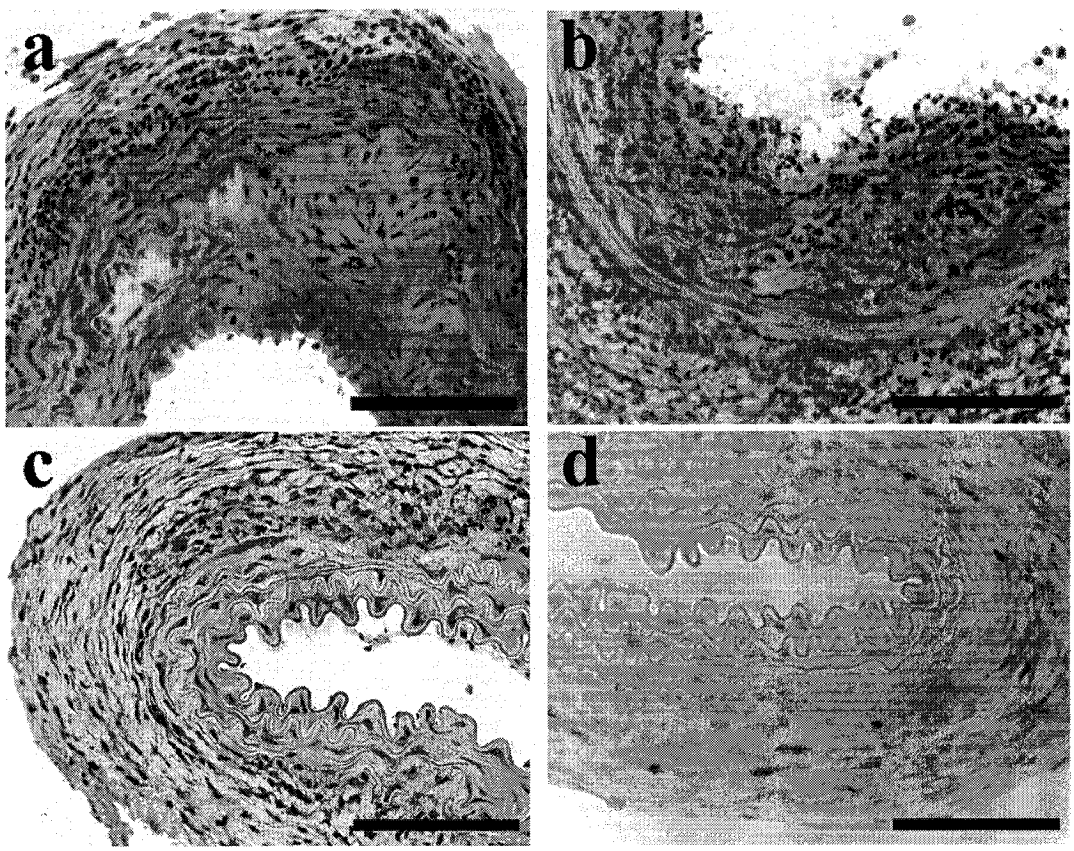


FIGURE 22

Figure 23. ***CD8$^+$ T cell-mediated AV is relatively resistant to cyclosporine A.***
Representative photomicrographs of wildtype C3H aortic allografts from B6 RAG-1$^{-/-}$ recipients reconstituted with primed, highly purified CD4$^+$ T cells (**a** and **c**) or primed, highly purified CD8$^+$ T cells (**b** and **d**) left untreated (**a** and **b**) or treated with cyclosporine A 50 mg/kg/d (**c** and **d**). Treatment with cyclosporine A ablates CD4$^+$ T cell mediated AV, whereas CD8$^+$ T cells are resistant; n=4-5 animals per group; scale bars = 200 μm.

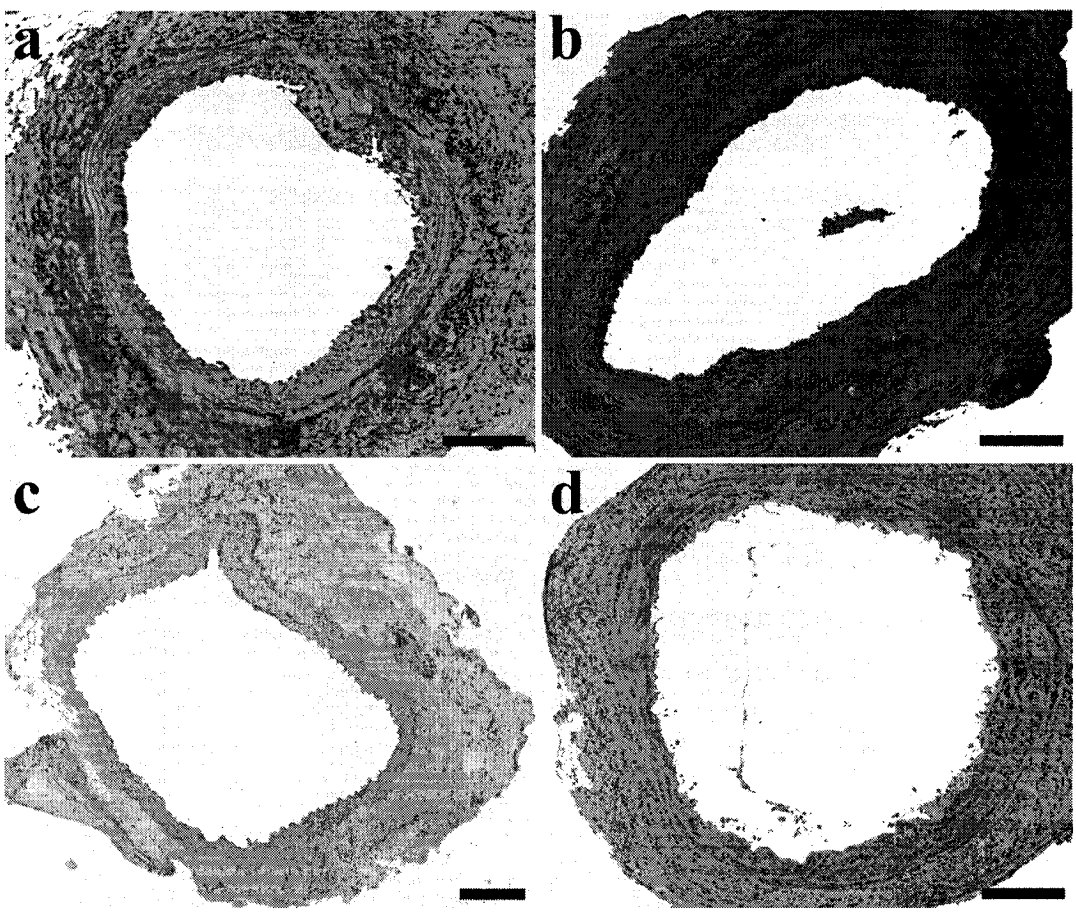


FIGURE 23

Figure 24. ***Differential sensitivity of CD4$^+$ and CD8$^+$ T cell subsets to cyclosporine A.*** Intimal area ratios of untreated to cyclosporine A-treated C57BL/6 RAG-1$^{-/-}$ allograft recipients reconstituted with primed, highly purified CD4$^+$ T cells or primed, highly purified CD8$^+$ T cells. n=4-5 for the groups (*, p=0.0308, Unpaired T-test).

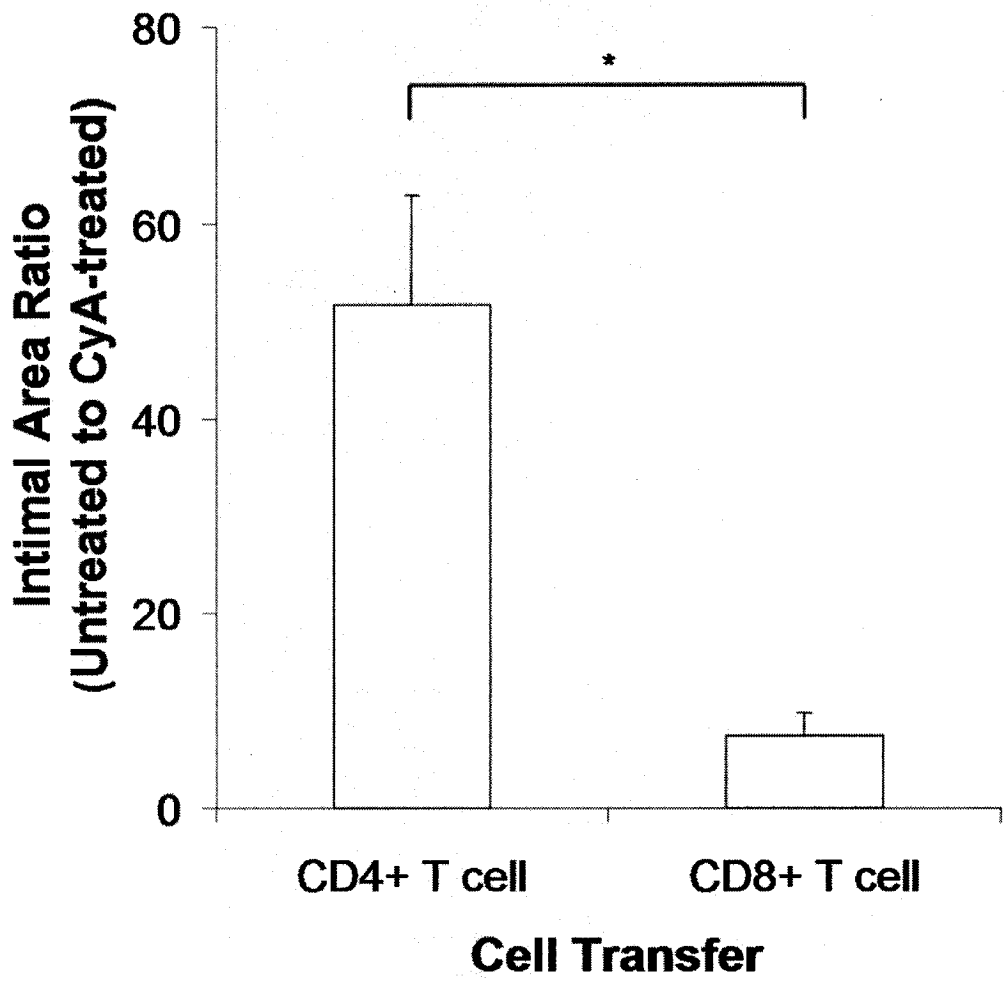

80
60
40
20
0
*
Intimal Area Ratio
(Untreated to CyA-treated)
CD4+ T cell
CD8+ T cell
Cell Transfer

FIGURE 24

Chapter 6

6.0 Discussion

In this thesis, I have demonstrated that $CD8^+$ T cells play a significant role in the development of AV. In fully MHC-mismatched murine aortic interposition allografts, $CD8^+$ T cells generated medial smooth muscle cell loss and intimal hyperplasia by contact-dependent cytotoxicity and by an interferon-γ-dependent indirect effector pathway. Moreover, in the presence of clinically-relevant immunosuppression with cyclosporine A, the effector activity of $CD8^+$ T cells was demonstrated to be absolutely essential to the generation AV. This strongly suggests that calcineurin inhibitor-resistant $CD8^+$ T cells may be implicated in the failure of modern transplant pharmacotherapy.

The murine aortic allograft model pioneered by Pekka Häyry and colleagues (Mennander *et al*, 1991) has been used by many investigators over the years, but recently it has come under criticism because some feel it does not adequately represent the pathophysiology of AV occurring within whole organ transplants (Pober, 2002). The aortic interposition graft lacks the parenchyma of a heart allograft and as such does not require immunosuppression in order to prolong graft survival sufficiently to form AV. In addition, it has been argued that intimal expansion in vessel allografts can result from any form of injury, including those better described as acute or subacute rather than chronic (Pober, 2002).

Contrary to this proposition, I have demonstrated in this thesis that AV still occurs in the presence of optimal immunosuppression capable of prolonging cardiac allograft survival greater than 60 d. Furthermore, others (Hillebrands *et al*, 2001, Légaré *et* al, 2001, Johnson *et al*, 2002) have found that immunosuppression using cyclosporine A,

which ameliorates acute rejection in this model, does not affect the generation of AV. In fact, data from both immunosuppressed (Hillebrands *et al*, 2001, Johnson *et al*, 2002) and non-immunosuppressed (Shimizu *et al*, 2001, Johnson *et al*, 2002) animal models which included aortic allografts (Shimizu *et al*, 2001, Hillebrands *et al*, 2001, Johnson *et al*, 2002) and whole organ transplants (Shimizu *et al*, 2001, Hillebrands *et al*, 2001, Saiura *et al*, 2001) have conclusively demonstrated a similar origin of the neointimal lesion cells (Skaro *et al*, 2002). Further, AV only occurs in the presence of robust allo-immunity and not in unreconstituted immunodeficient $RAG^{-/-}$ mice, which is consistent with data from cardiac allografts (Delfs *et al*, 2001). Moreover, it has been shown that the degree of intimal hyperplasia is highly concordant between aortic allografts and whole organ transplants in a combined heterotopic heart and aortic allograft model (Ensminger *et al*, 2000). A similar correlation was found in allograft aortas and coronary arteries of human transplants undergoing AV (Mehra *et al*, 1997). Taken together, these data support the position that the etiology of the lesion is similar in solid organ and aortic allografts.

To date, AV research has focused on allo-recognition and destruction of graft endothelium. This was thought to initiate leukocyte infiltration, production of cytokines, chemokines and growth factors (Häyry *et al*, 1993, Libby and Pober, 2001) and subsequent vascular SMC de-differentiation and migration to form an occlusive neointima (Häyry *et al*, 1993, Häyry *et al*, 1995, Bojakowski *et al*, 2000). Loss of SMC from the media is undoubtedly immune mediated since it does not occur in syngeneic transplants or immune-incompetent animals (Koulack *et al*, 1995, Koulack *et al*, 1996, Shi *et al*, 1996, Russell *et al*, 1997), but what remains unanswered is the mechanism by which it occurs and its pathological significance.

Few studies have addressed allo-immune mediated smooth muscle cell injury in the de-population of the vascular media. For example, we have previously demonstrated that the loss of SMC in the media of grafts undergoing AV is due to apoptosis, and not migration (Hirsch *et al*, 1998). Others (Koglin and Russell, 1999) have also demonstrated apoptosis in AV. Upon close examination of human pathologic specimens from hearts explanted due to severe AV, striking thinning and loss of medial smooth muscle cells is apparent.

In an aortic interposition graft model, the degree of medial SMC loss and intimal hyperplasia were directly proportional to the duration of allogeneic exposure (Mennander and Hayry, 1996, Hare *et al*, 1999), suggesting that medial destruction occurs by allo-immune mechanisms and may be linked to the development of intimal hyperplasia. Despite the suggestion that medial damage and intimal proliferation are differentially regulated (Mennander *et al*, 1993), other provocative evidence from arterial injury models has long suggested otherwise (Reidy and Silver, 1985, Reidy, 1985).

In arterial injury models, robust intimal hyperplasia is dependent on and proportional to the extent of medial SMC injury (Reidy, 1985). When endothelial cells are injured alone by mechanical means, no intimal thickening or medial SMC proliferation is observed (Reidy and Silver, 1985, Reidy, 1985). Moreover, after mechanical endothelial injury, even in the presence of sustained damage through lipid stress in hypercholesterolemic rats, no intimal thickening is observed (Clowes *et al*, 1976). However, when both endothelial and medial damage are incurred there is increased expression of PDGF, transforming growth factor (TGF)-β, insulin like growth factor (IGF) and epidermal growth factor (EGF) concurrent with the development of

intimal thickening (Häyry *et al*, 1995). In contrast to AV, the intimal lesion observed in mechanical injury models is transient, presumably because the lesion is allowed to heal in the absence of further injury, whereas AV manifests as a result of ongoing allo-immunity.

We have previously demonstrated that medial smooth muscle cell loss within allograft vessels occurs by apoptotic cell death and that CTL-derived mediators of apoptosis including Fas ligand, perforin and granzyme B are concomitantly up-regulated (Hirsch *et al*, 1998). This led us to believe that medial de-population might be the result of allo-CTL responses directed against the vascular media instead of transmigration. In a subsequent study, we depleted $CD8^+$ T cells from rat aortic allograft recipients and found that medial preservation was indeed apparent suggesting an important role for $CD8^+$ T cells in the medial smooth muscle cell loss observed in AV (Légaré *et al*, 2000). However, the development of intimal hyperplasia in these allografts, despite the depletion of $CD8^+$ T cells, suggested that these two pathologies may be differentially regulated (Légaré *et al*, 2000). Alternatively, residual $CD8^+$ T cells or other cytotoxic effectors might have generated sufficient medial damage to trigger intimal hyperplasia. This latter explanation is consistent with arterial injury models where medial destruction need not be complete for lesion formation to occur (Reidy and Silver, 1985, Reidy, 1985). Thus, a potential relationship between smooth muscle cell loss and intimal hyperplasia provided the impetus to more closely examine the mechanism by which medial destruction occurs in relation to the development of AV.

In this thesis we sought to elucidate the contribution of $CD4^+$ and $CD8^+$ T cell subsets in the generation of AV. In particular, we paid careful attention to medial events as they relate to the induction of intimal hyperplasia. In these experiments aortic

allografts from C3H/HeJ mice were transplanted into high responder C57BL/6 mice which were either wildtype or disrupted for $CD4^+$ or $CD8^+$ T cells. We also evaluated the involvement of effector mechanisms employed by $CD8^+$ T cells in the destruction of target cells. For example, allografts were transplanted into Fas ligand-deficient (*gld*) and perforin-deficient ($pfp^{-/-}$) mice to determine the role of the Fas-Fas ligand (FasL) and granule-exocytosis pathways in medial SMC apoptosis.

Allografts placed into wildtype C57BL/6 recipients developed maximal apoptosis, measured by *in situ*-TUNEL staining, at 14 d post-transplant. Therefore, allografts placed into immune-deficient recipients were analyzed at this time point. Allografts from mice lacking $CD8^+$ T cells by CD8-α and $\beta_2$microglobulin gene deletion, showed a marked reduction in apoptosis within the medial cell compartment. Allografts transplanted into immune environments with impaired CTL pathways (either by perforin or Fas ligand gene deletion), showed a similar reduction in medial cell apoptosis at this time point.

The residual apoptotic activity can likely be explained by the redundancy of the major CTL-killing pathways. Alternatively, other potential mediators of this early apoptosis include residual CTL activity through minor pathways of cytotoxicity, which can be mediated by $CD4^+$ CTL and innate immune effectors. Recently, antibody-dependent pathways of apoptosis have been described which might be active in our experiments (Plissonnier *et al*, 2000). Nonetheless, these data confirm that $CD8^+$ CTL are involved in the induction of apoptosis within the vascular media. This evidence is consistent with our previous data from the rat aortic allograft model where medial apoptosis was inhibited by depletion of $CD8^+$ T cells using anti-CD8 monoclonal antibody treatment (Légaré *et al*, 2000).

Further indirect evidence regarding the mechanism of medial SMC de-population has emerged after careful analysis of the nature and origin of the lesion cells, previously purported to be the result of donor-derived SMC migration (Häyry *et al*, 1993, Häyry *et al*, 1995, Bojakowski *et al*, 2000). The evidence to support this hypothesis is circumstantial and recently we (Johnson *et al*, 2002) and others (Shimizu *et al*, 2001, Saiura *et al*, 2001, Li *et al*, 2001, Hillebrands *et al*, 2001, Hillebrands *et al*, 2003) have provided substantial contradictory data. In a rat aortic allograft model, using strain specific PCR of the MHC Class I haplotype, we have demonstrated that the α-actin$^+$ smooth muscle-like cells in the expanding neointima are of recipient, not donor origin (Johnson *et al*, 2002). This confirms that donor SMC migration is not responsible for the accumulation of smooth muscle-like cells in the subendothelial space forming the occlusive neointima. Recently, other groups have provided further compelling evidence, in other rodent models (Shimizu *et al*, 2001, Saiura *et al*, 2001, Li *et al*, 2001, Hillebrands *et al*, 2001, Hillebrands *et al*, 2003) and humans (Grimm*et al*, 2001), of a recipient-derived lesion. Thus, it can no longer be convincingly argued that medial SMC loss is the result of transmigration. Taken together these data indicate that the medial smooth muscle cell loss observed in allografts undergoing AV is the result of CTL-induced apoptosis.

Given that medial apoptotic activity was not completely ablated by depletion of CD8$^+$ T cells or cytolytic impairment, significant redundancy in the early anti-graft response exists. It is well-established that the two dominant mechanisms of contact-dependent, lymphocyte-mediated cytotoxicity are the granule-exocytosis and the Fas-FasL pathways (Kagi *et al*, 1994). Moreover, it has been demonstrated in experimental

systems that these pathways of CTL killing are redundant, such that inhibition of either pathway alone is ineffective in disrupting all killing activity *in vitro* (Kagi *et al*, 1994). However, these studies are limited by the fact that the lymphoblast target cells utilized in these experiments do not necessarily reflect the mechanisms involved in smooth muscle cell killing. Moreover, the *in vitro* CTL assay is also limited by the artificial nature in which it approximates complex *in vivo* events. Nonetheless, valuable insight into the mechanisms involved in CTL-mediated killing of vascular smooth muscle cells can be gained by *in vitro* analysis.

As such, we isolated vascular smooth muscle cells from aortic explants derived from wildtype and Fas-deficient *lpr* C3H mice for use as targets in our killing assay. Wildtype vascular SMC targets were killed by primed CTL from wildtype and perforin-deficient mice in a dose-dependent manner. In contrast, *lpr* SMC targets were less-susceptible to killing by both wildtype and perforin-deficient $CD8^+$ T cells. These *lpr* targets were, however, killed effectively when treated with anti-H-$2D^k$ and complement. These data suggest that the Fas-FasL pathway of cytotoxicity is particularly important in CTL-mediated killing of vascular smooth muscle cells *in vitro*.

Fukuo and colleagues have shown that nitric oxide release in response to macrophage-derived IL-1β causes up-regulation of vascular smooth muscle cell Fas expression increasing their susceptibility to apoptosis (Fukuo *et al*, 1996). Furthermore, Schaub and co-workers have demonstrated that smooth muscle cell apoptosis mediated by Fas/FADD activation isnot silent (Schaub *et al*, 2000). Fas-induced apoptosis leads to up-regulation of the chemokines monocyte-chemoattractant protein-1 and interleukin-8, which cause massive infiltration of macrophages *in vivo* (Schaub *et al*, 2000). The

induction of a pro-inflammatory program by apoptotic vascular smooth muscle cells may contribute to the pathogenesis of AV. Thus, in models of AV within vessels with a large muscular component, a more pronounced role for $CD8^+$ T cells can be postulated, given that here we have demonstrated the involvement of $CD8^+$ CTL in medial smooth muscle cell apoptosis.

In the experiments outlined in this thesis, the impairment of recipient cytolytic activity *in vivo* was effective in reducing early medial apoptosis. However, it is possible that the inhibition of apoptosis is transient and that the progression of medial damage is only delayed by interruption of the major pathways of cytotoxicity. Moreover, the assessment of medial apoptosis at 14 d post-transplant is a static image of a dynamic process. To confirm that attenuation of early apoptotic events translated into preservation of medial elements at later stages, medial area measurements performed at 60 d post-transplant were expressed as a percentage of the medial area of an undamaged syngeneic aortic graft. Medial damage was most profound in wildtype and CD4 knockout recipients, whereas inhibition of $CD8^+$ T cells or CTL effector pathways conferred protection against medial destruction. Interruption of the Fas-FasL pathway had the most profound impact on medial sparing and was consistent with the data obtained from the *in vitro* CTL assay, confirming an important role for this pathway in the development of medial damage.

To confirm that a reduction in the observed medial area measurements reflected the degree of medial damage, α-smooth muscle actin immunostaining was used to identify residual smooth muscle cells. Allografts from wildtype and CD4 knockout mice showed complete medial de-population with no visible α-actin immunoreactivity. In

contrast, inhibition of $CD8^+$ T cells or impairment of cytotoxic mediators resulted in partial sparing of α-$actin^+$ medial smooth muscle cells. These data suggest that the medial damage observed in AV is mediated in part by $CD8^+$ T cells through contact-dependent cytotoxicity. Moreover, the Fas-FasL pathway is particularly implicated in the development of medial damage in non-immunosuppressed aortic allografts. The elucidation of the mechanisms involved in the destruction of the vascular media leading to structural collapse could have profound implications since these events might exacerbate the inward vessel remodelling which contributes to the hemodynamic compromise of AV (Pethig *et al*, 1998, Pasterkamp *et al*, 2000). Nonetheless, intimal hyperplasia remains the *sine qua non* of allograft vasculopathy.

Graft loss from AV is primarily due to the arteriosclerotic lesion which impedes blood flow within the transplanted heart eventually leading to ischemic organ failure (Shirwan *et al*, 1999). We have clearly implicated $CD8^+$ T cells and CTL effector mechanisms in the allo-immune destruction of medial smooth muscle cells. A possible link between medial damage and the evolution of intimal hyperplasia may exist. To this end, we assessed the development of AV in fully MHC disparate mouse aortic allografts transplanted into animals lacking $CD4^+$ T cells ($CD4^{-/-}$), $CD8^+$ T cells ($CD8^{-/-}$ and $\beta 2m^{-/-}$), and CTL effector molecules. We performed computer-assisted digital morphometric analysis and calculated a ratio of the intimal to medial area. The lack of $CD8^+$ T cells in $CD8^{-/-}$ and $\beta 2m^{-/-}$ mice resulted in significant attenuation of lesion formation compared to allografts from wildtype and $CD4^{-/-}$ mice. These data support an important role for $CD8^+$ T cells in the development of the neointimal lesion. However, these results contradict the

popular viewpoint that, similar to acute rejection, $CD4^+$ T cells are paramount in the initiation of AV (Shi *et al*, 1996, Ensminger *et al*, 2002, Yamada *et al*, 2003).

Shi and co-workers showed that AV does not occur in CD4 knockout mice, implicating a critical role for $CD4^+$ T cells in AV (Shi *et al*, 1996). Moreover, in their experiments with $\beta 2m^{-/-}$ mice they observed no reduction in AV (Shi *et al*, 1996). Ensminger and colleagues, using adoptive transfer of T cell subsets into immunodeficient mice, demonstrated that $CD4^+$ T cells and not $CD8^+$ T cells were capable of inducing AV within MHC class I-mismatched aortic allografts, via the indirect pathway of allo-recognition (Ensminger *et al*, 2002). Similarly, Yamada *et al* confirmed that indirectly-primed $CD4^+$ T cells are capable of mediating AV in murine cardiac allografts (Yamada *et al*, 2003).

Discrepancies in the published literature might reflect differences between the aortic and carotid loop models. In particular, the muscular component of the media is substantially larger in the mouse aorta (and in the human coronary artery) than in the mouse carotid loop. Our data have implicated medial smooth muscle cells as a target for $CD8^+$ CTL. In the event that CTL-mediated smooth muscle cell apoptosis fuels the intimal hyperplastic response, AV in the aorta would be predictably more dependent on $CD8^+$ CTL activity. In addition, the inconsistencies might be related to the different strain combination used in the experiments, which may tend to favourcertain responses. For example, high responder mice possess helper-independent $CD8^+$ T cells which contribute to immunopathology (Sun *et al*, 2001). Ensminger and co-workers used $CD8^+$ T cells from the relatively low responder strain CBA/Ca mice, which might be dependent on $CD4^+$ T cell help for activation and effector function (Ensminger *et al*, 2002). Similarly,

Shi and co-workers transplanted B10.A(2R) (H-2^{h2}) carotid allografts into B6129F_2 (H-2^{b}) recipients (Shi *et al*, 1996).

These studies used non-immunosuppressed allografts (Shi *et al*, 1996, Ensminger *et al*, 2002, Yamada *et al*, 2003) where potentially dominant CD4-mediated acute responses might manifest as vasculopathy. Indeed, the prevailing hypothesis is that $CD4^+$ T cells play a central role in acute cardiac allograft rejection. For example, long-term graft acceptance occurs in CD4 knockout recipient mice, whereas CD8 knockout mice develop typical acute rejection responses (Krieger *et al*, 1996, Krieger *et al*, 1997). Thus, it is imperative that immunosuppression, capable of abrogating acute cardiac allograft rejection, be employed when assessing the contribution of $CD4^+$ T cells to the development of clinically relevant AV.

In the third study, Yamada and colleagues interrupted $CD4^+$ T cell direct allo-recognition concurrently with $CD8^+$ T cell-depletion which allowed for prolongation of cardiac allograft survival sufficient for AV formation (Yamada *et al*, 2003). However, they did not evaluate the contribution of these cells in the presence of pharmaceutical immunosuppression. Moreover, in their model, directly primed $CD8^+$ T cells generated acute rejection, yet they did not examine the potential involvement of indirectly-primed $CD8^+$ T cells in AV.

To more accurately assess the impact of $CD4^+$ T cells in AV, Han and co-workers transplanted cardiac allografts into anti-CD4 transgenic mice which are completely devoid of $CD4^+$ T cells by endogenous antibody depletion (Han *et al*, 2000). These mice had significantly less peripheral $CD4^+$ T cells in comparison to CD4 and MHC class II knockout mice by flow cytometric analysis (Han *et al*, 2000). When these mice were used

as recipients, skin graft survival was significantly prolonged compared to CD4 and MHC class II knockout mice confirming the functional significance of improved CD4 depletion *in vivo* (Han *et al*, 2000). This skin graft survival was further prolonged by the addition of anti-CD8 treatment verifying the integrity of CD8 responses in these high responder mice (Han *et al*, 2000). BALB/c hearts into these mice and wildtype mice treated with anti-CD4 survived >100 d, but had striking evidence of AV, which was associated with a $CD8^+$ T cell infiltration of the allograft (Han *et al*, 2000). This data indicates that $CD8^+$ T cell-mediated AV occurs in the absence of peripheral $CD4^+$ T cells and suggests that the development of AV seems less dependent than acute rejection on $CD4^+$ T cell helper function.

I believe that $CD4^+$ T cells are sufficient to initiate AV and that they play a crucial role in this pathology under experimental conditions which necessitate their activity in the helper-dependent activation of CTL. However, our data supports the hypothesis that, while not necessary, $CD8^+$ T cells play a more prominent role than $CD4^+$ T cells in the development of AV within this non-immunosuppressed high responder aortic allograft model. In these mice exists a population of $CD4^-$ $CD8^-$ $\alpha\beta TCR^+$ cells with helper activity capable of supporting the activation of $CD8^+$ T cells in the absence of $CD4^+$ T cells (Locksley *et al*, 1993, Han *et al*, 2000). Nevertheless, data from wildtype C57BL/6 transplant recipients depleted of $CD4^+$ cells by monoclonal antibody treatment and anti-CD4 transgenic mice, in which this helper population are not present, develop AV, further supporting our observations (Han *et al*, 2000).

Although the effector mechanisms employed by allo-specific T cells have been well characterized, little is known regarding their role in the development of intimal

hyperplasia. It was therefore of interest to examine the role of CTL activity in the generation of the neointimal lesion, in lieu of the involvement of these effectors in medial apoptosis. We transplanted wildtype C3H allografts into perforin- and Fas Ligand-deficient C57BL/6 mice, as well as C3H Fas-deficient allografts into perforin-deficient recipients, where both major pathways of cytotoxicity are interrupted simultaneously. In these experiments, inhibition of the granule-exocytosis pathway alone showed no reduction in intimal lesion formation compared to wildtype recipients. Interruption of Fas-FasL mediated killing resulted in some inhibition of intimal hyperplasia supporting an important role for this pathway in the development of AV. However, simultaneous inhibition of the major pathways of cytotoxicity resulted in the greatest reduction in intimal hyperplasia, even surpassing the reduction in AV observed in CD8-deficient ($CD8^{-/-}$ and $\beta 2m^{-/-}$) animals.

Interestingly, $CD4^+$ CTL are known to kill target cells predominantly by the Fas-FasL pathway (Russell and Ley, 2002). Despite the fact that relatively few graft cells express MHC class II, $CD4^+$ CTL can be activated to initiate bystander killing after priming by graft endothelium, which up-regulate MHC class II expression in response to inflammatory mediators (Russell and Ley, 2002). Thus, the interruption of the Fas-FasL pathway also limits $CD4^+$ CTL activity, conferring an additional benefit to the inhibition of $CD8^+$ T cells alone. In particular, a population of $CD4^-$ cytotoxic T cells, which kill primarily by the Fas-FasL pathway, have been described in CD8-deficient mice (Dalloul *et al*, 1996). Taken together, the data presented in this thesis supports an important role for the Fas-FasL pathway in the destruction of medial SMC and the induction of intimal hyperplasia. Some involvement of the granule-exocytosis pathway is implied given that a

further reduction in AV is observed when this pathway is inhibited in addition to the Fas-FasL interaction. This data is consistent with accumulating evidence suggesting that $CD8^+$ T cell cytotoxic mechanisms play a significant role in AV by inducing apoptotic events in the transplant vasculature (Hirsch *et al*, 1998, Koglin and Russell, 1999, White *et al*, 1997).

Subbotin and colleagues have suggested a critical role for FasL-mediated killing in the development of AV by demonstrating the complete abrogation of lesion formation in recipient *gld* mice which lack FasL (Subbotin *et al*, 1999). Although our data supports an important role for the Fas-FasL interaction, AV still develops in our model using *gld* mice. Their results can be explained by the age-dependent immune hypo-responsiveness observed in *gld* mice. In fact, Djamali *et al* showed that 6 wk old *gld* mice develop typical acute rejection of cardiac allografts, whereas 13 wk old *gld* mice exhibit significantly prolonged graft survival (Djamali *et al*, 1998). Since the animals used by Subbotin *et al* were up to 12 wk old at the time of transplant and nearly 18 wk old at harvest, immune dysregulation and not Fas-FasL interruption is implicated in the abrogation of AV in their study (Subbotin *et al*, 1999). In our experiments, we used *gld* mice which were 6 wk old at the time of transplant. $CD8^+$ T cells derived from these mice exhibited robust killing activity in our *in vitro* assay confirming the intact nature of their CTL effector function. Thus, while our data supports an important role for the Fas-FasL interaction, it demonstrates that this interaction is not required for medial cell destruction or intimal hyperplasia during the progression of AV.

Recently, Krupnick and colleagues have suggested that the granule-exocytosis pathway is important in the killing of allogeneic endothelial cells *in vitro* with only a

minor contribution by the Fas-FasL pathway (Krupnick *et al*, 2002). However, susceptibility to killing in a 4 h *in vitro* CTL assay does not necessarily reflect *in vivo* killing, which occurs in an inflammatory milieu. Other groups have also showed that endothelium bear a striking resistance to apoptosis induced by the Fas-FasL interaction despite significant Fas expression (Zheng *et al*, 2000, Zheng *et al*, 2002). Similarly, Yasukawa *et al* have suggested that granule-exocytosis is the primary killing pathway utilized by $CD4^+$ and $CD8^+$ T cells in humans (Yasukawa *et al*, 2000). However, Li *et al* showed that interferon-γ, commonly present in an allograft environment, induces sensitivity of human endothelium to Fas-FasL mediated apoptosis *in vitro* (Li *et al*, 2002). Here, I have demonstrated that the Fas-FasL interaction is important in the induction of medial smooth muscle cell apoptosis *in vitro* and *in vivo* during the generation of AV. However, the most marked reduction in clinically significant intimal hyperplasia was observed when both pathways of direct cytolysis were inhibited simultaneously indicating that both killing pathways are involved in the pathogenesis of AV.

Since intimal hyperplasia and medial destruction are not completely inhibited when both killing pathways were blocked, either minor pathways of killing or other redundant non-CTL effector pathways are capable of inducing AV. These potentially include DTH-like responses through the secretion of the pro-inflammatory cytokine IFN-γ and allo-antibody mediated humoral responses. To investigate the contribution of allo-antibody in the medial SMC destruction and intimal hyperplasia observed in the absence of CTL activity, we passively transferred anti-serum from allo-sensitized wildtype mice into immunodeficient $RAG^{-/-}$ allograft recipients. This anti-serum was shown to be

specific for allogeneic cells and exhibited marked complement-dependent cytotoxicity *in vitro*. However, there was no medial damage or intimal hyperplasia observed in these animals indicating that allo-antibody is not involved. In contrast, Russell and colleagues, using a similar dose of anti-serum, showed that allo-antibody was capable of inducing AV in severe combined immunodeficient (SCID) recipients of cardiac allografts (Russell *et al*, 1994). However, they observed $CD3^+$ cells in their experimental animals and these residual T cells could easily be responsible for the development of AV in their model (Russell *et al*, 1994). In our experiments with $RAG^{-/-}$ animals no residual T cells could be demonstrated. Current experimentation in our laboratory is underway to confirm these results by performing adoptive transfer of allo-activated antibody producing non-T cells into $RAG^{-/-}$ mice.

It is important to remember that non-$CD8^+$ T cell sources of cytotoxic mediators have been associated with apoptosis (Ogura *et al*, 2001). In humans, endothelium exhibit constitutive expression of MHC class II, whereas mice up-regulate its expression in response to inflammation (Hasegawa *et al*, 1998, Pober, 1999). Thus, allograft endothelium can stimulate or elicit responses from $CD4^+$ T cells. Indeed, allo-recognition of graft endothelium by T cell subsets has been identified as an important step in the initiation of graft rejection (Fyfe *et al*, 1994). Although, the current accepted hypothesis regarding the etiology of AV suggests that endothelial disruption is a necessary step in the development of AV, our data link smooth muscle cell damage as a possible inciting stimulus for the vascular remodelling process observed in AV. It is likely that CTL-mediated allo-responses against both the graft endothelium and vascular smooth muscle cells occur during the initiation of the AV process. Fas-sensitive smooth muscle cells are

susceptible to apoptosis via CTL-derived FasL, while damage to Fas-resistant endothelium is largely mediated by granule-exocytosis (Geng *et al*, 1997, Krupnick *et al*, 2002).

The human coronary artery, much like the mouse aorta, has many layers of medial smooth muscle cells, which serve as a CTL target and might augment responses directed against the graft endothelium leading to the development of AV. Studies in arterial injury models suggest that a threshold of medial damage, as opposed to endothelial disruption, predicts the extent of intimal thickening during vessel remodelling (Bjorkerud *et al*, 1969, Clowes *et al*, 1983, Reidy, 1985). Thus, even in the absence of $CD8^+$ CTL, the residual medial damage caused by redundant immune effectors could be sufficient to cause the neointimal lesion formation observed during cytolytic impairment.

Current experimentation in our laboratory is focused on determining the impact of endothelial *versus* medial damage in the progression of AV. In the situation where the graft endothelium and media are of different strain origin, by creating a chimeric aortic segment, we can evaluate allo-responses directed against the media or intima in isolation. To achieve this we treated aortic segments with intralumenal ethylenediaminetetraacetic acid (EDTA) to denude the intimal layer. We confirmed successful denudation by submitting EDTA-treated and untreated aortic segments to anti-platelet-endothelial cell adhesion molecule (PECAM) immunocytochemistry and both transmission and scanning electron microscopy. By scanning microscopy EDTA-treated aortas showed a complete loss of the endothelial layer, whereas untreated vessels contained a relatively normal intima (Appendix 1). Similarly, anti-PECAM immunostaining clearly demonstrated the presence of intact endothelium in untreated vessels. In contrast, EDTA treatment resulted

in a complete loss of anti-PECAM immunoreactivity (Appendix 1). These denuded C3H aortic segments will then be transplanted into immunodeficient $RAG^{-/-}$ mice, which will result in re-endothelialization in the absence of further immune injury. After 21 d, we will confirm re-endothelialization by anti-PECAM and MHC class I immunocytochemistry and SEM. Once re-endothelialization has been confirmed, similarly treated aortic segments will be re-transplanted into either wildtype C3H or C57BL/6 mice. Thus, when these chimeric grafts are re-transplanted into C3H recipients, the endothelium will be recognized as foreign and the media as self. In contrast, when re-transplanted into C57BL/6 recipients, the endothelium is self and the media will be recognized as foreign. This will allow for the evaluation of whether damage to endothelium, or medial smooth muscle cells, or both are required for lesion formation.

If the redundant pathways involved in the generation of AV in the absence of $CD8^+$ CTL activity are $CD4^+$ T cell mediated, they may play a less significant role in clinical transplantation where CyA-based immunosuppression is common. There is anecdotal evidence which suggests that $CD4^+$ T cells are sensitive to the effects of CyA, whereas $CD8^+$ T cells are relatively resistant (Horsburgh *et al*, 1980, Bishop *et al*, 1992, Bujan *et al*, 2001, Hruban *et al*, 1990, Libby and Pober, 2001). For example, $CD4^+$ T cell mediated allo-DTH responses are down-regulated by CyA (Bretscher and Havele, 1992), while $CD8^+$ T cell mediated allo-CTL responses remain unaffected (Kroczek *et al*, 1987). In fact, a CyA-resistant CTL population was identified more than twenty years ago (Horsburgh *et al*, 1980). Further evidence that $CD4^+$ T cell, but not $CD8^+$ T cell, thymic maturation events are affected by CyA suggests a potential global down-regulation of $CD4^+$ T cell activity beyond peripheral T cell activation (Takeuchi *et al*, 1989). Thus, in

the clinical situation, $CD8^+$ T cell-dependent mechanisms may play a dominant role in the development of AV.

The evidence provided in this thesis suggests that the AV observed in non-immunosuppressed aortic allografts is mediated mostly, but not exclusively, by $CD8^+$ T cells. However, $CD8^+$ T cells also contribute to acute rejection under normal circumstances, given that graft rejection occurs more rapidly in the presence of both $CD4^+$ and $CD8^+$ T cells in adoptive transfer experiments (Hall *et al*, 1990). More recently, there is evidence that $CD8^+$ T cells can be activated to trigger acute allograft rejection by APCs in a helper-independent manner (Jones *et al*, 2000, Halamay*et al*, 2002, Kreisel *et al*, 2002).

Jones and co workers demonstrated that anti-CD154 treatment is capable of inhibiting $CD4^+$ T cell function, whereas $CD8^+$ T cells are spared and remain active, capable of mediating acute rejection (Jones *et al*, 2000). Halamay and colleagues, using 2C transgenic mice depleted of residual $CD4^+$ T cells as recipients, showed that $CD8^+$ T cells are sufficient to mediate acute cardiac allograft rejection (Halamay *et al*, 2002). Moreover, Kreisel *et al* observed that endothelial cells are capable of activating $CD8^+$ T cells *in vitro* and *in vivo* (Kreisel *et al*, 2002). In a separate study, they found that $CD8^+$ T cells activated by non-hematopoietic cells, in the absence of $CD4^+$ T cells, were capable of triggering the acute rejection of cardiac allografts (Kreisel *et al*, 2002). These data indicate that while not necessary, $CD8^+$ T cells contribute to the acute rejection of cardiac allografts and can do so in the absence of $CD4^+$ T cell influence.

Given that $CD8^+$ T cells can mediate acute rejection responses against cardiac allografts in the absence of $CD4^+$ T cells (Kreisel *et al*, 2002), it is important to rule out

the involvement of $CD8^+$ T cell mediated acute responses in the rejection of aortic allografts in our non-immunosuppressed system. Therefore, allo-transplantation was performed under CyA-based immunosuppression to inhibit responses which would normally be involved in the acute rejection of whole-organ transplants. In our experiments wildtype recipients developed significant AV despite CyA treatment with up to 70 mg/kg/d. In contrast, animals which lack $CD8^+$ T cells showed no evidence of pathology. It is important to note that a similar dose of CyA was successful in eliminating the acute rejection response to a cardiac allograft in the identical strain combination confirming that $CD8^+$ T cell-mediated acute rejection responses are not implicated in the vasculopathy observed in our aortic allografts. The differential susceptibility of $CD4^+$ and $CD8^+$ T cell subsets to CyA-based immunosuppression might explain the limited efficacy of current transplant pharmacotherapy in ameliorating AV. This data demonstrates that the AV observed in our system is dependent on $CD8^+$ T cell activity in the presence of CyA-based immunosuppression.

Despite our data, the involvement of $CD8^+$ T cells and CTL effector mechanisms in the development of the intimal proliferative lesion remains a contentious issue. Some have suggested that $CD8^+$ T cells are not involved in the progression of AV (Forbes *et al*, 1994, Shi *et al*, 1996, Szeto *et al*, 2002, Ensminger *et al*, 2002). Others have shown that depletion of $CD8^+$ T cells prevents the development of intimal hyperplasia (Allan *et al*, 1997). While our experimentation using CD4 and CD8 knockout mice provides useful evidence regarding the role of T cell subsets in the development of AV, this data is limited by well described compensatory mechanisms in these animals. For instance, CD4 knockout mice possess a population of $CD4^-$ $CD8^-$ $\alpha\beta TCR^+$ cells which exhibit

significant helper activity capable of contributing to the alleged helper-independent activation of $CD8^+$ T cells (Locksley *et al*, 1993). Similarly, CD8 knockout mice contain a population of $CD4^-$ αβTCR ^{intermediate} cytotoxic T cells which kill allogeneic targets primarily by the Fas-FasL pathway (Dalloul *et al*, 1996).

The use of antibody depletion strategies in wildtype mice has the advantage of avoiding such problems. However, the difficulty with this type of experimentation is that cells which escape depletion can complicate the interpretation of the results. The adoptive transfer of T cell subsets into immunodeficient $RAG^{-/-}$ mice offers a complementary line of experimentation, which facilitates the precise evaluation of effector mechanisms employed by $CD8^+$ T cells in the generation of AV. Moreover, this model addresses the question of whether $CD8^+$ T cells are alone sufficient to induce AV. This type of experimentation is, however limited by the ability to isolate and purify T cell subsets from splenocyte populations of wildtype mice. Indeed, any contaminating cells would render the data uninterpretable.

A further consideration when utilizing adoptive cell transfer into immunodeficient mice is the contribution of innate effectors stimulated by ischemic graft injury. Ischemic injury during organ procurement and transplantation has been identified as a particularly important factor (Gaudin *et al*, 1994). For instance, Gaudin *et al* showed that ischemic graft injury promotes the generation of AV in human heart transplants (Gaudin *et al*, 1994). Although many immune and non-immune factors are involved in the pathogenesis of AV (Schmid *et al*, 1997), in the absence of effective T cell activity (by using athymic nude, SCID, RAG-deficient animals or very high dose immunosuppression) AV does not occur (Hall, 1991, Russell *et al*, 1994, Delfs *et al*, 2001, Hillebrands *et al*, 2001,

Ensminger *et al*, 2002). This is confirmed in our experiments where immunodeficient RAG-1$^{-/-}$ mice did not exhibit AV in the absence of reconstitution. Moreover, when adoptive transfer of primed CD8^{+} T cells was performed 94 and 168 h after transplantation, when inflammation due to ischemia had subsided, there was no difference in AV observed. These data indicate that while ischemia is an important factor, AV in our model is dependent on potent allo-immune responses triggered by the transfer of primed CD8^{+} T cells.

Another caveat of cell transfer systems into immunodeficient hosts is the potential for homeostasis-driven proliferation of elements within the cell transfer population (Goldrath *et al*, 2000). In immunodeficient RAG$^{-/-}$ mice the B and T lymphocyte compartments are empty such that innate signals which act to maintain the homeostasis of these cell populations become active. The exposure to growth promoting cytokines, such as IL-7 is usually limited in a crowded cell compartment, but is not in these immunodeficient mice (Murali-Krishna *et al*, 1999). Thus, upon transfer into the immunodeficient environment, naïve and memory T cells proliferate in response to these signals in order to fill the void compartment. Such homeostasis-driven proliferation results in cells with a functional state similar to that of the memory CD8^{+} T cell phenotype capable of cytokine production and effector function (Schluns *et al*, 2000, Ge *et al*, 2002). For this reason we restricted our observations to the effector limb, by allowing primary allo-antigen activation to occur in the wildtype host and subsequently transferring effector T cell populations; thus avoiding the issue of homeostasis-driven proliferation. Memory T cell populations also exhibit this phenomenon; however a stable effector phenotype is maintained (Tan *et al*, 2002).

Prior to the*in vivo* adoptive transfer experiments, we confirmed that our allo-immunization protocol was effective in generating CTL capable of killing allogeneic targets *in vitro*. Allo-primed CTL exhibited efficient killing of allogeneic but not third party targets, while naïve $CD8^+$ T cells did not kill. Interestingly, deletion of $CD8^+$ or asialo $GM1^+$ cells from allo-primed T cell populations abrogated *in vitro* killing, indicating that a population of asialo $GM1^+$ $CD8^+$ T cells are responsible for the cytotoxic activity observed. This is consistent with data from other groups who have demonstrated that antigen-specific $CD8^+$ T cells up-regulate surface expression of NK cell associated markers upon activation, particularly in high responder C57BL/6 mice (Oughton and Kerkvliet, 1999, Slifka *et al*, 2000, Assarsson *et al*, 2000). This subset of CTL has been implicated in co-stimulatory blockade resistant rejection of skin allografts (Trambley *et al*, 1999). Moreover, the generation of mature CTL in C57BL/6 mice (Roopenian *et al*, 1983, Andrus *et al*, 1984) and in humans (Inaba *et al*, 1987, Young and Steinman, 1990) is under certain circumstances T helper cell-independent.

In this thesis we examined whether the adoptive transfer of primed, purified $CD8^+$ T cells was sufficient to induce AV within aortic allografts transplanted into immunodeficient recipients. The purified effector $CD8^+$ T cells when transferred into RAG-$1^{-/-}$ mice, which lack both T cells and B cells, induced robust AV, demonstrating that primed $CD8^+$ T cells are sufficient to generate AV. When $CD8^+$ cells were deleted from this transfer population AV induction was abrogated, confirming that the AV lesions seen are completely dependent on effector $CD8^+$ T cells. Moreover, when highly purified $CD8^+$ T cells, isolated by positive selection, were used in the adoptive transfer AV was again observed. These data are in contrast to other published reports where $CD4^+$

T cells but not $CD8^+$ T cells were capable of inducing AV (Ensminger *et al*, 2002). However, Ensminger and colleagues used naïve $CD8^+$ T cells in their adoptive transfer experiments. Thus, in their strain combination the activation of $CD8^+$ T cells was dependent on $CD4^+$ T cell helper function (Ensminger *et al*, 2002). To confirm that our results did not represent a strain specific phenomenon, we performed $CD8^+$ T cell adoptive transfer using the reverse strain combination. Indeed, C3H $CD8^+$ T cells generated robust AV in C57BL/6 aortic allografts. Even when primed $CD8^+$ T cells from type 2 responder BALB/c mice were used in the adoptive transfer, AV was still apparent further substantiating our observations.

The participation of $CD8^+$ T cells in the development of the neointimal lesion has been extensively studied, but has yielded conflicting results (Forbes *et al*, 1994, Shi *et al*, 1996, Allan *et al*, 1997, Han *et al*, 2000, Ensminger *et al*, 2000, Szeto *et al*, 2002, Ensminger *et al*, 2002). Depletion of $CD8^+$ T cells by antibody treatment in a rat heterotopic heart model had no impact on lesion formation (Forbes *et al*, 1994). Forbes and colleagues used a non-immunosuppressed minor-mismatch model where indirectly-primed $CD4^+$ T cells remain active. In the presence of immunosuppression, where $CD4^+$ T cell function is inhibited, CD8-depletion would undoubtedly have a greater impact on the development of AV.

Shi and colleagues used β2microglobulin-deficient mice, which lack surface MHC class I expression such that only few $CD8^+$ T cells undergo positive selection, as recipients of loop carotid allografts (Shi *et al*, 1996). They observed that β2m-deficient mice develop robust AV indicating that $CD8^+$ T cells are not required (Shi *et al*, 1996).

However, these animals have a population of $CD4^+$ CTL, as well as residual $CD8^+$ T cells which complicates the interpretation of their data.

Szeto and colleagues developed a model whereby donor hearts are re-populated with recipient type APCs by bone marrow transplantation (Szeto *et al*, 2002). Interestingly, these chimeric hearts do not reject acutely, but develop cardiac allograft vasculopathy (Szeto *et al*, 2002). Given that all the graft APCs are of recipient haplotype, the authors theorized that direct allo-recognition by $CD4^+$ T cells is inhibited leaving only the indirect pathway for $CD4^+$ T cells. They then demonstrated that depletion of $CD4^+$ T cells, but not $CD8^+$ T cells inhibited the development of AV (Szeto *et al*, 2002). However, this model is in direct conflict with their earlier work which implicated direct allo-recognition by $CD8^+$ T cells in acute cardiac allograft rejection (Kreisel *et al*, 2002). Moreover, other groups have described the acute rejection of heart allografts mediated by $CD8^+$ T cells with help provided by indirectly primed $CD4^+$ T cells (Yamada *et al*, 2003). This discrepancy in $CD8^+$ T cell function can potentially be explained by the low responder strain of recipients used in their experimentation (Stepkowski and Ito, 1990, Alfrey *et al*, 1995, Qi *et al*, 1997).

Allan and colleagues performed heterotopic heart transplants between MHC class I-disparate partially inbred miniature swine treated with CyA immunosuppression (Allan *et al*, 1997). Animals treated with CyA alone had evidence of severe parenchymal rejection, as well as marked intimal hyperplasia characteristic of AV evident by 28 d (Allan *et al*, 1997). However, when anti-CD8 monoclonal antibody therapy was added to CyA treatment, AV was completely inhibited despite the persistence of severe parenchymal rejection (Allan *et al*, 1997). These results would indicate that $CD8^+$ T cells

are essential for the development of early intimal hyperplasia in this model. However, the persistence of parenchymal rejection despite concurrent CyA and anti-CD8 treatment implies the ongoing activity of indirectly-primed $CD4^+$ T cells, or alternatively residual $CD8^+$ T cells in their system. While, the inhibition of $CD8^+$ T cells abrogated early AV, likely mediated by direct recognition of foreign MHC class I, they did not rule out the possibility that indirectly primed $CD4^+$ T cells might trigger intimal hyperplasia in a delayed manner. Furthermore, by using sub-therapeutic immunosuppression, AV can be potentiated by vessel injury mediated by the acute responses of $CD8^+$ T cells which might be silenced by a more clinically relevant dose of CyA.

In numerous experimental models, blockade of the CD40-CD154 co-stimulatory interaction leads to prolonged allograft survival through the inhibition of acute rejection mediated by $CD4^+$ T cells (Ensminger *et al*, 2000,) depending on the donor and recipient strain combination (van Maurik *et al*, 2002) and the ability of $CD8^+$ T cells to mediate acute rejection (Jones *et al*, 2000, Zhai *et al*, 2002). Experimental models of AV using anti-CD154 co-stimulatory blockade have suggested an important role for $CD8^+$ T cells in the development of AV (Ensminger *et al*, 2000). For example, BALB/c aortic allografts transplanted into C57BL/6 recipients treated with anti-CD154 develop the hallmark features of AV (Ensminger *et al*, 2000). However, the addition of anti-CD8 treatment prevented the development of intimal hyperplasia, indicating that co-stimulatory resistant $CD8^+$ are involved in the development of AV in this model (Ensminger *et al*, 2000). However, the question arises in high responder strain combinations, where co-stimulatory resistant $CD8^+$ T cells are capable of mediating acute allograft rejection (Jones *et al*, 2000, Zhai *et al*, 2002), whether acute CD8 responses

initiated the rejection of the aortic tissue manifesting as vasculopathy in the absence of immunosuppression. Taken together, these data indicate that $CD8^+$ T cells are not required for lesion formation in non-immunosuppressed models of AV. However, under certain circumstances, where effector CTL develop independently of $CD4^+$ T cell help, $CD8^+$ T cells play a more prominent role in the development of AV.

The development of a model in which primed $CD8^+$ T cells induce AV in the absence of other lymphocytic components of the immune system allows for the precise discrimination of effector mechanisms involved in $CD8^+$ T cell mediated AV. $CD8^+$ effector T cells exert contact-dependent cytotoxicity (Hirsch *et al*, 1998, Légaré *et* al, 2000), as well as producing cytokines which mediate DTH-like immune responses (Nagano *et al*, 1998, Delfs *et al*, 2001) capable of mediating AV. Our previous experimentation using knockout mice demonstrated that AV occurred through redundant immune pathways despite the inhibition of $CD8^+$ CTL. Although other groups have implicated $CD4^+$ T cells, innate and humoral immunity in AV (Russell *et al*, 1994, Shi *et al*, 1996, Russell *et al*, 2001, Ensminger *et al*, 2002, Yamada *et al*, 2003), $CD8^+$ T cell non-CTL effector mechanisms could potentially be involved.

Having established an important role for contact-dependent CTL effector function using knockout mice, we first examined the role of direct killing in $CD8^+$ T cell-mediated AV in our adoptive transfer system. Interestingly, when either the granule-exocytosis or the Fas-FasL pathways were blocked independently, AV lesion formation was unimpeded. These data confirm that AV proceeds normally despite the interruption of the Fas-FasL interaction and refute the observations of Subbotin and co-workers (Subbotin *et al*, 1999). While blockade of the Fas-FasL pathway conferred some medial protection,

damage in this compartment was still evident and might be involved in the induction of the intimal lesion. It is likely that prior allo-sensitization generates a robust CTL response capable of triggering graft damage by the granule exocytosis-pathway. Moreover, these primed $CD8^+$ T cells exhibit an increased capacity for the early production of interferon-γ and enhanced potential to mediate DTH which might be involved in graft injury and AV induction (Walzer *et al*, 2000). Alternatively, medial injury and intimal hyperplasia might be differentially regulated such that distinct $CD8^+$ T cell effector mechanisms are involved in the induction of these two potentially separate events. For example, our data suggests that the Fas-FasL interaction is involved in the destruction of medial smooth muscle cells, whereas Krupnick and co-workers have suggested that the granule-exocytosis pathway is primarily involved in endothelial cell apoptosis *in vitro* (Krupnick *et al*, 2002).

To evaluate the involvement of the major pathways of contact-dependent cytotoxicity in $CD8^+$ T cell mediated AV, we transplanted Fas-deficient aortic allografts into RAG-$1^{-/-}$ recipients reconstituted with primed $CD8^+$ T cells from perforin-deficient mice. When both pathways were blocked simultaneously in the adoptive transfer model, AV was significantly attenuated, indicating that contact-dependent direct killing plays a major role in the induction of AV. However, since AV was not completely inhibited, other redundant $CD8^+$ T cell effector pathways must be active. It is possible that minor pathways of cytotoxicity including apoptosis induced by TRAIL and TNF might be active in our system. To examine this possibility we performed *in vitro* CTL assays. The simultaneous blockade of the granule-exocytosis and Fas-FasL pathways eliminated direct killing of allogeneic lymphoblast and vascular smooth muscle cell targets *in vitro*,

indicating that minor pathways of cytotoxicity are unlikely to be active within our system. To further clarify this, we could have used TNF receptor superfamily 1 and 2 double knockout mice as aortic allograft donors in conjunction with primed $CD8^+$ T cells from perforin-deficient mice. These donors are not susceptible to killing by FasL, TRAIL or TNF. However, limited availability of this mouse precluded its use in our experimentation.

Alternatively, NK cells of the immunodeficient host could potentially be involved in the generation of AV in the absence of $CD8^+$ CTL activity, since these recipient cells maintain an intact perforin/granzyme system capable of injuring the allograft. Indeed, Russell and colleagues have implicated innate effectors in the development of cardiac allograft vasculopathy in there immunodeficient mouse model (Russell *et al*, 2001). However, only a small proportion of $RAG^{-/-}$ animals exhibited evidence of AV (Russell *et al*, 2001). Interestingly, 25% of syngeneic transplants into $RAG^{-/-}$ also developed proximal coronary lesions complicating the interpretation of their results (Russell *et al*, 2001). This might have been the result of excessive graft ischemic times which are capable of promoting AV even in isografts (Furukawa *et al*, 2002). In our experimentation, given that unreconstituted recipients do not develop lesions, NK cells of the host are not alone implicated in the induction of AV. Although, indirect $CD8^+$ T cell effectors, through secretion of IFN-γ, might recruit and activate $RAG\text{-}1^{-/-}$-derived macrophages or NK cells in a DTH-like manner capable of damaging the allograft. Taken together this data indicates that non-CTL effector mechanisms might contribute to $CD8^+$ T cell mediated AV.

To more definitively clarify the existence of an indirect $CD8^+$ T cell effector pathway we designed a system whereby CTL are unable to stably interact with target cells such that all contact-dependent killing including the minor pathways of cytotoxicity are inhibited. MHC class I-restricted $CD8^+$ T cells are unable to directly interact with β2microglobulin-deficient allografts since they lack surface MHC class I expression (Zijlstra *et al*, 1990, Glas *et al*, 1992, Apasov *et al*, 1993, Qian *et al*, 1996). However, some have suggested that a small percentage of class I heavy chains, not detectable by conventional immunocytochemistry or flow cytometric analysis, are present on the cell surface in the absence of β2microglobulin (Glas *et al*, 1992, Apasov *et al*, 1993). The functional significance of these residual heavy chains is a contentious issue. β2microglobulin-deficient target cells are generally not susceptible to CTL-mediated lysis after priming with wildtype cells (Zijlstra *et al*, 1990, Glas *et al*, 1992, Apasov *et al*, 1993). Even CTL primed with β2microglobulin-deficient cells showed significantly reduced killing of MHC class I-deficient targets *in vitro* (Glas *et al*, 1992). In our hands, primed $CD8^+$ T cells generated by immunization with allogeneic wildtype cells do not kill these β2microglobulin-deficient targets *in vitro*. However, susceptibility to killing in vitro does not necessarily reflect the complex events of target recognition and CTL killing *in vivo*. Glas and co-workers have speculated that the β2microglobulin-deficient mouse is not a suitable donor for models of transplantation, since recipient derived β2microglobulin might allow for proper folding and surface MHC class I expression (Glas *et al*, 1992). However, even when we performed CTL assays in the presence of fetal bovine serum or normal mouse serum, no killing of β2microglobulin-deficient targets was observed. Indeed, these target cells were not killed when incubated with anti-

H-2D^k and complement. Moreover, Qian *et al* demonstrated that β2microglobulin-deficient cardiac and hepatic allografts exhibit prolonged survival likely on the basis of impaired CD8$^+$ T cell direct allo-recognition (Qian *et al*, 1996). Taken together, these data indicate that the β2microglobulin-deficient allograft is suitable for preventing direct CD8$^+$ T cell responses, allowing for the evaluation of contact-independent non-CTL CD8$^+$ T cell effectors.

We investigated this non-CTL effector pathway by evaluating the ability of allo-primed CD8$^+$ T cells to reject MHC I-deficient aortic allografts. In our study, primed CD8$^+$ T cells generated AV in these allografts despite the mitigation of direct killing. This data strongly supports the hypothesis that non-CTL effectors are implicated in the generation of AV in MHC I-deficient allografts, as well as in *lpr* grafts of *pfp* reconstituted RAG-1$^{-/-}$ mice in which pathways of direct cytolysis are attenuated.

Alternatively, NK cells of the immunodeficient host might be directly involved in the generation of AV, since MHC-deficient cells are particularly targeted by these innate effectors (Bix *et al*, 1991, Liao *et al*, 1991). However, MHC class I-deficient allografts from unreconstituted RAG-1$^{-/-}$ mice showed no evidence of AV with normal vessel morphology, indicating that NK cells are unable to mediate AV in the absence of primed CD8$^+$ T cells in this system. Thus, the residual class I heavy chains may not be functional as a CTL target, but may inhibit lysis mediated by NK cells. Chung and colleagues have suggested that the TCR and NK receptors recognize distinct portions of MHC molecules (Natarajan *et al*, 1999, Chung *et al*, 1999). This same group later demonstrated that the inhibitory NK cell receptor Ly49A has crucial contacts with both H-2D^d and β2microglobulin (Wang *et al*, 2002). Moreover, some groups have suggested that

allogeneic MHC class I molecules confer partial protection against NK cell reactivity (Ohlen *et al*, 1995). In addition RAG-deficient mice exhibit reduced cytotoxicity which might explain the absence of rejection in our model (Shultz *et al*, 2000).

The type 1 cytokine interferon-γ (IFN-γ) has been strongly implicated in the development of native atherosclerosis, especially in the evolution of plaque rupture leading to myocardial infarction (Gupta *et al*, 1997). Similarly, IFN-γ is involved in the development of AV, since IFN-γ-deficient recipients exhibit either no AV or markedly attenuated lesions (Russell *et al*, 1994, Raisanen-Sokolowski *et al*, 1997, Nagano *et al*, 1997, Raisanen-Sokolowski *et al*, 1998, Nagano *et al*, 1998). As such, IFN-γ is a potential candidate cytokine involved in contact-independent $CD8^+$ T cell effector mechanisms (Nagano *et al*, 1998, Delfs *et al*, 2001).

In various transplant models, graft infiltrating $CD8^+$ T lymphocytes have been demonstrated to produce significant amounts of IFN-γ (Stinn *et al*, 1998, Valujskikh *et al*, 2001). IFN-γ is integral to the recruitment and activation of macrophages, as well as NK cells, and is involved in the induction of DTH-like cell mediated responses which might potentially trigger AV (Muller *et al*, 1994). More recently, it has been suggested that IFN-γ might exert its effect on lesion cells through the potentiation of PDGF-induced mitogenesis leading to the recruitment and expansion of the neointima (Tellides *et al*, 2000). However, the experiments performed by Tellides and co-workers would predict for a donor-derived lesion (Tellides *et al*, 2000), which is in direct conflict with convincing data suggesting a recipient origin (Hillebrands *et al*, 2001, Saiura *et al*, 2001, Shimizu *et al*, 2001, Johnson *et al*, 2002, Skaro *et al*, 2002). Delfs and colleagues demonstrated that IFN-γ^{hi} Tc1 cells, when transferred into immunodeficient hosts,

generated early graft vasculitis followed by AV, whereas Tc2 cells promoted graft infiltration by eosinophils with no evidence of AV (Delfs *et al*, 2001).

In our study, the transfer of primed $CD8^+$ T cells from IFN-γ-deficient mice into RAG-$1^{-/-}$ recipients of wildtype allografts generated robust AV. This is in contrast to data from other groups which supports a critical role for host-derived IFN-γ in lesion development, as IFN-γ-deficient recipients exhibit almost complete abrogation of AV (Nagano *et al*, 1997, Raisanen-Sokolowski *et al*, 1997, Raisanen-Sokolowski *et al*, 1998). Interestingly, these IFN-γ-deficient recipients mount typical acute rejection responses directed against cardiac allografts (Nagano *et al*, 1997). Moreover, CTL from IFN-γ-deficient mice exhibit exaggerated CTL activity (Saleem *et al*, 1996), which is likely the result of reduced expression of inhibitory NK receptors (McMahon and Raulet, 2001, Malmberg *et al*, 2002). These iNKR, when ligated, potently inhibit CTL activity while inducing further IFN-γ expression (Lukacher, 2002). Thus, it is feasible that IFN-γ-deficient $CD8^+$ T cells mediate the acute injury of aortic allografts which manifests as vasculopathy in the absence of immunosuppression.

Strikingly, the adoptive transfer of allo-primed $CD8^+$ T cells from IFN-γ-deficient mice into RAG-$1^{-/-}$ recipients of MHC class I-deficient aortic allografts, where the contact-dependent direct CTL effector pathways are inhibited, did not induce AV, thus demonstrating that the observed non-CTL effector pathway is IFN-γ-dependent. These data suggest that $CD8^+$ T cell-mediated AV occurs at least in part by classical contact-dependent cytotoxicity and by an IFN-γ-dependent non-CTL effector pathway in the non-immunosuppressed aortic allograft model. Alternatively, IFN-γ-dependent up-regulation of the alleged residual MHC class I within β2microglobulin-deficient grafts might be

necessary for direct recognition and contact-dependent killing. This possibility can be ruled out by using β2microglobulin and K^b D^b double knockout mice as graft donors, such that there is no residual surface expression of MHC class Ia or Ib molecules, preventing $CD8^+$ T cell direct allo-recognition and CTL triggering. Nonetheless, Tellides and co-workers have demonstrated that injection of immunodeficient recipients with rIFN-γ leads to the generation of AV (Tellides *et al*, 2000). Taken together, this evidence supports a role for $CD8^+$ T cell-derived IFN-γ as an inciting stimulus in the development of AV.

Another possible role for IFN-γ in the development of AV might relate to chemokines which are specifically induced by this pro-inflammatory cytokine. The importance of chemokines and chemokine receptors in the recruitment and infiltration of allografts by leukocytes, which are the main effectors of AV, has been the focus of intense research (Hancock, 2002). The CXCR3 chemokine receptor has been shown to be important in the infiltration of Th1 and Tc1 cells into inflamed tissues (Nakajima *et al*, 2002). CXCR3 recognizes a family of three interferon-γ inducible chemokines which include IFN-γ-inducible protein of 10 kD (IP-10), monokine induced by IFN-γ (MIG) and interferon-inducible T cell α chemoattractant (I-TAC) (Nelson and Krensky, 2001, Hancock, 2002).

Hancock's group first examined the role of CXCR3 in the development of acute rejection and AV in cardiac allografts using CXCR3-deficient mice (Hancock *et al*, 2000). They found that CyA-treated (10 mg/kg/d) $CXC3^{-/-}$ mice exhibited permanent engraftment of fully mismatched cardiac allografts with no histological evidence of AV (Hancock *et al*, 2000). This dose of CyA was sub-therapeutic as it was only able to

prolong graft survival by 3 d in wildtype recipients (Hancock *et al*, 2000). They found a significantly reduced number of infiltrating T lymphocytes ($CD4^+$ and $CD8^+$) and macrophages in allografts from $CXCR3^{-/-}$ mice (Hancock *et al*, 2000). Similarly, they found that anti-CXCR3 monoclonal antibody was able to indefinitely prolong allograft survival in CyA-treated wildtype mice even if started after the onset of rejection (Hancock *et al*, 2000). Concurrently, rejection in wildtype mice was associated with a profound increase in the expression of IFN-γ, IP-10, I-TAC and MIG (Hancock *et al*, 2000). These data suggest that CXCR3 and its family of chemokines are important in the recruitment and activation of allo-reactive T lymphocytes capable of mediating acute rejection and AV.

The data above indicate that the important role of IFN-γ is exerted through the release of CXCR3-binding chemokines resulting in the recruitment of leukocytes which act to damage the graft (Nelson and Krensky, 2001, Hancock, 2002). Alternatively, these chemokines might be involved in the recruitment of recipient-derived lesion forming cells (α-$actin^+$ smooth muscle-like cells) from the circulation to the subendothelial space. These lesion cells may be homing to the same chemokine signals which act as chemoattractants for effector leukocytes. In support of this, Wang and colleagues demonstrated that IP-10 is a potent mitogenic and chemotactic factor for vascular smooth muscle cells *in vitro* (Wang *et al*, 1996). Moreover, IP-10 mRNA was up-regulated when smooth muscle cells were challenged *in vitro* with either IFN-γ or IL-1β (Wang *et al*, 1996). Similarly, enhanced expression of IP-10 mRNA is observed in rat carotid artery after injury mediated by balloon angioplasty (Wang *et al*, 1996). However, this latter hypothesis remains to be directly evaluated in humans or animal models of

transplantation, particularly in the setting of immunosuppression where some of these signals may be dampened.

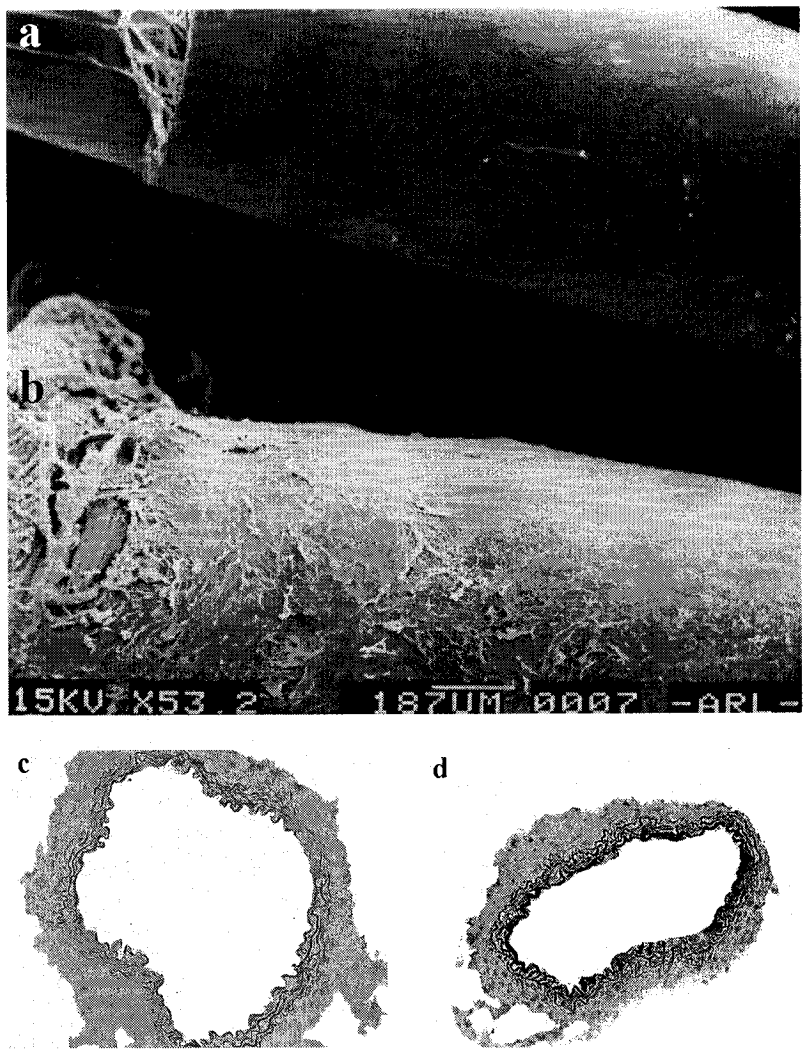
A role for $CD8^+$ effector T cells in AV may explain its resistance to calcineurin inhibitor based immunosuppressive treatments which primarily target $CD4^+$ T cell activation (Bishop *et al.*, 1992, Bujan *et al.*, 2001, Hruban *et al.*, 1990, Libby and Pober, 2001). This correlates with our evidence (Johnson *et al.*, 2002, Légaré *et al*, 2001) and the evidence of others (Hillebrands *et al.*, 2001) that in animal models moderate calcineurin inhibition ablates $CD4^+$ T cell mediated acute rejection but has little to no effect on AV. Indeed, we have found that C57BL/6 CD8-deficient recipients of C3H aortic allografts exhibit no AV when treated with CyA (≥50 mg/kg/d), whereas wildtype C57BL/6 mice develop significant lesions at even higher doses (70 mg/kg/d) of CyA. To confirm the differential susceptibility of $CD4^+$ and $CD8^+$ T cell subsets to CyA, we generated primed T cell subsets under CyA-based immunosuppression (50 mg/kg/d). Following allo-activation these T cell subsets were adoptively transferred into $RAG^{-/-}$ recipients of aortic allografts treated with CyA (50 mg/kg/d). As expected, adoptive transfer of both $CD4^+$ and $CD8^+$ T cells into untreated $RAG^{-/-}$ recipients generated significant AV. However, CyA-treated $CD8^+$ T cells remained capable of inducing AV, whereas similarly treated $CD4^+$ T cells did not. This demonstrates a relationship between $CD8^+$ T cell effector function and AV in the context of effective $CD4^+$ T cell immunosuppression. These results will need clarification in the presence of more clinically-relevant triple therapy, which is widely used after clinical heart transplantation. However, given the non-specific modes of action of anti-proliferative agents (mycophenolate mofetil and azathioprine) and corticosteroids, more complete $CD8^+$ T

cell inhibition is unlikely. Moreover, the activity of non-CTL effector $CD8^+$ T cells are particularly implicated in the failure of modern transplant pharmacotherapy, since indirect pathways, in general, are thought to be most resistant to calcineurin inhibition (Lee *et al*, 2001, Sawyer *et al*, 1993, Hornick *et al*, 2000).

Appendix

7.0 Appendix 1. *Creation of a chimeric aortic segment.*

Representative scanning electron photomicrographs of C3H aortic segments treated with EDTA (**a**) or left untreated (**b**). To confirm de-endothelialization, EDTA-treated (**c**) and untreated (**d**) aortas were immunostained with anti-PECAM monoclonal antibody. EDTA-treated vessels exhibit complete denudation of the intima. These denuded aortic segments will be re-endothelialized in $RAG^{-/-}$ and then re-transplanted into either wildtype C57BL/6 or C3H mice to assess the role of endothelial *versus* medial damage in the development of AV.



APPENDIX 1

References

8.0 References

1. Abraham N, Miceli MC, Parnes JR, Veillette A. Enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase p56lck. *Nature.* 1991;350:62-6.

2. Abraham RT, Wiederrecht GJ. Immunopharmacology of rapamycin. *Annu Rev Immunol.* 1996;14:483-510.

3. Adams DH, Tilney NL, Collins JJ Jr, Karnovsky MJ. Experimental graft arteriosclerosis. I. The Lewis-to-F-344 allograft model. *Transplantation.* 1992;53:1115-9.

4. Aktas O, Waiczies S, Smorodchenko A, Dorr J, Seeger B, Prozorovski T, Sallach S, Endres M, Brocke S, Nitsch R, Zipp F. Treatment of relapsing paralysis in experimental encephalomyelitis by targeting Th1 cells through atorvastatin. *J Exp Med.* 2003;197:725-33.

5. Akyurek LM, Paul LC, Funa K, Larsson E, Fellstrom BC. Smooth muscle cell migration into intima and adventitia during development of transplant vasculopathy. *Transplantation.* 1996;62:1526-9.

6. Alfrey EJ, Wang X, Lee L, Holm B, Kim J, Adams G, Dafoe DC. Tolerance induced by direct inoculation of donor antigen into the thymus in low and high responder rodents. *Transplantation.* 1995;59:1171-6.

7. Allan J, Cho J, Vesga L, Arn J, Pins M, Sachs D, Madsen J. Cardiac allograft vasculopathy is abrogated by anti-CD8 monoclonal antibody therapy. *Ann Thorac Surg.* 1997;64:1019-25.

8. Allison AC, Eugui EM. Purine metabolism and immunosuppressive effects of mycophenolate mofetil (MMF). *Clin Transplant.* 1996;10:77-84.

9. Alonso DR, Starek PK, Minick CR. Studies on the pathogenesis of atheroarteriosclerosis induced in rabbit cardiac allografts by the synergy of graft rejection and hypercholesterolemia. *Am J Pathol.* 1977;87:415-42.

10. Alpert S, Lewis NP, Ross H, Fowler M, Valantine HA. The relationship of granzyme A and perforin expression to cardiac allograft rejection and dysfunction. *Transplantation.* 1995;60:1478-85.

11. Andersen HO, Holm P, Stender S, Hansen BF, Kjeldsen K, and Nordestgaard BG. Relative importance of ischemic injury and immunological injury on the development of transplant arteriosclerosis in rabbit aortic allografts. *Transplantation.* 1995;60:631-8.

12. Andrus L, Granelli-Piperno A, Reich E. Cytotoxic T cells both produce and respond to interleukin 2. *J Exp Med.* 1984;159:647-52.

13. Apasov S, and Sitkovsky M. Highly lytic CD8+ alpha beta T-cell receptor cytotoxic T cells with major histocompatibility complex (MHC) class I antigen-directed cytotoxicity in beta 2-microglobulin, MHC class I-deficient mice. *Proc Natl Acad Sci U S A.* 1993;90:2837-41.

14. Assarsson E, Kambayashi T, Sandberg JK, Hong S, Taniguchi M, Van Kaer L, Ljunggren HG, Chambers BJ. CD8+ T cells rapidly acquire NK1.1 and NK cell-associated molecules upon stimulation in vitro and in vivo. *J Immunol.* 2000;165:3673-9.

15. Attur MG, Patel R, Thakker G, Vyas P, Levartovsky D, Patel P, Naqvi S, Raza R, Patel K, Abramson D, Bruno G, Abramson SB, Amin AR. Differential anti inflammatory effects of immunosuppressive drugs: cyclosporin, rapamycin and FK-506 on inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2 and PGE2 production. *Inflamm Res.* 2000;49:20-6.

16. Azuma H, Nadeau K, Mackenzie HS, Brenner BM, Tilney NL. Nephron mass modulates the hemodynamic, cellular, and molecular response of the rat renal allograft. *Transplantation.* 1997;63:519-28.

17. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. *Annu Rev Immunol.* 1994;12:141-79.

18. Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol.* 1996;14:649-83.

19. Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S. The CD40 antigen and its ligand. *Annu Rev Immunol.* 1994;12:881-922.

20. Barnes PJ, Karin M. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med.* 1997;336:1066-71.

21. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol.* 2002;2:401-9.

22. Baxter AG, Hodgkin PD. Activation rules: the two-signal theories of immune activation. *Nat Rev Immunol.* 2002;2:439-46.

23. Baylis C, Qiu C. Importance of nitric oxide in the control of renal hemodynamics. *Kidney Int.* 1996;49:1727-31.

24. Bennett WM, DeMattos A, Meyer MM, Andoh T, Barry JM. Chronic cyclosporine nephropathy: the Achilles' heel of immunosuppressive therapy. *Kidney Int.* 1996;50:1089-100.

25. Berke G. The CTL's kiss of death. *Cell.* 1995;81:9-12.

26. Billingham ME. Pathology and etiology of chronic rejection of the heart. *Clin Transplant.* 1994;8:289-292.

27. Binder CJ, Chang MK, Shaw PX, Miller YI, Hartvigsen K, Dewan A, Witztum JL. Innate and acquired immunity in atherogenesis. *Nat Med.* 2002;8:1218-26.

28. Binder J, Lehmann M, Graser E, Hancock WW, Watschinger B, Onodera K, Sayegh MH, Volk HD, Kupiec-Weglinski JW. The effects of nondepleting CD4 targeted therapy in presensitized rat recipients of cardiac allografts. *Transplantation.* 1996;61:804-11.

29. Bishop DK, and Li W. Cyclosporin A and FK506 mediate differential effects on T cell activation in vivo. *J Immunol.* 1992;148:1049-54.

30. Bishop DK, Chan S, Li W, Ensley RD, Xu S, Eichwald EJ. CD4-positive helper T lymphocytes mediate mouse cardiac allograft rejection independent of donor alloantigen specific cytotoxic T lymphocytes. *Transplantation.* 1993;56:892-7.

31. Bishop DK, Chan Wood S, Eichwald EJ, Orosz CG. Immunobiology of allograft rejection in the absence of IFN-gamma: CD8+ effector cells develop independently of CD4+ cells and CD40-CD40 ligand interactions. *J Immunol.* 2001;166:3248-55.

32. Bishop DK, Li W, Chan SY, Ensley RD, Shelby J, Eichwald EJ. Helper T lymphocyte unresponsiveness to cardiac allografts following transient depletion of CD4-positive cells. Implications for cellular and humoral responses. *Transplantation.* 1994;58:576-84.

33. Bix M, Liao NS, Zijlstra M, Loring J, Jaenisch R, Raulet D. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. *Nature.* 1991;349:329-31.

34. Bjorkerud, S. Reaction of aortic wall of the rabbit after superficial longitudinal mechanical trauma. *Virchows Arch.* 1969;347:197-210.

35. Blazar BR, Taylor PA, Noelle RJ, Vallera DA. CD4(+) T cells tolerized ex vivo to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. *J Clin Invest.* 1998;102:473-82.

36. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, Buhlman J, Xu J, Flavell RA, Korngold R, Noelle R, Vallera DA. Blockade of CD40 ligand-CD40 interaction impairs CD4+ T cell-mediated alloreactivity by inhibiting mature donor T cell expansion and function after bone marrow transplantation. *J Immunol.* 1997;158:29-39.

37. Bojakowski K, Reglia P, Bojakowska M, Hedin U, Gaciong Z, Thyberg J. Arteriosclerosis in rat aortic allografts: early changes in endothelial integrity and smooth muscle phenotype. *Transplantation.* 2000;70:65-72.

38. Bolton EM, Gracie JA, Briggs JD, Kampinga J, Bradley JA. Cellular requirements for renal allograft rejection in the athymic nude rat. *J Exp Med.* 1989;169:1931-46.

39. Borrow P, Tishon A, Lee S, Xu J, Grewal IS, Oldstone MB, Flavell RA. CD40L-deficient mice show deficits in antiviral immunity and have an impaired memory CD8+ CTL response. *J Exp Med.* 1996;183:2129-42.

40. Bourgeois C, Rocha B, Tanchot C. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science.* 2002;297:2060-3.

41. Bretscher P, Cohn M. A theory of self-nonself discrimination. *Science.* 1970;169:1042-9.

42. Bretscher PA, Havele C. Cyclosporin A can switch the immune response induced by antigen from a humoral to a cell-mediated mode. *Eur J Immunol.* 1992;22:349-55.

43. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, Lane WS, Schreiber SL. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature.* 1994;369:756-8.

44. Buller RM, Holmes KL, Hugin A, Frederickson TN, Morse HC 3rd. Induction of cytotoxic T-cell responses in vivo in the absence of CD4 helper cells. *Nature.* 1987;328:77-9.

45. Bujan J, Jurado F, Gimeno MJ, Rodriguez M, Bellon JMFunction of inflammatory cells and neoral cyclosporin-A in heart transplant-associated coronary vasculopathy. *Histol Histopathol.* 2001;16:197-203.

46. Calne RY, White DJ, Thiru S, Evans DB, McMaster P, Dunn DC, Craddock GN, Pentlow BD, Rolles K. Cyclosporin A in patients receiving renal allografts from cadaver donors. *Lancet.* 1978;2:1323-7.

47. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol.* 2002;20:29-53.

48. Chan AC, Desai DM, Weiss A. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu Rev Immunol.* 1994;12:555-92.

49. Chen RH, Bushell A, Fuggle SV, Wood KJ, Morris PJ. Expression of granzyme A and perforin in mouse heart transplants immunosuppressed with donor-specific transfusion and anti-CD4 monoclonal antibody. *Transplantation.* 1996;61:625-9.

50. Chen RH, Ivens KW, Alpert S, Billingham ME, Fathman CG, Flavin TF, Shizuru JA, Starnes VA, Weissman IL, Griffiths GM. The use of granzyme A as a marker of heart transplant rejection in cyclosporine or anti-CD4 monoclonal antibody-treated rats. *Transplantation.* 1993;55:146-53.

51. Chen WF, Zlotnik A. IL-10: a novel cytotoxic T cell differentiation factor. *J Immunol.* 1991;147:528-34.

52. Chirathaworn C, Kohlmeier JE, Tibbetts SA, Rumsey LM, Chan MA, Benedict SH. Stimulation through intercellular adhesion molecule-1 provides a second signal for T cell activation. *J Immunol.* 2002;168:5530-7.

53. Chong AS, Finnegan A, Jiang X, Gebel H, Sankary HN, Foster P, Williams JW. Leflunomide, a novel immunosuppressive agent. The mechanism of inhibition of T cell proliferation. *Transplantation.* 1993;55:1361-6.

54. Chow LH, Huh S, Jiang J, Zhong R, Pickering JG. Intimal thickening develops without humoral immunity in a mouse aortic allograft model of chronic vascular rejection. *Circulation.* 1996;94:3079-82.

55. Chung DH, Dorfman J, Plaksin D, Natarajan K, Belyakov IM, Hunziker R, Berzofsky JA, Yokoyama WM, Mage MG, Margulies DH. NK and CTL recognition of a single chain H-2Dd molecule: distinct sites of H-2Dd interact with NK and TCR. *J Immunol.* 1999;163:3699-708.

56. Clarke SR. The critical role of CD40/CD40L in the CD4-dependent generation of CD8+ T cell immunity. *J Leukoc Biol.* 2000;67:607-14.

57. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. *Annu Rev Immunol.* 1988;6:629-62.

58. Clowes A, Reidy M, Clowes M. Kinetics of cellular proliferation after arterial injury. I. Smooth muscle growth in the absence of endothelium. *Lab Invest.* 1983;49:327-33.

59. Clowes AW, Ryan GB, Breslow JL, Karnovsky MJ. Absence of enhanced intimal thickening in the response of the carotid arterial wall to endothelial injury in hypercholesterolemic rats. *Lab Invest.* 1976;35:6-17.

60. Cobbold SP, Graca L, Lin CY, Adams E, Waldmann H. Regulatory T cells in the induction and maintenance of peripheral transplantation tolerance. *Transpl Int.* 2003;16:66-75.

61. Combates NJ, Degiannis D, Raskova J, Raska K Jr. Direct inhibition of human CD8+ lymphocyte activation by cyclosporine A and Rapamune-Sirolimus. *Clin Immunol Immunopathol.* 1995;77:221-8.

62. Cooper D, Bansal-Pakala P, Croft M. 4-1BB (CD137) controls the clonal expansion and survival of CD8 T cells in vivo but does not contribute to the development of cytotoxicity. *Eur J Immunol.* 2002;32:521-9.

63. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973;16:343-50.

64. Crespo-Leiro MG, Paniagua MJ, Mosquera I, Tabuyo T, De la Fuente L, Bouzas B, Rodriguez JA, Hermida LF, Juffe A, Castro-Beiras A. Replacement of cyclosporine by tacrolimus for immunosuppression in heart transplant patients: safety and efficacy. *Transplant Proc.* 2002;34:113-4.

65. Dai Z, Konieczny BT, Lakkis FG. The dual role of IL-2 in the generation and maintenance of CD8+ memory T cells. *J Immunol.* 2000;165:3031-6.

66. Dallman MJ. Cytokines as mediators of organ graft rejection and tolerance. *Curr Opin Immunol.* 1993;5:788-93.

67. Dalloul AH, Ngo K, Fung-Leung WP. CD4-negative cytotoxic T cells with a T cell receptor alpha/beta intermediate expression in CD8-deficient mice. Eur *J Immunol.* 1996;26:213-8.

68. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science.* 1993;259:1739-42.

69. Dansky HM, Charlton SA, Harper MM, Smith JD. T and B lymphocytes play a minor role in atherosclerotic plaque formation in the apolipoprotein E-deficient mouse. *Proc Natl Acad Sci U S A.* 1997;94:4642-6.

70. Daugherty A, Pure E, Delfel-Butteiger D, Chen S, Leferovich J, Roselaar SE, Rader DJ. The effects of total lymphocyte deficiency on the extent of atherosclerosis in apolipoprotein E-/- mice. *J Clin Invest.* 1997;100:1575-80.

71. Davies MJ. Acute coronary thrombosis--the role of plaque disruption and its initiation and prevention. *Eur Heart J.* 1995;16:3-7.

72. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature.* 1988;334:395-402.

73. de Groot-Kruseman HA, Baan CC, Mol WM, Niesters HG, Maat AP, Balk AH, Weimar W. *Transpl Immunol.* 1999;7:201-5.

74. Delfs MW, Furukawa Y, Mitchell RN, Lichtman AH. $CD8^+$ T cell subsets TC1 and TC2 cause different histopathologic forms of murine cardiac allograft rejection. *Transplantation.* 2001;71:606-10.

75. Dengler TJ, Pober JS. Cellular and molecular biology of cardiac transplant rejection. *J Nucl Cardiol.* 2000;7:669-85.

76. Denton MD, Magee CC, Sayegh MH. Immunosuppressive strategies in transplantation. *Lancet.* 1999;353:1083-91.

77. Djamali A, and Odorico JS. Fas-mediated cytotoxicity is not required for rejection of murine nonvascularized heterotopic cardiac allografts. *Transplantation.* 1998;66:1793-801.

78. Eich D, Thompson JA, Ko DJ, Hastillo A, Lower R, Katz S, Katz M, Hess ML. Hypercholesterolemia in long-term survivors of heart transplantation: an early marker of accelerated coronary artery disease. *J Heart Lung Transplant.* 1991;10:45-9.

79. Ensminger S, Witzke O, Spriewald B, Morrison K, Morris P, Rose M, Wood K. CD8+ T cells contribute to the development of transplant arteriosclerosis despite CD154 blockade. *Transplantation.* 2000;69:2609-12.

80. Ensminger SM, Billing JS, Morris PJ, Wood KJ. Development of a combined cardiac and aortic transplant model to investigate the development of transplant arteriosclerosis in the mouse. *J Heart Lung Transplant.* 2000;19:1039-46.

81. Ensminger SM, Spriewald BM, Witzke O, Pajaro OE, Yacoub MH, Morris PJ, Rose ML, Wood KJ. Indirect allorecognition can play an important role in the development of transplant arteriosclerosis. *Transplantation.* 2002;73:279-286.

82. Eriksson H. Heart failure: a growing public health problem. *J Intern Med.* 1995;237:135-41.

83. Ewel CH, Foegh ML. Chronic graft rejection: accelerated transplant arteriosclerosis. *Immunol Rev.* 1993;134:21-31.

84. Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R. Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science.* 1991;253:1129-32.

85. Ferns GA, Reidy MA, Ross R. Balloon catheter de-endothelialization of the nude rat carotid. Response to injury in the absence of functional T lymphocytes. *Am J Pathol.* 1991;138:1045-57.

86. Ferraresso M, Tian L, Ghobrial R, Stepkowski SM, Kahan BD. Rapamycin inhibits production of cytotoxic but not noncytotoxic antibodies and preferentially activates T helper 2 cells that mediate long-term survival of heart allografts in rats. *J Immunol.* 1994;153:3307-18.

87. Ferrari-Lacraz S, Zheng XX, Kim YS, Li Y, Maslinski W, Li XC, Strom TB. An antagonist IL-15/Fc protein prevents costimulation blockade-resistant rejection. *J Immunol.* 2001;167:3478-85.

88. Forbes R, Zheng S, Gomersall M, Al-Saffar M, Guttmann R. Evidence that recipient $CD8^+$ T cell depletion does not alter development of chronic vascular rejection in a rat heart allograft model. *Transplantation.* 1994;57:1238-46.

89. Fukuo K, Hata S, Suhara T, Nakahashi T, Shinto Y, Tsujimoto Y, Morimoto S, Ogihara T. Nitric oxide induces upregulation of Fas and apoptosis in vascular smooth muscle. *Hypertension.* 1996;27:823-6.

90. Furukawa Y, Becker G, Stinn JL, Shimizu K, Libby P, Mitchell RN. Interleukin-10 (IL-10) augments allograft arterial disease: paradoxical effects of IL-10 in vivo. *Am J Pathol.* 1999;155:1929-39.

91. Furukawa Y, Libby P, Stinn JL, Becker G, Mitchell RN. Cold ischemia induces isograft arteriopathy, but does not augment allograft arteriopathy in non-immunosuppressed hosts. *Am J Pathol.* 2002;160:1077-87.

92. Fyfe AI, Stevenson LW, Harper CM, Drinkwater DC, Laks H, Fogelman AM, Berliner JA. Recipient mononuclear cell recognition and adhesion to graft endothelium after human cardiac transplantation. Lymphocyte recognition leads to monocyte adhesion. *J Clin Invest.* 1994;94:2142-7.

93. Gao D, Li J, Orosz CG, Bumgardner GL. Different costimulation signals used by CD4(+) and CD8(+) cells that independently initiate rejection of allogenic hepatocytes in mice. *Hepatology.* 2000;32:1018-28.

94. Garcia KC, Scott CA, Brunmark A, Carbone FR, Peterson PA, Wilson IA, Teyton L. CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature.* 1996;384:577-81.

95. Gaudin PB, Rayburn BK, Hutchins GM, Kasper EK, Baughman KL, Goodman SN, Lecks LE, Baumgartner WA, and Hruban RH. Peritransplant injury to the myocardium associated with the development of accelerated arteriosclerosis in heart transplant recipients. *Am J Surg Pathol.* 1994;18:338-346.

96. Ge Q, Hu H, Eisen HN, and Chen J. Different contributions of thymopoiesis and homeostasis-driven proliferation to the reconstitution of naive and memory T cell compartments. *Proc Natl Acad Sci USA.* 2002;99:2989-2994.

97. Geng YJ, Henderson LE, Levesque EB, Muszynski M, Libby P. Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1997;17:2200-8.

98. Geraghty JG, Stoltenberg RL, Sollinger HW, Hullett DA. Vascular smooth muscle cells and neointimal hyperplasia in chronic transplant rejection. *Transplantation.* 1996;62:502-9.

99. Gett AV, Sallusto F, Lanzavecchia A, Geginat J. T cell fitness determined by signal strength. *Nat Immunol.* 2003;4:355-60.

100. Ghosh P, Buchholz MA, Yano S, Taub D, Longo DL. Effect of rapamycin on the cyclosporin A-resistant CD28-mediated costimulatory pathway. *Blood.* 2002;99:4517-24.

101. Glas R, Franksson L, Ohlen C, Hoglund P, Koller B, Ljunggren HG, Karre K. Major histocompatibility complex class I-specific and -restricted killing of β_2-microglobulin-deficient cells by $CD8^+$ cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A.* 1992;89:11381.

102. Gobin SJ, van Zutphen M, Woltman AM, van den Elsen PJ. Transactivation of classical and nonclassical HLA class I genes through the IFN-stimulated response element. *J Immunol.* 1999;163:1428-34.

103. Goldrath AW, Bogatzki LY, and Bevan MJ. Naive T cells transiently acquire a memory-like phenotype during homeostasis-driven proliferation. *J Exp Med.* 2000;192:557-564.

104. Gould DS, Auchincloss H Jr. Direct and indirect recognition: the role of MHC antigens in graft rejection. *Immunol Today.* 1999;20:77-82.

105. Gregory CR, Huie P, Billingham ME, Morris RE. Rapamycin inhibits arterial intimal thickening caused by both alloimmune and mechanical injury. Its effect on cellular, growth factor, and cytokine response in injured vessels. *Transplantation.* 1993;55:1409-18.

106. Grewal IS, Foellmer HG, Grewal KD, Xu J, Hardardottir F, Baron JL, Janeway CA Jr, Flavell RA. Requirement for CD40 ligand in costimulation induction, T cell activation, and experimental allergic encephalomyelitis. *Science.* 1996;273:1864-7.

107. Grewal IS, Xu J, Flavell RA. Impairment of antigen-specific T-cell priming in mice lacking CD40 ligand. *Nature.* 1995;378:617-20.

108. Grimm PC, Nickerson P, Heffrey J, Savani RC, Gough J, McKenna RM, Stern E, Rush DN. Neointimal and tubulointerstitial infiltration by recipient mesenchymal cells in chronic renal allograft rejection. *N Engl J Med.* 2001;345:93-7.

109. Grusby M, Auchincloss HJ, Lee R, Johnson RS, Spencer JP, Zijlstra M, Jaenisch R, Papaioannou VE, Glimcher LH. Mice lacking major histocompatibility complex class I and class II molecules. *Proc Natl Acad Sci U S A.* 1993;90:3913.

110. Guo Z, Meng L, Kim O, Wang J, Hart J, He G, Alegre ML, Thistlethwaite JR Jr, Pearson TC, Larsen CP, Newell KA. CD8 T cell-mediated rejection of intestinal allografts is resistant to inhibition of the CD40/CD154 costimulatory pathway. *Transplantation.* 2001;71:1351-4.

111. Gupta S, Pablo AM, Jiang X, Wang N, Tall AR, Schindler C. IFN-gamma potentiates atherosclerosis in ApoE knock-out mice. *J Clin Invest.* 1997;99:2752-61.

112. Ha J, Bingaman AW, Durham MM, Pearson TC, Larsen CP. Aggressive skin allograft rejection in CD28-/- mice independent of the CD40/CD40L costimulatory pathway. *Transpl Immunol.* 2001;9:13-7.

113. Halamay KE, Kirkman RL, Sun L, Yamada A, Fragoso RC, Shimizu K, Mitchell RN, McKay DB. CD8 T cells are sufficient to mediate allorecognition and allograft rejection. *Cell Immunol.* 2002;216:6-14.

114. Hall BM, Dorsch S, Roser B. The cellular basis of allograft rejection in vivo. I. The cellular requirements for first-set rejection of heart grafts. *J Exp Med.* 1978;148:878-89.

115. Hall BM, Gurley KE, Dorsch SE. The possible role of cytotoxic T cells in the mediation of first-set allograft rejection. *Transplantation.* 1985;40:336-9.

116. Hall BM, Pearce NW, Gurley KE, Dorsch SE. Specific unresponsiveness in rats with prolonged cardiac allograft survival after treatment with cyclosporine: III. Further characterization of the CD4+ suppressor cell and its mechanisms of action. *J Exp Med.* 1990;171:141-57.

117. Hall BM. Cells mediating allograft rejection. *Transplantation.* 1991;51:1141-51.

118. Halloran PF, Miller LW, Urmson J, Ramassar V, Zhu LF, Kneteman NM, Solez K, Afrouzian M. IFN-gamma alters the pathology of graft rejection: protection from early necrosis. *J Immunol.* 2001;166:7072-81.

119. Han WR, Zhan Y, Murray-Segal LJ, Brady JL, Lew AM, Mottram PL. Prolonged allograft survival in anti-CD4 antibody transgenic mice: lack of residual helper T cells compared with other CD4-deficient mice. *Transplantation.* 2000;70:168-74.

120. Hancock WW, Buelow R, Sayegh MH, Turka LA. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti- apoptotic genes. *Nat Med.* 1998;4:1392-6.

121. Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD. Donor-derived IP-10 initiates development of acute allograft rejection. *J Exp Med.* 2001;193:975-80.

122. Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, Smiley ST, Ling M, Gerard NP, Gerard C. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J Exp Med.* 2000;192:1515-20.

123. Hancock WW. Chemokines and transplant immunobiology. *J Am Soc Nephrol.* 2002;13:821-4.

124. Hansson GK, Hellstrand M, Rymo L, Rubbia L, and Gabbiani G. Interferon gamma inhibits both proliferation and expression of differentiation-specific alpha-smooth muscle actin in arterial smooth muscle cells. *J Exp Med.* 1989;170:1595-608.

125. Hansson GK, Libby P, Schonbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. *Circ Res.* 2002;91:281-91.

126. Hansson GK. Immune mechanisms in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2001;21:1876-90.

127. Hare GM, Lee TD, Jordan JL, Hirsch GM. Medial cell apoptosis precedes the development of intimal hyperplasia after transient allogeneic exposure in a rat aortic allograft model of chronic rejection. *Transplant Proc.* 1999;31:1384-5.

128. Hasegawa S, Becker G, Nagano H, Libby P, Mitchell RN. Pattern of graft- and host-specific MHC class II expression in long-term murine cardiac allografts: origin of inflammatory and vascular wall cells. *Am J Pathol.* 1998;153:69-79.

129. Hathcock KS, Laszlo G, Pucillo C, Linsley P, Hodes RJ. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J Exp Med.* 1994;180:631-40.

130. Häyry P, Alatalo S, Myllarniem M, Raisanen-Sokolowski A, Lemstrom L. Cellular and molecular biology of chronic rejection. *Transplant Proc.* 1995;27:71-4.

131. Häyry P, Isoniemi H, Yilmaz S, Mennander A, Lemstrom K, Raisanen-Sokolowski A, Koskinen P, Ustinov J, Lautenschlager I, Taskinen E. Chronic allograft rejection. *Immunol Rev.* 1993;134:33-81.

132. Häyry P, Mennander A, Yilmaz S, Ustinov J, Raisanen A, Miettinen A, Lautenschlager I, Lemstrom K, Bruggeman CA, Paavonen T. Towards understanding the pathophysiology of chronic rejection. *Clin Invest.* 1992;70:780-90.

133. Häyry P, Myllarniemi M, Aavik E, Alatalo S, Aho P, Yilmaz S, Raisanen-Sokolowski A, Cozzone G, Jameson BA, Baserga R. Stabile D-peptide analog of insulin-like growth factor-1 inhibits smooth muscle cell proliferation after carotid ballooning injury in the rat. *FASEB J.* 1995;9:1336-44.

134. Heinzel FP, Sadick MD, Holaday BJ, Coffman RL, Locksley RM. Reciprocal expression of interferon gamma or interleukin 4 during the resolution or progression of murine leishmaniasis. Evidence for expansion of distinct helper T cell subsets. *J Exp Med.* 1989;169:59-72.

135. Hermiston ML, Xu Z, Weiss A. CD45: A Critical Regulator of Signaling Thresholds in Immune Cells. *Annu Rev Immunol.* 2003;21:107-37.

136. Hillebrands JL, Klatter FA, Rozing J. Origin of vascular smooth muscle cells and the role of circulating stem cells in transplant arteriosclerosis. *Arterioscler Thromb Vasc Biol.* 2003;23:380-7.

137. Hillebrands JL, Klatter FA, van den Hurk BM, Popa ER, Nieuwenhuis P, Rozing J. Origin of neointimal endothelium and alpha-actin-positive smooth muscle cells in transplant arteriosclerosis. *J Clin Invest.* 2001;107:1411-22.

138. Hirsch G, Kearsey J, Burt T, Karnovsky MJ, Lee TDG. Medial smooth muscle cell loss in allografted arteries occurs by cytotoxic cell induced apoptosis. *Eur J Cardiothorac Surg.* 1998;14:89-97.

139. Honey K, Cobbold SP, Waldmann H. CD40 ligand blockade induces CD4+ T cell tolerance and linked suppression. *J Immunol.* 1999;163:4805-10.

140. Hornick PI, Mason PD, Baker RJ, Hernandez-Fuentes M, Frasca L, Lombardi G, Taylor K, Weng L, Rose ML, Yacoub MH, Batchelor R, Lechler RI. Significant frequencies of T cells with indirect anti-donor specificity in heart graft recipients with chronic rejection. *Circulation.* 2000;101:2405-10.

141. Horsburgh T, Wood P, Brent L. Suppression of in vitro lymphocyte reactivity by cyclosporin A: existence of a population of drug-resistant cytotoxic lymphocytes. *Nature.* 1980;286:609-11.

142. Horwitz MS, Evans CF, McGavern DB, Rodriguez M, Oldstone MB. Primary demyelination in transgenic mice expressing interferon-gamma. *Nat Med.* 1997;3:1037-41.

143. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: Eighteenth Official Report-2001. *J Heart Lung Transplant.* 2001;20:805-15.

144. Hruban RH, Beschorner WE, Baumgartner WA, Augustine SM, Ren H, Reitz BA, Hutchins GM. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. *Am J Pathol.* 1990;137:871-82.

145. Huang LR, Chen FL, Chen YT, Lin YM, Kung JT. Potent induction of long-term CD8+ T cell memory by short-term IL-4 exposure during T cell receptor stimulation. *Proc Natl Acad Sci U S A.* 2000;97:3406-11.

146. Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, Kroczek RA. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature*. 1999;397:263-6.

147. Ikonen TS, Gummert JF, Hayase M, Honda Y, Hausen B, Christians U, Berry GJ, Yock PG, Morris RE. Sirolimus (rapamycin) halts and reverses progression of allograft vascular disease in non-human primates. *Transplantation*. 2000;70:969-75.

148. Ikonen TS, Gummert JF, Serkova N, Hayase M, Honda Y, Kobayase Y, Hausen B, Yock PG, Christians U, Morris RE. Efficacies of sirolimus (rapamycin) and cyclosporine in allograft vascular disease in non-human primates: trough levels of sirolimus correlate with inhibition of progression of arterial intimal thickening. *Transpl Int*. 2000;13:314-20.

149. Illigens BM, Yamada A, Fedoseyeva EV, Anosova N, Boisgerault F, Valujskikh A, Heeger PS, Sayegh MH, Boehm B, Benichou G. The relative contribution of direct and indirect antigen recognition pathways to the alloresponse and graft rejection depends upon the nature of the transplant. *Hum Immunol*. 2002;63:912-25.

150. Inaba K, Young JW, Steinman RM. Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. *J Exp Med*. 1987;166:182-94.

151. Isakov N, Altman A. Protein kinase C(theta) in T cell activation. A*nnu Rev Immunol*. 2002;20:761-94.

152. Isoniemi HM, Ahonen J, Tikkanen MJ, von Willebrand EO, Krogerus L, Eklund BH, Hockerstedt KV, Salmela KE, Hayry PJ. Long-term consequences of different immunosuppressive regimens for renal allografts. *Transplantation*. 1993;55:494-9.

153. Jamieson SW, Burtn NA, Bieber CP, Reitz BA, Oyer PE, Stinson EB, Shumway NE. Cardiac-allograft survival in primates treated with cyclosporin A. *Lancet*. 1979;1:545.

154. Janeway CA Jr. The T cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T cell activation. *Annu Rev Immunol*. 1992;10:645-74.

155. Jinquan T, Larsen CG, Gesser B, Matsushima K, Thestrup-Pedersen K. Human IL-10 is a chemoattractant for CD8+ T lymphocytes and an inhibitor of IL-8-induced CD4+ T lymphocyte migration. *J Immunol*. 1993;151:4545-51.

156. Johansen H, Strauss B, Arnold JM, Moe G, Liu P. On the rise: The current and projected future burden of congestive heart failure hospitalization in Canada. *Can J Cardiol.* 2003;19:430-5.

157. Johnson MR. Transplant coronary disease: nonimmunologic risk factors. *J Heart Lung Transplant.* 1992;11:124-32.

158. Johnson P, Carpenter M, Hirsch G, Lee T. Recipient cells form the intimal proliferative lesion in the rat aortic model of allograft arteriosclerosis. *Am J Transplant.* 2002;2:207-215.

159. Jones ND, Van Maurik A, Hara M, Gilot BJ, Morris PJ, Wood KJ. T-cell activation, proliferation, and memory after cardiac transplantation in vivo. *Ann Surg.* 1999;229:570-8.

160. Jones ND, van Murik A, Hara M, Spreiweld BM, Witzke O, Morris PJ, Wood KJ. CD40-CD40 ligand-independent activation of CD8+ T cells can trigger allograft rejection. *J Immunol.* 2000;165:1111-18.

161. Jones TR, Ha J, Williams MA, Adams AB, Durham MM, Rees PA, Cowan SR, Pearson TC, Larsen CP. The role of the IL-2 pathway in costimulation blockade-resistant rejection of allografts. *J Immunol.* 2002;168:1123-30.

162. Ju ST, Cui H, Panka DJ, Ettinger R, and Marshak-Rothstein A. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. *Proc Natl Acad Sci USA.* 1994;91:4185-89.

163. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature.* 1994;369:31-7.

164. Kagi D, Vignaux F, Ledermann B, Burki K, Depraetere V, Nagata S, Hengartner H, Golstein P. Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. *Science.* 1994;265:528-30.

165. Kanegane H, Tosato G. Activation of naive and memory T cells by interleukin-15. *Blood.* 1996;88:230-5.

166. Karck M, Meliss R, Hestermann M, Mengel M, Pethig K, Levitzki A, Banai S, Golomb G, Fishbein I, Chorny M, Haverich A. Inhibition of aortic allograft vasculopathy by local delivery of platelet-derived growth factor receptor tyrosine-kinase blocker AG-1295. *Transplantation.* 2002;74:1335-41.

167. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol.* 2000;18:621-63.

168. Katznelson S, Kobashigawa JA. Dual roles of HMG-CoA reductase inhibitors in solid organ transplantation: lipid lowering and immunosuppression. *Kidney Int Suppl.* 1995;52:112-5.

169. Katznelson S, Wang XM, Chia D, Ozawa M, Zhong HP, Hirata M, Terasaki PI, Kobashigawa JA. The inhibitory effects of pravastatin on natural killer cell activity in vivo and on cytotoxic T lymphocyte activity in vitro. *J Heart Lung Transplant.* 1998;17:335-40.

170. Kawai K, Shahinian A, Mak TW, Ohashi PS. Skin allograft rejection in CD28-deficient mice. *Transplantation.* 1996;61:352-5.

171. Kearney JF. Immune recognition of OxLDL in atherosclerosis. *J Clin Invest.* 2000;105:1683-5.

172. Kennedy LJ Jr, Weissman IL. Dual origin of intimal cells in cardiac-allograft arteriosclerosis. *N Engl J Med.* 1971;285:884-7.

173. Khanna A, Plummer M, Bromberek C, Bresnahan B, Hariharan S. Expression of TGF-beta and fibrogenic genes in transplant recipients with tacrolimus and cyclosporine nephrotoxicity. *Kidney Int.* 2002;62:2257-63.

174. Khanna AK, Hosenpud JS, Plummer MS, Hosenpud JD. Analysis of transforming growth factor-beta and profibrogenic molecules in a rat cardiac allograft model treated with cyclosporine. *Transplantation.* 2002;73:1543-9.

175. Kieper WC, Tan JT, Bondi-Boyd B, Gapin L, Sprent J, Ceredig R, Surh CD. Overexpression of interleukin (IL)-7 leads to IL-15-independent generation of memory phenotype CD8+ T cells. *J Exp Med.* 2002;195:1533-9.

176. Kishimoto K, Sandner S, Imitola J, Sho M, Li Y, Langmuir PB, Rothstein DM, Strom TB, Turka LA, Sayegh MH. Th1 cytokines, programmed cell death, and alloreactive T cell clone size in transplant tolerance. *J Clin Invest.* 2002;109:1471-9.

177. Knight RJ, Dikman S, Liu H, Martinelli GP. Cold ischemic injury accelerates the progression to chronic rejection in a rat cardiac allograft model. *Transplantation.* 1997;64:1102-7.

178. Kobashigawa JA, Katznelson S, Laks H, Johnson JA, Yeatman L, Wang XM, Chia D, Terasaki PI, Sabad A, Cogert GA, et al. Effect of pravastatin on outcomes after cardiac transplantation. *N Engl J Med.* 1995;333:621-7.

179. Koglin J, Glysing-Jensen T, Gadiraju S, Russell ME. Attenuated cardiac allograft vasculopathy in mice with targeted deletion of the transcription factor STAT4. *Circulation.* 2000;101:1034-9.

180. Koglin J, Glysing-Jensen T, Raisanen-Sokolowski A, Russell ME. Immune sources of transforming growth factor-beta1 reduce transplant arteriosclerosis: insight derived from a knockout mouse model. *Circ Res.* 1998;83:652-60.

181. Koglin J, Russell M. Alloimmune mediated apoptosis: comparison in mouse models of acute and chronic cardiac rejection. *Transplantation.* 1999;67:904-9.

182. Kohlmeier JE, Rumsey LM, Chan MA, Benedict SH. The outcome of T-cell costimulation through intercellular adhesion molecule-1 differs from costimulation through leucocyte function-associated antigen-1. *Immunology.* 2003;108:152-7.

183. Kolb F, Heudes D, Mandet C, Plissonnier D, Osborne-Pellegrin M, Bariety J, Michel JB. Presensitization accelerates allograft arteriosclerosis. *Transplantation.* 1996;62:1401-10.

184. Koller BH, Marrack P, Kappler JW, and Smithies O. Normal development of mice deficient in beta-2 m MHC class I proteins, and $CD8^+$ T cells. *Science.* 1990;248:1227-1229.

185. Konieczny BT, Dai Z, Elwood ET, Saleem S, Linsley PS, Baddoura FK, Larsen CP, Pearson TC, Lakkis FG. IFN-gamma is critical for long-term allograft survival induced by blocking the CD28 and CD40 ligand T cell costimulation pathways. *J Immunol.* 1998;160:2059-64.

186. Koskinen PK, Lemström KB, Häyry PJ. How Cyclosporine modifies histological and molecular events in the vascular wall during chronic rejection of rat cardiac allografts. *Am J Pathol.* 1995;146:972-80.

187. Koulack J, McAlister V, Giacomantonio C, Bitter-Suermann H, MacDonald A, Lee T. Development of a mouse aorta transplant model of chronic rejection. *Microsurgery.* 1995;16:110-3.

188. Koulack J, McAlister V, MacAulay M, Bitter-Suermann H, MacDonald A, Lee T. Importance of the role of minor histocompatibility antigens in the development of allograft arteriosclerosis. *Clin Immuno Immunopathol.* 1996;80:273-7.

189. Krammer PH. CD95's deadly mission in the immune system. *Nature.* 2000;407:789-95.

190. Krams SM, Hayashi M, Fox CK, Villanueva JC, Whitmer KJ, Burns W, Esquivel CO, Martinez OM. CD8+ cells are not necessary for allograft rejection or the induction of apoptosis in an experimental model of small intestinal transplantation. *J Immunol.* 1998;160:3673-80.

191. Kreisel D, Krupnick AS, Balsara KR, Riha M, Gelman AE, Popma SH, Szeto WY, Turka LA, Rosengard BR. Mouse vascular endothelium activates CD8+ T lymphocytes in a B7-dependent fashion. *J Immunol.* 2002;169:6154-61.

192. Kreisel D, Krupnick AS, Gelman AE, Engels FH, Popma SH, Krasinskas AM, Balsara KR, Szeto WY, Turka LA, Rosengard BR. Non-hematopoietic allograft cells directly activate CD8+ T cells and trigger acute rejection: an alternative mechanism of allorecognition. *Nat Med.* 2002;8:233-9.

193. Krieger NR, Fatham CG. The use of CD4 and CD8 knockout mice to study the role of T-cell subsets in allotransplant rejection. *J Heart Lung Transplant.* 1997;16:263-7.

194. Krieger NR, Yin D, Fatham CG. CD4+ but not CD8+ cells are essential for allo-rejection. *J Exp Med.* 1996;184:2013-18.

195. Kroczek RA, Black CDV, Barbet J, Shevach EM. Mechanism of action of cyclosporin A in vivo. I. Cyclosporin A fails to inhibit T lymphocyte activation in response to alloantigens. *J Immunol.* 1987;139:3597-603.

196. Krupnick AS, Kreisel D, Popma SH, Balsara KR, Szeto WY, Krasinskas AM, Riha M, Wells AD, Turka LA, Rosengard BR. Mechanism of T cell-mediated endothelial apoptosis. *Transplantation.* 2002;74:871-6.

197. Ku CC, Murakami M, Sakamoto A, Kappler J, Marrack P. Control of homeostasis of CD8+ memory T cells by opposing cytokines. *Science.* 2000;288:675-8.

198. Kuang AA, Diehl GE, Zhang J, and Winoto A. FADD is required for DR4- and DR5-mediated apoptosis: lack of trail-induced apoptosis in FADD-deficient mouse embryonic fibroblasts. *J Biol Chem.* 2000;275:25065-68.

199. Kung L, Batiuk TD, Palomo-Pinon S, Noujaim J, Helms LM, Halloran PF. Tissue distribution of calcineurin and its sensitivity to inhibition by cyclosporine. *Am J Transplant.* 2001;1:325-33.

200. Kuo P, Monaco AP. Chronic rejection and suboptimal immunosuppression. *Transplant Proc.* 1993;25:2082-84.

201. Laden AM. The effects of treatment on the arterial lesions of rat and rabbit cardiac allografts. Transplantation. 1972;13:281-90.

202. Laderach D, Movassagh M, Johnson A, Mittler RS, Galy A. 4-1BB co-stimulation enhances human CD8(+) T cell priming by augmenting the proliferation and survival of effector CD8(+) T cells. *Int Immunol.* 2002;14:1155-67.

203. Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. *Aust J Exp Biol Med Sci.* 1975;53:27-42.

204. Lakkis FG, Konieczny BT, Saleem S, Baddoura FK, Linsley PS, Alexander DZ, Lowry RP, Pearson TC, Larsen CP. Blocking the CD28-B7 T cell costimulation pathway induces long term cardiac allograft acceptance in the absence of IL-4. *J Immunol.* 1997;158:2443-8.

205. Lancki DW, Weiss A, Fitch FW. Requirements for triggering of lysis by cytolytic T lymphocyte clones. *J Immunol.* 1987;138:3646-53.

206. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, Cho HR, Aruffo A, Hollenbaugh D, Linsley PS, Winn KJ, Pearson TC. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature.* 1998;393:480-3.

207. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. *J Exp Med.* 1990;171:307-14.

208. Latour S, Veillette A. Proximal protein tyrosine kinases in immunoreceptor signaling. *Curr Opin Immunol.* 2001;13:299-306.

209. Lattime EC, Stutman O. WEHI-164 clone 2F: in vitro antitumor effects of tumor necrosis factor and gamma-interferon. *Nat Immun.* 1992;11:34-45.

210. Lechler R, Ng WF, Steinman RM. Dendritic cells in transplantation--friend or foe? *Immunity.* 2001;14:357-68.

211. Lee HW, Park SJ, Choi BK, Kim HH, Nam KO, Kwon BS. 4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1. *J Immunol.* 2002;169:4882-8.

212. Lee RS, Yamada K, Houser SL, Womer KL, Maloney ME, Rose HS, Sayegh MH, Madsen JC. Indirect recognition of allopeptides promotes the development of cardiac allograft vasculopathy. *Proc Natl Acad Sci U S A.* 2001;98:3276-81.

213. Légaré JF, Haddad H, Haddad S, Buth KJ, Sullivan JA, Hirsch G. Ten-year heart transplantation experience at the MARITIME HEART CENTER: does volume affect results? *Can J Cardiol.* 1999;15:1212-6.

214. Légaré JF, Issekutz T, Lee TDG, Hirsch G. CD8+ T lymphocytes mediate destruction of the vascular media in a model of chronic vascular rejection. *Am J Pathol.* 2000;157:859-65.

215. Légaré JF, Ross DB, Issekutz TB, Ruigrok W, Creaser K, Hirsch GM, Lee TD. Prevention of allograft heart valve failure in a rat model. *J Thorac Cardiovasc Surg*2001;122:310 -7.

216. Legros-Maida S, Soulie A, Benvenuti C, Wargnier A, Vallee N, Berthou C, Guillet J, Sasportes M, Sigaux N. Granzyme B and perforin can be used as predictive markers of acute rejection in heart transplantation. *Eur J Immunol.* 1994 Jan;24(1):229-33.

217. Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, Linsley PS, Bluestone JA. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science.* 1992;257:789-92.

218. Lewis RS. Calcium signaling mechanisms in T lymphocytes. *Annu Rev Immunol.* 2001;19:497-521.

219. Li J, Han X, Jiang J, Zhong R, Williams GM, Pickering JG, Chow LH. Vascular smooth muscle cells of recipient origin mediate intimal expansion after aortic allotransplantation in mice. *Am J Pathol.* 2001;158:1943-7.

220. Li JH, Kluger MS, Madge LA, Zheng L, Bothwell AL, Pober JS. Interferon-gamma augments CD95(APO-1/Fas) and pro-caspase-8 expression and sensitizes human vascular endothelial cells to CD95-mediated apoptosis. *Am J Pathol.* 2002;161:1485-95.

221. Li L, Sad S, Kagi D, Mosmann TR. CD8Tc1 and Tc2 cells secrete distinct cytokine patterns in vitro and in vivo but induce similar inflammatory reactions. *J Immunol.* 1997;158:4152-61.

222. Li XC, Demirci G, Ferrari-Lacraz S, Groves C, Coyle A, Malek TR, Strom TB. IL-15 and IL-2: a matter of life and death for T cells in vivo. *Nat Med.* 2001;7:114-8.

223. Li XC, Strom TB, Turka LA, Wells AD. T cell death and transplantation tolerance. *Immunity.* 2001;14:407-16.

224. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, Strom TB. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat Med.* 1999;5:1298-302.

225. Liao NS, Bix M, Zijlstra M, Jaenisch R, Raulet D. MHC class I deficiency: susceptibility to natural killer (NK) cells and impaired NK activity. *Science.* 1991;253:199-202.

226. Libby P, and Pober JS. Chronic rejection. *Immunity.* 2001;14:387-97.

227. Libby P, Zhao DX. Allograft arteriosclerosis and immune-driven angiogenesis. *Circulation.* 2003;107:1237-9.

228. Libby P. Inflammation in atherosclerosis. *Nature.* 2002;420:868-74.

229. Linder V, Fingerle J, Reidy M. Mouse model of arterial injury. *Circ Res.* 1993;73:792-6.

230. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA. CTLA-4 is a second receptor for the B cell activation antigen B7. *J Exp Med.* 1991;174:561-9.

231. Linsley PS, Greene JL, Brady W, Bajorath J, Ledbetter JA, Peach R. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity.* 1994;1:793-801.

232. Lipman ML, Stevens AC, Strom TB. Heightened intragraft CTL gene expression in acutely rejecting renal allografts. *J Immunol.* 1994;152:5120-7.

233. Liu X, Bai XF, Wen J, Gao JX, Liu J, Lu P, Wang Y, Zheng P, Liu Y. B7H costimulates clonal expansion of, and cognate destruction of tumor cells by, CD8(+) T lymphocytes in vivo. *J Exp Med.* 2001;194:1339-48.

234. Liwski RS, and Lee TD. Nematode infection enhances survival of activated T cells by modulating accessory cell function. *J Immunol.* 1999;163:5005-12.

235. Liwski R, Zhou J, McAlister V, Lee TD. Prolongation of allograft survival by Nippostrongylus brasiliensis is associated with decreased allospecific cytotoxic T lymphocyte activity and development of T cytotoxic cell type 2 cells. *Transplantation.* 2000;69:1912-22.

236. Locksley RM, Reiner SL, Hatam F, Littman DR, Killeen N. Helper T cells without CD4: control of leishmaniasis in CD4-deficient mice. *Science.* 1993;261:1448-51.

237. Lohning M, Hutloff A, Kallinich T, Mages HW, Bonhagen K, Radbruch A, Hamelmann E, Kroczek RA. Expression of ICOS in vivo defines CD4+ effector T cells with high inflammatory potential and a strong bias for secretion of interleukin 10. J Exp Med 2003 Jan 20;197(2):181-93.

238. Lowry RP, Gurley KE, Forbes RD. Immune mechanisms in organ allograft rejection. I. Delayed-type hypersensitivity and lymphocytotoxicity in heart graft rejection. *Transplantation.* 1983;36:391-401.

239. Lubaroff DM. Cellular requirements for the rejection of skin allografts in rats. *J Exp Med.* 1973;138:331-41.

240. Lukacher AE. IFN-gamma suspends the killing license of anti-tumor CTLs. *J Clin Invest.* 2002;110:1407-9.

241. Lutgens E, Cleutjens KB, Heeneman S, Koteliansky VE, Burkly LC, Daemen MJ. Both early and delayed anti-CD40L antibody treatment induces a stable plaque phenotype. *Proc Natl Acad Sci U S A.* 2000;97:7464-9.

242. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Koteliansky VE, Flavell RA. Requirement for CD154 in the progression of atherosclerosis. *Nat Med.* 1999;5:1313-6.

243. MacDonald AS, Sabr K, MacAuley MA, McAlister VC, Biter-Suermann, Lee T. Effects of leflunomide and cyclosporine on aortic allograft chronic rejection in the rat. *Transplant Proc.* 1994;26:3244-5.

244. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, Luster AD. Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. *J Clin Invest.* 1999;104:1041-50.

245. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature.* 1998;394:200-3.

246. Mallat Z, Besnard S, Duriez M, Deleuze V, Emmanuel F, Bureau MF, Soubrier F, Esposito B, Duez H, Fievet C, Staels B, Duverger N, Scherman D, Tedgui A. Protective role of interleukin-10 in atherosclerosis. *Circ Res.* 1999;85:17-24.

247. Malmberg KJ, Levitsky V, Norell H, de Matos CT, Carlsten M, Schedvins K, Rabbani H, Moretta A, Soderstrom K, Levitskaya J, Kiessling R. IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. *J Clin Invest.* 2002;110:1515-23.

248. Marrero MB, Schieffer B, Paxton WG, Heerdt L, Berk BC, Delafontaine P, Bernstein KE. Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature*. 1995;375:247-50.

249. Marx SO, Jayaraman T, Go LO, Marks AR. Rapamycin-FKBP inhibits cell cycle regulators of proliferation in vascular smooth muscle cells. *Circ Res*. 1995;76:412-7.

250. Mason DW, Dallman MJ, Arthur RP, Morris PJ. Mechanisms of allograft rejection: the roles of cytotoxic T-cells and delayed-type hypersensitivity. *Immunol Rev*. 1984;77:167-84.

251. Matzinger P. The JAM test. A simple assay for DNA fragmentation and cell death. *J Immunol Methods*. 1991;145:185-92.

252. McDiarmid SV, Farmer DG, Kuniyoshi JS, Robert M, Khadavi A, Shaked A, Busuttil RW. Perforin and granzyme B. Cytolytic proteins up-regulated during rejection of rat small intestine allografts. *Transplantation*. 1995;59:762-6.

253. McMahon CW, Raulet DH. Expression and function of NK cell receptors in CD8+ T cells. Curr Opin Immunol. 2001;13:465-70.

254. Mehra MR, Ventura HO, Stapleton DD, Karsan AK, Smart FW, Ramee SR, Collins TJ. Allograft aortopathy: an in vivo study of donor aorta involvement in cardiac allograft vasculopathy. *Am Heart J*. 1997;133:698-702.

255. Meiser BM, Billingham ME, Morris RE. Effects of cyclosporin, FK506, and rapamycin on graft-vessel disease. *Lancet*. 1991;338:1297-8.

256. Mennander A, Hayry P. Reversibility of allograft arteriosclerosis after retransplantation to donor strain. *Transplantation*. 1996;62:526-9.

257. Mennander A, Paavonen T, Hayry P. Intimal thickening and medial necrosis in allograft arteriosclerosis (chronic rejection) are independently regulated. *Arterioscler Thromb*. 1993;13:1019-25.

258. Mennander A, Tiisala S, Halttunen J, Yilmaz S, Paavonen T, Hayry P. Chronic rejection in rat aortic allografts. An experimental model for transplant arteriosclerosis. *Arterioscler Thromb*1991;11:671 -80.

259. Mennander A, Tiisala S, Paavonen T, Halttunen J, Hayry P. Chronic rejection of rat aortic allograft. II. Administration of cyclosporin induces accelerated allograft arteriosclerosis. *Transpl Int*. 1991;4:173-9.

260. Minami Y, Kono T, Miyazaki T, Taniguchi T. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol*. 1993;11:245-68.

261. Mohacsi PJ, Tuller D, Hulliger B, Wijngaard PL. Different inhibitory effects of immunosuppressive drugs on human and rat aortic smooth muscle and endothelial cell proliferation stimulated by platelet-derived growth factor or endothelial cell growth factor. *J Heart Lung Transplant.* 1997 May;16(5):484-92.

262. Mondino A, Jenkins MK. Surface proteins involved in T cell costimulation. *J Leukoc Biol.* 1994;55:805-15.

263. Morton AL, Bell EB, Bolton EM, Marshall HE, Roadknight C, McDonagh M, Bradley JA. CD4+ T cell-mediated rejection of major histocompatibility complex class I-disparate grafts: a role for alloantibody. *Eur J Immunol.* 1993;23:2078-84.

264. Moser JM, Gibbs J, Jensen PE, Lukacher AE. CD94-NKG2A receptors regulate antiviral CD8(+) T cell responses. *Nat Immunol.* 2002;3:189-95.

265. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986;136:2348-57.

266. Mottram PL, Han WR, Purcell LJ, McKenzie IF, Hancock WW. Increased expression of IL-4 and IL-10 and decreased expression of IL-2 and interferon-gamma in long-surviving mouse heart allografts after brief CD4-monoclonal antibody therapy. *Transplantation.* 1995;59:559-65.

267. Mueller DL, Jenkins MK, Schwartz RH. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J Immunol.* 1989;142:2617-28.

268. Mullbacher A, Lobigs M, Hla RT, Tran T, Stehle T, Simon MM. Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. *J Immunol.* 2002;169:145-50.

269. Muller I, Kropf P, Louis JA, Milon G. Expansion of gamma interferon-producing CD8+ T cells following secondary infection of mice immune to Leishmania major. *Infect Immun.* 1994;62:2575-81.

270. Murali-Krishna K, Lau LL, Sambhara S, Lemonnier F, Altman J, Ahmed R. Persistence of memory CD8 T cells in MHC class I-deficient mice. *Science.* 1999;286:1377-81.

271. Murphy GJ, Bicknell GR, Nicholson ML. Rapamycin inhibits vascular remodeling in an experimental model of allograft vasculopathy and attenuates associated changes in fibrosis-associated gene expression. *J Heart Lung Transplant*. 2003;22:533-41.

272. Murray CJ, and Lopez AD. Global mortality, disability, and the contribution of risk factors: Global Burden of Disease Study. *Lancet*. 1997;349:1436-42.

273. Nagano H, Libby P, Taylor MK, Hasegawa S, Stinn JL, Becker G, Tilney NL, Mitchell RN. Coronary arteriosclerosis after T-cell-mediated injury in transplanted mouse hearts: role of interferon-gamma. *Am J Pathol*. 1998;152:1187-97.

274. Nagano H, Mitchell RN, Taylor MK, Hasegawa S, Tilney NL, Libby P. Interferon-gamma deficiency prevents coronary arteriosclerosis but not myocardial rejection in transplanted mouse hearts. *J Clin Invest*. 1997;100:550-7.

275. Nagata S, Golstein P. The Fas death factor. *Science*. 1995;267:1449-56.

276. Nair RV, Cao W, Morris RE. Inhibition of smooth muscle cell proliferation in vitro by leflunomide, a new immunosuppressant, is antagonized by uridine. *Immunol Lett*. 1995;47:171-4.

277. Nair RV, Morris RE. Immunosuppression in cardiac transplantation: a new era in immunopharmacology. *Curr Opin Cardiol*. 1995;10:207-17.

278. Nakajima C, Mukai T, Yamaguchi N, Morimoto Y, Park WR, Iwasaki M, Gao P, Ono S, Fujiwara H, Hamaoka T. Induction of the chemokine receptor CXCR3 on TCR-stimulated T cells: dependence on the release from persistent TCR-triggering and requirement for IFN-gamma stimulation. *Eur J Immunol*. 2002;32:1792-801.

279. Natarajan K, Boyd LF, Schuck P, Yokoyama WM, Eliat D, Margulies DH. Interaction of the NK cell inhibitory receptor Ly49A with H-2Dd: identification of a site distinct from the TCR site. *Immunity*. 1999;11:591-601.

280. Nelson PJ, Krensky AM. Chemokines, chemokine receptors, and allograft rejection. *Immunity*. 2001;14:377-86.

281. Newell KA, He G, Guo Z, Kim O, Szot GL, Rulifson I, Zhou P, Hart J, Thistlethwaite JR, Bluestone JA. Cutting edge: blockade of the CD28/B7 costimulatory pathway inhibits intestinal allograft rejection mediated by CD4+ but not CD8+ T cells. *J Immunol*. 1999;163:2358-62.

282. Nolan CR, Saenz KP, Thomas CA 3rd, Murphy KD. Role of the eosinophil in chronic vascular rejection of renal allografts. *Am J Kidney Dis.* 1995;26:634-42.

283. Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature.* 1988;336:79-81.

284. Nunokawa Y, Tanaka S. Interferon-gamma inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation. *Biochem Biophys Res Commun.* 1992;188:409-15.

285. O'Connell JB, Bristow MR. Economic impact of heart failure in the United States: time for a different approach. *J Heart Lung Transplant.* 1994;13:S107-12.

286. Ogura Y, Martinez OM, Villanueva JC, Tait JF, Strauss HW, Higgins JPT, Tanaka K, Esquivel CO, Blankenberg FG, Krams SM. Apoptosis and allograft rejection in the absence of CD8+ T cells. *Transplantation.* 2001;71:1827-34.

287. Ohlen C, Hoglund P, Sentman CL, Carbone E, Ljunggren HG, Koller B, Karre K. Inhibition of natural killer cell-mediated bone marrow graft rejection by allogeneic major histocompatibility complex class I, but not class II molecules. *Eur J Immunol.* 1995;25:1286-91.

288. Olivari MT, Homans DC, Wilson RF, Kubo SH, Ring WS. Coronary artery disease in cardiac transplant patients receiving triple-drug immunosuppressive therapy. *Circulation.* 1989;80:111-5.

289. Oughton JA, Kerkvliet NI. Novel phenotype associated with in vivo activated CTL precursors. *Clin Immunol.* 1999;90:323-33.

290. Ozkaynak E, Gao W, Shemmeri N, Wang C, Gutierrez-Ramos JC, Amaral J, Qin S, Rottman JB, Coyle AJ, Hancock WW. Importance of ICOS-B7RP-1 costimulation in acute and chronic allograft rejection. *Nat Immunol.* 2001;2:591-6.

291. Pasterkamp G, de Kleijn DP, Borst C. Arterial remodeling in atherosclerosis, restenosis and after alteration of blood flow: potential mechanisms and clinical implications. *Cardiovasc Res.* 2000;45:843-852.

292. Pescovitz MD, Thistlethwaite JR Jr, Auchincloss H Jr, Ildstad ST, Sharp TG, Terrill R, Sachs DH. Effect of class II antigen matching on renal allograft survival in miniature swine. *J Exp Med.* 1984;160:1495-508.

293. Pethig K, Heublein B, Wahlers T, Haverich A. Mechanism of luminal narrowing in cardiac allograft vasculopathy: inadequate vascular remodeling rather than intimal hyperplasia is the major predictor of coronary artery stenosis. Working Group on Cardiac Allograft Vasculopathy. *Am Heart J.* 1998;135:628 - 633.

294. Piccirillo CA, Shevach EM. Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol.* 2001;167:1137-40.

295. Pinderski LJ, Fischbein MP, Subbanagounder G, Fishbein MC, Kubo N, Cheroutre H, Curtiss LK, Berliner JA, Boisvert WA. Overexpression of interleukin-10 by activated T lymphocytes inhibits atherosclerosis in LDL receptor-deficient Mice by altering lymphocyte and macrophage phenotypes. *Circ Res.* 2002;90:1064-71.

296. Pinderski Oslund LJ, Hedrick CC, Olvera T, Hagenbaugh A, Territo M, Berliner JA, Fyfe AI. Interleukin-10 blocks atherosclerotic events in vitro and in vivo. *Arterioscler Thromb Vasc Biol.* 1999;19:2847-53.

297. Plissonnier D, Henaff M, Poncet P, Paris E, Tron F, Thuillez C, Michel JB. Involvement of antibody-dependent apoptosis in graft rejection. *Transplantation.* 2000;69:2601-8.

298. Plissonnier D, Nochy D, Poncet P, Mandet C, Hinglais N, Bariety J, Michel JB. Sequential immunological targeting of chronic experimental arterial allograft. *Transplantation.* 1995;60:414-24.

299. Pober JS. Aortic interposition grafts: a model of rejection or rejection of the model? *Am J Transplant.* 2002;2:201-2.

300. Pober JS. Immunobiology of human vascular endothelium. *Immunol Res.* 1999;19:225-32.

301. Poston RS, Billingham M, Hoyt EG, Pollard J, Shorthouse R, Morris RE, Robbins RC. Rapamycin reverses chronic graft vascular disease in a novel cardiac allograft model. *Circulation.* 1999;100:67-74.

302. Prowse SJ, Warren HS, Agostino M, Lafferty KJ. Transfer of sensitised Lyt 2+ cells triggers acute rejection of pancreatic islet allografts. *Aust J Exp Biol Med Sci.* 1983;61:181-5.

303. Qi Z, Riesbeck K, Ostraat O, Tufveson G, Ekberg H. Single dose anti-CD4 monoclonal antibody for induction of tolerance to cardiac allograft in high- and low-responder rat strain combinations. *Transpl Immunol.* 1997;5:204-11.

304. Qian S, Fu F, Li Y, Lu L, Rao AS, Starzl TE, Thomson AW, Fung JJ. Impact of donor MHC class I or class II antigen deficiency on first- and second-set rejection of mouse heart or liver allografts. *Immunology*. 1996;88:124-9.

305. Quill H, Schwartz RH. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J Immunol*. 1987;138:3704-12.

306. Raisanen-Sokolowski A, Glysing-Jensen T, Koglin J, Russell ME. Reduced transplant arteriosclerosis in murine cardiac allografts placed in interferon-gamma knockout recipients. *Am J Pathol*. 1998;152:359-65.

307. Raisanen-Sokolowski A, Mottram PL, Glysing-Jensen T, Satoskar A, Russell ME. Heart transplants in interferon-gamma, interleukin 4, and interleukin 10 knockout mice. Recipient environment alters graft rejection. *J Clin Invest*. 1997;100:2449-56.

308. Raisanen-Sokolowski A, Vuoristo P, Myllarniemi M, Yilmaz S, Kallio E, Hayry P. Mycophenolate mofetil (MMF, RS-61443) inhibits inflammation and smooth muscle cell proliferation in rat aortic allografts. *Transpl Immunol*. 1995;3:342-51.

309. Rao A, Luo C, Hogan PG. Transcription factors of the NFAT family: regulation and function. *Annu Rev Immunol*. 1997;15:707-47.

310. Reidy MA, Silver M. Endothelial regeneration. VII. Lack of intimal proliferation after defined injury to rat aorta. *Am J Pathol*. 1985;118:173-7.

311. Reidy MA. Biology of disease. A reassessment of endothelial injury and arterial lesion formation. *Lab Invest*. 1985;53:513-20.

312. Richter M, Skupin M, Grabs R, Schramm D, Richter H, Olbrich HG. New approach in the therapy of chronic rejection? ACE- and AT1-blocker reduce the development of chronic rejection after cardiac transplantation in a rat model. *J Heart Lung Transplant*. 2000;19:1047-55.

313. Richter M, Skupin M, Schramm D, Weinert M, Richter H, Mohr FW, Olbrich HG. Cyclosporine does not enhance the development of accelerated coronary artery disease: experimental study in a rat cardiac transplant model. *J Heart Lung Transplant*. 2002;21:425-34.

314. Roopenian DC, Widmer MB, Orosz CG, Bach FH. Helper cell-independent cytolytic T lymphocytes specific for a minor histocompatibility antigen. *J Immunol*. 1983;130:542-5.

315. Rosenberg AS, Mizuochi T, Singer A. Analysis of T-cell subsets in rejection of Kb mutant skin allografts differing at class I MHC. *Nature.* 1986;322:829-31.

316. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999;340:115-26.

317. Ruegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med.* 1998;4:408-14.

318. Russell JH, Ley TJ. Lymphocyte-mediated cytotoxicity. *Annu Rev Immunol.* 2002;20:323-70.

319. Russell JH, Masakowski VR, and Dobos CB. Mechanisms of immune lysis. I. Physiological distinction between target cell death mediated by cytotoxic T lymphocytes and antibody plus complement. *J Immunol.* 1980;124:1100-5.

320. Russell P, Chase C, Colvin R. Contributions of cellular and humoral immunity to arteriopathic lesions in transplanted mouse hearts. *Transplant Proc.* 1997;29:2527-8.

321. Russell PS, Chase CM, Colvin RB. Alloantibody- and T cell-mediated immunity in the pathogenesis of transplant arteriosclerosis: lack of progression to sclerotic lesions in B cell-deficient mice. *Transplantation.* 1997;64:1531-6.

322. Russell PS, Chase CM, Sykes M, Ito H, Shaffer J, and Colvin RB. Tolerance, mixed chimerism, and chronic transplant arteriopathy. *J Immunol.* 2001;167:5731-40.

323. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts. II. Importance of humoral immunity. *J Immunol.* 1994;152:5135-41.

324. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts. III. Effects of recipient treatment with a monoclonal antibody to interferon-gamma. *Transplantation.* 1994;57:1367-71.

325. Saiura A, Sata M, Hirata Y, Nagai R, and Makuuchi M. Circulating smooth muscle progenitor cells contribute to atherosclerosis. *Nat Med.* 2001;7:382-3.

326. Saleem S, Konieczny BT, Lowry RP, Baddoura FK, Lakkis FG. Acute rejection of vascularized heart allografts in the absence of IFNgamma. *Transplantation.* 1996;62:1908-11.

327. Samelson LE, Phillips AF, Luong ET, Klausner RD. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc Natl Acad Sci U S A*. 1990;87:4358-62.

328. Samelson LE. Signal transduction mediated by the T cell antigen receptor: the role of adapter proteins. *Annu Rev Immunol*. 2002;20:371-94.

329. Sano H, Sudo T, Yokode M, Murayama T, Kataoka H, Takakura N, Nishikawa S, Nishikawa SI, Kita T. Functional blockade of platelet-derived growth factor receptor-beta but not of receptor-alpha prevents vascular smooth muscle cell accumulation in fibrous cap lesions in apolipoprotein E-deficient mice. *Circulation*. 2001;103:2955-60.

330. Sawyer GJ, Dalchau R, and Fabre JW. Indirect T cell allorecognition: a cyclosporin A resistant pathway for T cell help for antibody production to donor MHC antigens. *Transpl Immunol*. 1993;1:77-81.

331. Sayegh MH, Akalin E, Hancock WW, Russell ME, Carpenter CB, Linsley PS, Turka LA. CD28-B7 blockade after alloantigenic challenge in vivo inhibits Th1 cytokines but spares Th2. *J Exp Med*. 1995;181:1869-74.

332. Sayegh MH, and Turka LA. The role of T-cell costimulatory activation pathways in transplant rejection. *N Engl J Med*. 1998;338:1813-21.

333. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. 1998;17:1675-87.

334. Schaub FJ, Han DK, Liles WC, Adams LD, Coats SA, Ramachandran RK, Seifert RA, Schwartz SM, Bowen-Pope DF. Fas/FADD-mediated activation of a specific program of inflammatory gene expression in vascular smooth muscle cells. *Nat Med*. 2000;6:790-6.

335. Schluns KS, Kieper WC, Jameson SC, and Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. *Nat Immunol*. 2000;1:426-32.

336. Schluns KS, Williams K, Ma A, Zheng XX, Lefrancois L. Cutting edge: requirement for IL-15 in the generation of primary and memory antigen-specific CD8 T cells. *J Immunol*. 2002;168:4827-31.

337. Schmid C, Heemann U, and Tilney NL. Factors contributing to the development of chronic rejection in heterotopic rat heart transplantation. *Transplantation*. 1997;64:222–228.

338. Schmid C, Heemann U, Azuma H, Tilney NL. Rapamycin inhibits transplant vasculopathy in long-surviving rat heart allografts. *Transplantation.* 1995;60:729-33.

339. Schmidt CS, Mescher MF. Peptide antigen priming of naive, but not memory, CD8 T cells requires a third signal that can be provided by IL-12. *J Immunol.* 2002;168:5521-9.

340. Schnetzler B, Pavie A, Dorent R, Camproux AC, Leger P, Delcourt A, Gandjbakhch I. Heart retransplantation: a 23-year single-center clinical experience. *Ann Thorac Surg.* 1998;65:978-83.

341. Schocken DD, Arrieta MI, Leaverton PE, Ross EA. Prevalence and mortality rate of congestive heart failure in the United States. *J Am Coll Cardiol.* 1992;20:301-6.

342. Schoenberger SP, Toes RE, van der Voort EI, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature.* 1998;393:480-3.

343. Schonbeck U, Sukhova GK, Shimizu K, Mach F, Libby P. Inhibition of CD40 signaling limits evolution of established atherosclerosis in mice. *Proc Natl Acad Sci U S A.* 2000;97:7458-63.

344. Schwartz RH. T cell anergy. *Annu Rev Immunol.* 2003;21:305-34.

345. Schwartz RH. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annu Rev Immunol.* 1985;3:237-61.

346. Sepulveda H, Cerwenka A, Morgan T, Dutton RW. CD28, IL-2-independent costimulatory pathways for CD8 T lymphocyte activation. *J Immunol.* 1999;163:1133-42.

347. Shaddy RE. Apoptosis in heart transplantation. *Coron Artery Dis.* 1997;8:617-21.

348. Sharpe AH, Freeman GJ. The B7-CD28 superfamily. *Nat Rev Immunol.* 2002;2:116-26.

349. Shelton MW, Walp LA, Basler JT, Uchiyama K, Hanto DW. Mediation of skin allograft rejection in scid mice by CD4+ and CD8+ T cells. *Transplantation.* 1992;54:278-86.

350. Shevach EM. The effects of cyclosporin A on the immune system. *Annu Rev Immunol.* 1985;3:397-423.

351. Shi C, Lee S, He Q, Zhang D, Fletcher D, Newell J, Haber E. Immunologic basis of transplant-associated arteriosclerosis. *Proc Nat Acad Sci USA*. 1996;93:4051-6.

352. Shimizu K, Sugiyama S, Aikawa M, Fukumoto Y, Rabkin E, Libby P, Mitchell RN. Host bone-marrow cells are a source of donor intimal smooth-muscle-like cells in murine aortic transplant arteriopathy. *Nat Med*. 2001;7:738-41.

353. Shimokado K, Yokota T, Kato N, Kosaka C, Sasaguri T, Masuda J, Ogata J, Numano F. Bidirectional regulation of smooth muscle cell proliferation by IFN-gamma. *J Atheroscler Thromb*. 1994;1:29-33.

354. Shin GT, Khanna A, Ding R, Sharma VK, Lagman M, Li B, Suthanthiran M. In vivo expression of transforming growth factor-beta1 in humans: stimulation by cyclosporine. *Transplantation*. 1998;65:313-8.

355. Shirwan H. Chronic allograft rejection. Do the Th2 cells preferentially induced by indirect alloantigen recognition play a dominant role? *Transplantation*. 1999;68:715-26.

356. Shoskes DA, Wood KJ. Indirect presentation of MHC antigens in transplantation. *Immunol Today*. 1994;15:32-8.

357. Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A, Mittler RS. 4-1BB costimulatory signals preferentially induce CD8+ T cell proliferation and lead to the amplification in vivo of cytotoxic T cell responses. *J Exp Med*. 1997;186:47-55.

358. Shultz LD, Lang PA, Christianson SW, Gott B, Lyons B, Umeda S, Leiter E, Hesselton R, Wagar EJ, Leif JH, Kollet O, Lapidot T, Greiner DL. NOD/LtSz-Rag1null mice: an immunodeficient and radioresistant model for engraftment of human hematolymphoid cells, HIV infection, and adoptive transfer of NOD mouse diabetogenic T cells. *J Immunol*. 2000;164:2496-507.

359. Siegling A, Lehmann M, Riedel H, Platzer C, Brock J, Emmrich F, Volk HD. A nondepleting anti-rat CD4 monoclonal antibody that suppresses T helper 1-like but not T helper 2-like intragraft lymphokine secretion induces long-term survival of renal allografts. *Transplantation*. 1994;57:464-7.

360. Sigal NH, Dumont FJ. Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction. *Annu Rev Immunol*. 1992;10:519-60.

361. Skaro AI, Liwski RS, Johnson P, Légaré JF, Lee TDG, Hirsch GM. Donor versus recipient: neointimal cell origin in allograft vascular disease. *Graft.* 2002;5:390-397.

362. Slavik JM, Lim DG, Burakoff SJ, Hafler DA. Uncoupling p70(s6) kinase activation and proliferation: rapamycin-resistant proliferation of human CD8(+) T lymphocytes. *J Immunol.* 2001;166:3201-9.

363. Slifka MK, Pagarigan RR, Whitton JL. NK markers are expressed on a high percentage of virus-specific CD8+ and CD4+ T cells. *J Immunol.* 2000;164:2009-15.

364. Srivastava R, Keck BM, Bennett LE, Hosenpud JD. The results of cardiac retransplantation: an analysis of the Joint International Society for Heart and Lung Transplantation/United Network for Organ Sharing Thoracic Registry. *Transplantation.* 2000;70:606-12.

365. Steiger J, Nickerson PW, Steurer W, Moscovitch-Lopatin M, Strom TB. IL-2 knockout recipient mice reject islet cell allografts. *J Immunol.* 1995;155:489-98.

366. Steiger JU, Nickerson PW, Hermle M, Thiel G, Heim MH. Interferon-gamma receptor signaling is not required in the effector phase of the alloimmune response. *Transplantation.* 1998;65:1649-52.

367. Stepkowski SM, Ito T. Frequency of alloantigen-specific T cytotoxic cells in high- and low-responder recipients of class I MHC-disparate heart allografts. *Transplantation.* 1990;50:112-9.

368. Stinchcombe JC, Bossi G, Booth S, Griffiths GM. The immunological synapse of CTL contains a secretory domain and membrane bridges. *Immunity.* 2001;15:751-61.

369. Stinn JL, Taylor MK, Becker G, Nagano H, Hasegawa S, Furakawa Y, Shimizu K, Libby P, Mitchell RN. Interferon-gamma-secreting T-cell populations in rejecting murine cardiac allografts: assessment by flow cytometry. *Am J Pathol.* 1998;153:1383-92.

370. Strehlau J, Pavlakis M, Lipman M, Shapiro M, Vasconcellos L, Harmon W, Strom TB. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Natl Acad Sci U S A.* 1997;94:695-700.

371. Su B, Jacinto E, Hibi M, Kallunki T, Karin M, Ben-Neriah Y. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell.* 1994;77:727-36.

372. Subbotin V, Sun H, Aitouche A, Salam A, Valdivia LA, Fung JJ, Starzl TE, Rao AS. Marked mitigation of transplant vascular sclerosis in FasLgld (CD95L) mutant recipients. The role of alloantibodies in the development of chronic rejection. *Transplantation.* 1999;67:1295-300.

373. Sun D, Whitaker JN, Huang Z, Liu D, Coleclough C, Wekerle H, Raine CS. Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. *J Immunol.* 2001;166:7579-87.

374. Sutton GC. Epidemiologic aspects of heart failure.*Am Heart J .* 1990;120:1538-40.

375. Szeto WY, Krasinskas AM, Kreisel D, Krupnick AS, Popma SH, Rosengard BR. Depletion of recipient CD4+ but not CD8+ T lymphocytes prevents the development of cardiac allograft vasculopathy. *Transplantation.* 2002;73:1116-22.

376. Szot GL, Zhou P, Rulifson I, Wang J, Guo Z, Kim O, Newel KA, Thistlethwaite JR, Bluestone JA, Alegre ML. Different mechanisms of cardiac allograft rejection in wildtype and CD28-deficient mice. *Am J Transplant.* 2001;1:38-46.

377. Takahashi C, Mittler RS, Vella AT. Cutting edge: 4-1BB is a bona fide CD8 T cell survival signal. *J Immunol.* 1999;162:5037-40.

378. Takeuchi T, Lowry RP, Konieczny B. Heart allografts in murine systems. The differential activation of Th2-like effector cells in peripheral tolerance. *Transplantation.* 1992;53:1281-94.

379. Takeuchi Y, Okumura K, Suzuki G. Cyclosporin A and anti-Ia antibody cause a maturation defect of $CD4^{+}8^{-}$ cells in organ-cultured fetal thymus. *Immunology* 1989;66:362-7.

380. Tan JT, Ernst B, Kieper WC, LeRoy E, Sprent J, Surh CD. Interleukin (IL)-15 and IL-7 jointly regulate homeostatic proliferation of memory phenotype CD8+ cells but are not required for memory phenotype CD4+ cells. *J Exp Med.* 2002;195:1523-32.

381. Tan JT, Whitmire JK, Ahmed R, Pearson TC, Larsen CP. 4-1BB ligand, a member of the TNF family, is important for the generation of antiviral CD8 T cell responses. *J Immunol.* 1999;163:4859-68.

382. Tellides G, Tereb DA, Kirkiles-Smith NC, Kim RW, Wilson JH, Schechner JS, Lorber MI, Pober JS. Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature.* 2000;403:207-11.

383. Thomas DA, Du C, Xu M, Wang X, and Ley TJ. DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis. *Immunity.* 2000;12:621-32.

384. Tilney N, Whitley W, Diamond J, Kupiec-Weglinski J, Adams D. Chronic rejection-an undefined conundrum. *Transplantation.* 1991;52:389-98.

385. Trambas CM, Griffiths GM. Delivering the kiss of death. *Nat Immunol.* 2003;4:399-403.

386. Trambley J, Bingaman AW, Lin A, Elwood ET, Waitze SY, Ha J, Durham MM, Corbascio M, Cowan SR, Pearson TC, Larsen CP. Asialo GM1(+) CD8(+) T cells play a critical role in costimulation blockade-resistant allograft rejection. *J Clin Invest.* 1999;104:1715-22.

387. Turka LA, Linsley PS, Lin H, Brady W, Leiden JM, Wei RQ, Gibson ML, Zheng XG, Myrdal S, Gordon D, *et al.* T-cell activation by the CD28 ligand B7 is required for cardiac allograft rejection in vivo. *Proc Natl Acad Sci U S A.* 1992;89:11102-5.

388. Turvey SE, Wood KJ. Immunobiology of solid organ transplantation. *Int Surg.* 1999;84:279-90.

389. Tuzcu EM, Kapadia SR, Tutar E, Ziada KM, Hobbs RE, McCarthy PM, Young JB, Nissen SE. High prevalence of coronary atherosclerosis in asymptomatic teenagers and young adults: evidence from intravascular ultrasound. *Circulation.* 2001;103:2705-10.

390. Ugolini S, Arpin C, Anfossi N, Walzer T, Cambiaggi A, Forster R, Lipp M, Toes RE, Melief CJ, Marvel J, Vivier E. Involvement of inhibitory NKRs in the survival of a subset of memory-phenotype CD8+ T cells. *Nat Immunol.* 2001;2:430-5.

391. Uretsky BF, Kormos RL, Zerbe TR, Lee A, Tokarczyk TR, Murali S, Reddy PS, Denys BG, Griffith BP, Hardesty RL, et al. Cardiac events after heart transplantation: incidence and predictive value of coronary arteriography. *J Heart Lung Transplant.* 1992;11:S45-51.

392. Valujskikh A, Hartig C, and Heeger PS. Indirectly primed $CD8^+$ T cells are a prominent component of the allogeneic T-cell repertoire after skin graft rejection in mice. *Transplantation.* 2001;71:418-21.

393. Valujskikh A, Lantz O, Celli S, Matzinger P, Heeger PS. Cross-primed CD8(+) T cells mediate graft rejection via a distinct effector pathway. *Nat Immunol.* 2002;3:844-51.

394. Van Den Broek ME, Kagi D, Ossendorp F, Toes R, Vamvakas S, Lutz WK, Melief CJ, Zinkernagel RM, Hengartner H. Decreased tumor surveillance in perforin-deficient mice. *J Exp Med.* 1996;184:1781-90.

395. Van Der Merwe PA, Davis SJ. Molecular interactions mediating T cell antigen recognition. *Annu Rev Immunol.* 2003;21:659-84.

396. Van Der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation.* 1994;89:36-44.

397. Van Maurik A, Herber M, Wood KJ, Jones ND. Cutting edge: CD4+CD25+ alloantigen-specific immunoregulatory cells that can prevent CD8+ T cell-mediated graft rejection: implications for anti-CD154 immunotherapy. *J Immunol.* 2002;169:5401-4.

398. Van Maurik A, Wood KJ, Jones ND. Impact of both donor and recipient strains on cardiac allograft survival after blockade of the CD40-CD154 costimulatory pathway. *Transplantation.* 2002;74:740-3.

399. Van Riemsdijk IC, Baan CC, Balk AH, Vantrimpont PM, Maat LP, Weimar W. Improvement of cardiovascular risk factors in heart transplant recipients after conversion from cyclosporine to tacrolimus: a role of the TGF-beta system. *Transplant Proc.* 2002;34:1864-5.

400. Van Stipdonk MJ, Lemmens EE, Schoenberger SP. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. *Nat Immunol.* 2001;2:423-9.

401. Von Scheidt W. Cardiac allograft vasculopathy-problem and model. *Z Kardiol.* 2000;89:2-5.

402. Waldmann H. Transplantation--caught in the crossfire! *Nat Immunol.* 2002;3:803-4.

403. Wallin JJ, Liang L, Bakardjiev A, Sha WC. Enhancement of CD8+ T cell responses by ICOS/B7h costimulation. *J Immunol.* 2001;167:132-9.

404. Walzer T, Joubert G, Dubois PM, Tomkowiak M, Arpin C, Pihlgren M, Marvel J. Characterization at the single-cell level of naive and primed CD8 T cell cytokine responses. *Cell Immunol.* 2000;206:16-25.

405. Wang J, Guo Z, Dong Y, Kim O, Hart J, Adams A, Larsen CP, Mittler RS, Newell KA. Role of 4-1BB in Allograft Rejection Mediated by CD8+ T Cells. *Am J Transplant.* 2003;3:543-51.

406. Wang J, Whitman MC, Natarajan K, Tormo J, Mariuzza RA, Margulies DH. Binding of the natural killer cell inhibitory receptor Ly49A to its major histocompatibility complex class I ligand. Crucial contacts include both H-2Dd AND beta 2-microglobulin. *J Biol Chem.* 2002;277:1433-42.

407. Wang R, Rogers AM, Ratliff TL, and Russell JH. CD95-dependent bystander lysis caused by $CD4^+$ T helper 1 effectors. *J Immunol.* 1996;157:2961-68.

408. Wang X, Yue TL, Ohlstein EH, Sung CP, Feuerstein GZ. Interferon-inducible protein-10 involves vascular smooth muscle cell migration, proliferation, and inflammatory response. *J Biol Chem.* 1996;271:24286-93.

409. Weng NP, Liu K, Catalfamo M, Li Y, Henkart PA. IL-15 is a growth factor and an activator of CD8 memory T cells. *Ann N Y Acad Sci.* 2002;975:46-56.

410. Wever PC, Boonstra JG, Laterveer JC, Hack CE, van der Woude FJ, Daha MR, ten Berge IJ. Mechanisms of lymphocyte-mediated cytotoxicity in acute renal allograft rejection. *Transplantation.* 1998;66:259-64.

411. White WL, Zhang YL, Trautman MS, Perkins SL, Hammond EH, Shaddy RE. Myocardial apoptosis in a heterotopic murine heart transplantation model of chronic rejection graft vasculopathy. *J Heart Lung Transplant.* 1997;16:250-5.

412. Whitmire JK, Flavell RA, Grewal IS, Larsen CP, Pearson TC, Ahmed R. CD40-CD40 ligand costimulation is required for generating antiviral CD4 T cell responses but is dispensable for CD8 T cell responses. *J Immunol.* 1999;163:3194-201.

413. Whitmire JK, Slifka MK, Grewal IS, Flavell RA, Ahmed R. CD40 ligand-deficient mice generate a normal primary cytotoxic T-lymphocyte response but a defective humoral response to a viral infection. *J Virol.* 1996;70:8375-81.

414. Williams MA, Trambley J, Ha J, Adams AB, Durham MM, Rees P, Cowan SR, Pearson TC, Larsen CP. Genetic characterization of strain differences in the ability to mediate CD40/CD28-independent rejection of skin allografts. *J Immunol.* 2000;165:6849-57.

415. Williams NS, Engelhard VH. Perforin-dependent cytotoxic activity and lymphokine secretion by CD4+ T cells are regulated by CD8+ T cells. *J Immunol.* 1997;159:2091-9.

416. Wood KJ, Sakaguchi S. Regulatory T cells in transplantation tolerance. *Nat Rev Immunol.* 2003;3:199-210.

417. Workalemahu G, Foerster M, Kroegel C, Braun RK. Human gamma delta-T lymphocytes express and synthesize connective tissue growth factor: effect of IL-15 and TGF-beta 1 and comparison with alpha beta-T lymphocytes. *J Immunol.* 2003;170:153-7.

418. Yamada A, Laufer TM, Gerth AJ, Chase CM, Colvin RB, Russell PS, Sayegh MH, Auchincloss H Jr. Further analysis of the T-cell subsets and pathways of murine cardiac allograft rejection. *Am J Transplant.* 2003;3:23-7.

419. Yang Y, Wilson JM. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science.* 1996;273:1862-4.

420. Yasukawa M, Ohminami H, Arai J, Kasahara Y, Ishida Y, Fujita S. Granule exocytosis, and not the fas/fas ligand system, is the main pathway of cytotoxicty mediated by alloantigen -specific CD4(+) as well as CD8(+) cytotoxic T lymphocytes in humans. *Blood.* 2000;95:2352-5.

421. Young JW, Steinman RM. Dendritic cells stimulate primary human cytolytic lymphocyte responses in the absence of CD4+ helper T cells. *J Exp Med.* 1990;171:1315-32.

422. Youssef S, Stuve O, Patarroyo JC, Ruiz PJ, Radosevich JL, Hur EM, Bravo M, Mitchell DJ, Sobel RA, Steinman L, Zamvil SS. The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature.* 2002;420:78-84.

423. Yun JJ, Fischbein MP, Whiting D, Irie Y, Fishbein MC, Burdick MD, Belperio J, Strieter RM, Laks H, Berliner JA, Ardehali A. The role of MIG/CXCL9 in cardiac allograft vasculopathy. *Am J Pathol.* 2002;161:1307-13.

424. Zand MS, Li Y, Hancock W, Li XC, Roy-Chaudhury P, Zheng XX, Strom TB. Interleukin-2 and interferon-gamma double knockout mice reject heterotopic cardiac allografts. *Transplantation.* 2000;70:1378-81.

425. Zhai Y, Meng L, Gao F, Busuttil RW, Kupiec-Weglinski JW. Allograft rejection by primed/memory CD8+ T cells is CD154 blockade resistant: therapeutic implications for sensitized transplant recipients. *J Immunol.* 2002;169:4667-73.

426. Zheng L, Ben LH, Pober JS, Bothwell AL. Porcine endothelial cells, unlike human endothelial cells, can be killed by human CTL via Fas ligand and cannot be protected by Bcl-2. *J Immunol.* 2002;169:6850-5.

427. Zheng L, Dengler TJ, Kluger MS, Madge LA, Schechner JS, Maher SE, Pober JS, Bothwell AL. Cytoprotection of human umbilical vein endothelial cells against apoptosis and CTL-mediated lysis provided by caspase-resistant Bcl-2 without alterations in growth or activation responses. *J Immunol.* 2000;164:4665-71.

428. Zhou J, Carr RI, Liwski RS, Stadnyk AW, Lee TD. Oral exposure to alloantigen generates intragraft $CD8^+$ regulatory cells. *J Immunol.* 2001;167:107-13.

429. Zhou X, Nicoletti A, Elhage R, Hansson GK. Transfer of CD4(+) T cells aggravates atherosclerosis in immunodeficient apolipoprotein E knockout mice. *Circulation.* 2000;102:2919-22.

430. Zijlstra M, Bix M, Simister NE, Loring JM, Raulet DH, Jaenisch R. Beta 2-microglobulin deficient mice lack CD4-8+ cytolytic T cells. *Nature.* 1990;344:742-6.