CD8+ T CELLS IN THE DEVELOPMENT OF ALLOGRAFT VASCULOPATHY
AND DE NOVO ALLOSPECIFIC MEMORY FORMATION

by

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~Dedicated to Mr. Nick Mason and Ms. Maxine Widmeyer~
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ABSTRACT

Long-term survival of cardiac transplant recipients continues to be severely limited by the development of a pathological, chronic rejection process, termed allograft vasculopathy (AV). This remains to be the case despite dramatic improvements in the areas of surgical techniques, pre- and post-operative care, and immunosuppression.

To model the clinical setting we used calcineurin inhibitor (CNI) immunosuppression, the cornerstone of post-transplant immunosuppression, in a murine aortic interposition transplant model for our analysis of AV development. This model mimics the presentation of AV in human cardiac transplants through the development of a progressively occlusive neointimal lesion. Our previous work in this model has demonstrated that CD8+, but not CD4+, T cells play a role in neointimal lesion formation. Further investigation also highlighted a specific requirement for either CD8+ T cell-derived IFN-γ or direct cytotoxicity in the development of lesion formation. In the current study we confirmed that CD8+ T cell-derived IFN-γ also leads to the loss of medial smooth muscle cells, an event which inversely correlates with lesion formation. The Fas/FasL direct cytotoxic pathway was also significantly involved in neointimal lesion formation and medial remodeling. This work clarified the pathways utilized by CD8+ T cells in their role as mediators of AV development.

Recognizing the threat that CD8+ T cells pose to cardiac transplant recipients in the presence of CNI immunosuppression, and a growing concern with the presence of anti-donor memory T cells in transplant recipients, we next explored the development of memory CD8+ T cells in the presence of CNI immunosuppression. We first established that memory CD8+ T cells could not develop when CNI immunosuppression was initiated immediately post-challenge. Next, we hypothesized that the clinical practice of CNI delay post-transplant would permit the development of de novo memory CD8+ T cells. Immediate and early initiation was sufficient to prevent the development of de novo memory CD8+ T cells. However, later delay to within a clinically practiced timeframe did permit the development of de novo memory CD8+ T cells. Our analysis revealed that this population demonstrated equivalent functionality to de novo memory CD8+ T cells generated in the absence of CNI immunosuppression.
<table>
<thead>
<tr>
<th>Symbol</th>
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<tr>
<td>α</td>
<td>Alpha</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<td>Theta</td>
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<td>κ</td>
<td>Kappa</td>
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<td>°C</td>
<td>Degrees Celsius</td>
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<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>ACK</td>
<td>Ammonium-Chloride-Potassium</td>
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<tr>
<td>ACR</td>
<td>Acute Cellular Rejection</td>
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<tr>
<td>ALG</td>
<td>Anti-Lymphocyte Globulin</td>
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<tr>
<td>AMR</td>
<td>Antibody Mediated Rejection</td>
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<tr>
<td>AP-1</td>
<td>Activator Protein-1</td>
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<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>ATG</td>
<td>Anti-Thymocyte Globulin</td>
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<tr>
<td>AV</td>
<td>Allograft Vasculopathy</td>
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<tr>
<td>BH</td>
<td>Bcl-2 Homology</td>
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<tr>
<td>BID</td>
<td>Bcl-2 Homology3 interacting Domain Molecule</td>
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<tr>
<td>BIT</td>
<td>Benign Intimal Thickening</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CACF</td>
<td>Carlton Animal Care Facility</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CNI</td>
<td>Calcineurin Inhibitor</td>
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<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
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<td>CyA</td>
<td>Cyclosporine</td>
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<td>Day</td>
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<tr>
<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
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<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
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<tr>
<td>DISC</td>
<td>Death Inducing Silencing Complex</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EC</td>
<td>Endothelial Cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>EMB</td>
<td>Endomyocardial Biopsy</td>
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<tr>
<td>FACS</td>
<td>Fluorescent Activated Cell Sorting</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated-Death-Domain</td>
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<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
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<tr>
<td>GAPDH</td>
<td>Glyeraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-Guanine Phosphoribosyltransferase</td>
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<td>hr</td>
<td>Hour</td>
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<tr>
<td>ICAM</td>
<td>Intracellular Adhesion Molecule</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2,3-deoxygenase</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>IL-2R</td>
<td>IL-2 Receptor</td>
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<tr>
<td>IL-2RA</td>
<td>IL-2 Receptor Antagonist</td>
</tr>
<tr>
<td>IMDPH</td>
<td>Inosine Monophosphate Dehydrogenase</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol Trisphosphate</td>
</tr>
<tr>
<td>ISHLT</td>
<td>International Society for Heart and Lung Transplantation</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosine-based Activation Motif</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular Ultrasound</td>
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<tr>
<td>JAK</td>
<td>Janus Activated Kinases</td>
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<tr>
<td>kDa</td>
<td>KiloDalton</td>
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<tr>
<td>LAT</td>
<td>Linker for Activation of T Cells</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MLR</td>
<td>Mixed Lymphocyte Reaction</td>
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<tr>
<td>MMF</td>
<td>Mycophenolate Mofetil</td>
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<td>mo</td>
<td>Month</td>
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<tr>
<td>MPA</td>
<td>Mycophenolic Acid</td>
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<tr>
<td>MPEC</td>
<td>Memory Precursor Effector Cell</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTORC</td>
<td>Mammalian Target of Rapamycin Complex</td>
</tr>
<tr>
<td>mTORi</td>
<td>Mammalian Target of Rapamycin Inhibitor</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells</td>
</tr>
<tr>
<td>O.C.T</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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PE  Phycoerythrin
PI3K  Phosphatidylinositol 3-Kinase
PLCγ  Phospholipase C gamma
pMHC  Peptide Major Histocompatibility Complex
PTK  Protein Tyrosine Kinase
qPCR  Quantitative PCR
RPMI  Roswell Park Memorial Institute
RT  Room Temperature
s  Second
sf  Serum Free
SH2  Src Homology 2
SLEC  Short Lived Effector Cell
SLP76  Src Homology 2-Domain-Containing Leukocyte Phosphoprotein of 76kDa
SMC  Smooth Muscle Cell
STAT  Signal Transducer and Activator of Transcription
Tac  Tacrolimus
TCR  T Cell Receptor
TNFR  Tumor Necrosis Factor Receptor
TNF-α  Tumor Necrosis Factor Alpha
v/v  Volume per Volume
VAD  Ventricular Assist Device
VCAM  Vascular Cell Adhesion Molecule
wk  Week
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<th>y</th>
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<tr>
<td>Zap70</td>
<td>Zeta-Chain-Associated Phoshoprotein of 70 kDa</td>
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ACKNOWLEDGEMENTS

I would like to begin by expressing my deepest gratitude to my supervisor and mentor Dr. Timothy Lee. Seven and a half years after beginning with Tim as an honours student I have graduated as one of his last Ph.D students. Throughout this journey Tim has passed a wealth of knowledge to me for which I will be forever grateful.

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CHAPTER 1 INTRODUCTION

1.1 CARDIAC TRANSPLANTATION

Cardiac transplantation is a mainstay of treatment for patients with end-stage heart disease. The International Society for Heart and Lung Transplantation (ISHLT) has reported more than $1 \times 10^5$ heart transplants have been performed at its member centers since 1982, which it estimates represents just over two-thirds of heart transplants performed globally.\(^1\) The data from ISHLT reveal that more than 90% of recipients over the past two decades have needed a cardiac transplant because of either the progression of coronary artery disease or cardiomyopathy.\(^1\) For many of these patients cardiac transplantation is the conclusion of a treatment path that contained pharmacologic or mechanical attempts to treat their disease. In some instances, mechanical circulatory support, which unlike cardiac transplantation is not limited by a donor pool, is attempted as a destination therapy rather than a bridge to transplantation. An early multi-center study investigating left ventricular assist devices (VAD) as a destination therapy reported a significant increase in survival compared to patients treated with medical therapy alone.\(^2\) However, survival with the VAD was still quite low at less than 1.5y post-implantation. Although, more recent studies have reported improved long-term survival with VAD support,\(^2,3\) it is still inferior to cardiac transplantation.\(^4\) Therefore, survival with a VAD is superior to medical therapy, but not cardiac transplantation. This notion may partly explain the increase in patients bridged to cardiac transplantation with a VAD.\(^1\)
In the decade following the first successful human heart transplant, cardiac transplant patients experienced a 50% chance of surviving to 5y post-transplant. After almost half a century of refining surgical techniques, post-operative care, and immunosuppression, survival for post-cardiac transplantation has increased significantly, with 50% of patients now surviving to 11y post-transplant. Pertinent to this thesis are acute and chronic rejection which over the last 2 decades have accounted for up to 21% of deaths post-transplant. Interestingly, this percentage may under represent death due to rejection since some deaths attributed to another leading category of death, graft failure, are thought to have been consequences of rejection.

1.2 Acute Rejection

From the mid to late 1990s, admission to hospital because of an acute rejection episode within the first year post-transplant was carried out for 42% of recipients, whereas a decade later it was only carried out for 22% of recipients. However, these data do not merely reflect a decrease in the number of acute rejection episodes requiring treatment, but also reflect a trend toward less aggressive treatment. For instance, since 2004 ISHLT has been collecting data on whether an acute rejection episode was detected and whether it was treated. These data demonstrate an increasing trend toward not treating acute rejection episodes. This change in clinical care is thought to reflect the finding that many lower grade rejection events resolve without treatment. Data from ISHLT reveals that recipients who experience an untreated rejection event have significantly better survival compared to those with a treated rejection event. However, this comparison lacks
adjustment for the rejection grade and therefore attributing the survival advantage solely to the absence of treatment is not possible.

Diagnosis of an acute rejection event is made by surveillance endomyocardial biopsy (EMB) taken from the right ventricle at various time-points post-transplant. The diagnosis can be classified as either acute cellular rejection (ACR) or antibody-mediated rejection (AMR). In ACR, interstitial or perivascular lymphocytes and macrophages are observed with increasing areas of myocyte damage as the severity increases. In contrast, AMR is diagnosed following the observation of inflammatory changes in the absence of lymphocytic infiltrate and of unexplained cardiac dysfunction. The additional defining histological feature of AMR is immunofluorescence staining for deposition of the complement split product, C4d. Once a diagnosis of either ACR or AMR is made the severity of the patient's symptoms dictates the aggressiveness of treatment.

Typically, treatment ranges from minimal intervention where the calcineurin inhibitor (CNI) immunosuppression dosage is increased in addition to treatment with steroids, to very aggressive treatment with a variety of treatment options. Interestingly, it is still not clear whether surviving an acute rejection episode predisposes one to the development of chronic rejection. A significant body of evidence exists supporting and refuting the notion that acute rejection predisposes patients to the development of chronic rejection.
1.3 Chronic Rejection

The hallmark feature of chronic rejection in cardiac transplants occurs in the coronary arteries of the graft, which has earned it the title of cardiac allograft vasculopathy (AV). Its presence ranges from the proximal epicardials (Figure 1.1) to the microvasculature. In the epicardials, AV is often described as a diffuse concentric neointimal lesion,\textsuperscript{12–15} whereas, in the microvasculature it alternatively presents as stenosis caused by either intimal or medial thickening.\textsuperscript{16}

The etiology of AV is still not completely understood, but both immune and non-immune mediated injury is widely acknowledged to play a part.\textsuperscript{14,17,18} While non-immune injury certainly contributes to the development of AV, injury mediated by the adaptive immune system is recognized to be absolutely essential, since immunodeficient animal recipients of allogeneic grafts do not develop AV.\textsuperscript{19,20} This requirement has been explored further and found to involve both humoral and cellular arms of the adaptive immune response. For instance, clinically relevant murine models, which incorporate immunosuppression, have revealed involvement of anti-donor antibody and CD8+ T cells in the development of AV.\textsuperscript{21–24} Similar observations have been made in models without immunosuppression.\textsuperscript{25–31} In human cardiac transplant recipients, the development of donor-specific antibody post-transplant is significantly associated with the development of AV.\textsuperscript{32–34} Similarly, several studies have reported increased allospecific T cell activation in cardiac transplant patients with AV compared to those without AV.\textsuperscript{35–37} Taken together, these studies reinforce a role for adaptive immunity in the development
of AV, but for the purposes of this thesis only involvement by CD8+ T cells will be discussed.

The cellular targets and outcome of T-cell mediated injury have been debated in the literature. Initial observations by Libby's group detailed the activation of endothelial cells (ECs) lining human AV lesions, and the close association between them, T cells, and macrophages. Libby and colleagues likened this response to a chronic delayed-type hypersensitivity reaction and proposed that it may be a driving force in AV development. Alternatively, our group and others using experimental animal transplant models have provided evidence revealing a link between medial smooth muscle cell (SMC) loss, the recipient’s alloimmune response, and the development of AV (Figure 1.1). Based on these observations, we speculated that neointimal lesion formation during AV development is analogous to the response to medial SMC injury described by Reidy and colleagues using a mechanical injury model. Interestingly, while there are instances of medial SMC loss in the coronaries of human cardiac transplants with AV, the extent to which this occurs in transplant models of human coronaries is much less pronounced than in experimental animal models. Therefore, the extent to which medial SMC loss occurs in the coronaries of human cardiac transplants with developing AV remains unclear.

Alternatively, instead of SMC loss occurring as a result of cytotoxicity some have proposed dedifferentiation and migration of medial SMCs into the neointima. In experimental rodent transplant models, this proposal does not seem to apply since the neointimal lesion has been shown to be composed of cells that are recipient in origin. However, in stark contrast, human cardiac AV lesions contain almost solely donor
SMCs and therefore migrating medial SMCs could theoretically be contributing as well. In addition to migration, neointimal SMCs of AV lesions may originate from pre-existing intimal donor SMCs which exist in human coronaries between the media and intimal ECs. This layer, which our group has termed benign intimal thickening (BIT), persists early post-transplant in human cardiac allografts and may contribute to neointimal lesion formation (Figure 1.1).

In addition to BIT serving as a foundation for neointimal lesion formation, pre-existing atherosclerotic disease can also become incorporated into cardiac AV. Studies using two-dimensional intravascular ultrasound (IVUS) (discussed below) have found that pre-existing disease does not accelerate the development of cardiac AV. In contrast, a recent analysis using three-dimensional IVUS, which is more sensitive and accurate, found that pre-existing disease did significantly accelerate plaque progression. Owing to the relatively small patient population of this study further validation of the contradictory finding is necessary.

The primary method for the detection of cardiac AV, and that recommended by an ISHLT working group, is coronary angiography. Diagnosis of AV with this method is accomplished by comparing the observed luminal diameter in a primary or branch vessel to the diameter from previous angiograms or a normal reference. AV is then categorized as mild, moderate or severe, depending on the degree of stenosis and graft dysfunction. Despite the popularity of angiography, several decades ago Johnson et al. reported cardiac AV of less than 25% stenosis in angiographically silent individuals upon post-mortem examination. This observation illustrates the lack of sensitivity of coronary
angiography. In contrast to angiography, IVUS provides a high resolution cross-section of the vessel, thereby enabling measurement of luminal diameter, vessel remodelling, intimal thickening and in some cases intimal composition.\textsuperscript{18,67–69} IVUS is also able to detect vessels with less than 25% stenosis which would otherwise be missed by angiography.\textsuperscript{70} Despite the increased sensitivity offered by IVUS, it was considered insufficient by the ISHLT working group to replace angiography as the primary method for the detection of cardiac AV, especially because angiography is capable of identifying flow-limiting epicardial disease.\textsuperscript{12} This opinion is not shared by other experts in the field who recommend that IVUS should be used in all post-transplant cardiac AV screening.\textsuperscript{18}

Regardless of the method used to detect cardiac AV, its detection in the epicardials or microvasculature does not bode well for the transplant recipient. The detection of cardiac AV in the epicardials within the first year post-transplant strongly predicts a poor outcome.\textsuperscript{71,72} Similarly, microvascular cardiac AV detected within the later part of the first year post-transplant predicts poor survival.\textsuperscript{16} These findings are echoed in the most recent data from ISHLT which reveals a significant reduction in survival for those recipients with cardiac AV.\textsuperscript{1} Taken together, these data reveal the significant long-term threat that cardiac AV poses to recipient survival.

Unfortunately, very few treatment options exist for patients with cardiac AV.\textsuperscript{10} These include, statin therapy regardless of the recipient's lipid levels, in addition to including mycophenolate mofetil (MMF), sirolimus, or everolimus as a maintenance immunosuppressant (discussed below) if they can be tolerated. Lastly, stents can be used to treat discrete cardiac AV lesions, otherwise coronary artery bypass grafting or
retransplantation can be attempted for patients with severe cardiac AV. Overall, the continued absence of a very successful treatment for cardiac AV maintains it as one of the formidable opponents in the long-term survival of cardiac transplant recipients.

1.4 IMMUNOSUPPRESSION FOR CARDIAC TRANSPLANT RECIPIENTS

Over the past two decades ISHLT has reported that more than 60% of cardiac transplant recipients have received grafts mismatched at 3 or more loci of the human leukocyte antigen (HLA). This translates to a significant immunological disparity between donors and recipients, which in turn requires chronic immunosuppression to prevent rejection. The immunosuppression regimen for cardiac transplant recipients is generally divided into induction, maintenance and rejection regimens. In the following section select induction and maintenance agents will be discussed.

1.4.1 INDUCTION IMMUNOSUPPRESSION

Induction therapy is very often a brief and aggressive immunosuppressive treatment with a specialized agent, such as an antibody, to prevent early rejection. From 2005 to 2012, just over 50% of cardiac transplant recipients received induction immunosuppression in North America and Europe. Within this time frame the most popular agents used were interleukin (IL)-2 receptor antagonists (IL-2RAs) and polyclonal antilymphocyte/thymocyte globulin (ALG/ATG). Interestingly, the preference to use IL-2RAs in cardiac transplantation has occurred without strong evidence demonstrating a clear benefit over ATG. Retrospective single center and randomized multi-center trials have compared IL-2RAs to ATG. Taken together the studies found that IL-2RA
induction resulted in fewer infections, while ATG resulted in fewer rejection episodes. Notably these differences did not translate to a difference in post-transplant survival.75,76,79

1.4.1.1 IL-2 Receptor Antagonists

The two IL-2RAs which have been used to induce cardiac transplant recipients are daclizumab and basiliximab. Daclizumab is a humanized monoclonal antibody (10% murine, 90% human) directed against the IL-2R alpha sub-unit, CD25.80 Basiliximab, in contrast, is a chimeric monoclonal antibody (30% murine 70% human) that is also directed against CD25.81 The human portion of these IL-2RAs increases their circulating half-life and prevents the development of serum sickness which can be associated with animal-derived preparations.74 Literature from the transplantation community attributes the immunosuppressive effects of IL-2RAs to the competitive inhibition of the high-affinity IL-2R, CD25, on the surface of activated T cells.74,81,82 However, novel mechanisms of action have described in recent trials where daclizumab was tested as a treatment for multiple sclerosis.83 Daclizumab treatment increased the frequency and function of a natural killer (NK) cell subset that is believed to be immunosuppressive84,85 and also prevented trans-presentation of IL-2 to T cells by dendritic cells (DCs) expressing CD25.86,87 These novel mechanisms of immunosuppression have not yet been reported for basiliximab.
1.4.1.2 Anti-lymphocyte/Anti-thymocyte Globulin

Polyclonal ALG refers to any anti-lymphocyte serum generated against human lymphocytes. ATG is a type of ALG that is specifically generated using human thymocyte tissue. Currently, there are several preparations of ALG and ATG used to treat solid-organ transplant recipients across Europe and North America, but the most commonly used preparation is rabbit ATG, which is marketed as Thymoglobulin. It is prepared and purified from the pooled serum of thymocyte-immunized pathogen free rabbits, and has been used in transplantation for over 25 ys. Over this long period of application multiple mechanisms of immunosuppression have been noted for Thymoglobulin, of which T cell depletion is regarded as the primary mediator. It depletes CD3+, CD4+, and CD8+ T cells in the peripheral blood, lymph nodes and the spleen.

1.4.2 MAINTENANCE IMMUNOSUPPRESSION

Current maintenance immunosuppression protocols typically include a calcineurin inhibitor (CNI), mycophenolate mofetil (MMF), and tapered administration of prednisone. Unfortunately, CNI use is associated with immunosuppression mediated nephrotoxicity, and in an effort to limit this injury, CNI avoidance, minimization and conversion regimens have been investigated. In CNI avoidance protocols a mammalian target of rapamycin inhibitor (mTORi) is often used as a replacement. The ISHLT registry 1y follow-up data from 2006 to 2012 reveals that CNI-free protocols specifically utilizing a mTORi were used in less than 4% of cardiac transplant recipients. These data provide an indication of the limited scope of mTORi-based, CNI-free regimens. In the
following sections, the common maintenance therapeutics: cyclosporine (CyA), Tacrolimus (Tac), MMF, sirolimus, and everolimus, will be discussed in regards to their current application in cardiac transplantation, and primary mechanism of action.

1.4.2.1 Calcineurin Inhibitors

The introduction of CNIs, first with CyA and later Tac revolutionized solid-organ transplantation. For instance, in the context of cardiac transplantation widespread adoption of CyA as part of the maintenance regimen dramatically improved early post-transplant survival.\(^9^1\) In the following decades Tac would supplant CyA as the most widely used CNI in maintenance regimens. From 1999 to 2012, Tac would increase from use in 10% to more than 81% of maintenance regimens at the 1y post-transplant follow-up.\(^1^,9^2\) Several factors which may have contributed to this shift are problems with reliable dosing using CyA’s initial formulation,\(^9^3\) the increased potency of Tac,\(^9^4\) and the recognition that Tac offers equivalent survival with a different adverse effects profile.\(^9^5\)

CyA and Tac have similar mechanisms of action with a common outcome, interfering with T cell activation (Figure 1.2). Owing to its lipophilic nature CyA readily crosses the cell membrane and upon reaching the cytoplasm binds with high affinity to immunophilins, mainly cyclophilin A.\(^9^6\) Similarly, Tac complexes with immunophilins called FK binding proteins (FKBP), mainly FKBP12.\(^9^6\) In both cases, these immunophilin-drug complexes can individually inactivate calcineurin, which is a key player in the intracellular signalling pathway associated with signal one of T cell activation. Described in more detail below, signal one refers to signal transduction from the T cell receptor (TCR) signalosome, which activates a number of intracellular...
signalling pathways. One of the pathways leads to release of calcium stores from the endoplasmic reticulum and subsequent activation of calmodulin, which then activates calcineurin. Activated calcineurin is then able to dephosphorylate members of the nuclear factor of activated T cells (NFAT) family. Active NFAT translocates into the nucleus where it initiates transcription of a variety of genes. One of the critical genes targeted is interleukin-2 (IL-2), which is involved in the differentiation and survival of CD4+ and CD8+ T cells, and may also be required for the successful development of memory CD8+ T cells.

1.4.2.2 Mycophenolate Mofetil

Currently, either MMF or its active metabolite mycophenolic acid (MPA) is included in maintenance therapy for just over 85% of patients at their 1y follow-up. MPA attracted initial attention as an immunosuppressant because of its ability to disproportionately inhibit the proliferation of lymphocytes compared to other cell types. In general, MPA prevents proliferation by inhibiting the enzyme inosine monophosphate dehydrogenase (IMPDH), which is responsible for the de novo synthesis of the guanosine nucleotide that is required during DNA synthesis. T and B lymphocytes are particularly sensitive to treatment with MPA because they express the isoform of IMPDH which is preferentially targeted. Additionally, T and B lymphocytes do not synthesize guanosine nucleotide through the alternative salvage pathway and therefore solely rely on IMPDH for guanosine nucleotide synthesis.
1.4.2.3 Sirolimus and Everolimus

In 1999, and later in 2003, sirolimus and everolimus appeared as maintenance immunosuppressants in the field of cardiac transplantation.\textsuperscript{105,106} Since that period, their use in maintenance regimens has remained relatively constant with nearly 15\% of patients on one of them at their 1y follow-up.\textsuperscript{1} As was noted above, they have been investigated as replacements for CNIs. In one recent example their use in a CNI-free regimen was limited because they were poorly tolerated and trended toward a higher incidence of acute rejection episodes.\textsuperscript{107} Furthermore, their limited use in cardiac transplantation may also be influenced by concerns surrounding their potential deleterious effect on wound healing.\textsuperscript{108}

In the late-1970s, 2ys after its initial discovery, sirolimus was noted to exhibit potent \textit{in vivo} immunosuppressive activity.\textsuperscript{109} More recent publications have detailed its inhibition of \textit{in vitro} T lymphocyte proliferation induced by a variety of stimuli.\textsuperscript{110–112} These properties are attributable to the formation of a complex with the same target as Tac, FKBP12, and the subsequent inhibition of a protein complex known as the mammalian target of rapamycin complex (mTORC)\textsubscript{1}.\textsuperscript{113} The activity of mTORC2 is also inhibited by rapamycin but requires prolonged exposure or a higher dose.\textsuperscript{114,115} The inhibition of these complexes has differential effects on CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. For instance, the differentiation of effector CD4\textsuperscript{+} T cells into different helper subsets are strongly influenced by the activity of mTORC\textsubscript{1} or mTORC\textsubscript{2}.\textsuperscript{115} Alternatively, inhibition of mTORC\textsubscript{1} and mTORC\textsubscript{2} causes activated CD4\textsuperscript{+} T cells to differentiate into Foxp3\textsuperscript{+} regulatory T cells.\textsuperscript{116} In contrast to CD4\textsuperscript{+} T cells, the consequence of mTORC inhibition for CD8\textsuperscript{+} T cell responses depends on the dosing and timing of inhibition. When low
doses are administered during the priming and expansion of CD8+ T cells, memory 
CD8+ T cell development is enhanced.117 Similarly, if high doses are administered during 
the contraction phase of effector CD8+ T cells, formation of memory is again enhanced. 
Only when high doses of rapamycin are administered during the expansion phase of an 
effector CD8+ T cell response does inhibition of the effector and memory population 
occur. 117

1.5 T Lymphocytes
The two major lineages of T lymphocytes are identified based on their expression of 
either co-receptor, CD4 or CD8. In addition to phenotype this division represents their 
divergent roles in the adaptive immune response. CD4+ T cells are widely regarded to be 
central figures in adaptive immunity; they orchestrate the development of humoral and 
cell-mediated immunity.118 Alternatively, CD8+ T cells play a major part in cell-
mediated immunity through the targeted execution of compromised or foreign cells.119 In 
part, these roles are dictated by the specificity of CD4+ T cells for peptide presented in 
the context of major histocompatibility complex (pMHC) class II and the specificity of 
CD8+ T cells for pMHC class I. One proposal suggests that these specificities are 
directed by the co-receptors themselves, which are acquired during T cell precursor 
development in the thymus.120 In addition to this lineage commitment during 
development in the thymus, T cell precursors also genetically rearrange their TCR.121 T 
cell precursors expressing a rearranged TCR are then selected for on the basis of 
recognizing but not strongly responding to self-pMHC.122 The T cell precursors which 
fulfill these requirements become peripheral T cells and populate secondary lymphoid
tissue. As a consequence of thymic education and TCR gene rearrangement, these peripheral T cells are limited in the total number of pMHCs that can be recognized. Recent estimates for mice suggest that the naïve CD4+ T cell population can recognize $1 \times 10^6$ distinct pMHCs, whereas the naïve CD8+ T cells can recognize $1 \times 10^5$ distinct pMHCs. $^{123}$ These estimates have been extrapolated from studies which have measured the precursor frequencies of CD4+ and CD8+ T cells to a variety of peptides. $^{124,125}$ Interestingly, of these peptides, a single allo-peptide was used and revealed a CD4+ T cell precursor frequency 10 times larger than two conventional CD4+ T cell precursor frequencies that were obtained. This finding aligns with traditional estimates which suggest that alloreactive T cell populations may be 10 to 100 times larger than conventional T cell populations. $^{126}$

In the following sections T cell activation will be presented in the context of allo-immunity. T cell allo-recognition and activating signals 1 and 2 will be discussed generally with a later focus on CD8+ T cell differentiation and function.

1.5.1 INDIRECT, DIRECT AND SEMI-DIRECT ALLO-RECOGNITION

In the context of solid-organ transplantation, indirect, direct, and semi-direct are the three pathways through which activation of allo-reactive T cells occurs. Indirect allo-recognition follows the same manner of antigen presentation as a conventional immune response, whereby allo-antigen is taken up by recipient antigen presenting cells (APCs), processed, and then presented in the context of recipient-MHC. $^{127}$ Potential antigens presented in this manner include allo-MHC$^{128,129}$ and minor histocompatibility
antigens. In the direct pathway, allo-recognition occurs when donor-derived APCs present peptide in the context of allo-MHC. In this setting, the recipient TCR interacts to some degree with both the peptide and MHC presented by the donor cell. It should be noted that peptide specificity for this pathway has not been clearly determined. Semi-direct allo-recognition is the final pathway of allo-recognition described to date. Through this pathway, allo-MHC from donor cells gets taken up by the recipient APCs, and instead of being processed, is presented to recipient T cells. In theory, CD4+ and CD8+ T cell lineages can recognize allo-antigen through these three pathways; however, there is some opposition to the notion that indirect allo-recognition by CD8+ T cells has a role in the development of solid-organ transplant rejection. Regardless of the pathway, TCR engagement by cognate pMHC represents the first signal in the subsequent activation, proliferation and differentiation of CD4+ and CD8+ T cells.

1.5.2 T CELL ACTIVATION SIGNAL 1: TCR ENGAGEMENT

The first of two signals leading to T cell activation and proliferation is initiated following sufficient engagement of the TCR by cognate pMHC presented by an APC (Figure 1.2). This event involves signalling from engaged TCR microclusters, which are TCRs associated with CD3 dimers and the co-stimulatory molecule CD28, as well as intracellular signalling molecules. Over time, as signalling continues the microclusters and other associated membrane bound signalling molecules are organized into discrete regions called supramolecular activation clusters, which may serve to reinforce continued signalling. Inside the T cell, TCR engagement triggers the
protein tyrosine kinase (PTK), LCK, maintained in an active state by CD45 and
associated with the cytoplasmic tails of CD4 and CD8, to phosphorylate immunoreceptor
tyrosine-based activation motifs (ITAMs) on CD3 dimers that are non-covalently linked
to the engaged TCR.\textsuperscript{134} Phosphorylated ITAMs recruit another PTK called the zeta-chain-
associated phosphoprotein of 70kDa (Zap70) which is then also activated by
phosphorylation.\textsuperscript{135} Activated Zap70 phosphorylates the two molecules that form the
nucleus of the proximal signaling complex, the linker for activation of T cells (LAT) and
src homology 2 (SH2)-domain-containing leukocyte phosphoprotein of 76kDa (SLP-
76).\textsuperscript{134} Together phosphorylated LAT and SLP-76 form the scaffold for the signalosome
which through interactions with other signalling molecules propagate and integrate the
TCR activation signal to mediate several events, notably the activation of transcription
factors NFAT, activator protein-1 (AP-1), and nuclear factor κ-light-chain-enhancer of
activated B cells (NF-κB).\textsuperscript{97,134}

As part of the LAT signalosome active phospholipase Cγ (PLCγ) hydrolyzes membrane
phosphotidylinositol 4, 5-bisphosphate yielding second messengers inositol trisphosphate
(IP\textsubscript{3}) and diacylglycerol (DAG).\textsuperscript{97} The second messenger IP\textsubscript{3} subsequently engages
receptors on the endoplasmic reticulum where it triggers the release of Ca\textsuperscript{2+} ion stores
into the cytoplasm. This rapid increase in intracellular Ca\textsuperscript{2+} activates additional signalling
molecules eventually leading to the activation of transcription factors.\textsuperscript{136} For instance,
calcineurin, which is inhibited by CyA and Tac, is one of the critical signalling molecules
activated as a result of the cytoplasmic increase in Ca\textsuperscript{2+} ions. Following activation,
calcineurin dephosphorylates NFAT family members, revealing nuclear localization
sequences, which initiates NFAT translocation into the nucleus where it complexes with additional transcription factors, to form homo or heterodimers, and DNA.\textsuperscript{98}

The second messenger DAG recruits protein kinase C (PKC) isoforms to the LAT signalosome where they become activated. Each of these isoforms plays an important role in shaping TCR mediated signalling.\textsuperscript{137} Notably, active PKCθ leads to the formation and recruitment of a large complex which subsequently activates the transcription factor NF-κB by releasing it from its inhibitor.\textsuperscript{138} In addition to PKC activation, DAG recruits a factor to the signalosome which activates the mitogen-activated protein kinase Ras.\textsuperscript{97} Ras is responsible for the generation of the transcription factor AP-1, through the activation of mitogen-activated protein kinase (MAPK) pathway.\textsuperscript{97}

1.5.3 T cell activation signal 2: Co-stimulation

TCR engagement alone is insufficient to trigger T cell activation, and will instead induce a state of unresponsiveness.\textsuperscript{139} The additional activation signals required for initiation of proliferation and differentiation are received at the same time as, or shortly after, TCR signalling and are mediated through engagement of co-stimulatory molecules by their ligands. Thus far the majority of co-stimulatory molecules can be classified on the basis of structural homology as members of either the immunoglobulin (Ig) or tumor necrosis factor receptor (TNFR) superfamily.\textsuperscript{140} While a number of these possess unique qualities a review of the canonical co-stimulator, CD28, will provide sufficient information on the process of co-stimulation.
The expression of CD28 on naïve T cells permits the opportunity for its role in the early signalling events following TCR engagement by pMHC. In fact, the role provided by CD28 co-stimulation supports and refines the outcome of TCR induced signalling. This was revealed through an analysis of the gene expression profile of activated T cells receiving CD28 co-stimulation, where the strength of expression of many of the same genes induced by TCR signalling alone was influenced by the addition of CD28 co-stimulation.\(^{141}\) This profile includes upregulating expression of cytokines, chemokines, and their receptors, in addition to other inducible receptors involved in consolidating T cell activation.\(^{142}\)

Similar to the proximal signalling events following TCR engagement, residues on the cytoplasmic tail of CD28 are phosphorylated by the PTK, LCK (Figure 1.2).\(^{142}\) In contrast to phosphorylated ITAMS which recruit Zap70, the phosphorylated residues on the cytoplasmic tail of CD28 recruit and activate phosphatidylinositide 3-kinase (PI3K).\(^{142}\) Activated PI3K through the generation of 3' phosphoinositide lipids, is involved in the activation of Akt and glycogen synthase kinase 3 (GSK3) which then contribute to NF-κB and NFAT activation, respectively. The activation of these transcription factors serve as an example of the CD28 co-stimulatory support for TCR-mediated signal transduction.

Recognizing that CD28 is an important co-stimulatory molecule for T cell activation, its role in transplant rejection was investigated using experimental animal models. Knockdown of CD28 signalling was attempted with antibodies which targeted CD28’s ligands CD80 and CD86.\(^{143}\) Alternatively, the recombinant fusion protein CTLA-4-Ig, which binds to the ligands with a higher affinity than CD28, was also investigated in an attempt to mitigate CD28 co-stimulation and ultimately alleviate T cell mediated
transplant rejection.\textsuperscript{144} While these had promising results, further investigations with more robust strain combinations or large animal models did not achieve protection.\textsuperscript{145–147} Together these studies have informed the use of CD28 blockade as a method to be used in combination with other immunosuppressive agents which so far has yielded promising clinical outcomes for kidney transplant patients, but has yet to be investigated in cardiac transplant patients.\textsuperscript{148,149}

1.5.4 Helping the Primary CD8+ T Cell Response

The requirement for CD4+ T cell help in the optimal activation and differentiation of CD8+ T cells has for some time been a matter of debate. In recent years it has generally been agreed upon that CD4+ T cell help is required in the development of effector CD8s under non-inflammatory conditions,\textsuperscript{150–152} as is the case with adoptive transfer of peptide-pulsed DCs.\textsuperscript{153–155} Under these circumstances CD4+ T cell help is believed to be mediated through DCs that have become activated during antigen presentation. The expression of CD154 (CD40L) by the activated CD4+ T cells interacts with CD40 expressed by the DC and "licenses" it to subsequently activate cognate CD8+ T cells.\textsuperscript{156}

In contrast to non-inflammatory conditions, there does not appear to be a requirement for CD4+ T cell help in the activation and differentiation of CD8+ T cell into effector cells under inflammatory conditions.\textsuperscript{150–152} This finding has been demonstrated in a variety of settings including allogeneic skin transplants,\textsuperscript{157} viral\textsuperscript{150,158–160} and bacterial infections.\textsuperscript{158,161} The prevailing view is that danger signals produced by pathogens, and possibly in response to cellular stress or death, can alone activate and license DCs to induce CD8+ T cell proliferation and differentiation.\textsuperscript{150,151} Recently, evidence in favor of
this hypothesis has revealed that certain pathogens can license DCs through certain Toll-like receptors to sufficiently activate CD8+ T cells independently of CD4+ T cell help.\textsuperscript{162} Furthermore, in the absence of CD4+ T cell help the development of effector CD8+ T cells was dependent on this route of activation.

1.5.5 \textbf{Effector CD8+ T cells}

At the peak of the immune response the effector CD8+ T cell population is believed to be a heterogeneous population composed of short lived effector cells (SLECs) and memory precursor cells (MPECs) (Figure 1.3).\textsuperscript{163} This population arises from sufficiently activated naïve CD8+ T cells that have undergone dramatic clonal expansion. The extent to which division occurs relies on a number of factors, but has been estimated to be able to reach up to 19 cell divisions, representing a $4 \times 10^5$-fold expansion.\textsuperscript{164} To achieve this level of expansion activated CD8+ T cells must divide extremely quickly. Depending on the stimulus used, early divisions have been reported to occur within 2 to 6h of each other.\textsuperscript{165} Interestingly, before proliferation is even initiated some activated CD8+ T cells show indications of future effector molecule expression, such as IFN-γ.\textsuperscript{166} In fact, the subsequent clones of these CD8+ T cells develop into poly-functional effector cells, expressing IFN-γ in addition to IL-2 and TNF-α, whereas the clones of CD8+ T cells that did not demonstrate future signs of IFN-γ expression exhibit limited cytokine expression.\textsuperscript{166} This observation speaks to an ongoing controversy in the literature concerning the timing of effector CD8+ T cell commitment to either the SLEC or MPEC lineage and is outside the scope of this thesis. Furthermore, regardless of when this cellular fate decision is made the resulting effector population is certainly composed of
these two distinct lineages. As their name implies SLECs develop into cytotoxic T lymphocytes (CTLs), which upon resolution of the activating agent undergo dramatic contraction by apoptosis.\textsuperscript{151,167} In contrast, the remaining 5 to 10% of effectors represent MPECs which over time further differentiate into a diverse population of memory cells.

1.5.6 CD8+ T CELL-MEDIATED CYTOTOXICITY

The CTLs which develop from effector CD8+ T cells are efficient biological killers that directly terminate their targets using lytic granules and by engaging death receptors. In order to minimize the damage to healthy tissue, target cells are specifically identified through recognition of pMHC class I. Reminiscent of activation through the TCR, engagement by a target cell’s pMHC complex leads to rapid formation of an immunological synapse.\textsuperscript{168} However, this event does not necessarily lead to granule exocytosis, and therefore efficient killing.\textsuperscript{169,170} Instead, the strength of TCR signalling, influenced by the concentration and quality of the stimulus, appears to regulate the speed and extent of granule release. The outcome of successful granule release, known as the 'kiss of death', triggers programmed cell death through delivery of the granule contents to target cells.

The molecules contained within lytic granules that are critical for initiating target cell apoptosis are perforin and granzymes, notably granzyme B.\textsuperscript{171} Following exocytosis of the granule contents into the immunological synapse, perforin monomers combine to form pores of variable sizes, averaging between 130-200Å, in the target cell membrane.\textsuperscript{172} Interestingly, even though perforin pores could be lytic in the same way as the complement membrane attack complex,\textsuperscript{173} target cells instead die by apoptosis.\textsuperscript{174}
which is thought to be caused by cytosolic granzyme B.\textsuperscript{175} The mechanism through which granzyme B translocates from the immunological synapse into the cytosol of a target cell is a matter of ongoing debate.\textsuperscript{175} Pores composed of oligomerized perforin are large enough to theoretically permit the passage of granzyme monomers,\textsuperscript{172} such as granzyme B, but alternative evidence suggests that access to the target cells cytosol may instead be granted from inside endocytic vesicles.\textsuperscript{175} Regardless of the exact mechanism of entry, once inside the target cell, granzyme B mediates apoptosis.\textsuperscript{176} Both human and murine granzyme B are thought to mediate apoptosis through activation of the executioner caspase-3.\textsuperscript{175} In contrast, human granzyme B also cleaves and activates Bcl-2 homology (BH)3 interacting domain (BID),\textsuperscript{177} which leads to destabilization of the mitochondrial membrane and eventual formation of the apoptosome, a complex which also drives programmed cell death.\textsuperscript{171,178}

Granzyme B and perforin have both been detected in cardiac allograft tissue undergoing acute rejection.\textsuperscript{179,180} Granzyme B has also been detected in human AV lesions in close proximity to T cells, SMCs, and cells undergoing apoptosis.\textsuperscript{181} Furthermore, direct cytotoxicity of graft endothelial cells has been demonstrated using CTLs cultured from EMBs.\textsuperscript{182} Murine transplant models have been used to examine the role of the lytic granules pathway in the development of AV. In these models, AV is characterized by a loss of medial SMCs and the development of a neointimal lesion.\textsuperscript{17} Minor MHC-mismatched transplant recipients deficient in either perforin or granzyme B demonstrated significantly reduced AV development compared to control recipients.\textsuperscript{30,31} These findings, however, do not accurately reflect the clinical scenario in which the majority of cardiac transplant recipients are HLA mismatched and receiving maintenance
immunosuppression. Our studies have more closely mimicked this setting by using fully
MHC-mismatched aortic transplants, with recipients receiving CNI immunosuppression. Under these conditions the perforin and granzyme B pathway is a minor contributor to AV development.

In addition to the perforin and granzyme B pathway, CTLs directly initiate apoptosis by engaging the death receptor, Fas, on target cells. Initiation of this pathway is regulated through the management of Fas ligand (FasL) expression, which is induced by stimulation through the TCR. Following sufficient stimulation there are two waves of FasL expression by the CTL. In the first wave, preformed FasL stored in granules, distinct from those used in the lytic granule pathway, is rapidly translocated to the cell surface, whereas later on a second wave of newly synthesized FasL is transported to the surface of the CTL. Similar to the induction of lytic granule exocytosis the expression of FasL is influenced by the strength of signalling from the TCR. Interestingly, the pool of preformed FasL can be mobilized to the CTL surface by stimulation with a lower concentration of cognate pMHC than is required for lytic granule exocytosis. Alternatively, the de novo FasL expression appears to be more important for killing bystander Fas expressing cells. In either case, the binding of FasL to Fas supports its clustering and consequently a conformational change in its cytoplasmic tail. This permits stable interaction with the adaptor molecule Fas-associated-death-domain (FADD) which exposes a binding site for procaspase-8. In this complex, caspase-8 becomes activated, thereby forming the death inducing silencing complex (DISC), which subsequently activates effector caspases that mediate the extrinsic apoptotic pathway.
and BID which mediates mitochondrial injury and the apoptotic pathway discussed above.

Similar to perforin and granzyme B, both Fas and FasL have been detected in EMB specimens undergoing rejection.\textsuperscript{190–192} Histological analysis of vessels with AV also revealed the presence of Fas positive endothelial cells undergoing apoptosis.\textsuperscript{45} Although Fas expression was not observed by SMCs, grafts with severe AV did exhibit damaged regions within the medial compartment that were positive for apoptosis.\textsuperscript{45} In experimental transplant models, Fas expression by graft cells has also been implicated in the development of AV.\textsuperscript{23,41,193} Using clinically relevant transplant models we,\textsuperscript{23} and others,\textsuperscript{41} reported an absence of damage to the medial compartment in grafts genetically deficient for Fas. Furthermore, we reported on neointimal lesion formation in our study and observed its absence in the fas deficient grafts.\textsuperscript{23}

IFN-\(\gamma\) is a pleiotropic cytokine that is expressed by a number of cells, including natural killer cells, macrophages, and T cells.\textsuperscript{194,195} From CTLs it is secreted in a polarized fashion towards the target cell, and then leaks from the immunological synapse into the surrounding environment.\textsuperscript{196} In this setting, target and non-target cells in the vicinity encounter IFN-\(\gamma\) which binds to a heterodimeric receptor on their surface.\textsuperscript{194} The IFN-\(\gamma\) receptor then signals through Janus activated kinases (JAKs) and signal transducers and activators of transcription (STATs) to initiate transcription of a variety of genes.

HERE In the terms of cytotoxicity, IFN-\(\gamma\) alone or in combination with other cytokines has been reported to directly induce apoptosis of some primary cells and cell lines.\textsuperscript{197–200} More widely studied is the indirect contribution of IFN-\(\gamma\) to CTL-mediated killing, which
occurs through various mechanisms. For instance, IFN-γ induces the expression of chemokines, specifically CXCL9 and CXCL10, by endothelial cells and SMCs, which recruits CXCR3 positive lymphocytes. Following recruitment of these cells, IFN-γ induced target cell expression of cellular adhesion molecules, such as intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, can contribute to killing by reinforcing the immunological synapse and focusing cytolyltic granule delivery. IFN-γ also sensitizes a variety of cells, including endothelial cells and SMCs, for killing by increasing peptide-MHC class I surface expression as well as Fas expression.

Together with the direct cytotoxic pathways discussed above, the indirect cytotoxic activities of IFN-γ may also play a role in the development of AV. For instance, IFN-γ expression is significantly increased in EMBs and coronary arteries from human cardiac grafts with AV compared to grafts without AV or normal tissue controls. Similarly, IFN-γ expression by circulating peripheral blood mononuclear cells is elevated in recipients with AV compared to those without AV. Strong expression of the IFN-γ-inducible chemokines CXCL9 and CXCL10, and their receptor CXCR3, is also observed in EMBs or coronary arteries with AV. Together these clinical findings reinforce the notion that IFN-γ may be involved in the development of AV. Further supporting this role are findings from experimental transplant models, which have provided mechanistic insight into how IFN-γ may contribute to AV development.

In a model which involves transplantation of human epicardial coronary arteries into immunodeficient mice as aortic interposition grafts, IFN-γ expression led to strong
expression of MHC class I and II and the formation of a neointimal lesion in the human vessel.\textsuperscript{207} In our clinically relevant transplant model we pinpointed the source of IFN-γ to the CD8+ T cell compartment.\textsuperscript{23,24} This was accomplished by reconstituting immunodeficient recipient mice of murine aortic transplants with IFN-γ-deficient CD8+ T cells, while maintaining CNI immunosuppression. In this setting neither neointimal lesion formation or SMC loss occurs. Taken together, these studies reveal a critical role for IFN-γ in the development of AV.

\subsection*{1.5.7 The role of IL-2 in effector and memory CD8+ T cell development}
One important outcome of CD8+ T cell activation by signal one and two is the expression of IL-2 and subsequently the IL-2 high-affinity receptor subunit, CD25.\textsuperscript{100,208} Shortly after the onset of an acute viral infection, the surface expression of CD25 begins to wane and a dichotomy in expression levels becomes apparent in the responding CD8+ T cell population.\textsuperscript{209} Interestingly, this split phenotype translated to a split function profile as well, with the CD25-high CD8+ T cells expressing higher levels of IL-2 and effector molecules. Similar functional observations have been made with CD8+ T cells cultured in high concentrations of IL-2 compared to low concentrations. Furthermore, in three separate studies using bone marrow chimeras, which possessed CD25 deficient and competent cells, at least a two-fold reduction in the expansion of CD25 deficient compared to competent antigen-specific CD8+ T cells was observed.\textsuperscript{101–103,210} Taken together, these studies illustrate that IL-2 signalling early following stimulation supports the development of effector CD8+ T cells.
The studies discussed above have yielded contradictory results regarding the role of IL-2 signalling in the development of memory CD8+ T cells. Several of the studies which used bone marrow chimeras found CD25-deficiency substantially reduced the size of the secondary effector population following re-challenge, whereas another study did not observe this effect. It does not seem plausible that slight differences in the models used would yield such dramatically different results, but a better explanation is not offered, nor clearly discernible.

1.5.8 Effector and Central Memory T Cells

Memory T cells are quiescent antigen-experienced cells that persist in the host long after contraction of the primary immune response and rapidly respond to antigenic challenge upon re-exposure. In humans and mice, quiescent memory cells are recognized to express CD45RO and CD44, respectively. Within this general memory population several different subsets can be further identified on the basis of phenotype. One of the most common schemas describes effector and central memory T cells, which in addition to CD45RO (human) or CD44 (mice), are CCR7-negative and CD62L-negative, or CCR7-positive and CD62L-positive, respectively. In addition to phenotype, these subsets demonstrate different functional responses as well. Following brief ex vivo stimulation, effector memory T cells rapidly express effector molecules such as IFN-γ and perforin, and exhibit cytotoxic activity. In contrast, central memory T cells exhibit robust proliferation and compared to other T cell subsets, produce the most IL-2 following stimulation.
1.5.8.1 Memory T cells in solid-organ transplant graft rejection

In the context of transplantation, memory T cells which recognize and respond to donor antigens pose a threat to the maintenance and survival of that organ. Particularly concerning is the recognition that memory T cells have lower activation thresholds and appear more resistant than their naïve counterparts to current immunosuppressive therapies.\textsuperscript{216,217} Currently, as a measure of immunological sensitization, transplant candidates are assessed for the presence of donor specific antibody,\textsuperscript{218} but not for the presence of alloreactive T cell memory. In recognition of this deficiency and the potential role that memory T cells play in transplant rejection, multiple centers have supported the development of a clinical assay for the evaluation of alloreactive effector and memory T cells in solid-organ transplant recipients.\textsuperscript{219,220}

Pre-transplant candidates waiting for a solid-organ transplant may already possess a pre-existing population of memory T cells that respond to potential donor antigens. These pre-existing populations could either be allo-specific or cross-reactive in nature.\textsuperscript{221} Pre-existing allo-specific memory T cells develop in response to previous sensitizing events, such as blood transfusions, pregnancies, or transplants.\textsuperscript{216,222} Alternatively, cross-reactive memory T cells develop in response to previous infectious agents, but are able to also respond to donor antigens.\textsuperscript{223} This phenomenon, which is explained by the ability of a given TCR to cross-react with different pMHC complexes, is believed to compensate for the relatively low number of unique TCRs compared to possible antigenic challenges, thus expanding the immunological repertoire.\textsuperscript{224}
In addition to pre-existing populations of memory T cells, *de novo* memory T cells, which develop following stimulation by donor antigen, could also pose a threat to the graft. Currently, the clinical practices of cytolytic induction therapy (e.g. ALG/ATG) and delayed CNI initiation may foster the development of *de novo* memory T cells in solid-organ transplant recipients. This suspicion is based on the observation that the T cell compartment is mostly composed of memory T cells following cytolytic induction therapy, which is thought to result from superior resistance to induction agents as well as the subsequent lymphopenia-induced proliferation. Additionally, while the specific timing of CNI initiation is not widely reported in clinical transplant literature, a recent multi-center study from the United Kingdom has provided some insight. They reported initiation of CNI immunosuppression ranging from day 0 to 6 post-transplant in more than 2x10^3 cardiac transplant recipients. In this setting, the development of specifically allo-specific memory CD8+ T cells may be permitted because of their requirement, albeit controversial, for early IL-2 signalling (discussed above). However, this has not yet been evaluated in clinical transplantation or experimental animal models.

Accumulating clinical evidence demonstrates a correlation between the presence of anti-donor effector and memory T cells and rejection. One of these reports presented histological findings in which up to a third of T cells in the neointima and adventitia from cardiac transplants with AV expressed memory phenotype markers. There was also very high expression of IFN-γ transcript in these compartments. These findings have been corroborated by studies that measured the activity of effector and memory T cells in transplant recipients. In one study, a significantly higher frequency of
anti-donor reactive effector and memory T cells was observed in cardiac transplant patients with AV compared to those without AV.\textsuperscript{230} Similarly, in several studies looking at renal transplant recipients, anti-donor effector and memory T cells were observed in recipients prior to the onset of a rejection episode.\textsuperscript{220,229,231} Moreover, reinforcing the resiliency of this anti-donor cellular immunity is the recognition that some of these recipients received cytolytic induction therapy.\textsuperscript{229,231} Together, these studies demonstrate the correlation between effector and memory T cells and the development of rejection, but do not speak to the presence of pre-existing anti-donor memory. This idea was explored in an earlier study which reported a correlation between the presence of pre-existing effector and memory T cells in kidney transplant candidates and subsequent rejection episodes\textsuperscript{232} and has been observed in recent studies.\textsuperscript{233–236} Lastly, the assessment of cross-reactive memory T cells in the context of transplant recipients has been limited, but one recent study has positively identified cross-reactive memory T cells in a small cohort of lung-transplant recipients.\textsuperscript{237} Taken together, these studies illustrate the growing body of literature demonstrating the involvement of anti-donor memory in the development of post-transplant rejection.

The contributions of pre-existing allo-specific and cross-reactive anti-donor memory T cells to solid-organ transplant rejection have also been examined in experimental animal models. For instance, immunosuppression protocols which induce tolerance in naïve mice are unsuccessful in mice that have been sensitized to the donor with a previous transplant.\textsuperscript{238–241} In regards to memory CD8+ T cells, they have been observed to infiltrate into heterotopic heart transplants within two days post-transplant\textsuperscript{242,243} and enhance subsequent recruitment of primary effector T cells.\textsuperscript{242} Furthermore, they are the
most resistant T cell population to treatment with an otherwise successful tolerogenic protocol.\textsuperscript{241} In addition to pre-existing allo-specific memory T cells, cross-reactive populations also pose a threat to graft maintenance. For instance, a tolerogenic protocol that is successful in naïve mice cannot completely prevent rejection of skin grafts from mice that have previously recovered from an acute viral infection and developed cross-reactive memory as a result.\textsuperscript{241} This rejection effect was more substantial when the recipient mice had previously recovered from serial infections with different strains of viruses. Together, these studies clearly demonstrate that pre-existing memory T cells, particularly of the CD8 lineage, can mediate rejection of allografts in the presence of immunosuppression.

1.6 RATIONALE AND OBJECTIVES

Cardiac AV continues to be a limiting factor in the long-term survival of transplant recipients.\textsuperscript{1} The etiology of AV is still not completely understood, but is appreciated to involve immune and non-immune factors.\textsuperscript{14,17,18} Allo-specific CD8+ T cells are recognized as one of the immunological mediators of this pathological process,\textsuperscript{19,23,24,28,183,244,245} and yet how they specifically contribute to its development is unclear. Previous evidence from our lab using a clinically relevant animal model demonstrated separate requirements for CD8+ T cell-derived IFN-\(\gamma\) and cytotoxic pathways in the development of AV.\textsuperscript{24} However, cooperation between these pathways was not assessed. Based on the literature discussed above, we hypothesized that CD8+ T cell-derived IFN-\(\gamma\) would support the direct cytotoxic pathway in mediating AV development.
Our analysis of the mechanism through which CD8+ T cells contribute to the development of AV arises from the recognition that anti-donor CD8+ T cells pose a threat to cardiac transplant recipients. Others have demonstrated that pre-existing memory CD8+ T cells aggressively respond to donor antigens in the context of experimental cardiac transplantation. However, the development of de novo alloreactive memory CD8+ T cells has not been thoroughly explored in this setting. The potential for de novo memory CD8+ T cell development in this setting is based on evidence from models of viral infection which revealed an early role for IL-2 signalling in the development of memory CD8+ T cells. In the context of cardiac transplantation, the mainstay of maintenance therapy, CNI immunosuppression is initiated at various time-points post-transplant. Taken together, we hypothesized that the clinical practice of delaying CNI immunosuppression post-transplant would permit early expression of IL-2, thus permitting the development de novo allo-specific memory CD8+ T cells.

The objectives of this study are:

1) To determine what cytotoxic pathways are employed by CD8+ T cells to mediate AV development in the presence of CNI immunosuppression (Chapter 3, Manuscript 1), and

2) To determine if de novo allo-specific memory CD8+ T cells develop following allogeneic challenge in the presence of CNI immunosuppression (Chapter 4, Manuscript 2).
Figure 1.1 The morphology of the native murine aortic graft and the native proximal human epicardial coronary compared to those that have developed AV. In the murine aortic graft there is a dramatic loss of area and SMCs from the media, which correlates with the development of a progressively occlusive neointimal lesion. In the proximal human epicardial coronary artery medial area may change due to outward and/or constrictive remodeling, but is thought to maintain its population of SMCs. There is also development of a progressively occlusive neointimal lesion, which may involve elements of pre-existing BIT and/or atherosclerosis. The vessel lumen is demarcated by the intimal ECs (blue). The media is demarcated by the internal and external elastic lamina (red) and contains SMCs and infiltrating leukocytes during the development of AV (blue). The neointimal lesion develops between the intima and media and contains infiltrating immune cells (blue) and α-actin+ myofibroblastic cells (blue). The adventitia also contains infiltrating leukocytes, which may in some cases form clusters.
Figure 1.2 T cell activation signal 1 and 2. Engagement of pMHC by TCR and co-receptor CD8 precipitates sufficient intracellular activation of LCK to phosphorylate ITAMs on CD3 dimers. This leads to recruitment, phosphorylation, and activation of Zap70. Zap70 is then responsible for the phosphorylation and activation of multiple signaling molecules, including LAT, SLP-76, which form the nucleus of the LAT signalosome. One of the molecules that is recruited to the LAT signalosome and activated is PLCγ, which then hydrolyzes membrane phosphotidylinositol 4, 5-bisphosphate to yield IP3 and DAG. IP3 mediates the release of Ca2+ stores from the endoplasmic reticulum, which leads to activation of calcineurin and consequently NFAT family members. DAG recruits PKC isoforms, and molecules for the activation of Ras. Recruited PKC0 becomes activated and catalyzes the formation of a large complex which leads to the release of active NF-κB. Activated Ras mediates activation of the MAPK pathway and consequently AP-1. Positive co-stimulation through CD28 leads to the recruitment and activation of PI3K, when is then involved in the activation of Akt and GSK3. These two molecules support TCR mediated signal transduction in the activation of NF-κB and NFAT, respectively. Black arrows represent activation, recruitment and/or phosphorylation. Red bar-headed lines represent inhibition.
Figure 1.3 Primary CD8+ T cell response. Naïve CD8+ T cells responding to a primary (1°) challenge develop into early effector cells (EECs). Further expansion and differentiation yields a heterogeneous population of 1° effectors containing memory precursor effector cells (MPECs) and short lived effector cells (SLECs). The SLECs give rise to a population of cytotoxic T lymphocytes (CTLs) which kill compromised or foreign cells. Consequently, the inciting agent is cleared from the host which leads to contraction of the 1° effector population by apoptosis. Instead of undergoing apoptosis, a small proportion of the 1° effector population persists as long lived memory (T_M).
CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS

Donor Balb/cJ (Balb/cJ; H-2d) C3H/HeJ (C3H;H-2k), C3.MRL-Tnfrsf6lpr (Fas−/−; H-2k), and recipient C57BL/6 (B6; H-2b), and B6.129S7-RAG-1tm1Mom (B6 RAG-1−/−; H-2b) mice, of 7–12wk old were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were maintained in the Carleton Animal Care Facility (CACF), Dalhousie University, with food and water given ad libitum. All procedures involving animals were completed in accordance with the Canadian Council for Animal Care and under protocols approved by the Carleton Animal Care Committee at Dalhousie University.

2.2 REAGENTS

Ammonium chloride, beta-mercaptoethanol, bovine serum albumin (BSA), chloroform, Eosin Y disodium salt, ethylenediaminetetraacetic acid disodium salt dehydrate, Harris’s hematoxylin solution modified, mitomycin C suitable for cell culture, potassium bicarbonate, potassium chloride, potassium phosphate monobasic, sodium azide, sodium bicarbonate, sodium phosphate dibasic, sucrose, and trypan blue (0.4%) were purchased from Sigma-Aldrich (Oakville, ON). Diethylpyrocarbonate (DEPC)-treated water, fetal bovine serum (FBS), L-glutamine (200mM), penicillin and streptomycin (1x10^4 units/mL and 1x10^4 μg/mL, respectively), SuperScript® III first-strand synthesis supermix, the TURBO DNA-free™ kit, TRIzol reagent, and 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (1M; HEPES) were purchased from Life Technologies Inc. (Burlington, ON). Roswell Park Memorial Institute (RPMI)-1640 medium was purchased
from MP Biomedical (Santa Ana, CA). Acetic acid, formaldehyde, and cell culture plastics were purchased from Thermo Fisher Scientific (Ottawa, ON). Optimal cutting temperature (O.C.T.) compound (Sakura Finetek USA Inc, Torrance, CA), and anhydrous ethyl and isopropyl alcohol (Commercial Alcohols Inc., Brampton, ON) were purchased from Dalhousie University Tupper Building Central Stores (Halifax, NS). Isoflurane USP (99.9%; Pharmaceutical Partners of Canada, Richmond Hill, ON) and 0.9% sodium chloride (Hospira, Montreal, QC) were purchased from the CACF. iQ™ SYBR® green supermix and Tween-20 were purchased from Bio-Rad Laboratories (Hercules, CA). Brefeldin A was purchased from eBioscience, Inc. (San Diego, CA). Somnotol was purchased from the Animal Resource Center at McGill University (Montreal, QC).

2.3 Stock solutions and buffers

Solutions used for non-cell culture applications were prepared on-site using double de-ionized water. These include phosphate buffered saline (PBS), 20% sucrose prepared in 0.1M phosphate buffer, enzyme-linked immunosorbent assay (ELISA) buffer (PBS, 0.05% Tween-20), and fluorescent activated cell sorting (FACS) buffer (PBS, 1% FBS, 0.09% sodium azide). Solutions used for cell culture were prepared on-site with ultrapure water from the Milli-Q® Integral water purification system (EMD Millipore, Billerica, MA) and filter-sterilized(0.22μm; Fisher Scientific). These include PBS, complete RMPI supplemented with 5% FBS, 25mM HEPES, 2g/L sodium bicarbonate, and 50μM betamercaptoethanol (cRMPI-5), serum free (sf)RMPI, ammonium-chloride-potassium (ACK) lysis buffer, MACS® buffer (PBS, 0.5% BSA and 2mM EDTA), EasySep™ buffer (PBS + 2% FBS + 1mM EDTA). Lyophilized mitomycin C was dissolved in
sfRMPI to 0.25mg/mL and stored at 4°C for up to 1mo. 0.4% Trypan blue was diluted to 0.1% with sfRMPI (v/v).

2.4 Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-CD4, anti-CD8α and their isotype was purchased from Cedarlane Laboratories Ltd. (Burlington, ON). Phycoerythrin (PE)-conjugated anti-H2-Kd was purchased from BioLegend Inc. (San Diego, CA). Allophycocyanin (APC)-conjugated anti-IFN-γ, the remaining isotypes, and Brefeldin A (3mg/mL) was purchased from eBioscience, Inc. (San Diego, CA). BDPharmigen™ rat anti-mouse CD8α and biotin goat anti-rat immunoglobulin for immunohistochemistry (IHC) were purchased from BD Biosciences (Missisauga, ON).

2.5 Murine splenocyte and CD8+ T cell isolation

C3H/HeJ, Balb/cJ, and C5BL/6J mice were anaesthetized with isoflurane (2L/min isoflurane and 1L/min oxygen) and subsequently euthanized by cervical dislocation. Spleens were then excised using aseptic technique. Splenocytes were released into cRMPI-5 by mechanical disruption. Cell debris was removed with cell strainers (70μm; Thermo Fisher Scientific) before washing by centrifugation at 300 x g for 10min. Erythrocytes were lysed with ACK lysis buffer for 2min before the addition of cold cRMPI-5. Splenocytes were subsequently washed 3 times with cRPMI-5 by centrifugation. Viability and cell counts were assessed with 0.1% trypan blue dye.

In chapter 3, highly purified CD8+ T cell were isolated from splenocytes using positive selection anti-CD8α magnetic microbeads (Miltenyi Biotec, Auburn, CA) as per the
manufacturer's instructions. Splenocytes, isolated as described above, were resuspended in MACS® buffer and anti-Ly2 CD8 magnetic microbeads (MACS®, Miltenyi Biotec, Sunnyvale, CA), and incubated at 4°C for 15min. Splenocytes were then washed in MACs buffer by centrifugation and resuspended in MACs buffer. Labelled splenocyte suspensions were passed through a pre-rinsed MACS® Pre-Separation Filter (30μm nylon mesh; Miltenyi Biotec) and MACS® LS column (Miltenyi Biotec) suspended in the magnetic field of the MACS® separator magnet (Miltenyi Biotec). Columns were washed three times, removed from the magnetic field, and plunged to recover CD8+ T cells, which were counted as described above. Purity of ≥95% CD8+ T cells was confirmed by flow cytometry with ≤1% CD4+ contaminating cells.

In chapter 4, highly purified CD8+ T cells were isolated from splenocytes using the negative selection EasySep™ Mouse CD8+ T cell Enrichment Kit (Stemcell Technologies, Vancouver, BC) as per the manufacturer's special applications suggested protocol. This kit was chosen because it dramatically increased the number of individual replicates that could be simultaneously isolated. Splenocytes, isolated as described above, were resuspended in EasySep™ buffer and pipetted into the perimeter wells of a 24-well plate. Each well with splenocytes received rat serum EasySep™ Mouse CD8+ T cell enrichment cocktail followed by incubation at 4°C for 15min. This cocktail contains biotinylated antibodies directed against CD4, CD11b, CD11c, CD19, CD45R (B220), CD49b, and TER119, which together will bind CD4+ T cells, DCs, macrophages, granulocytes, B cells, NK cells, and erythroid cells. EasySep™ Biotin Selection Cocktail was added to each well with splenocytes, incubated at 4°C for 15min, followed by
addition of EasySep™ D Magnetic Particles and incubation 4°C for 5min. Well volumes were increased to 1.5mL with EasySep™ buffer and incubated at 4°C for 5min on the "EasyPlate" EasySep™ magnet. Supernatants were removed from wells by pipetting and cells enumerated as described above. Cells were washed by centrifugation, and resuspended in cRPMI-5. Purity of ≥85% CD8+ T cells was confirmed by flow cytometry with ≤1% CD4+ contaminating cells.

### 2.6 Mitomycin C-treatment

Stimulator splenocytes for allo-priming were resuspended in sfRMPI and incubated with mitomycin C (13.9μg/mL) for 30min at 37°C. They were then washed by centrifugation, resuspended in sfRMPI, counted, washed again, and resuspended in sfRMPI to 2.5×10^8 cells/mL. Stimulator splenocytes for one-way mixed lymphocyte reactions (MLRs) were resuspended in sfRMPI and incubated with mitomycin C (13.9μg/mL) for 20min at 37°C. They were then washed by centrifugation, resuspended in PBS, incubated at 37°C for 10min, washed by centrifugation, resuspended in PBS, incubated at 37°C for 30min, washed by centrifugation three times, and resuspended in cRPMI-5 prior to counting as described above.

### 2.7 Allo-priming and Adoptive Cell Transfer

C57BL/6J mice received intraperitoneal injection of 5×10^7 mitomycin C-treated C3H/HeJ (chapter 3) or Balb/cJ (chapter 4) splenocytes. This represents the alloprime (chapter 3 and 4) and boost (chapter 4). Some of the mice receiving this alloprime treatment also received 50mg/kg/d CyA.
Adoptive transfer experiments were performed as previously described. One week post allo-priming highly purified CD8+ T cells were isolated from splenocytes as described above. 1×10^7 CD8+ T cells were resuspended in 200μl serum-free RPMI and injected into the peritoneum of RAG1−/− recipient mice 24h after aortic transplantation (see below). Some of the RAG1−/− recipients also received daily injections of CyA for the duration of the experiment.

2.8 AORTIC TRANSPLANTATION

Segments of abdominal aorta were transplanted by our microsurgeon Ms. Brenda Ross as has been previously reported. Briefly, a 1-2mm section of the abdominal aorta was harvested from an anaesthetized (6.5mg/100g Somnotol) donor mouse, flushed and maintained in cold 0.9% saline until transfer into the recipient mouse. Cold ischemia never exceeded 25min. At this point, the infrarenal abdominal aorta was isolated and following isolation, the proximal and distal regions of the recipient aorta were clamped to allow space for the donor aorta. This space was transected and the donor aorta was interposed with end to end anastamoses using 11-0 sutures (Keir Surgical Ltd., Vancouver, BC) in an interrupted fashion. The clamps were removed and the graft monitored for blood flow and patency.

2.9 IMMUNOSUPPRESSION

Cyclosporin A (CyA; 50mg/mL; Sandimmune® I.V., Novartis) purchased from the Victoria General hospital pharmacy stores, Halifax, was diluted in 0.9% sterile saline to
50mg/kg and administered subcutaneously once daily to recipient mice. We recognize that this dose is higher than what is used clinically,\textsuperscript{250} but we\textsuperscript{22,183} and others\textsuperscript{251} have demonstrated robust activation of the allo-immune response at lower doses. CyA was initiated on day 0 (chapter 3 and 4), 2, 6 or 10 and maintained until harvest, or 3d prior to harvest/boost in the case of 5wk and 6wk time-points (chapter 4).

### 2.10 One-way mixed lymphocyte reaction (MLR)

Splenocytes were isolated from allogeneic recipient mice at 1wk, 10d, 2wk, 5wk, and 6wk post-prime as described above. Splenocytes resuspended in cRPMI-5 were cultured in 96-well round bottom plates with mitomycin C-treated Balb/cJ splenocytes in a 1:1 ratio and maintained for 5h at 37°C. In these MLRs, 0.6μg Brefeldin A (eBioscience, Inc.) was included to halt intracellular protein transport. Purified CD8+ T cells were alternatively cultured with mitomycin C-treated Balb/cJ splenocytes in a 1:1 ratio and maintained for 18h at 37°C. Culture supernatants were collected from 18h MLRs and frozen at -20°C for analysis by ELISA.

### 2.11 Histology and digital image analysis

Aortic grafts were fixed in buffered 10% formalin and paraffin-embedded using standard protocols. Cross sections (5μm) of paraffin-embedded aortic grafts were mounted on Fisherbrand\textsuperscript{TM} Superfrost\textsuperscript{TM} Plus glass slides (Fisher Scientific). Approximately 30-50 slides with 4 sections each were collected for each graft. Five slides with sections that were not within the regions of the proximal and distal anastomoses were randomly selected for hematoxylin and eosin (H&E) staining and immunohistochemistry (see
Digital images of stained sections were captured using the Zeiss AxioCam HRc (Carl Zeiss, Thornwood, NY). Adobe Photoshop CS5 Extended Academic Edition software was used to measure graft neointimal lesion and medial cross-sectional area, as well as enumerate SMCs and CD8+ T cells (Adobe Systems Canada, Ottawa, ON). Variation in the cross-sectional area between grafts was controlled for by calculating the medial area relative to the total cross-sectional area surrounded by the external elastic lamina.

### 2.12 IMMUNOHISTOCHEMISTRY

Aortic grafts were cryoprotected in O.C.T.: 20% sucrose (2:1), frozen in liquid nitrogen and stored at −80 °C. Cross sections (6-7 µm) were cut on a cryostat and mounted as above. Sections were fixed in ice cold acetone and stored at −20°C. Endogenous peroxidase activity was blocked using hydrogen peroxide in PBS. Protein blocking was accomplished with PBS supplemented with 10% normal goat serum (Cedarlane) for 1h at RT. Sections were then incubated with the primary monoclonal antibody, rat anti-mouse CD8a (1:25; BD Biosciences) for 30min at RT. For negative controls the primary antibody was omitted. Sections were then washed twice with PBS and incubated with the secondary polyclonal antibody, biotinylated mouse anti-rat (1:100; BD Biosciences) for 30min at RT. Sections were then washed three times with PBS and incubated with a peroxidase avidin/biotin complex from a VECTASTAIN® ABC kit (Vector Labs, Inc., Burlington, ON) for 30min at RT. Sections were washed twice with PBS and incubated with the peroxidase enzyme substrate, 3, 3’-diaminobenzidine (DAB) from a DAB peroxidase substrate kit (Vector Labs, Inc.) for 45s before being counterstained with
hematoxylin (Sigma-Aldrich) for 2min. Slides were coverslipped and 5 sections were selected as above for analysis. For each section 4 fields were randomly assigned and CD8+ T cells counted.

2.13 Detection of apoptosis

Paraffin embedded aortic grafts from 2, 4 and 6wk post-transplant were sectioned as above. Deparaffinized sections were treated with Ready-To-Use Proteinase K (20μg/mL; Dako Inc., Burlington, ON) for 10min prior to washing with dH2O. Endogenous peroxidase activity was blocked as above followed by washing with dH2O. After a short incubation with Equilibration Buffer (ApopTag®) sections were treated with terminal deoxytransferase (TdT) diluted in reaction buffer from the ApopTag® kit (1:3; EMD Millipore) for 1 h at 37°C. For negative controls TdT was omitted. The reaction was stopped, sections washed and digoxigenin-labeled nucleotides were detected by incubation with an anti-digoxigenin peroxidase-conjugated antibody (Aptoptag™). Sections were washed four times before incubation with DAB for 5min (Vector Labs, Inc.). Sections were counterstained with hematoxylin (Sigma-Aldrich) prior to being coverslipped.

2.14 RNA isolation and RT-PCR

Total RNA was extracted from grafts using TRIzol reagent (Life Technologies Inc.) following the manufacturer's instructions. In brief, grafts were homogenized in TRIzol® and incubated at room temperature (RT) for 5min before being mixed with chloroform. After 3min at RT samples were centrifuged at 12x10³ xg for 15min at 4°C. The aqueous
phase was isolated, mixed with isopropyl alcohol to precipitate the RNA, and incubated for 10 min at RT. After centrifugation at 12x10^3 xg for 10 min at 4°C the pellet was washed with 75% ethanol prepared with DEPC-treated water and centrifuged again, but at 7.5x10^3 x g for 5 min at 4°C. The supernatant was discarded and pellet allowed to dry before resuspension in DEPC-treated water. Contaminating genomic DNA was degraded with TURBO™ DNase treatment from the TURBO DNA-free™ kit (Applied Biosystems, USA) for 30 min at 37°C, as per the manufacturer's instructions. The concentration and purity of RNA in recovered isolates was assessed using the ND1000 spectrophotometer (Fisher Scientific, Ottawa, ON) and standardized to 8.3 ng/μl, which was the isolate with the lowest yield. Quality of RNA replicates was not performed because the quantity was too low to perform gel electrophoresis. RT-PCR was performed on the RNA isolates using SuperScript® III first-strand synthesis supermix following manufacturer's instructions. Briefly, RNA was incubated with oligo(dT)_20 primers and annealing buffer at 65°C for 5 min before the addition of annealing buffer and SuperScript® III/RNaseOUT™ enzyme mix and subsequent incubation at 50°C for 5 min. cDNA (2.37 ng/μL) was stored at -80°C until later use.

### 2.15 Quantitative PCR

Amplification reactions (25 μl) contained 4.74 ng template cDNA, iQ™ SYBR® Green Supermix (12.5 μl) (Bio-Rad Laboratories Inc.) and 300 nM forward and reverse primer (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at 100 nM to avoid primer dimers) (Table 2.1) (Integrated DNA technologies, USA). Primers used in qPCR were designed using Primer3Plus. The cycling conditions comprised 3 min polymerase
activation at 95°C and 40 cycles at 95°C for 10s, 60 °C for 30s and 72°C for 45s. Each assay included (in duplicate): a standard curve of four 1/5 serial dilution points of C57BL/6 J splenocyte cDNA, a no template control and 4.7ng of each template cDNA. Amplification efficiencies were above 95%. Results were exported from iQ5 Optical System Software (version 2.0) (Bio-Rad Laboratories, Inc.) and normalized to the geometric mean of GAPDH, β-actin and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) using qBase software.253

2.16 Flow Cytometry

One-way MLR cultured splenocytes or purified CD8+ T cells were washed with FACS buffer and stained with antibodies specific for CD4, CD8 (Cedarlane Laboratories Ltd) and H2Kd-PE (BioLegend), or their respective isotype controls. Stained cells were washed with flow cytometry buffer, fixed in 1% formalin and read on a BD FACSCalibur flow cytometer (BD Biosciences). Immediately following surface labeling, splenocytes from 5h MLR cultures were fixed and permeabilized overnight using the FoxP3/Transcription factor staining buffer set (eBioscience, Inc.). Permeabilized cells were stained for intracellular IFN-γ using anti-mouse IFN-γ-APC or isotype-APC (eBioscience, Inc.). Data was collected as above. All flow cytometry data was analyzed using WinList (Verity Software House, Topsham, ME, USA). Frequencies of IFN-γ+ CD8+ T cells were obtained by enriching for live, CD8+, H2Kd negative events and using the naïve controls to establish the IFN-γ gate. The total number of IFN-γ+ CD8+ T cells was calculated using the percentages of live and H2Kd negative enriched, IFN-γ+CD8+ events and the total number of splenocytes for each corresponding recipient.
2.17 ELISA

The concentration of IL-2 was analyzed in supernatants collected from 18h one-way MLRs using the IL-2 Ready-Set-Go!® ELISA kit (eBioscience, Inc.) following the manufacturer’s instructions. ELISAs were read on a Tecan Infinite M200 Pro plate reader (Tecan Systems, Inc., San Jose, CA, USA) and analyzed using Gen5 software (BioTek, Winooski, VT, USA).

2.18 Statistics

Data are presented as mean ± standard deviation. Statistical analyses were performed using GraphPad Prism version 4.03 (GraphPad Software, La Jolla, CA, USA). Results comparing groups of two were analyzed using a Student's two-tailed t-test. Results comparing groups of three or more were analyzed using one-way analysis of variance with a Bonferonni, Dunnett’s or linear trend post-test. The p values are represented in figures as follows: ***p<0.001; **p<0.01; *p<0.05.

Table 2.1 qPCR Primers. Primer pairs for genes of interest and housekeeping genes

<table>
<thead>
<tr>
<th>Transcripts</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
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<td>IFN-γ</td>
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CHAPTER 3 CD8+ T CELLS UTILIZE IFN-γ TO CONTRIBUTE TO FAS/FASL MEDIATED ALLOGRAFT VASCULOPATHY

Portions of this chapter have been submitted for publication:

*Contributions:* Surgeries were performed by Ms. B Ross. Tissue was isolated, embedded, and cut by S Nejat and M Hart-Matyas. Data for Fig 3.4 was collected and analysed by S Nejat. Data for Fig 3.5 was collected and analysed by S Nejat and M Hart-Matyas. Data for Fig 3.1, 3.2, and 3.3 was collected and analysed by M Hart-Matyas. All figures were completed by M Hart-Matyas
3.1 INTRODUCTION

Allograft vasculopathy (AV) remains as a leading cause of late death after cardiac transplantation. Intravascular ultrasound studies have provided evidence that 75% of recipients show significant AV by 3ys post-transplant and 90% show robust AV by 10ys. CNI based immunosuppression, with CyA or Tac, is still the mainstay of treatment regimens for cardiac transplantation, but has proven ineffective at preventing AV. In contrast, adjuvant immunosuppression with mTORis such as sirolimus and everolimus, has been shown to be beneficial, but there is very significant variation in response to these drugs and the longer term outcomes are less promising. The antiproliferative MMF has had a similar positive, but limited, outcome in preventing AV. A better understanding of the mechanisms involved in the etiology of this vascular pathology will provide novel potential targets for therapeutic intervention.

AV is characterized by extensive vascular remodeling of the transplant coronary arteries resulting in a progressive neointimal lesion that becomes occlusive over time. The most recent computer assisted IVUS and contrast-enhanced echocardiography studies suggest that lesion formation begins in the large proximal epicardial vessels and spreads distally. The neointimal lesion consists of α-actin+ myofibroblastic cells and infiltrating leukocytes. Macrophage infiltration is ubiquitous but lipid rich foamy macrophages are not seen in the developing neointimal lesions. Evidence from animal models has confirmed that the α-actin+ myofibroblastic cells in the lesion are overwhelmingly of recipient origin, even in the presence of CNI immunosuppression. The exact origin of these recipient myofibroblastic cells is uncertain but convincing data
of their bone marrow origin has been presented.\textsuperscript{260} In contrast to the animal models, human vascular lesions have been found to contain donor elements.\textsuperscript{54–56}

The immunological mechanisms that initiate and maintain AV remain to be fully elucidated but there is ample evidence that the dominant allo-immune response involves T cells.\textsuperscript{14,17,18} The importance of B cell function and antibody in AV remains controversial.\textsuperscript{261–263} We have recently conclusively demonstrated that B cells are not required for AV but that alloantibody, if produced, can contribute to the progression of the disease.\textsuperscript{21} We, and others, have shown that CD8+ T cells can initiate AV in animals not treated with immunosuppression.\textsuperscript{19,30,31,183,264} In contrast, in the presence of therapeutic levels of CNI immunosuppression, CD4+ T cell mediated effects are ablated and AV is entirely dependent on active CD8+ T cell responses.\textsuperscript{183} The manner by which these CD8+ T cells contribute to AV is yet to be elucidated.

Our working hypothesis is that cardiac AV is a “response to injury” process, the purpose of which is to restore structural support to high pressure, epicardial proximal coronary arteries that have been weakened by immunological damage to the media. We believe that this immunological damage is mediated by CD8+ T cells that escape the effects of current immunosuppressive therapies. These CD8+ T cells induce medial SMC apoptosis, the pivotal event that initiates the vascular remodelling characterized as AV. As such, immune damage to the media is critical to the development and progression of AV. The nature of the CD8+ T cell effector mechanisms responsible for medial SMC loss and lesion formation is the focus of this study.
3.2 Results
3.2.1 CD8+ T cell influx and induction of medial apoptosis

We have suggested that CD8+ T cells cause medial SMC loss by inducing SMC apoptosis. To confirm this, we sought to establish a temporal link between CD8+ T cell infiltration, presence of known cytotoxic mediators and SMC apoptosis. We first examined the influx of CD8+ T cells into the allograft over time. Lesion formation in the strain combination used, in the presence of CyA, begins at approximately 6wk post-transplantation so we examined T cell influx immediately prior to that time point. Immunohistochemistry was performed on sections of grafts harvested at 2, 3, 4, 5 and 6wk post-transplantation. CD8+ T cells were absent at 2 and 3wk post-transplant (data not shown). As shown in Fig. 1, CD8+ T cell infiltration was barely evident by 4wk. In contrast, T cell influx was evident by 5wk and reached maximal levels by 6wk post-transplant (p<0.001). By 6wk, CD8+ T cells could be seen in the adventitia, the media and in the developing neointima (Fig. 1). The absence of infiltration at the earlier time points (2, 3 and 4wk) serves as an internal negative control for this experiment.

To provide supportive mechanistic data to link the presence of CD8+ T cells with cytotoxic activity we assessed the mRNA expression (by qPCR) of the markers of effector CD8+ T cell function, IFN-γ and granzyme B. Grafts were harvested at 5wk post-transplantation. mRNA expression was compared to the negative control of a syngeneic aortic segment harvested at the same time point post-transplantation under identical conditions. Our findings (Fig. 2) verified an upregulation of these markers of cytotoxicity at this time point. These CD8+ T cell effector mediators were not present at detectable levels 2, 3 or 4wk post-transplantation (data not shown).
We have previously provided evidence of a positive correlation between medial SMC loss and lesion formation in allografts of CyA treated mice.\textsuperscript{24} In this study, to link CD8+ T cell influx with SMC loss we examined apoptosis in allografts of CyA treated recipients at 4, 5 and 6wk post-transplantation using the TUNEL assay. Apoptotic nuclei were absent in the medial layer of grafts at 4wk post-transplant but were evident in the medial SMC compartment by 6wk post-transplant (Fig. 3). These data support the hypothesis of CD8+ T cell mediated apoptosis of SMCs resulting in SMC loss from the media. The absence of apoptotic events at 4wk post-transplant provides an internal negative control for this experiment.

3.2.2 CD8+ T CELL EFFECTOR MECHANISMS REQUIRED FOR MEDIAL CELL LOSS

We suggest above a direct relationship between CD8+ T cell effector function and SMC death. We have previously shown that alloprimed CD8+ T cells require both the direct and indirect effector pathways, simultaneously, to mediate neointimal lesion formation in the presence of CNI immunosuppression (either pathway is sufficient in the absence of immunosuppression).\textsuperscript{24} It has yet to be determined which pathway is primarily responsible for medial SMC loss in these grafts. To elucidate this, we designed an experiment to block either the indirect IFN-γ-dependent pathway, or the direct CTL pathway, of CD8+ T cell activity and investigate medial SMC loss under these conditions.

To block the IFN-γ mediated pathway, alloprimed CD8+ T cells were generated in IFN-γ deficient B6 mice and transferred into immunodeficient B6 RAG1\textsuperscript{−/−} recipients of wild type (WT) C3H aortic grafts. To block the direct CTL pathway, alloprimed CD8+ T cells
were generated in perforin deficient B6 mice and transferred into RAG1−/− recipients of Fas−/− aortic grafts. All recipients were treated with therapeutic doses of CyA for 8wk at which time the grafts were harvested and medial SMC were enumerated. The data shown in Fig. 4 demonstrate that SMC were preserved in the media of RAG1−/− recipients in which either the CTL pathway or the IFN-γ pathway were ablated. In contrast, WT CD8+ T cells that are competent in both effector pathways were capable of depleting SMCs from the media by 8wk (positive control). This data on SMC loss is in direct agreement with our earlier published data measuring intimal lesion formation.24 It confirms a link between CD8+ T cell effector activity, medial SMC loss and neointimal lesion formation.

3.2.3 IFN-γ COOPERATES WITH THE FAS/FASL PATHWAY TO INDUCE SMC LOSS

We have now demonstrated that both CTL and IFN-γ mediated effector pathways are required for SMC loss and lesion formation. We have yet to determine if one of the two CTL pathways predominate in the generation of AV. In this final experiment we tested the hypothesis that IFN-γ cooperates with the Fas/FasL pathway to induce SMC loss. Such cooperation has been suggested in naturally occurring arteriosclerosis.193 To test the hypothesis, we isolated Fas/FasL mediated killing from perforin/granzyme mediated killing by using genetically deficient mice. To ablate Fas/FasL interactions we transferred alloprimed WT CD8+T cells into RAG−/−recipients of Fas−/−allografts. In this case, the transferred CD8+T cells had an intact IFN-γ response and the ability to kill via the perforin/granzyme pathway, but they could not kill via the Fas/FasL pathway. To assess the role of the perforin/granzyme effector pathway we transplanted WT grafts into RAG1−/− mice and reconstituted them with alloprimed CD8+T cells derived from perforin deficient (prf−/−) mice. Under these conditions the IFN-γ and the Fas/FasL
pathways were active, but the perforin/granzyme pathway was ablated. Both of these experiments were conducted in the presence of CyA immunosuppression. Positive controls for the Fas/FasL experiment included WT, rather than Fas−/−, grafts. Positive controls for the perforin/granzyme pathway included transfer of WT, rather than perforin deficient CD8+T cells.

Representative photomicrographs of our results at 8 wk post-transplantation are shown in Fig. 5. Those recipients with intact IFN-γ and Fas/FasL pathways, but a blocked perforin/granzyme pathway, exhibited robust AV. This was characterized by both medial cell loss and robust lesion formation. In contrast, those recipients with intact IFN-γ and perforin/granzyme pathways, but a blocked Fas/FasL pathway, showed no evidence of AV.

This was confirmed by quantitative digital image analysis of the graft sections (Fig. 5). Both the perforin/granzyme B blocked group and the WT (positive) control group demonstrated significant (p<0.01) loss of SMC in comparison to native, untransplanted (negative) control sections. In contrast, relative medial area was preserved in the group with Fas/FasL blockade. Both the native (negative) control group and the Fas/FasL blocked group showed significantly (p<0.01) larger relative medial areas than the WT (positive) control group or the perforin/granzyme blocked group. These data support a hypothesis that the Fas/FasL effector pathway, in cooperation with CD8+ T cell IFN-γ mediated pathway, is the dominant effector pathway for the induction of SMC apoptosis.

Digital image analysis of neointimal lesions showed a similar pattern (Fig. 5). In this experiment, those recipients lacking an effective perforin/granzyme pathway undergo
lesion formation that appears somewhat lower, but is not statistically different from WT allografts (p>0.05). In contrast, those recipients with ablated Fas/FasL activity showed no lesion formation (p<0.001).

3.3 DISCUSSION

Our working hypothesis is that cardiac AV is a “response to injury” process, the purpose of which is to restore structural support to high pressure, epicardial proximal coronary arteries that have been weakened by immunological damage. Based on our previous experiments, we believe that this immunological damage is mediated by CD8+ T cells that escape the effects of current CNI based immunosuppressive therapies.

There remains controversy over the dominant target of alloimmune damage. Some have postulated that damage to the endothelium initiates this response. In contrast, we have suggested medial damage plays a dominant role in the induction of AV. We base this conclusion primarily on the work of Reidy, with mechanical injury models and on our novel chimeric model. Indeed, we now have an expanding body of information that suggests, in our clinically relevant model (fully disparate transplants with CNI immunosuppression) a direct link between medial SMC loss and lesion formation.

The mechanisms by which CD8+ T cells induce this SMC loss are unclear. Akyurek et al. demonstrated Fas expression on medial SMC after transplantation and expression of high levels of FasL on infiltrating T cells. In their model of tolerance induction they noted that reduction of lesion size correlated with a decrease in SMC loss and a decrease in FasL expression on infiltrating T cells. Others have also implicated Fas/FasL killing
in the death of SMC and generation of AV. We have shown the expression of granzyme B, FasL and IFN-γ in the allograft prior to lesion formation in CyA immunosuppressed animals. We\textsuperscript{19,264}, and others,\textsuperscript{268} have shown that IFN-γ is not critical for the development of AV in non immunosuppressed mice. However, IFN-γ is critical in animals treated with therapeutic levels of CNI immunosuppression.\textsuperscript{24} Moreover, in a number of animal models, levels of IFN-γ and IFN-γ-related molecules, such as ITAC and IP-10, parallel lesion formation. Protocols that reduce lesion formation also reduce IFN-γ and/or related molecules.\textsuperscript{244,269–272} There is even evidence that IFN-γ may play a direct role in disease progression.\textsuperscript{273,274} Our cell transfer model has allowed us to confirm that the cellular source of this IFN-γ is the CD8+ T cell. In our model at least, non-T cell sources of IFN-γ do not induce AV.\textsuperscript{24} We speculate that this may be due to the guided expression of IFN-γ by CD8+ T cells in response to antigen-specific interactions, presumably with target graft cells.

IFN-γ has recently been shown to potentiate SMC apoptosis in vitro by activation of the intracellular signal STAT3.\textsuperscript{275} Whether this also holds true for SMC in the vascular media is, as yet, untested. It is known that IFN-γ renders SMC more sensitive to FasL killing\textsuperscript{193} in vitro and, as such, may contribute to SMC loss in more than one way. The data presented here lead us to speculate that one of the important roles of IFN-γ is to upregulate Fas/FasL killing of SMC.

In this study we provide further evidence in support of our working hypothesis that CD8+ T cells mediate graft vasculopathy by inducing medial damage. We first demonstrated that CD8+ T cells infiltrate the graft media by 5 wk post-transplantation, at which time
we also see an upregulation in mRNA expression of CD8+ T cell effector mediators, such as IFN-γ and Granzyme B, in the grafts. This correlates with the initiation of SMC apoptosis in the graft vascular media. We and others have demonstrated that SMC apoptosis is observed as early as 2wk post-transplantation in the absence of effective CNI immunosuppression. Under such conditions the tissue damage is a direct result of CD4+ T cell mediated acute rejection events. In this study, where acute rejection events are eliminated by CNI immunosuppression, SMC apoptosis is not seen until 5–6wk post-transplantation.

To ascertain the primary pathway of SMC loss we examined SMC loss in donor/recipient combinations that resulted in either CTL or IFN-γ blockade. These experiments confirmed that CD8+ T cells require both pathways together to induce SMC loss in the face of CNI immunosuppression. These data are consistent with our previous evidence that both pathways of CD8+ T cell cytotoxicity are required for neointima lesion formation under CNI immunosuppression. This confirms a correlation between SMC loss and neointimal formation and suggests collaboration between the two pathways of CD8+ T cell cytotoxicity.

The Fas/FasL killing pathway has been implicated in the development of AV and more specifically in SMC apoptosis. In one study, all apoptotic cells detected in human transplant coronary arteries were shown to be Fas positive. In 1998, Moldovan et al. demonstrated that medial SMCs are induced to undergo apoptosis in AV and that this apoptosis requires a functional Fas pathway. More interestingly, there is evidence that IFN-γ increases Fas surface expression from internal stores in to vascular SMCs and
therefore renders them more sensitive to Fas/FasL mediated apoptosis.\textsuperscript{193,277} Such a change in Fas expression could occur in medial SMCs rendering them more susceptible to CTL mediated apoptosis.

In contrast, there is abundant evidence that the primary mode of killing by CTL is by granzyme activation of intracellular caspases.\textsuperscript{265,278,279} Choy et al.,\textsuperscript{30} in a series of elegant experiments, demonstrated a significant reduction in cardiac AV in granzyme deficient animals.

To clarify this issue we compared the relative contribution of the Fas/FasL pathway and the perforin/granzyme pathway in SMC loss and lesion formation. When we blocked the Fas/FasL pathway (leaving the IFN-γ and perforin/granzyme pathway intact), SMC loss and lesion formation was ablated. When we blocked the perforin/granzyme pathway (leaving the IFN-γ and Fas/FasL pathways intact), AV was still seen. In this latter group, there was no significant change in SMC loss or lesion size when compared to fully immunocompetent WT recipient controls.

The finding that the perforin/granzyme pathway is of limited importance is at variance with the data from the work of Choy et al., who demonstrated a reduced, although not ablated, lesion in the absence of granzyme activity.\textsuperscript{30} This variance might result from the fact that the relatively small group size used here demonstrated a numerical, but not statistical, decrease in the mean lesion size in the perforin/granzyme pathway blocked group. Perhaps larger group sizes would result in similar results as seen by Choy et al., such that a reduced, but not ablated, AV response would be demonstrated. This variance might also be explained by the fact that the two models are very different. In our model,
we use fully disparate (at MHC class I, II, and minor antigens) strain combinations and ablate acute rejection events with CNI immunosuppression. This reflects the clinical reality of human heart transplantation. Choy et al.\textsuperscript{30} used minor antigen disparity only in their transplant combination. The resulting weak response does not require CNI immunosuppression to prevent acute rejection. In contrast to our model, this latter widely used model leaves CD4\textsuperscript{+} T cell activities intact and does not provide foreign Class I MHC as a primary target for the CTL.

In contrast, the data here clearly demonstrate that the Fas/FasL pathway, in cooperation with the IFN-\(\gamma\) mediated pathway, is critical for the development of AV in the presence of effective levels of CNI immunosuppression. Lesion formation did not occur in recipients in which the Fas/FasL pathway was blocked. It is noteworthy that vascular SMC are normally resistant to Fas mediated killing by internal sequestration of this molecule to maintain the integrity of the vessel wall.\textsuperscript{280} Fas-induced apoptosis of these cells appears only after additional priming.\textsuperscript{281} This priming can take place in response to the high levels of IFN-\(\gamma\) produced by activated CD8\textsuperscript{+} T cells.

Evidence provided in this study is consistent with our hypothesis that AV is the result of an allo-immune response mediated by CD8\textsuperscript{+} T cells that escape the effects of calcineurin inhibitor immunosuppression. The data suggests that the IFN-\(\gamma\) mediated pathway acts in cooperation with the Fas/FasL killing pathway to mediate SMC loss. This leads to extensive medial damage which is crucial to the development of the neointimal lesion and progression of AV.
Figure 3.1 CD8+T cell influx in AV under CyA immunosuppression. (a) Representative photomicrographs of allografts from CyA treated recipient mice grafts at 4 and 6wks post-transplant probed with anti-CD8 antibody. (n=5). Arrows indicate internal and external elastic lamina. (b) Quantification of infiltrating CD8+T cells at 4, 5 and 6wk post-transplant. Aortic sections were probed with anti-CD8 antibody and positive cells were quantified by digital image analysis (n=5/group).
Figure 3.2 CD8+T cell effector mediator expression in AV. mRNA expression (by qPCR) of IFN-γ (a) and granzyme B (Gzm B) (b) in allografts of CyA treated recipient mice at 5wk post-transplant compared to syngeneic grafts. mRNA levels are expressed relative to three housekeeping genes. (n=3 for syngeneic controls, n=6 for IFN-γ; n=7 for GzmB)
Figure 3.3 Medial cell apoptosis. Representative photomicrographs from allografts of CyA treated recipient mice at 4 wk or 6 wk post-transplantation. Sections stained using TUNEL technique to detect apoptotic nuclei. Red arrows point to apoptotic SMC nuclei in the media (M). Black arrows point to non-apoptotic nuclei. “A” indicates adventitia, and “N” indicates neointimal lesion.
Figure 3.4 CD8+ T cell effector pathways required for medial cell loss. Wild type allografts were transplanted into RAG1−/− recipients reconstituted with wild type CD8+ T cells (WT); allografts from Fas−/− mice were transplanted into RAG1−/− recipients reconstituted with CD8+ T cells from perforin−/− mice (CTL−/−); wild type allografts were transplanted into RAG1−/− recipients reconstituted with IFN-γ−/− CD8+ T cells (IFN-γ−/−). Medial SMC numbers per cross section of were enumerated at 8wks post-transplantation. All recipients were treated with CyA immunosuppression (n=3 per group).
Figure 3.5 IFN-γ cooperates with Fas/FasL pathway to induce AV. (a) Representative photomicrographs of aortic section from a transplant combination where the perforin/granzyme pathway is blocked. (b) Representative photomicrographs of aortic section from a transplant combination where the Fas/FasL pathway is blocked. Scale bars represent 50μm. (c) Digital image analysis of sections measuring the relative medial area in: a WT combination (WT); a combination with the perforin (prf)/granzyme pathway blocked (Prf−/−); a combination with the Fas/FasL pathway blocked (Fas−/−) or in native untransplanted aortas. n=4 per group. (d) Digital image analysis of sections measuring the extent of intimal lesion formation in: a wild type combination (WT); a combination where the prf/granzyme pathway is blocked (Prf−/−); or in a combination where the Fas/FasL pathway is blocked (Fas−/−). n=4 per group.
CHAPTER 4 CHARACTERIZATION OF DE NOVO MEMORY CD8+ T CELL DEVELOPMENT IN THE PRESENCE OF CNI IMMUNOSUPPRESSION

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Hart-Matyas M, Gareau AJ, Hirsch GM, Lee TD. De novo allospecific memory CD8+ T cells develop in response to allogeneic challenge when calcineurin inhibitor immunosuppression is delayed. JHLT.

Contributions: Allopriming and CyA administration were completed by M Hart-Matyas. Tissue isolations were completed by M Hart-Matyas, AJ Gareau, T Myers, and B Ross. Assays were set-up by M Hart-Matyas, AJ Gareau and T Myers. Data was collected, analysed, and figures completed by M Hart-Matyas.
4.1 Introduction

Interest in the measurement of pre-existing anti-donor effector and memory T cells in solid-organ transplant recipients continues to increase. This interest has arisen because of the recognition that a large portion of an otherwise protective memory T cell population can cross-react with donor antigens and potentially contribute to rejection. For instance, one estimate suggests that 45% of virus-specific T cells can engage with antigen presented in allo-HLA molecules and mediate death of their allogeneic targets. The presence of these T cells in recipients is further complicated by evidence which suggests that memory T cells demonstrate superior resistance to immunosuppression compared to naïve T cells. Recently, in addition to pre-existing populations, de novo allospecific memory T cells have also been recognized as a potential threat to transplant recipients, but only very limited investigation into this population exists. To address this issue, we investigated the in vivo development of de novo effector and central memory CD8+ T cells in response to allogeneic challenge in the presence of calcineurin inhibitor (CNI) immunosuppression, the cornerstone of immunosuppression protocols for cardiac transplantation.

Memory T cells can be discriminated from naïve T cell populations based on surface expression of CD45RO (in humans), or CD44 (in mice). Within this general memory population further discrimination between effector and central memory subsets can be performed using CCR7 and CD62L-positive. These distinct phenotypes also represent functional differences. For instance, effector memory can be measured through production of IFN-γ in response to brief ex vivo stimulation, whereas central memory can be measured through IL-2 expression following stimulation. In this study we focused
on the CD8+ lineage because of the evidence we, and others, have accumulated demonstrating that CD8+ T cells are less sensitive to CNI immunosuppression than CD4+ T cells.\textsuperscript{183,251,288}

Interestingly, the timing of CNI immunosuppression introduction into the therapeutic regimen appears to vary among patients and across centers.\textsuperscript{228,289–291} The extent to which this occurs has not been widely reported. We hypothesized that this delay would permit early interleukin (IL)-2 expression by alloreactive T cells, which is a requirement for memory CD8+ T cell development. To address this possibility, we assessed the development and functionality of memory CD8+ T cells in response to allogeneic challenge at various clinically relevant time-points of CNI initiation post-transplant.

We observed that immediate and early initiation of CyA immunosuppression prevented the development of memory CD8+ T cells. In contrast, delaying CyA initiation to within clinically accepted time-points permitted the development of memory CD8+ T cells. Given the available data regarding the threat that memory T cells pose in a transplant setting, our findings suggest that in settings of delayed CNI immunosuppression, clinical evaluation of \textit{de novo} allospecific memory CD8+ T cell development should be considered.

\textbf{4.2 Results}

\subsection*{4.2.1 Kinetics of the CD8 + T cell response to a MHC mismatched aloprime in the presence of immediate CyA initiation}

We examined the kinetics of primary effector CD8+ T cell development in our alloprime model (Fig. 1). Effector CD8+ T cells (as measured by IFN-\(\gamma\) production) were detected,
with a peak response at 1wk post-prime, in recipients not treated with CyA (p<0.001). In contrast, treating recipients with CyA at day 0 significantly prevented the development of effector CD8+ T cells by 1wk post-prime (p<0.001). These findings are in agreement with previous cytotoxicity studies using this model. The effector CD8+ T cell population that was observed at 1wk post-prime in recipients not treated with CyA exhibited significant contraction by 2wk post-prime (p<0.001).

4.2.2 Immediate CyA initiation prevents the development of effector memory CD8+ T cells

An equal frequency of IFN-γ+ CD8+ T cells at 2wk and 5wk post-priming (Fig. 2A) confirmed that contraction of the primary effector CD8+ T cell population was complete by 2wk post-prime. Therefore, CD8+ T cells which produced IFN-γ in response to brief ex vivo stimulation at 5wk post-prime were interpreted to represent effector memory CD8+ T cells (Fig. 2). At this time-point the frequency of effector memory CD8+ T cells in recipients treated with CyA at day 0 was significantly lower than recipients not treated with CyA (p<0.001) (Fig. 2B) Furthermore, the frequency of effector memory CD8+ T cells in recipients treated with CyA at day 0 was not significantly different from naïve non-primed controls (p>0.05). These data demonstrate that immediate CyA initiation post-prime prevents the development of functional effector memory CD8+ T cells.

4.2.3 Immediate CyA initiation prevents the development of secondary effector CD8+ T cells

We subsequently measured the generation of secondary effector CD8+ T cells in primed recipients that received CyA at day 0. To do so, primed recipients were boosted at 5wk post-prime with donor splenocytes and assessed 1wk later. Note that CyA was withdrawn
3d before the boost. This was done to purge CyA from the system so as not to influence the subsequent development of secondary effector CD8+ T cells. Secondary effector CD8+ T cells developed in boosted recipients not treated with CyA (Fig 3). In contrast, the development of secondary effectors was prevented in boosted recipients treated with CyA immediately following the prime (p<0.01) (Fig. 3). These results reveal that CyA initiation immediately following the prime abrogates subsequent generation of secondary effectors.

4.2.4 Delayed CyA initiation permits the development of effector CD8+ T cells

We hypothesized that delayed CNI initiation, resulting in delayed inhibition of IL-2 expression, would allow de novo allospecific memory CD8+ T cell formation. To test this hypothesis we first needed to characterize the development of primary effector CD8+ T cells under conditions of delayed CyA initiation. We initiated CyA on day 2 or day 6 post-prime, rather than at day 0, and harvested recipient splenocytes at 1wk and 10d post-prime (Fig. 4A and Fig. 4B, respectively). Effector CD8+ T cells were not detected at 1wk post-prime in recipients where CyA was initiated at day 2 (Fig. 4A). In contrast, effector CD8+ T cells developed in recipients when CyA was initiated at day 6 post-prime (p<0.001). Recipients sacrificed at 1wk and at 10d post-priming showed the development and contraction of substantial, albeit signifficantly reduced, effector CD8+ T cells even in the face of CyA given at day 6 (p<0.01)(Fig 4A and 4B). These results reveal that the timing of CyA initiation influences the development of primary allospecific effector CD8+ T cells.
4.2.5 Delayed CyA Initiation Permits Effector and Central Memory CD8+ T Cell Development

Since delaying CyA initiation permitted the formation of effector CD8+ T cells we assessed for the subsequent development of memory CD8+ T cells at 5wk post-prime. Primed mice treated with CyA from day 2 onwards did not exhibit effector memory CD8+ T cells at 5wk post-prime (Fig. 5 A). In contrast, primed recipients treated with CyA from day 6 onwards exhibited effector memory CD8+ T cells, the frequency of which were not significantly different from recipients not treated with CyA (p>0.05)(Fig. 5 A). This finding prompted us to assess for the development of functional central memory CD8+ T cells in these recipients. This was accomplished by measuring the concentration of IL-2, a hallmark of central memory cells, in MLR supernatants where purified CD8+ T cells from primed recipients represented the responding population. The concentrations of IL-2 from wells that contained purified CD8+ T cells from recipients receiving CyA from day 6 onwards or recipients not treated with CyA were not significantly different (p>0.05)(Fig 5B). Furthermore, these concentrations were significantly higher than in wells that contained CD8+ T cells isolated from naïve control mice (p<0.01 and p<0.001, respectively), thus confirming the presence of central memory CD8+ T cells in 5wk primed recipients. Taken together, these data suggest that the initiation of CyA 6ds post-priming permits the development of functional allospecific effector and central memory CD8+ T cells with equivalent functionality to those in recipients not treated with CyA.

4.2.6 Delayed CyA Initiation Permits the Development of Secondary Effector CD8+ T Cells
To confirm that the memory CD8+ T cell population which develops when CyA initiation is delayed (described above) is also functional \textit{in vivo}, we boosted these recipients, as before, and assessed for secondary effector CD8+ T cells 1wk later. As expected, secondary effector CD8+ T cells were not detected in recipients treated with CyA from day 2 onward. In contrast, secondary effector CD8+ T cells were detected in recipients treated with CyA from day 6 onwards as well as recipients not treated with CyA (Fig. 6A). Interestingly, the frequency of secondary effectors was significantly higher in recipients receiving CyA from day 6 onwards compared to recipients not receiving CyA (p<0.01). When the data was adjusted for the total number of effector cells this significant increase was no longer seen (Fig 6B). Note that some replicates in these three groups failed to respond to the prime and/or boost as indicated by secondary effector frequencies below that of the average primary effector population (3.45%), and were therefore removed. Taken together, these data demonstrate that delaying CyA initiation to 6 days after alloprime permits the development of secondary effectors CD8+ T cells within the range seen in recipients not treated with CyA.

\textbf{4.3 Discussion}

Throughout life a diverse repertoire of immunological memory develops in response to pathogens and environmental stimuli. Many authors have noted the presence of allospecific and cross-reactive memory T cells within this memory compartment that could potentially contribute to solid-organ transplant rejection.\textsuperscript{217,221,283,284} Recently, \textit{de novo} memory, developing in response to the transplant itself, has also been acknowledged as a potential contributor to graft loss.\textsuperscript{221} Cardiac transplant recipients may be particularly susceptible to \textit{de novo} memory formation because of the extent at which
they are HLA-mismatched from their donors. For instance, over the past two decades more than 60% of recipients have received cardiac transplants mismatched at 3 or more loci. Furthermore, a recent report suggests that this degree of mismatch leads to worse outcomes, even with modern immunosuppression regimens. Despite these findings there has been only limited investigation into the development of de novo memory T cells in the presence of CNI immunosuppression, the mainstay of immunosuppression for cardiac transplant recipients.

Evidence from in vitro studies have demonstrated successful, albeit attenuated, development of memory CD8+ T cells in the presence of CNI immunosuppression. In contrast, an in vivo study in the presence of CNI immunosuppression revealed a differentiation shift away from early memory precursors to terminally differentiated effectors without later evaluation of memory functionality. The functionality of memory CD8+ T cells which develop in response to an in vivo alloprime in the presence of CNI immunosuppression has not yet been assessed.

We observed complete inhibition of de novo effector memory CD8+ T cell development following immediate administration of CyA post-prime in our murine model. In further support of an absent memory compartment, we observed a complete lack of secondary effectors in boosted recipients treated with CyA immediately post-prime. These finding are in contrast to earlier data reported from in vitro studies. This may be due to the dosing schedules used. We mimicked the clinical situation by providing CyA on a daily basis, whereas others administered CNI immunosuppression to cultures only every other day.
Our data provide evidence that de novo memory CD8+ T cell development is abrogated if CNI immunosuppression is initiated immediately after allogeneic challenge. However, in some centers, CNI initiation is delayed, in an attempt to limit nephrotoxicity. Delayed CNI immunosuppression has been reported by others and was reviewed in a recent multi-center study from the UK which demonstrated variability in the timing of CNI initiation. In our in vivo rodent study we approached this issue with the view that such a delay in CNI initiation would allow a ‘window of opportunity’ for the development of de novo allospecific memory CD8+ T cells.

Delaying CyA initiation to day 2 post-prime did not allow the development of functional allospecific memory CD8+ T cells. In contrast, prolonging initiation to day 6 post-prime did allow for their development. Primed recipients treated with CyA from day 6 onward and harvested at 5wk post-prime exhibited effector memory CD8+ T cells, identified by the frequency of IFN-γ+ CD8+ T cells, and central memory CD8+ T cells, identified by the expression of IL-2 from purified CD8+ T cells. To the best of our knowledge, these data are the first to demonstrate that functional allospecific memory CD8+ T cells can develop in vivo when CNI immunosuppression is delayed within a clinically utilized timeframe.

In addition to direct involvement in graft tissue destruction, memory CD8+ T cells could give rise to a population of allospecific secondary effectors that would further exacerbate the rejection event. We sought to identify the capacity of de novo allospecific memory CD8+ T cells, which developed in the presence of CyA immunosuppression, to generate a population of secondary effector CD8+ T cells. Our data reveals the development of
secondary effector CD8+ T populations of similar size in boosted recipients treated with CyA from day 6 onward, and in boosted recipients not treated with CyA. These results reveal that *de novo* allospecific memory CD8+ T cells that develop in the presence of delayed CNI immunosuppression maintain the ability to develop robust secondary effector populations.

In summary, our study reveals that the timing of CNI immunosuppression initiation strongly influences the formation of memory CD8+ T cells in response to allogeneic challenge. The data suggest that the programming of functional memory CD8+ T cell precursors occurs after 2d and before 6d post-prime. The *de novo* memory CD8+ T cell population that develops represents a previously unrecognized pool within the memory compartment that needs to be acknowledged when considering the threat of memory T cells to the maintenance of allogeneic solid-organ transplants. We hypothesize that these *de novo* allospecific memory CD8+ T cells, in addition to pre-existing allospecific and cross-reactive populations, could hamper graft function by promoting the development of rejection events.
Figure 4.1 Administration of CyA at 0d post-prime inhibits the development of primary effector CD8+ T cells. Frequency of IFN-γ+ CD8+ T cells at 1 and 2wks post-prime in non-CyA treated recipients or at 1wk in CyA at 0d treated recipients, and naïve controls. Results are compiled from at least three independent experiments.
Figure 4.2 CyA initiation following alloprime prevents effector/memory and central memory CD8+ T cell development. A) Frequency of IFN-γ+ CD8+ T cells at 2wk and 5wk post-prime in non-CyA treated recipients. Results are from one experiment. B) Frequency of IFN-γ+ CD8+ T cells at 5wk post-prime in non-CyA treated or CyA at 0d treated recipients, and naïve controls. Results are compiled from three independent experiments.
Figure 4.3 Immediate initiation of CyA at 0d post-prime inhibits the development of secondary effector CD8+ T cells in response to a boost. Frequency of IFN-γ+ CD8+ T cells at 6wk post-prime (1wk post-boost) in non-CyA treated or CyA at 0d treated recipients. Results are compiled from two independent experiments.
Figure 4.4 The timing of delayed CyA initiation post-prime is pivotal in permitting the development of primary effector CD8+ T cells. A) Frequency of IFN-γ+ CD8+ T cells at 1wk post-prime in non-CyA treated, or CyA at 2d or 6d treated recipients. Results are compiled from two independent experiments. B) Frequency of IFN-γ+ CD8+ T cells at 10d post-prime in non-CyA or CyA at 6d treated recipients. Results are compiled from two independent experiments.
Figure 4.5 Prolonged delay of CyA initiation post-prime permits the development of functional effector and central memory CD8+ T cells. A) Frequency of IFN-γ+ CD8+ T cells at 5wk post-prime in non-CyA treated, or CyA at 2d or 6d treated recipients. Results are compiled from three independent experiments. B) Concentration of IL-2 in one-way MLR supernatants with responder purified CD8+ T cells from 5wk post-prime non-CyA treated or CyA at 2d or 6d treated recipients. Results are compiled from three independent experiments.
Figure 4.6 Prolonged delay of CyA initiation post-prime permits the development of secondary effector CD8+ T cells in response to a boost. A) Frequency of IFN-γ+ effector CD8+ T cells at 6wk post-prime (1wk post-boost) in non-CyA treated, or CyA at 2d or 6d treated recipients. B) Total number of IFN-γ+ CD8+ T cells at 6wk post-prime (1wk post-boost) in non-CyA treated, or CyA at 2d or 6d treated recipients. Results are compiled from three independent experiments.
CHAPTER 5 DISCUSSION

5.1 Significance of experimental findings
Immunosuppression has dramatically improved since the first successful human cardiac transplant in which irradiation, steroids and azathioprine were used in an attempt to prevent rejection. One of the most notable improvements has been the introduction of calcineurin inhibitors (CNIs), which following their widespread adoption have endured as the mainstay of post-transplant protocols with currently more than 90% of maintenance regimens including them at 1 and 5y post-transplant follow-ups. Despite the relative success of CNIs, there is evidence in human cardiac transplant recipients and more widely in experimental transplant models that the adaptive immune response may retain some functionality in the presence of calcineurin inhibition alone. This study focused on the CD8+ T cell lineage in this context.

5.1.1 The role of CD8+ T cells in the development of AV
Under the influence of immunosuppression the specific contribution of CD8+ T cells to the development of cardiac AV is still not completely known. We have proposed that immune-mediated killing of medial SMCs initiates a repair response which culminates in neointimal lesion formation similar to that reported by Reidy and colleagues. In our previous study we illustrated the requirement for CD8+ T cells in the development of the neointimal lesion in the presence of CNI immunosuppression. Further, we demonstrated neointimal lesion development is mirrored by the loss of medial SMCs. In the present study, we observed that neointimal lesion development and medial SMC loss also coincide with CD8+ T cell infiltration into allograft tissue. We revealed that the loss of medial SMC is intimately linked to direct and indirect cytotoxic pathways.
mediated by CD8+ T cells. Specifically, in the absence of the direct cytotoxic pathways mediated through perforin/granzyme B and Fas/FasL or CD8+ T cell-derived IFN-γ medial SMCs persist in the allograft. Furthermore, neointimal lesion did not develop in the absence of cytotoxicity mediated by the Fas/FasL pathway, but did develop in the absence of the perforin/granzyme B pathway. In contrast, in a murine heterotopic heart transplant model the perforin/granzyme B pathway plays a significant role in neointimal lesion formation.\textsuperscript{30,31} As was discussed above, the heterotopic heart transplant model was carried out using donor/recipient pairs mismatched at minor histocompatibility antigens only and in the absence of immunosuppression. This setting does not accurately reflect the clinical scenario where more than 60% of cardiac transplant recipients receive hearts mismatched at 5-6 HLA loci with more than 90% of recipients on CNI immunosuppression.\textsuperscript{1} In contrast, our model uses fully MHC mismatched donor/recipient pairs and incorporates the use of CNI immunosuppression, and therefore may more closely mimic the clinical development of neointimal lesion formation. In this model, SMC loss and neointimal lesion formation are linked to CD8+ T cell-derived IFN-γ and Fas engagement.\textsuperscript{23,183} Within the literature IFN-γ is reported to be involved in rejection using other transplant models.\textsuperscript{20,193,207,294} Specifically, IFN-γ-derived from recipient peripheral blood mononuclear cells has been shown to be required for neointimal lesion formation in a humanized mouse transplant model.\textsuperscript{20} As was observed in the humanized model,\textsuperscript{193} it may be the case that in our model IFN-γ induces the surface expression of Fas by SMCs facilitating death receptor driven apoptosis.
In the context of clinical cardiac transplantation, a number of recent studies have documented the presence of T cells in coronary tissue afflicted with cardiac AV.\textsuperscript{37,58,60} One of these studies reported on the activation status and observed a large proportion of activated T cells indicated by CD27, CD69 and HLA-DR expression.\textsuperscript{37} These findings lend further support to the notion that T cells are involved in the development of cardiac AV. In earlier experiments, infiltrating T cells cultured from endomyocardial biopsy (EMB) samples were functionally assessed.\textsuperscript{182,295} These studies revealed that the infiltrating CD8\textsuperscript{+} T cells were capable of killing donor-derived graft cells. This method has not been attempted with coronary tissue that has cardiac AV, but their results may represent what could be expected if this were accomplished. Certainly, our findings suggest that CD8\textsuperscript{+} T cell-mediated injury contributes to the development of AV.\textsuperscript{23,24,183} In part, this injury may be supported by the expression of IFN-\textgamma by CD8\textsuperscript{+} T cells.\textsuperscript{23,24} This may occur through IFN-\textgamma-induced surface expression of Fas by graft cells,\textsuperscript{193} thereby increasing susceptibility to death receptor driven apoptosis.\textsuperscript{187} Certainly, Fas expression by ECs undergoing apoptosis has been reported in cardiac AV specimens.\textsuperscript{45} However, this observation has not yet been reported for SMCs in cardiac AV specimens. This will be discussed below, and may reflect relative immunoprivilege of the human medial compartment.\textsuperscript{47}

5.1.2 THE DEVELOPMENT OF DE NOVO ALLOREACTIVE MEMORY CD8\textsuperscript{+} T CELLS

Increasingly alloreactive memory T cells are being recognized as a serious threat to the maintenance of solid-organ transplants.\textsuperscript{216,217} Coupled with our observations stated above, we hypothesized that a portion of this threat would be posed by alloreactive memory CD8\textsuperscript{+} T cells. As was discussed above, the alloreactive memory compartment may
already exist in a cardiac transplant recipient prior to transplant, or alternatively develop following transplantation. The development of this latter population, de novo memory CD8+ T cells, has not been thoroughly explored in the literature.

We have previously established that the in vitro cytotoxicity of CD8+ T cells alloprimed in vivo in the presence of CyA is greatly diminished, yet they are able to mediate the development of AV in vivo in the presence of CyA.183 This activity involves both IFN-γ expression and the direct cytotoxic pathways, with an emphasis on Fas death receptor engagement.23,24 We speculated that this apparent inconsistency could be due to the development of memory CD8+ T cells in vivo. In the present study, we addressed this notion and have found that effector memory CD8+ T cells do not develop following allopriming in vivo when CyA immunosuppression is used. Furthermore, given that these recipients do not develop secondary effectors we speculated that central memory CD8+ T cell development is also prevented under these conditions. Within the literature, similar observations have been made in an experimental murine model of viral infection.288 In this model, virus-specific CD8+ T cells expand in the presence of CNI immunosuppression, but most progeny develop into non-functional short lived effector cells (SLECs). Later assessment of virus-specific memory CD8+ T cells was not assessed, but would be unlikely to develop from a population of effectors lacking memory precursor cells (MPECs). In contrast, the in vitro development of functional human effector memory CD8+ T cells has been observed in the presence of CNI immunosuppression.285 Two considerable differences between this study and the in vivo models were the addition of CNI immunosuppression every other day, instead of daily, and the addition of low dose IL-2 to the cultures to improve yield of viable cells. These
differences cast doubt on the finding that memory CD8+ T cell generation truly occurred independently of IL-2 signalling. Taken together, it does not seem likely that functional memory CD8+ T cells can develop when they are challenged in the presence of CNI immunosuppression.

As was discussed above, cardiac transplant recipients are not necessarily started on CNI immunosuppression immediately post-transplant, but the exact prevalence of this practice in the transplantation community is not widely reported. Some insight, however, comes from a recent UK national study which described variability in the timing of CNI initiation across multiple centers and more than 2x10^3 recipients. In the present study, we speculated that under conditions of delayed CNI initiation the development of de novo memory CD8+ T cells may be possible. Certainly, when CNI immunosuppression was delayed to 6ds post-challenge, which is within the observed clinical range, alloprimed recipients developed a robust population of de novo alloreactive memory CD8+ T cells. Under these conditions both effector and central memory subsets were detected in recipients and following re-challenge in the absence of CNI immunosuppression developed functional secondary effectors. This latter observation demonstrates the ability of alloreactive memory CD8+ T cells which develop in the presence of CNI immunosuppression to generate functional secondary effectors. While we acknowledge that these findings have yet to be confirmed in human cardiac transplant recipients, we speculate that the de novo alloreactive memory CD8+ T cells could be among high avidity CD8+ T cells which become less inhibited when immunosuppression is tapered post-transplant.
5.2 Potential limitations of experimental models

5.2.1 Murine aortic interposition transplant model

There are two major differences between the development of AV in murine models and human models or transplants. Firstly, unlike the aortic graft, there is not a significant loss of medial SMCs from coronary grafts in the humanized mouse model.\textsuperscript{46,47} This discrepancy may be due to immunoprivilege in the medial compartment of human vasculature. One study reported the induction of indoleamine 2,3-dioxygenase (IDO), an enzyme which degrades an essential T cell metabolite, in the media of human coronary grafts.\textsuperscript{47} A direct link between SMC protection and IDO expression was demonstrated through the inhibition of IDO which led to a significant loss of $\alpha$-actin positive medial SMCs. Interestingly, IDO expression has also recently been observed in a fully-mismatched murine aortic transplant model.\textsuperscript{298} In this murine model medial collapse is histologically evident, but not assessed. Therefore, it would appear as though IDO expression in human and not murine grafts is sufficient to induce medial immunoprivilege. This difference may be due to different levels of IDO expression. In the human coronary transplant report, strong IDO expression was observed in SMCs. Therefore, a link may exist between medial immunoprivilege and the number of medial SMCs present. On the basis of this assumption, one would expect the medial layer in human coronaries to be better protected than in murine aortas because there are significantly more SMCs (~300\textsuperscript{47} versus ~150\textsuperscript{299}, per cross-section, respectively). There may be other explanations for this species specific difference, but without further investigation the solution remains a matter of speculation. Certainly, in the murine model we have evidence to suggest that medial injury is a stronger driving force in the development of AV than intimal injury.\textsuperscript{40} However, as was discussed above, in human
models a destroyed medial compartment is not required for the development of AV. The significance of this discrepancy to the development of AV is not immediately clear, and regardless of the explanation, it is clear that in both models adaptive immune injury, and particularly IFN-γ, play a significant role in the development of AV in murine and human models.

The origin of the neointimal lesion is the second major difference between AV in murine models and cardiac AV in humans. As was discussed above, the neointimal lesion is almost exclusively composed of recipient cells in murine models, whereas the neointimal lesion in cardiac AV is almost exclusively composed of donor cells. One recent hypothesis to explain this difference posits that in humans, the AV lesion is a combination of the pre-existing benign intimal thickening (BIT) present in donor hearts and atherosclerotic lesions that develop following transplantation. Theoretically, expansion of the benign intimal layer could account for the largely donor origins of the human AV lesion. It is noteworthy to point out that this intimal thickening has not been observed in the aorta or coronaries of rodents. Therefore, in the absence of BIT, the recipient origin of the neointimal lesion in rodent models of AV is unsurprising.

5.2.2 CELLULAR CHALLENGE MODEL

We used the cellular challenge model to mimic the alloantigenic challenge that a patient may experience following transplantation of a solid-organ. In terms of stimulating an immune response the cellular challenge model only provides an acute source of antigen since the donor splenocytes are mitomycin C treated and therefore unable to successfully divide and persist in the recipient. In comparison, a solid-tissue transplant could serve as
a chronic source of antigen. Whether these two models differ in their ability to stimulate the development of de novo alloreactive memory CD8+ T cell is not clear. We do have preliminary evidence to suggest that functional de novo alloreactive memory CD8+ T cells develop in recipients of murine aortic interposition grafts not treated with CNI immunosuppression post-transplant (Appendix 1 Figure 1.1). Further, these memory CD8+ T cells can be stimulated to produce a population of secondary effectors (Appendix 1 Figure 1.1). In contrast, immediate initiation of CyA post-transplant prevented the development of de novo alloreactive memory CD8+ T cells, and the development of secondary effectors (Appendix 1 Figure 1.1). These are the same findings that were reported in the above study using the cellular challenge model and may therefore support the notion that similar findings could be expected in recipients of solid-tissue transplant that were treated with delayed CNI immunosuppression.

5.3 Future directions

5.3.1 Determine if BIT is attacked by alloreactive CD8+ T cells

Taking into consideration the findings from the current study, the recognition that BIT may contribute to the development of AV begs the question of whether it is targeted by alloreactive CD8+ T cells. Certainly, there is good evidence to suggest that medial SMCs in human coronary arteries enjoy a position of immunoprivilege because of IDO expression. However, is the same true of SMCs within BIT? Within the same study, a cellular layer situated on top of the internal elastic, which may be BIT, is observed to be heavily infiltrated with memory T cells. In another study which used the same model, this layer stains α-actin positive, thereby reinforcing the notion that this layer may indeed be BIT. One approach our lab has undertaken to investigate this question is to develop an
animal model which incorporates a BIT-like lesion. The coronary transplant humanized mouse model is also being entertained as an option, although this strategy is limited by ease of access to fresh human coronary tissue.

5.3.2 DETERMINE IF DE NOVO MEMORY CD8+ T CELLS DEVELOP IN HUMAN CARDIAC TRANSPLANT RECIPIENTS

There is also interest in pursuing the development of de novo alloreactive memory CD8+ T cells in cardiac transplant recipients when CNI immunosuppression is delayed. In comparison to our model, cardiac transplant recipients may receive induction immunosuppression to support the CNI delay post-transplant. In the studies cited above, either ATG or Basiliximab (IL-2RA) were used for induction. Unlike Basiliximab, ATG does not prevent IL-2 signalling, but instead depletes circulating and peripheral T cells, resulting in a lymphopenic environment. The surviving T cell population undergoes homeostatic proliferation, which for the CD8+ compartment favours terminally differentiated effectors and effector memory subsets. Future study will be required to ascertain whether de novo memory T cells develop in this environment.
Appendix 1 Figure 1.1. CyA initiation following transplantation prevents effector memory CD8+ T cell development and secondary effector development following boost. Frequency of IFN-γ+ CD8+ T cells at 8wk post-transplant in non-CyA treated or CyA at 0d treated recipients, and naïve controls. Some transplant recipients were boosted in the absence of CyA and harvested 1wk later. Results are compiled from two independent experiments.
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