B CELLS AND ANTIBODY IN THE DEVELOPMENT OF LONG-TERM CARDIAC GRAFT REJECTION

by

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For my dad

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ABSTRACT

The long-term survival of heart transplants is limited by the development of allograft vasculopathy (AV), a vascular pathology that develops in spite of the use of modern immunosuppressive therapies. Although it is widely accepted that T cells play a major role in the development of AV, the contribution of B cells and antibody has been less well characterized.

A fully MHC-mismatched cell transfer model was used to mimic the antigenic stimulus of a cardiac graft, we examined the production of antibody under conditions of clinically relevant immunosuppression in the form of the calcineurin inhibitor cyclosporine A (CyA). Anti-donor antibody with the capacity to mediate complement-dependent cytotoxicity of donor strain cells, but not third-party cells, developed in the presence of two different doses of CyA (30 mg/kg and 50 mg/kg). When this antibody was passively transferred into immunodeficient B6.RAG1$^{-/-}$ abdominal aortic graft recipients, the antibody alone had the capacity to mediate formation of a neointimal lesion and induce the loss of medial smooth muscle cells. These are two hallmark characteristics of AV in this animal model. A wild-type model, where BALB/c grafts were transplanted into B6 recipients and received daily CyA immunosuppression was used to test the *de novo* antibody response to the transplant itself. Again, anti-donor antibody was produced with the capacity to mediate complement-dependent cytotoxicity of donor cells. In addition, grafts showed evidence of C4d deposition in the medial area, indicating that area as a sit of antibody binding and activation of the classical complement cascade.

The presence of anti-donor antibody has been demonstrated to correlate with poorer graft outcome and a higher risk of developing AV in patients. Examination of human epicardial coronary artery tissue from patients with cardiac transplants demonstrated the presence in the adventitia of ectopic lymphoid structures (ELS) containing CD20^{+} B cells, plasma cells, IgM, and IgG. These findings illustrate active, antibody-producing ELS in close proximity to the vessels developing AV. Of note was the finding of CD20^{+}CD27^{+} memory B cells in these ectopic lymphoid structures. Memory B cells are rapidly re-activated following exposure to their cognate antigen and easily differentiate into plasma cells. Taken together, these data suggest that memory B cells and antibody may be contributing to long-term allograft rejection and therapeutic options should be considered to target these immune mechanisms.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
Ab	Antibody
Abs	Antibodies
ADCC	Antibody-Dependent Cellular Cytotoxicity
AID	Activation-Induced Cytidine Deaminase
AMR	Antibody-Mediated Rejection
ANOVA	Analysis of Variance
AV	Allograft Vasculopathy
β	Beta
BAFF	B Cell Activating Factor
BAFF-R	B Cell Activating Factor Receptor
BCR	B Cell Receptor
BIT	Benign Intimal Thickening
BM	Bone Marrow
C	Constant
CAV	Cardiac Allograft Vasculopathy
CD	Cluster of Differentiation
CD40L	CD40 Ligand
CDR	Complementarity Determining Regions
CNI	Calcineurin Inhibitor
CyA	Cyclosporine A
d	Day

D	Diversity
DNA	Deoxyribonucleic Acid
DSA	Donor-Specific Antibody
ECM	Extracellular Matrix
ELS	Ectopic Lymphoid Structure(s)
FBS	Fetal Bovine Serum
FDC	Follicular Dendritic Cells
FGF	Fibroblastic Growth Factor
γ	Gamma
H&E	Haematoxylin and Eosin
HEV	High Endothelial Venules
HLA	Human Leukocyte Antigen
IEL	Internal Elastic Lamina
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
I/R	Ischemia/Reperfusion Injury
ISHLT	International Society for Heart and Lung Transplantation
J	Joining
LAD	Left Anterior Descending Artery
LCx	Left Circumflex Artery
LPS	Lipopolysaccharide

MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
min	Minute
MMF	Mycophenylate mofetil
mTOR	Mammalian Target of Rapamycin
NFAT	Nuclear Factor of Activated T cells
NK	Natural Killer
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived Growth Factor
PNAd	Peripheral Node Addressin
Pro-B	Progenitor B Cell
RAG	Recombination Activating Gene
RCA	Right Coronary Artery
RPMI	Roswell Park Memorial Institute
RTX	Rituximab
RSS	Recombination Signal Sequence
s.c.	Subcutaneous
SCF	Stem Cell Factor
SCID	Severe Combined Immunodeficiency
SEM	Standard Error of the Mean
sfRPMI	Serum-free RPMI
SMC	Smooth Muscle Cell
Tac	Tacrolimus

TdT	Terminal Deoxynucleotidyl Transferase
TGF	Transforming Growth Factor
Th	T Helper Cell
TLO	Tertiary Lymphoid Organ
TNF	Tumor Necrosis Factor
V	Variable
VCAM-1	Vascular Cell Adhesion Molecule-1
VLA-4	Very Late Antigen-4
wk	week
WT	Wild-type

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CHAPTER 1

INTRODUCTION

1.0 Overview

Cardiac transplantation is the primary long-term treatment for end-stage heart failure. Early survival rates are excellent, at approximately 85-90% at one year.[1] In contrast, long-term survival following transplantation is poor despite the use of modern immunosuppressive therapies. Late graft loss can be attributed to a variety of causes but one of the most important limitations to long term survival is the development of cardiac allograft vasculopathy (AV).[2] The expression of the pathology known as AV depends on the area of the vascular tree examined. For example, in the microvasculature the predominant pathology is associated with endothelial swelling.[3] In the intramyocardial arterioles, AV is characterized by medial thickening.[4] In the epicardial vessels, AV is characterized by a progressive, eventually occlusive, neointimal lesion.[5] This occlusive neointimal lesion impedes circulation and is considered to be the most important limitation of long-term graft survival. It is this lesion that is the topic of study of this thesis. Currently, there are no effective pharmaceutical treatments that prevent AV. Once AV is formed, the only definitive therapy is re-transplantation, but due to the severe shortage in suitable donor organs, this is not a practical treatment option. For this reason, the International Society for Heart and Lung Transplantation (ISHLT) have called cardiac AV the "Achilles heel" of cardiac transplantation.[2]

The etiology of AV is multi-factorial, although it is widely accepted that it is an immune-mediated graft pathology, and T cells play a major role.[6–8] Because the majority of clinical immunosuppressive regimens target T cell function, but rates of survival have not improved, attention has begun to shift to the role played by other immune elements. This paradigm shift has brought B lymphocytes and antibodies, their soluble effector

molecules, into the spotlight in the realm of transplant research. It is still unknown what the exact role of these lymphocytes are, but the thesis research discussed here contributes knowledge to this area.

1.1 Adaptive Immune Responses to Transplantation

Following transplantation of a donor organ into a recipient, a robust immune response will inevitably occur due to recognition by the recipient's immune system of the graft as foreign. Of these foreign antigens recognized by the recipient's immune system, the major histocompatibility complex (MHC), or human leukocyte antigen (HLA) system in humans, is the most important. The HLA system encodes class I and II cell surface glycoproteins that are essential in initiating an effective adaptive immune response.[9] Class I is expressed on the surface of all nucleated cells, while class II is expressed primarily on antigen presenting cells (macrophages, dendritic cells, and B cells).[10]

After transplantation the recipient immune response is presented with two immunological challenges. The first is the presence of donor antigen-presenting cells (primarily dendritic cells) which can mediate what has been referred to as "direct" antigen presentation.[11] The second is the presentation of donor antigen in the context of recipient MHC on recipient antigen-presenting cells, referred to as "indirect" antigen presentation.[12] The relative role that these types of antigen presentation in play in transplant rejection is still controversial and outside the scope of this thesis. In any event, $CD4^+$ and $CD8^+$ T cells are activated by this antigen presentation and activated effector T cells are developed. The evidence suggests that $CD4^+$ T cell-mediated responses most

likely play a leading role in the development of rejection.[8,13] Indeed, most immunosuppressive regimens target this cell type.

Activated T cells synthesize and release IL-2 and IFNγ.[6] IL-2 supports the growth and differentiation of T cells, including $CD8^+$ T cells. These $CD8^+$ T cells develop into mature cytotoxic effector cells that result in direct graft cell lysis.[14,15] Both $CD4^+$ and $CD8^+$ T cells secrete IFNγ which has pleotropic effects. The most important effects of IFNγ in the context of transplantation are the up-regulation of MHC class I on donor cells in the grafted organ (making them a target for direct killing) and the activation of macrophages (which can damage donor tissue in a non-specific way through frustrated phagocytosis). It is often overlooked that IFNγ is also the switch factor that drives B cells to switch from IgM to the more long-lived complement-fixing IgG.[16] A subset of these activated $CD4^+$ T cells will engage in B cell activation and support B cell differentiation. They accomplish this by secreting IL-4, IL-5, IL-6 and IL-10, important growth and differentiation factors for B cells.[17]

The majority of the immune mechanisms discussed above are controlled, to a great extent, by modern immunosuppressive regimens that target T cell function. The etiology of late graft rejection is less clear but it appears to have a similar starting point. Graft survival studies have implicated donor MHC antigens by showing that HLA matching is correlated with the risk of chronic rejection. In these studies, long-term graft survival is strongly correlated with the degree of histocompatibility between the transplant recipient and donor.[18–20] It is possible that the effect of matching results in a decreased incidence of acute rejection episodes and that this, in turn, leads to a decrease in chronic rejection. There is good evidence that the risk of chronic rejection increases as

the number of acute rejection episodes increases.[21] It is also possible that the mechanism of late graft rejection is significantly different from acute rejection and that although MHC disparity is common to both, the resulting immunological process is not identical.

One similarity between these two responses is the growing evidence that humoral elements are involved in both. Often referred to as antibody-mediated rejection (AMR), humoral responses have been shown to contribute to acute rejection in a number of studies.[22,23] More recently, humoral immune responses have been implicated in late graft rejection.[24–27] Several clinical studies have demonstrated a correlation between anti-HLA antibodies and poor graft outcome in both kidney and heart allografts, and animal models of transplantation have reinforced these findings.[28–32]

1.2 Antibody Structure and Gene Rearrangement

Humoral immunity is mediated by B lymphocytes and the antibody molecules produced by these lymphocytes following their activation and differentiation into plasma cells. This important part of the adaptive immune response protects against an almost limitless number of pathogens, resulting from several mechanisms occurring during B cell development that culminate in incredible immunoglobulin diversity.

Every antibody molecule consists of two heavy and two light chains,[33] and each of these chains are individually encoded by a separate multigene family.[33] The heavy chain gene consists of several variable (V), diversity (D), and joining (J) segments, while the light chain gene consists of only V and J segments.[34] Each of these genes also possesses a constant (C) domain consisting of individual exons.[34] Rearrangement of the

V, D, and J segments confer diversity to the antibody molecule, while the C domain dictates the effector function of the antibody molecule (Figure 1.1).[35]

The gene segments that can be recombined are flanked by specific nucleotide motifs, called recombination signal sequences (RSS).[36] The typical initiation event in recombination is the recognition of the RSS by the products of the recombination-activating genes (RAG-1 and RAG-2), which are critical lymphoid-specific proteins involved in gene rearrangement.[36] During the rearrangement process, two RSS and their adjacent coding sequences are brought together, and the RAG proteins cleave the DNA, leaving a hairpin loop adjacent to each gene segment.[34] Additional proteins, including DNA-dependent protein kinase (DNA-PK), Ku, Artemis, and a dimer of DNA ligase and XRCC4 are recruited into a large complex with the RAG proteins.[37] The DNA hairpins left at the ends of the desired gene segments are cleaved, and the enzyme terminal deoxynucleotidyl transferase (TdT) adds random non-germline encoded base pairs to the ends of these gene segments.[36] In addition to the random recombination of the gene segments, the addition of random nucleotides by TdT introduces junctional diversity into the recombined antibody genes, further increasing antibody diversity.[37] The other enzymes in the recombinase complex ligate the ends together.[38] The functional rearrangement status of the heavy and light chain genes is one of the defining characteristics of the various stages of B cell development.

1.3 B Cell Development in the Bone Marrow

As mentioned, the stages of B cell development can be defined by the functional rearrangement status of the heavy and light chain gene segments,[39] as well as the

expression of specific surface markers.[40] In humans and all mammals, B lymphocytes begin their development in the bone marrow (BM) from hematopoietic stem cells, where the BM stromal cells provide many of the critical signals necessary for maturation.[40]

Specific steps in the early maturation process often require direct physical contact with the BM stromal cells, mediated via anchoring of VCAM-1 on the HSC to VLA-4 on the BM stromal cell.[41] The initial interaction with the stromal cell, combined with heavy chain gene rearrangement where a D segment on the heavy chain (D_H), is joined with a J segment on the heavy chain (J_H) results in HSC differentiation into a progenitor B (pro-B) cell (Figure 1.2).[34] Pro-B cells begin to express CD19, a hallmark B cell marker which is part of the B cell co-receptor in conjunction with CD21 and CD81.[42]

Joining of the variable region of the heavy chain (V_H) to the rearranged D_H-J_H sequence, and interaction of c-Kit (CD117) with stem cell factor (SCF) on the BM stromal cell drives further differentiation into a pre-B cell.[34] Pre-B cells are defined by the appearance of cytoplasmic μ (IgM) heavy chains.[34] When these rearranged heavy chains associate with VpreB (CD179a) and λ5 (CD179b), which together form the surrogate light chain, cells begin to express a pre-B cell receptor (pre-BCR).[43] Appearance of this pre-BCR turns off the RAG-1 and RAG-2 genes, preventing further gene rearrangement.[37] Late pre-B daughter cells, produced after 4 to 6 cycles of cell division, reactivate RAG-1 and RAG-2 and undergo a productive rearrangement of the variable and joining regions of the light chain (V_L and J_L) (Figure 1.2).[37] This commits the B cell to a defined antigenic specificity determined by the random combination of the V_H-D_H-J_H and V_L-J_L sequences, and the lymphocyte is now defined as an immature B cell. The cell surface expression of IgM, along with Igα and Igβ, forms the B cell receptor

(BCR), capable of binding antigen and activating the B cell.[44] Splicing of the heavy chain constant region permits the co-production and simultaneous expression of IgM and IgD with the same antigenic specificity.[45] In addition to CD19, an immature B cell expresses CD20, IgM, low levels of IgD and low levels of the BAFF receptor (BAFF-R).[42] It leaves the bone marrow as a transitional B cell, maintaining the expression levels of IgM and increasing expression of IgD and BAFF-R.[34] These transitional B cells then become either a follicular B cell or a marginal zone B cell. Follicular B cells reside in the B cell follicle and have the capacity to mature into a germinal center B cell; these cells reduce expression of IgM and begin to express CD21 and CD23.[46] Marginal zone B cells begin to express CD21, but fail to express CD23.[47] They can also express CD27, and have the ability to develop into short-lived plasma cells expressing high levels of CD38 and CD138.[34]

These mature IgM^+IgD^+ B cells leave the BM, enter the blood and migrate to the periphery, forming the majority of the B cell pool in the secondary lymphoid organs.[48] Mature B cells expressing IgM and IgD have a short lifespan, and if unstimulated, live a few days to a few weeks, only surviving if activated through antigenic recognition.[49]

1.4 Activation of B Cells

Mature B cells constantly circulate throughout the bloodstream, lymph, and secondary lymphoid organs, and are classified as naïve until they encounter antigen and become activated in the secondary lymphoid organs.[34]

In almost all cases, B cell activation and differentiation is dependent on help from $CD4^+$ T cells. In a minority of cases, almost exclusively restricted to elements of

pathogenic microbial surfaces including LPS, direct activation of B cells occurs. Direct activation leads to a truncated development pathway and restriction to polyclonal IgM secretion.[50,51]

In general, B cell activation begins with antigen binding to antigen-specific BCR.[52] The BCR is internalized via receptor-mediated endocytosis, digested, and the resulting antigenic peptides are then inserted in the groove of MHC class II molecules and presented on the B cell surface to the cognate T cell receptor.[34] The second activation signal is delivered through interaction with a $CD4^+$ T helper cell[53] and involves ligation of CD40 on B cells with CD40L on T cells, and B7.1 (CD80) or B7.2 (CD86) binding to CD28 on the T cell. The activation signal is enhanced through engagement of the B cell co-receptor, comprised of three surface proteins: CD19, CD21 and CD81. Association of this complex with the BCR generates a signal through CD19 that activates a PI3-kinase signaling pathway and amplifies the cellular response.[54,55] Apart from these cellular interactions, $CD4^+$ T cells also produce cytokines that ultimately affect the isotypes of antibody produced during class switch recombination.[52] B cell activation invokes additional methods of antibody diversification, including the processes of somatic hypermutation and class switching within the germinal centers.[56]

1.5 Affinity Maturation

Germinal centers are well-organized, microanatomical structures within the secondary lymphoid organs that consist of rapidly dividing B cells and $CD4^+$ helper T cells within networks of follicular dendritic cells (FDC).[57] These FDCs not only provide a scaffold for the dividing cells, but they also have the capability to present large amounts

of antigen to T and B cells. B cells located in germinal centers express high levels of activation-induced cytidine deaminase (AID), an enzyme that is required for the process of somatic hypermutation and isotype switching.[56] AID deaminates cytidine residues within the DNA, generating uracil residues in their place and marking them for repair enzymes.[58] Somatic hypermutation introduces point mutations into complementarity-determining regions (CDR) of V_H and V_L gene sequences and ultimately results in greater immunoglobulin binding capacity.[56] Class switch recombination allows the replacement of the IgM isotype and gives rise to IgG, IgA, or IgE instead, resulting in the production of class-switched B cells.[57] Stimuli important in the induction of class switch recombination include the cytokines IFNγ, TGF-β**,** IL-2, IL-4, IL-5 and IL-10.[59] B cells that gain affinity for their cognate antigen through the process of affinity maturation are selected for and preferentially expand compared to their less avid counterparts.[40] During this reaction, some B cells become either antibody-secreting plasma cells or memory B cells, both of which are long-lived and contribute to host protection against re-exposure.[57,60]

CD27 is widely accepted as a marker of human memory cells,[61] and it is believed that many or most of the $CD27^+$ cells in a person at any time are relatively recently generated memory cells.[62] This has been disputed in a recent study on survivors of the 1918 influenza pandemic suggesting that memory B cells can last for decades without re-exposure to antigen.[63] Studies in mice have shown that germinal center B cells adopt a memory phenotype following ligation of CD40.[64] Cytokines have also been proposed as players in determining B cell fate. It has been proposed that IL-10 promotes plasma cell formation,[65] while IL-24 has the opposite effect and inhibits plasma cell formation.[66] IL-

2, IL-10, and CD40L have been shown to drive B cells to a memory phenotype,[67] while IL-21 has been shown to have PC-inducing effects on human naïve B cells *in vitro*.[68] Both memory B cells and plasma cells produced as a result of affinity maturation in the germinal centers are long-lived and contribute to host protection against re-exposure.[40]

1.6 Antibody Isotypes

Through the process of class switch recombination, antibodies with effector functions more closely suited to the eliminating the invading foreign pathogen or resolving infection are produced.[40] The five isotypes of antibody are IgM, IgG, IgE, IgA, and IgD – all of these isotypes are distributed differently throughout the body tissues and vary in their effectiveness against specific types of antigen. In humans, there are 4 subclasses of IgG: IgG1, IgG2, IgG3, and IgG4, each with its own effector functions.[35]

IgM is the subclass associated with primary immune responses, and their levels are often tested to diagnose acute exposure to a pathogen.[45] Due to the early expression of IgM during B cell development, the μ heavy chain associates with regions of genes that have not yet undergone extensive antigen-induced somatic mutation. This often results in IgM antibodies that are relatively low-affinity compared to their class-switched counterparts.[69] Due to the pentameric structure of secreted IgM, these large molecules are the most effective antibodies at activating the classical complement cascade, resulting in death of the target molecule due to formation of the membrane attack complex (MAC).[70]

IgG, which exists as a monomeric protein, is the most predominant isotype found in the serum, and is the most extensively studied Ig subclass.[71] It is often clinically associated with a secondary, or recall, immune response.[60] One of the key effector

functions of IgG is the facilitation of antibody-dependent cellular cytotoxicity (ADCC), a process where antigens coated with antibody activate effector cells through binding of the Fc region of the Ig to the corresponding Fcγ receptor on the effector cell. IgG can also activate the classical complement cascade; in humans IgG3 has the highest affinity for the C1q molecule, followed by IgG1 and IgG2, and IgG4 is not able to activate complement.[35] IgG1 and IgG3 are generally produced in response to protein antigens, such as viral protein capsids, while IgG2 is primarily manufactured in response to polysaccharide antigens, including those found in bacterial cell walls.[35] IgG4 is found in secretions, such as saliva or tears in conjunction with, or in place of IgA.[72] It can also be produced in response to extracellular parasites along with IgE, and it has been shown to be produced at high levels in response to allergen-specific immunotherapy.[71]

IgA has the second-highest serum level, behind IgG, although it is the predominant antibody subclass found at mucosal surfaces.[73] IgA exists as a monomer in the serum, but mucosal IgA exists as a dimer. The 2 subclasses of IgA differ in the length of their hinge regions; IgA1 has a longer hinge region than IgA2, which renders it more sensitive to bacterial proteases.[73] This could explain why IgA2 is predominant in mucosal secretions, whereas serum IgA is mainly IgA1.[73] It follows that IgA is imperative in protection against pathogens at mucosal surfaces through direct neutralization or prevention of binding to the mucosal surface. Complement fixation by IgA does not seems to be a major effector mechanism at the mucosal surface. The IgA receptor is expressed on neutrophils, which may cause local activation of ADCC.[35]

IgE is a very effective immunoglobulin, despite its extremely low serum concentration. It is commonly associated with hypersensitivity and allergic reactions, and

the immune response to parasitic worm infections.[71] The FcεRI, expressed on mast cells, basophils, and eosinophils, binds IgE with extremely high affinity.[74]

Circulating IgD is also found at very low levels, and its purpose remains unclear, as it has no detectable effector function.[45] The newest studies into the function of IgD demonstrate that $IgM^{-}IgD^{+}$ B cells are abundant in the upper respiratory tract and that secreted IgD can bind microbial virulence factors as well as pathogenic bacteria and viruses in this area. Taken together, these findings suggest a role for IgD in protection from respiratory tract pathogens.[75,76]

1.7 B Cell and Antibody Functions

In the context of transplantation, the most important role of a B cell is its ability to differentiate into an antibody-producing plasma cell, although it is important to recognize that B cells possess other important functions that may contribute to graft damage. B cells are garnering attention as extremely effective antigen-presenting cells having shown to be as effective as macrophages in presenting antigen due to high expression of class II MHC on their surface,[77] and also as producers of inflammatory cytokines which have been shown to be detrimental to graft survival, including IFNγ, IL-2, and TNFα.[78]

It is believed that alloantibodies mediate vascular destruction, and/or remodeling, through a variety of pathways. Through ADCC, antibodies can lyse target cells through the low affinity Fc receptor for IgG FcγIII (CD16) on the surface of NK cells and macrophages.[79,80] Moreover, stimulation of these receptors can promote the production of growth factors such as fibroblastic growth factor (FGF) and platelet-derived growth factor (PDGF)[80] which would contribute to vascular remodeling.

Another important method of vascular damage is through complement-mediated lysis of blood vessel cells. In this case antibodies bind to blood vessels cells and complement components directly mediate target lysis. The classical complement cascade is initiated when antibody binds to target cell antigens, recruiting the complement component C1q.[79] C1q associates with C1r and C1s, forming an enzyme complex that cleaves C4 into C4a and C4b. C4b binds to the membrane and to C2, which is cleaved into C2a and C2b. C2b and C4a are released, while C2a and C4b catalyze the formation of the C3 convertase. The cascade eventually culminates with the formation of the C5-9 membrane attack complex which results in cell lysis.[80] The formation of complement split products such as the anaphylatoxins C3a and C5a can influence the recruitment and effector functions of leukocytes, further contributing to the process of rejection.[81]

Although it has no influence on cellular recruitment, C4b remains bound to the antigenic surface, becoming C4d, and can remain here for up to two weeks.[82] A cryptic epitope on C4d has allowed the development of immunohistological staining techniques that produce sensitive and specific staining for this marker.[83] As a result, C4d is now routinely used in renal biopsies and endomyocardial biopsies from transplant tissue as a surrogate marker of antibody-mediated rejection.[83] The presence of C4d in grafts is strongly associated with both early and late graft failure, and is an indication that AMR is occurring.[84,85] Complement inhibitors are currently being developed as treatments for AMR; eculizumab is a monoclonal antibody to C5 that prevents formation of the MAC and thus cell lysis.[86]

1.8 Immunosuppression

To inhibit the post-transplant immune response, patients are prescribed an regimen that typically includes "triple therapy" consisting of a calcineurin inhibitor (CNI), an anti-proliferative agent, and a steroid.[87,88] These current regimes primarily target T cell effector functions. The calcineurin inhibitor used has traditionally been cyclosporine A (CyA), although tacrolimus (Tac) is now used in the majority of patients, due to less severe nephrotoxic effects.[89,90] Calcineurin inhibitors act by inhibiting the interaction between calcineurin and the nuclear factor of transcription (NFAT), preventing the translocation of NFAT into the nucleus, and subsequently, the transcription of the cytokine IL-2. Subsequently, this prevents the proliferation of $CD4^+$ T helper cells, which have been shown to play a key role in acute rejection.[8,12]

mTOR inhibitors, including sirolimus and everolimus, have been shown to be a beneficial therapy in the treatment of acute rejection.[89,91–93] These inhibitors work by inhibiting the IL-2-induced binding of transcription factors in the proliferating cell, subsequently impeding cell cycle progression.[94] By inhibiting the response to IL-2, the activation of T and B cells are blocked. Although some studies have suggested that they have a positive impact on AV,[95,96] the response to mTOR inhibitors varies widely among patients and their effectiveness in preventing late graft rejection is less promising than in preventing acute rejection.[97]

Another commonly used immunosuppressive agent is the anti-proliferative agent mycophenylate mofetil (MMF), which works by preventing the *de novo* pathway of purine synthesis. This results in the inhibition of lymphocyte proliferation and

activation.[98] MMF has exhibited a similar positive, but limited outcome in the prevention of AV.[91] Newer therapies now included in patient care can include rituximab (RTX), a monoclonal antibody against CD20 that depletes B cells through ADCC and complement-mediated cytotoxicity.[99] Although rituximab has been shown to be effective in depleting B cells from peripheral blood, it is not as effective in removing $CD27^+$ memory B cells from the secondary lymphoid organs.[100] Other studies have examined the use of RTX in desensitization protocols, finding that giving RTX diminishes the number of splenic naive B cells, but did not affect the $CD27^+$ memory B cell population.[101]

In summary, while these therapies are effective at ablating the development of acute rejection, they have limited effects on preventing long-term graft rejection which manifests as AV.

1.9 Antibodies in Clinical Transplantation

The contribution of B cells and antibody to long-term transplant rejection has been a source of contention for decades. Modern immunosuppressive agents that target the T cell compartment of the adaptive immune response have not been able to ablate long-term rejection. This is likely be why attention has gradually shifted to focus on the role of the humoral immune response. Since the completion of Paul Russell's seminal experimental study in 1994,[102] accumulating clinical evidence has confirmed that donor-specific antibodies play a detrimental role in solid organ rejection.[25,103–107] At the time of transplant, recipients may have pre-existing anti-HLA antibodies from past exposure to allogeneic HLA molecules through pregnancy, blood transfusions, or previous transplants.[108] In the past, these anti-HLA antibodies have caused hyperacute rejection,

which occurs within minutes to days following transplantation.[26] This type of rejection is prevented in the modern era of transplantation as a result of improved techniques to detect pre-existing antibodies, which reduces the patient's rejection risk and excludes incompatible donors.[109]

Perhaps the most detrimental type of antibodies are the ones that develop in response to the graft. *De novo* development of anti-HLA antibodies has been repeatedly associated with allograft rejection, decreased graft function, and increased risk of long-term rejection in kidney and heart transplant recipients.[103,110] In a large clinical study, Terasaki and colleagues demonstrated that kidney grafts in patients with anti-HLA antibodies had higher rates of failure than grafts in patients without antibodies.[111] Another retrospective study showed that 70% of patients with chronic kidney graft rejection had donor-specific antibodies (DSA).[112]

The presence of either pre-existing or *de novo* antibodies can result in AMR, defined as all rejection caused by antibodies against HLA molecules, blood group antigens, or endothelial cell antigens.[113] A recent study by Reinsmoen *et al*. demonstrated that at two years post-transplant, freedom from AMR was 95.4% for patients with no donor-specific antibody, 66.9% for patients who developed *de novo* DSA, and 25% for those with DSA at the time of transplant.[114] Tissue inflammation and remodeling following an alloimmune response can expose autoantigens or their antigenic determinants, and as a result, non-HLA molecules can be antibody targets as well. Antibodies against vimentin,[115,116] myosin,[117] MHC class I polypeptide-related sequence A (MICA),[118] collagen-V and K-α1-tubulin[119] have also been associated with AMR and decreased graft survival.

AMR can develop at early or late timepoints post-transplant, and the incidence of patients who experience episodes of AMR varies by center and the type of transplant. In renal transplantation, the rate of AMR ranges from 5.6% to 23% in the general recipient population, to 30-60% in patients receiving an ABO-incompatible transplant or transplant across a positive donor-specific crossmatch.[113] A study with a large patient cohort of cardiac transplant patients demonstrated an incidence of early asymptomatic AMR in 13.6% of biopsies from patients within the first year post-transplant and 5.2% of patients beyond the first-year post-transplant.[120] After an episode of AMR was resolved, the recurrence rate was 56.2% at one year.[120] Late cardiac AMR caused by *de novo* DSA is not widely prevalent, but it is a serious problem. A study out of the U.K. demonstrated that 46% of patients with late-onset AMR developed persistent cardiac dysfunction and their medium-term survival was poor.[121] The long-term survival of patients with asymptomatic AMR is comparable to patients without AMR, in that they have a higher risk of developing AV.[122]

In clinical cardiac transplantation, AMR is a significant risk factor for poor graft outcome[122–124] and is associated with hemodynamic compromise, increased graft loss, and a higher risk of developing CAV.[24,104,124,125] Risk factors that are associated with the development of AMR include female gender, elevated pre-transplant panel-reactive antibodies, positive donor-specific crossmatch, prior implantation of left ventricular assist device, and re-transplantation.[86]

AMR is diagnosed in cardiac transplant patients based on serological, histological, and functional findings, including the presence of DSA in the circulation, deposits of complement split product in the endomyocardial biopsy, and signs of cardiac

dysfunction.[109] Traditional therapies to treat AMR include plasmapheresis, intravenous immunoglobulin (IVIg), and immunoadsorption to specifically remove antibodies from the patient. Newer therapies, such as RTX and bortezomib, target B cells and plasma cells, respectively, to eliminate the cells responsible for antibody production.[126]

Taken together, this data implicates antibodies as a major impediment to the success of transplantation and a contributor to the development of AV.

1.10 Allograft Vasculopathy

AV is described as a remodeling of the coronary arteries following transplantation. A normal artery consists of 3 distinct layers: the adventitia, the media, and the intima. The adventitia is the outermost layer; it is highly vascularized and consists of loose connective tissue.[127] The media is the middle arterial layer, and is comprised of thick layers of smooth muscle cells (SMC) admixed with extracellular matrix (ECM).[127] This layer is demarcated by the internal and external elastic laminae. The intima is located adjacent to the vessel lumen, and in our animal model, consists of a single layer of endothelial cells resting on an extracellular matrix.[127] In contrast, epicardial coronary arteries from healthy human adults, who would be considered ideal candidates for transplant donation, have a seemingly harmless, concentric neointimal lesion overlaying the internal elastic lamina. This layer has been termed benign intimal thickening (BIT).[128]

During the development of AV in the animal models, vascular remodeling is characterized by a loss of medial smooth muscle cellularity.[129] Following the loss of SMC, a concentric neointimal lesion forms that occludes the vessel (Figure 1.3), leading to failure of the graft from ischemic injury.[129] This lesion is comprised of smooth muscle

cell-like (SMC-like), alpha-actin positive proliferating myofibroblasts and infiltrating recipient leukocytes.[11,15,130,131]

In humans, the neointimal lesions characteristic of cardiac allograft vasculopathy manifest themselves in the major epicardial coronary arteries.[128] Microarray profiles of such coronary arteries removed from patients with AV have showed that products of immunoglobulin genes are highly upregulated compared to control coronaries removed from patients with atherosclerosis.[79]

In our rodent model, the loss of SMC coupled with the development of an alpha-actin positive neointimal lesion are the hallmarks of AV.[11,15,131] Inflammatory cells can often be found in the adventitia, and in some cases lymphocytes can be organized into ectopic lymphoid structures.[132] In rodent models, the SMC-like cells found in the lesion have been shown to be exclusively of recipient origin.[133] In contrast, the composition of human AV lesions appears to be more chimeric in nature, with cells being predominantly donor,[134–136] most likely due to the BIT layer providing a platform for neointimal lesion formation.[128]

1.11 Role of T Cells in AV

It has been shown that the dominant alloimmune response that initiates AV involves T cells.[11,137,138] Researchers have reported the central importance of T cells in graft rejection, by demonstrating that rejection fails to develop in experimental models lacking T cells,[7,139] as well as in humans where T cell effector mechanisms have been suppressed.[137] As a result, most current clinical therapies target the functional components of T cells.[127] Both $CD4^+$ and $CD8^+$ T cells can play a role in AV

development in the absence of immunosuppression,[11,80,138] but in the presence of therapeutic levels of CyA, $CD4^+$ T cell-mediated effects are ablated, and active $CD8^+$ T cell responses mediate vasculopathy.[131] In this situation, both direct and indirect $CD8^+$ T cell effector pathways are involved[11] and may synergize to cause alloimmune damage to the media required for AV development.

Antigen-specific T cells also play a role in the production of antibody, as these T cells are required for the activation of B cells. In the venue of transplantation, the immunodominant antigens include donor class I and II MHC and minor histocompatibility antigens.[10] As mentioned, $CD4^+$ T cells are widely recognized as the T cell subset required for the activation of B cells to become antibody-producing plasma cells and memory B cells.[107] The development of antibody that binds to target antigens and mediates lysis by the activation of the complement cascade is therefore dependent on $CD4^+$ T cells. Because we have previously found that CNI immunosuppression in the form of CyA ablates the ability of $CD4^+$ T cells to induce allograft vasculopathy,[131] it is important to investigate what effect the presence of CyA may have on the production of alloantibody.

1.12 Role of B Cells in AV

Studies in animal models using B cell-deficient recipients have shown that although B cells are not required for the development of AV,[140–142] antibody alone can mediate lesion development.[102,141] Using a B cell-deficient strain of heterotopic heart recipients with a pre-operative course of anti-CD4 and anti-CD8 monoclonal antibody, Russell's group demonstrated that extensive AV did develop in these animals.[142]

However, the neointimal lesions found in these recipients had a different composition than the characteristic AV lesions found in WT mice. Specifically, the adventitia did contain an infiltration of macrophages and T cells, but lacked collagen deposition and an infiltration by alpha-actin positive SMC[142] in the lesion itself. This suggests that the development of fully fibrotic lesions is reliant on the presence of antibodies.[143] Both collagen deposition and the presence of alpha-actin positive SMC are typical within WT lesions.[80]

In addition, results from animal models have demonstrated that antibody alone can mediate lesion development. Russell *et al* were the first group to demonstrate that antibody alone can play a role in the induction of AV.[102] They demonstrated that transplants from B10.A to B10.BR mice, a combination in which only the MHC class I antigens differ, but one in which a robust antibody response can be produced, resulted in the formation of severe coronary lesions.[102] In the presence of antibody, the development of lesions in animals was much more severe than in animals where antibody was undetectable. Subsequent to these studies, the ability of alloantibody to directly cause intimal proliferation was investigated, using B10.BR heterotopic heart transplants into SCID mice and passively transferred pooled anti-donor antibody. This antibody was produced through skin graft presensitization, followed by administration of spleen and lymph node cells three times a week. They found that 4 out of 5 recipients of B10.BR donor hearts developed obtrusive vascular lesions when given pooled antibody.[102] These lesions showed marked intimal thickening and an infiltrate of mononuclear cells into the adventitia along with deposition of mouse IgG, IgM and the complement product C3 along the internal elastic lamina in the grafts. In generating AV in these SCID mice,

Russell illustrated that antibodies may be sufficient to aid in the development of AV without the involvement of T or B lymphocyte function. A major weakness of this study was the use of SCID mice as a model of immunodeficiency, as these mice can develop circulating antibody and T cells later in life. These adaptive immune mechanisms may have contributed to the development of the vascular pathology seen.[140] In fact, the mononuclear infiltrate in the intima stained positively for $CD3^+$ cells.[102] Despite the weaknesses identified in this study, it still provides an important piece of information regarding the role of antibody in AV development. We have also demonstrated that the passive transfer of anti-donor antibody into a $RAG1^{-/-}$ recipient of an abdominal aortic allograft can contribute to lesion formation that, albeit smaller than a wild-type lesion, is alpha-actin positive and is accompanied by medial SMC loss.[141] In the studies discussed above, the antibody that was either raised in response to transplant or passively transferred into the graft recipients was not produced in the presence of clinically relevant immunosuppression. We extend our studies in this thesis to address the contribution to AV of antibody raised in the presence of CyA.

1.13 Vascular Targets in AV

Currently, researchers have a thorough understanding of the arterial changes that occur during the development of AV, but the complete etiology of AV remains unclear despite decades of research studies in this area. Immediately following transplantation, ischemia/reperfusion (I/R) injury results in a loss of endothelial cells and damage of the medial SMC. The mechanical I/R injury, coupled with non-immune risk factors including hypertension, hyperlipidemia, increased donor age, and gender can contribute to AV

development.[21] Regardless of these factors, animal models of syngeneic transplantation between genetically identical donor-recipient combinations have demonstrated that these factors alone are not sufficient to cause AV.[102,116,140] AV can only develop when alloimmune factors are present to damage the grafted organ.[140,144] However, the major cellular target of this alloimmune damage remains controversial. Several groups believe that endothelial cells are the main target of the immune system;[145–147] while others have provided convincing evidence that the medial layer is the predominant target following transplantation.[148] In a series of experiments using mechanical injury, Reidy[148] differentiated endothelial damage from medial damage and concluded that damage to the endothelium alone was not sufficient to cause vasculopathy. Damage to the media was sufficient to initiate lesion development. Reidy's data shows that a threshold of between 20 – 25% of medial cell loss was needed for development of vasculopathy.[148] Similarly, Booth showed, using a carotid cuff model, that medial injury, rather than endothelial injury, propels the development of vasculopathy.[149] Our group has published data to support this work by illustrating that an allogeneic media is important in the development of AV when using a model of re-transplantation with chimeric graft.[133] Aortas were harvested from C3H/HeJ mice and stripped of the endothelium using an EDTA treatment. Grafts were then transplanted into B6 RAG1$^{-/-}$ mice so re-endothelialization could occur, resulting in a graft with a C3H media and B6 endothelium. These chimeric aortas were transplanted into B6 WT mice and robust lesion formation occurred, whereas chimeric grafts with allogeneic endothelium and syngeneic media showed no evidence of lesion formation, confirming that the media is the more important immunological target in AV.[133]

1.14 Vascular Targets of Antibodies

Based on several animal studies, it is believed by some groups that the primary cellular target of graft-damaging antibody is the intimal endothelial cell. Binding of antibody to endothelial cells results in their activation and secretion of growth factors that can lead to the proliferation of fibroblasts and SMC.[150,151] Our group hypothesizes, based on additional studies, that medial smooth muscle cells are also a likely target of damaging alloantibody. An *in vitro* study demonstrated that anti-MHC class I antibodies had the capacity to induce apoptosis in donor SMC.[152] We have previously shown, in an animal model of antibody-mediated AV, that medial smooth muscle cell dropout occurs in the presence of antibody.[141] Using a fully disparate aortic graft, Thaunat and colleagues showed that alloantibody deposition was found only in the medial layer of the recipient graft and that binding of this alloantibody led to a decrease in medial thickness.[153] In concert with the aforementioned study, these anti-class I MHC antibodies were also capable of mediating SMC death *in vitro*.[153] Other investigators claim that both the intimal endothelial cells and the medial SMC may be subject to the effects of alloantibody.[152]

On a cellular level, it is widely accepted that MHC molecules are the major antibody targets.[80] Vascular cells, such as endothelial cells, smooth muscle cells, and fibroblasts, do not express MHC class II, thus it is presumed that MHC class I is the predominant target of the antibody.[80,154] However, minor histocompatibility antigens may also be a target of antibody-mediated rejection. Antibodies targeting non-MHC molecules have been shown to be present during episodes of chronic rejection, and in some cases, these antibodies have been shown to mediate AV in transplant recipients in certain animal

models. Wu *et al* [155] showed, using an intra-abdominal cardiac graft, that passive transfer of non-MHC antibodies can mediate AV in the absence of T cells, indicating that non-MHC alloantibodies are pathogenic.

1.15 Ectopic Lymphoid Structures

Secondary lymphoid organs, including the spleen and lymph nodes, develop through a process termed lymphogenesis.[156] These conventional structures normally form during ontogeny, but can develop in adulthood in and around areas of persistent inflammation.[157] These non-traditional lymphoid structures are termed ectopic lymphoid structures (ELS),[156,158] and can vary drastically in their morphology from disorganized aggregates of lymphoid cells, to well-defined structures containing distinct T and B cell compartments and high endothelial venules (HEV) for the trafficking of lymphocytes.[159] They differ from lymph nodes in that they do not contain afferent lymphatic vessels, and they are not encapsulated, meaning they have direct exposure to cytokines and the antigens in the inflammatory area.[160] In some cases, the lymphoid cells organize themselves into tertiary lymphoid tissues (TLO) that have the same microarchitecture as secondary lymphoid organs. In these cases, there is a core of B cells that contain germinal centers, interspersed with follicular dendritic cells, surrounded by T cells, dendritic cells and specialized endothelial cells.[160] TLO but have stringent requirements for their classification. As such, ELS are named because they do not possess all of the morphological characteristics to define them as TLO.

Many hypotheses exist as to why these ELS form. One hypothesis is that they prevent chronic activity in secondary lymphoid organs and prevent systemic

dissemination of autoantigens.[157] Another hypothesis is that they maximize antigen presentation at the local site of inflammation, resulting in cytokines, effector cells, and immunoglobulins being concentrated near the inflammatory locus, in hopes of resolving the stimulus of the immune response.[156] It has also been proposed that presence of these ELS is an attempt by the influx of immune cells to organize into tertiary lymphoid organs (TLO).[161] However, the requirements of classification as a TLO are stringent, and not all of the characteristics of these structures are always present in the inflamed tissue. To be classified as a TLO, the lymphoid aggregates must fulfill all of the following criteria: distinct B and T cell areas, HEV expressing peripheral node addressin (PNAd), follicular dendritic cells expressing CD21, and the presence of germinal centers.[157]

The earliest observations of ELS occurred in patients with autoimmune diseases or chronic infections. Patients with Helicobacter pylori-induced gastritis have demonstrated ELS formation, leading to the local production of plasma cells producing IgA specific for H. pylori associated antigens.[162] ELS formation has also been observed in HCV-mediated chronic hepatitis and placental malaria.[163] In addition to infectious diseases, lymphoid neogenesis is routinely observed in autoimmune diseases, including type I diabetes, multiple sclerosis, rheumatoid arthritis and Sjogren's syndrome.[164–166] The persistent, insidious inflammation that accompanies chronic infection or autoimmune disease can also develop as a result of transplantation, and it follows that organs undergoing chronic rejection in animal models have also been sites of lymphoid neogenesis and ELS formation. In grafted tissue, ELS are generally characterized by HEV-like venules, T cells, and plasma cells producing DSA.[132,162,167] ELS has also been reported in human allografts.[168]

ELS has been shown to be fully functional with the capacity to prime naïve cells. Using an animal model of skin grafting, Nasr and colleagues demonstrated that the ELS had the capacity to support the local activation of naïve T cells, resulting in their differentiation into effector and memory cells.[159] In another study of a large grouping of murine heterotopic cardiac transplants, classical TLO were found in 78 out of 319 recipients. These TLO contained T and B cells with HEV or HEV only with no organized lymphoid accumulations.[167] In another animal model of heterotopic heart transplantation, the formation of these structures was shown to be dependent on humoral immunity as TLO did not develop in recipients lacking B cells.[169]

ELS have been previously found in kidney, heart, and lung grafts explanted due to chronic rejection.[168,170–172] In the study of heart allografts, infiltrates of B cells were found in the epicardial fat of patients with advanced chronic rejection,[168] although this study did not focus on identifying the phenotype of the B cells found. Based on our previous findings, the proximity of these ELS to vessels developing AV, and the continuously growing body of literature suggesting that antibody contributes to long-term cardiac graft rejection, we investigate here the phenotype and function of the B cells in these ELS.

1.16 Hypothesis and Objectives

The contribution of B cells and antibody to AV has been debated for several years. The hypothesis of the current study is that B cells and antibody are not required for the development of allograft vasculopathy, based on extensive experimentation that has shown T cells to be the main effector cell of AV development. However, based on previous experimental and clinical studies, where patients are treated with CNI

immunosuppression, it is evident that B cells and antibody can make some contribution to AV. Therefore, we hypothesize that the alloantibody can be produced under cover of CyA in response to a cellular immunization model and in response to a transplant. To extend our work into the clinical scenario, we examined epicardial coronary artery tissue from cardiac transplant recipients. There were ectopic lymphoid structures (ELS) present in close proximity to vessels developing CAV. We hypothesize that these ELS are active sites of antibody production that may be contributing to graft damage.

1.16.1 Objective 1: Assess the Influence of CyA on Alloantibody Production and Subsequent Effect of this Alloantibody on AV Development

CNI immunosuppression is included in over 90% of therapeutic regimens following cardiac transplantation, and patients developing alloantibody must do so under cover of CNI. We have previously shown that CyA, a CNI, affects the function of $CD4^+$ T cells, which are required for the activation of B cells and their subsequent production of antibody. Although $CD4^+$ T cells are present in CyA treated transplant recipients, I was unsure whether these cells still retained the capability to activate B cells and subsequently result in the production of alloantibody. I was also unsure whether this antibody, if it could be produced, would have the capacity to mediate killing of cells through the recruitment of complement proteins and activation of the classical pathway. I wished to investigate whether complement-fixing alloantibody can be produced in animals being treated with CyA and whether this antibody could mediate AV when transferred into immunodeficient mice. Finally, I wished to address the question of T cell help in the production of alloantibody in CyA treated animals, since we have previously shown

dramatic ablative effects of CyA on $CD4^+$ T cells, which would be expected to provide this help.

1.16.2 Objective 2: Determine the Phenotype and Effector Function of B Cells Found in Ectopic Lymphoid Structures

Upon the examination of epicardial coronary artery sections from patients with heart transplants, it was evident that there were ectopic lymphoid structures within the adventitia from these grafts. These ELS have been seen before, but a function has not yet been fully ascribed to them. There is debate within the transplant community whether these ELS develop as a result of transplantation and are benign, or whether they actively participate in the rejection response. In this thesis, we provide a contribution to the literature in that we assess the phenotype of the cells found in these ELS and ascribe a function based on that phenotype.

Figure 1.1 *The structure of an antibody molecule*. The variable (V) region of the light and heavy chains constitutes the antigen-binding portion of the antibody molecule, while the constant (C) region confers the effector function of the antibody.[173]

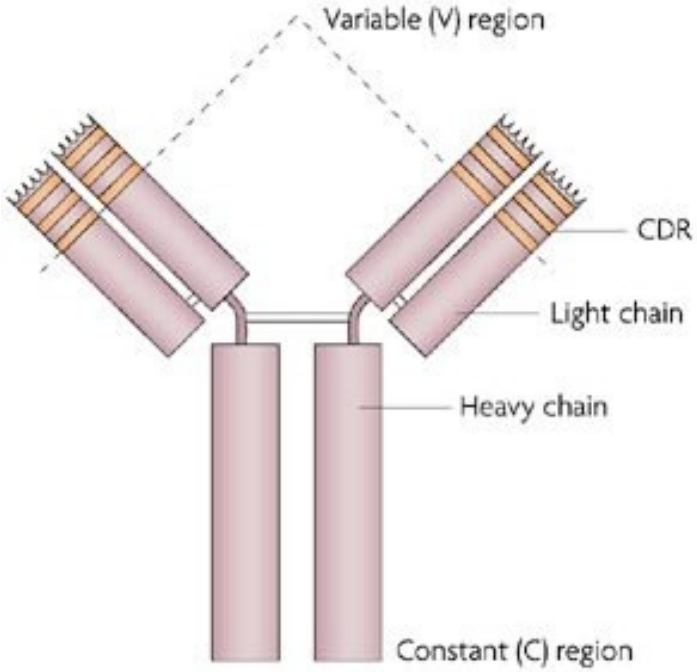


Figure 1.2 *Gene rearrangement in the B cell*. The left panel illustrates rearrangement of the light chain gene segments; the V and J segments are joined during the recombination process. The right panel illustrates rearrangement of the heavy chain gene segments; the D and J segments are joined first, followed by joining to the V segment.[174]

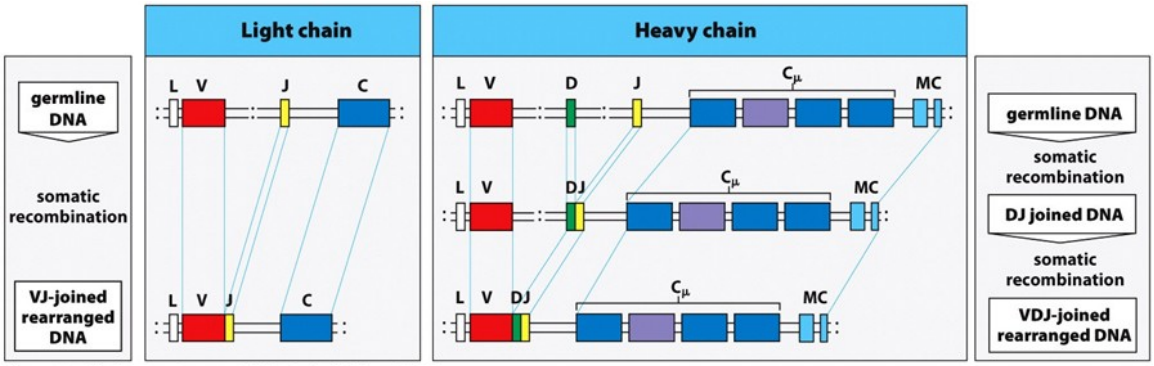
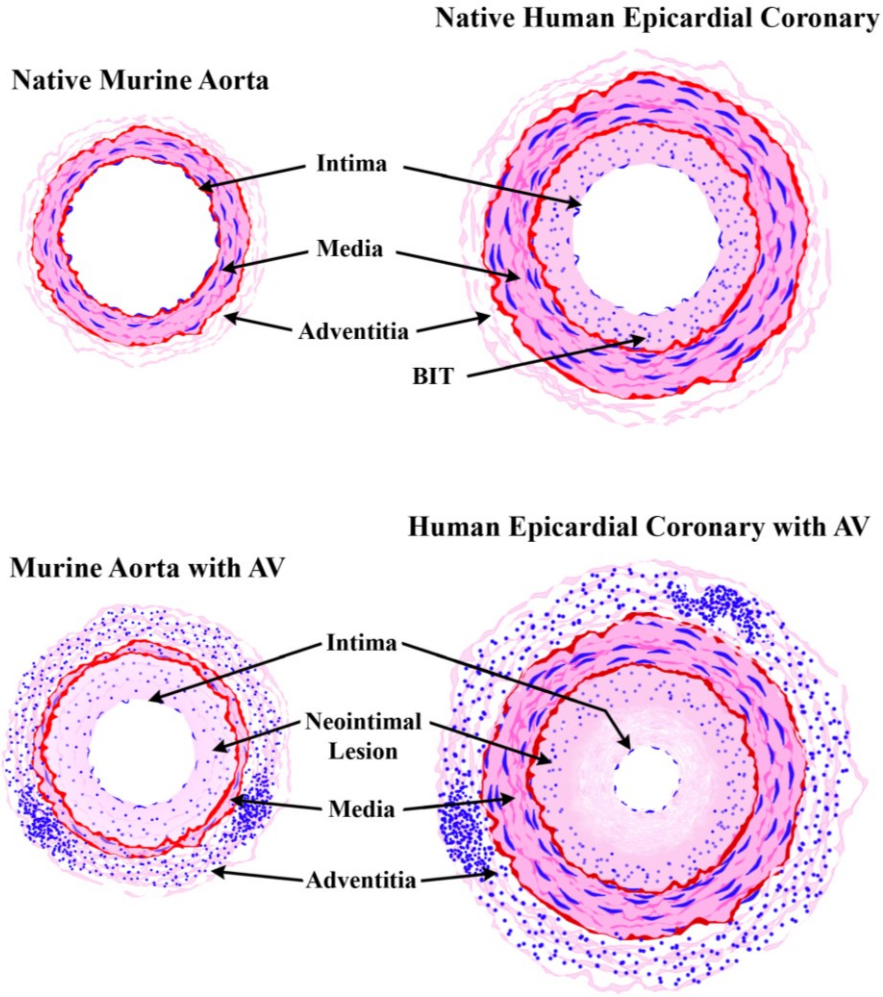


Figure 4.17 The Immune System, 3ed. (© Garland Science 2009)

Figure 1.3 *Allograft vasculopathy*. The upper left image illustrates a native murine aorta, with a thick, SMC-rich media and a single cell layer of endothelial cells comprising the intima. This is compared to the upper right image, depicting a native human epicardial coronary artery, which also has a thick SMC-rich media but also exhibits the BIT layer in contrast to a single cell thick intima. The bottom left image shows a typical murine aorta with AV, where the neointimal lesion is evident, as is the loss of medial SMC. The bottom right image illustrates a human coronary artery with AV which has developed a dual-layered neointimal lesion (Image courtesy of Dr. Michael Hart-Matyas).



CHAPTER 2

MATERIALS & METHODS

2.1 Animals

All animals were purchased from Jackson Laboratories (Bar Harbour, ME). Aortic transplant recipients included: wild-type C57BL/6J (H-2^b), referred to as B6 WT, and B6.129S7-$Rag1^{tmom}$, referred to as B6 $RAG1^{-/-}$. Donors for transplants were C3H/HeJ (H-2^k) mice, referred to as C3H, or BALB/c (H-2^d) mice, referred to as BALB. Animals immunized for the production of alloantibody included B6 WT and B6.129S2-*Cd4*^{tm1Mak}/J, referred to as $CD4^{-/-}$. C3H and BALB splenocytes were used for alloantibody production and complement-dependent cytotoxicity assays. Mice were used at 8–10 wk of age, weighing between 23 and 27 g. Animals were housed at the Carleton Animal Care Facility, at the Sir Charles Tupper Medical Building, in accordance with the guidelines of the Canadian Council for Animal Care, under an approved animal protocol. Food and water were given *ad libitum*.

2.2 Patient Population and Tissue Procurement

The autopsy database from the Capital District Health Authority was searched for patients who had died post-transplant and had available tissue from the following coronary arteries: left anterior descending (LAD), right coronary artery (RCA) and the left circumflex (LCx). Of the cases examined, eight were found to have usable coronary artery tissue. The tissue was obtained under approval of the Capital Health Research Ethics Board (REB #CDHA-RS2011-339).

2.3 Aortic Transplantation

Abdominal aortic segments were transplanted as we have previously described.[175] In this procedure, mice were anaesthetized with 55 mg/kg of sodium pentobarbital intraperitoneally (i.p.) prior to surgery. Sodium pentobarbital was purchased from the Animal Resource Center at McGill University (Montreal, QC).

Once anaesthetized, the abdominal area was shaved and cleaned and a midline incision was made in the abdomen. The abdominal aorta and vena cava of both the donor and recipient animal were isolated and two microvascular clamps were used to obtain proximal and distal control of the recipient aorta. With micro-scissors, a single cut was made to the recipient abdominal aorta to allow for placement of the donor aorta. The donor segment (approximately 1–2 mm in length) was then placed into the cut space of the recipient aorta targeted for transplant. Using an end-to-end microsurgical anastomotic technique, the donor segment was sutured with 11-0 nylon suture to the recipient aorta using single interrupted sutures.[175] Once suturing was complete, the clamps were removed and blood flow was re-established. Grafts were harvested eight weeks later.

2.4 Preparation of Solutions and Media

Phosphate buffered saline (PBS) used for general histology and other staining was prepared in the laboratory using double de-ionized water. All other solutions, including PBS used for incubation of Mitomycin C (Sigma-Aldrich, Oakville, ON) were prepared using ultrapure water from the Milli-Q Integral Water Purification System (EMD Millipore, Billerica, MA) and filter-sterilized with a 0.22 μm bottle-top filter (Fisher Scientific). Roswell Park Memorial Institute 1640-Media (RPMI) (MP Biomedicals,

Solon, OH) was supplemented with 10% fetal bovine serum ((FBS), Life Technologies Inc., Burlington, ON), 25mM 4-(2-hydroxyethyl)-piperazineethanesulfonic acid ((1M; HEPES), Life Technologies Inc., Burlington, ON), L-glutamine (200mM) (Life Technologies Inc.), penicillin and streptomycin ($(1x10_4$ units/mL and $1x10_4$ μg/mL, respectively) Life Technologies Inc.) and 50 μM beta-mercaptoethanol (Sigma-Aldrich) for splenocyte isolations (cRPMI-10). Serum-free RPMI (sfRPMI) was prepared in the same manner, without the addition of FBS.

2.5 Immunosuppression

CyA (Sandimmune i.v.^{TM}) was purchased from the QEII Pharmacy, Halifax, NS and diluted in 0.9 % sterile saline (Hospira, Montreal, QC), to obtain the desired concentration. The day following each transplant, CyA was administered subcutaneously (s.c.) at a concentration of 30 mg/kg/d or 50 mg/kg/d for the duration of each experiment. CyA was also given at a concentration of 30 mg/kg/d or 50 mg/kg/d for the duration of each cell transfer experiment (day 0 to day 28), according to the experiment.

2.6 Alloantibody Production

Alloantibody was produced using a modified protocol based on a method described by Russell *et al.* [102] C3H or BALB mice were anaesthetized i.p. with 55 mg/kg of sodium pentobarbital. Spleens were isolated under sterile conditions, and mechanical disruption was performed in cRPMI-10. The resulting cell suspension was transferred to a 15 ml conical polypropylene tube containing cRPMI-10. Cell suspensions were centrifuged at 300x*g* for 10 min and resuspended in 5 ml ammonium-chloride-potassium

(ACK) buffer for 2 min to lyse red blood cells. An equal volume of cRPMI-10 was added to prevent the lysis of lymphocytes and the cells were centrifuged for 10 min. The resulting erythrocyte-free C3H or BALB splenocytes were washed two additional times in cRPMI-10. Cells were resuspended in 5 ml serum-free RPMI (sfRPMI) and 4 ml of Mitomycin C solution (500 µl of a 250 mg/ml Mitomycin C (Sigma-Aldrich)) added to 3.5 ml sterile phosphate buffered saline (PBS)) was added to inhibit cell proliferation. Tubes were incubated in a water bath at 37°C for 30 min. Cells were washed two times in cRPMI-10. 20 µl of the cell suspension was diluted 1:20 in a 1% trypan blue solution (Sigma-Aldrich), and cell counts were performed, using a hemocytometer. WT B6 mice were injected i.p with 5x 10^7 C3H splenocytes suspended in 200 µl sfRPMI on day 0. Immunized mice were boosted with the same quantity of C3H splenocytes on days 7, 14, and 21. One week after the final immunization (day 28), mice were anaesthetized with sodium pentobarbitol and blood was collected, under sterile conditions, by cardiac puncture. Serum from abdominal aortic graft recipients was collected in the same manner, at the 8 wk timepoint prior to graft harvest. Blood was left to clot overnight at 4 °C. Serum was collected and pooled the following morning, and centrifuged at 200x*g* for 10 min to separate any remaining erythrocytes. Serum was aliquoted into 50 µl portions and stored at -20°C until use.

2.7 Antibody-Mediated Cytotoxicity

C3H or BALB splenocytes (2x10^5/well) were placed in 96-well plates along with doubling dilutions of pooled serum from B6 WT alloprimed mice or B6 WT abdominal aortic graft recipients. Plates were incubated at 4°C for 45 min, and centrifuged at 500x*g*

for 5 min. Supernatant was removed, 25 µl sfRPMI and 25 µl Low-tox rabbit Complement (Cedarlane Labs, Burlington, ON) were added, and plates were incubated for a further 45 min at 37°C. Subsequently, the plates were washed by centrifugation (500xg) and 25 µl of supernatant was removed. 100 µl trypan blue solution ((1% in sfRPMI), Sigma-Aldrich) was added to each well. Percent lysis was calculated as number of dead cells out of total counted cells x100.

2.8 Passive Transfer

B6 RAG1$^{-/-}$ recipients of a C3H aortic interposition graft were injected i.p. with 200 µl of pooled alloimmune serum the day following transplantation. This was followed by three weekly injections of 200 µl of a 1:4 dilution (in sterile sfRPMI) of alloimmune serum over an 8 wk period.

2.9 General Histology

2.9.1 Animal Tissue

All aortas were harvested at 8 wk post-transplantation. Allografts were flushed with heparinized saline and fixed in 10% formalin at 4°C overnight. Grafts were subsequently transferred to cold (4°C) PBS for 1-2 h and then transferred into 70% ethanol until processing. Grafts were embedded in paraffin, 5 µm serial sections were cut and mounted on Fisherbrand™ Superfrost™ Plus glass slides (Fisher Scientific). Approximately 50 slides with 3 – 4 sections per slide were collected. 5 – 6 slides were stained per specimen. Sections were deparaffinized in xylene, hydrated through an ethanol series, and stained with Harris' Haematoxylin (Sigma-Aldrich) and 0.5% Eosin

(Sigma-Aldrich) for general histology (H&E), to visualize the extent of allograft vasculopathy and to perform digital-assisted morphometric analysis and medial SMC counts.

2.9.2 Human Coronary Artery Tissue

Paraffin-embedded tissue samples were obtained from the Capital District Health Authority. 5 µm serial sections were prepared and mounted on Fisherbrand™ Superfrost™ Plus glass slides (Fisher Scientific). Sections were deparaffinized in xylene, hydrated through an ethanol series, and stained with Harris' Haematoxylin (Sigma-Aldrich) and 0.5% Eosin (Sigma-Aldrich; H&E) for general histology, to visualize the extent of cardiac allograft vasculopathy and the location and quantification of ectopic lymphoid structures, and to perform digital-assisted morphometric analysis.

2.10 Immunohistochemistry

2.10.1 Animal Tissue

Sections were deparaffinized in xylene, hydrated through an ethanol series, and cleared in running water. Sections were then digested with proteinase K (Dako, Carpenteria, CA), and endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Avidin/biotin blocking was performed using a Vector® avidin/biotin blocking kit following the manufacturer's instructions (Vector Labs, Inc.). The primary antibodies used were a monoclonal anti-alpha smooth muscle actin Ab (clone IA4; Sigma, St. Louis, MO), at a dilution of 1:1500, and a polyclonal anti-C4d (Hycult Biotech, Plymouth Meeting, PA) at a dilution of 1:50. Secondary antibodies included a

polyclonal biotinylated anti-mouse IgG reagent (Vector Labs, Inc.) at a dilution of 1:250, and a polyclonal biotinylated anti-rabbit IgG (Vector Labs, Inc.) at a dilution of 1:500. Sections were washed in PBS and incubated with a peroxidase avidin/biotin complex (Vector Labs, Inc.). One drop of prepared 3, 3'-diaminobenzidine (DAB; Dako) in 1 ml of substrate buffer was used as the chromogen. Sections were counterstained with Mayer's Haematoxylin. WT B6 mouse aorta was used as a positive and negative (no primary antibody) control for the alpha-actin staining, and WT B6 mouse kidney was used as a positive and negative (no primary antibody) control for the C4d stain.

2.10.2 Human Coronary Artery Tissue

Arterial sections were deparaffinized in xylene, hydrated through an ethanol series and cleared in running water. Antigen retrieval for all primary antibodies was performed using a heat-mediated method in sodium citrate buffer (pH 6.0) with a 2100-Retriever (Electron Microscopy Sciences, Hatfield, PA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 1X PBS. Sections of post-transplant tissue were incubated with various primary antibodies diluted in 5% normal goat serum, including: a mouse monoclonal antibody to identify B cells [anti-CD20 (clone L26) at a 1:50 dilution; Abcam (San Francisco, CA)], a rabbit monoclonal antibody to detect IgM [anti-IgM (clone EPR5539 at a 1:100 dilution; Abcam), a rabbit monoclonal antibody to detect IgG [anti-IgG (clone EPR4421 at a 1:500 dilution; Abcam], a rabbit polyclonal antibody to detect $CD8^+$ T cells (anti-CD8 at a 1:200 dilution; Abcam), a rabbit monoclonal antibody to detect $CD4^+$ T cells [anti-CD4 (clone EPR6855) at a 1:100 dilution; Abcam], a mouse monoclonal antibody to detect macrophages [anti-CD68 (clone 514H12) at a 1:40

dilution; Abcam], and a rabbit polyclonal antibody to detect plasma cells (anti-syndecan at a dilution of 1:50; Abcam). Secondary antibodies included a polyclonal biotinylated anti-mouse IgG reagent (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:500, and a polyclonal biotinylated anti-rabbit IgG (Vector Labs, Inc.) at a dilution of 1:500. Sections were washed in 1X PBS and incubated with a peroxidase avidin/biotin complex (Vector Labs, Inc.). One drop of commercially prepared DAB (Dako) in 1 mL of substrate buffer was used as the chromogen. Sections were counterstained with Mayer's Haematoxylin. Human tonsil tissue was used as both a positive and negative (no primary antibody) control for all primary antibodies.

2.11 Immunofluorescence

Antigen retrieval for all primary antibodies was performed using a heat-mediated method in sodium citrate buffer (pH 6.0) with a 2100-Retriever (Electron Microscopy Sciences). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 1X PBS. To identify memory B cells, sections of post-transplant tissue were incubated with two primary antibodies diluted in 5% normal goat serum, including a mouse monoclonal anti-CD20 at a 1:50 dilution (clone L26, Abcam), and a rabbit monoclonal anti-CD27 antibody at a 1:100 dilution (clone EPR8569, Abcam). Secondary antibodies included a goat anti-mouse conjugated to Alexa 488 (Invitrogen, Eugene, OR) and a goat anti-rabbit conjugated to Alexa 555 (Invitrogen), both used at a dilution of 1:500. Hoescht 33342 at a dilution of 1:5000 (Invitrogen) was used as a nuclear counterstain. Human tonsil tissue was used as both a positive and negative (no primary antibody) control for all primary antibodies.

2.12 Image Analysis

Digital images of immunohistochemistry were captured using a Zeiss AxioImager.A1 microscope and AxioCam camera (Carl Zeiss, Thornwood, NY). Intimal and medial areas were measured using the Zeiss Axiovision 4.6 digital image analysis program (Carl Zeiss). Mean intimal area was calculated using intimal area from 4-6 individual specimens, depending on the experimental group. Smooth muscle cells (SMC) were quantified by counting nuclei within the medial area of H&E stained sections. Mean SMC number was calculated using number of SMCs from 4-6 specimens, depending on the experimental group. C4d quantification was performed using Adobe Photoshop CS5 Extended Academic Edition (Adobe Systems Canada, Ottawa, ON). Digital images of immunofluorescence were captured using a Zeiss Axiovert 200M microscope and a Hamamatsu Orca R2 camera (Hamamatsu, Boston, MA).

2.13 Statistics

Data are presented as mean ± SEM for each experimental group. All statistics were performed using GraphPad Prism® (GraphPad software; San Diego CA). Results comparing groups of two were analyzed using a student's two-tailed t test. Results comparing groups of three or more were analyzed by one-way ANOVA and the Tukey-Kramer multiple comparisons test was used. Values of $p<0.05$ were considered significant.

CHAPTER 3

CYCLOSPORINE IMMUNOSUPPRESSION DOES NOT PREVENT THE PRODUCTION OF DONOR-SPECIFIC ANTIBODY CAPABLE OF MEDIATING ALLOGRAFT VASCULOPATHY

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*Brenda Ross performed all abdominal aortic transplants. All other experimental studies were completed by the candidate unless otherwise indicated and manuscript was prepared by candidate under editorial guidance of supervisor Tim Lee

3.1 Introduction

Currently, cardiac transplantation results in excellent early outcomes due to the ability of calcineurin inhibition (CNI) immunosuppression to limit acute rejection, including Cyclosporine A (CyA). In contrast, CNI immunosuppression has had little impact on long-term graft survival, which remains poor.[2] Allograft vasculopathy (AV) is a primary contributor to late graft failure and, as such, has been referred to as the "Achilles heel" of cardiac transplantation.[176] AV is characterized by coronary artery remodeling and the formation of an occlusive neointimal lesion within the coronary vessels.[177]

Previous studies have demonstrated the contribution of T cells in AV development.[131,137–139] However, antibody-mediated rejection (AMR) is now appreciated as a distinct phenomenon correlating with both poorer early graft survival and the development of AV in longer surviving grafts.[178,179]

We have previously shown that antibody can contribute to AV lesion development in a mouse model using fully disparate strain combinations and CNI to ablate acute rejection.[141] In this model, we demonstrated that CNI inhibits $CD4^+$ T cell effector function such that AV occurs as a consequence of $CD8^+$ T cell function.[131] Because antibody production requires $CD4^+$ T cell help,[180] it follows that alloantibody production would be difficult to achieve under CNI, leading one to deduce that alloantibody plays a limited role in AV development in patients receiving CNI. In contrast, detectable alloantibody titres are found post-transplant,[24,27,181–183] where the majority of patients are receiving CNI immunosuppression.[2] Some suggest a link between this alloantibody and late graft rejection.[123] This link has come from evidence of

complement deposition in cardiac grafts from both experimental models and human biopsy specimens.[79,184,185] C4d, a long-lived breakdown product of the classical complement pathway, has been used as a surrogate marker for antibody-mediated effector function.

Given that alloantibody production is a T cell-dependent process, and CNI limits $CD4^+$ T helper cell activity, the generation of an effective alloantibody response in the presence of CNI is counterintuitive. In this study, we examine the ability of mice to develop complement-fixing alloantibody in response to allochallenge in the presence of CNI. We further examine the ability of this alloantibody to contribute to AV.

3.2 Results

3.2.1 CNI Immunosuppression Allows for the Production of Complement-Fixing Antiserum

Pooled serum from mice immunized in the presence of 30 mg/kg/d or 50 mg/kg/d CyA (n=10 per group) was tested in an *in vitro* assay for complement-mediated cytotoxicity against allogeneic splenocytes. Percent cytotoxicity was compared to control serum (from unimmunized mice or from immunized mice untreated with CyA). Figure 3.1 demonstrates that antiserum raised in the presence of either CyA dose mediated complement-dependent lysis of donor cells, albeit to a more limited degree when compared to serum from mice immunized in the absence of CyA ($p<0.05$). As the dose of CyA was diminished, higher levels of antibody-mediated lysis was observed ($p<0.05$).

3.2.2 Antiserum Raised in the Presence of CNI is Donor-Specific

Antiserum was also tested for complement-mediated cytotoxicity against third party (BALB) splenocytes to ensure donor specificity. Figure 3.2 demonstrates that antiserum raised in both the presence and absence of 30 mg/kg/d CyA was able to mediate lysis of donor cells but not third party cells ($p<0.001$). No significant difference was seen between killing of third party cells by immune serum versus control serum ($p>0.05$).

3.2.3 Alloantibody Raised in the Presence of CNI Contributes to AV

To examine the impact of alloantibody raised in the presence of CNI on AV, we passively transferred alloantiserum raised in the presence of 50 mg/kg/d CyA into B6

RAG1$^{-/-}$ mice bearing C3H grafts. Control recipient mice received no alloantibody (n=4 per group). Grafts were examined for neointimal lesion formation and medial SMC loss, characteristics of AV in this model.[127] Figure 3.3 (a & b) demonstrates that immunodeficient B6.RAG1$^{-/-}$ mice that received alloantibody showed intimal lesions whereas control (no antibody transfer) immunodeficient B6.RAG1$^{-/-}$ mice did not develop lesions. Image analysis (Fig 3.3c) confirmed this finding of a significant ($p<0.05$) difference between the two groups. Grafts in the B6.RAG1$^{-/-}$ mice that received alloantibody had significantly ($p<0.001$) fewer SMC than grafts from control animals (Fig 3.3d). To confirm that alloantibody raised in the presence of CNI resulted in lesions phenotypically similar to those described in other animal models, we confirmed the presence of alpha-actin positive myofibroblasts in the neointima, characteristic of rodent AV (Fig 3.4).

3.2.4 Transplants Present a Sufficient Allochallenge to Generate Complement-Fixing Alloantibody in the Presence of CNI

The data above confirm that, in the presence of CNI, allochallenge with donor splenocytes engenders the generation of complement-fixing donor specific alloantibody. In this section, we compare the ability of allopriming with a transplant (aortic interposition graft) to priming by injections of splenocytes. Grafts were transplanted as above in the presence of CyA to prevent acute rejection. Serum and grafts were harvested 8 wk post-transplant. We demonstrate here that complement-fixing alloantibody was induced at equal levels by allografts and alloimmunization (Fig 3.5).

3.2.5 C4d is Located in the Medial Area of Grafts

The data presented above suggest a link between complement-fixing alloantibody and AV. To clarify this link, we performed immunohistochemical staining for the complement split product C4d. Grafts from animals treated with 50 mg/kg/d CyA showed deposition of C4d primarily in the medial but also in the neointimal area (Fig 3.6b). As expected, C4d was absent in the native aorta (Fig 3.6a). Digital image analysis confirmed the localization of C4d primarily to the media compared to the intima (Fig 3.6c; $p<0.01$).

Figure 3.1 *Alloimmunization in the presence of CNI results in antibody formation***.** Donor splenocytes were incubated with a 1:16 dilution of: serum from unimmunized animals (control); or antiserum raised in the presence of either 50 mg/kg or 30 mg/kg of CyA; or antiserum from immunized mice without CNI present. Results shown are mean ± SEM of three experiments (*p<0.05, ***p<0.001 compared to control).

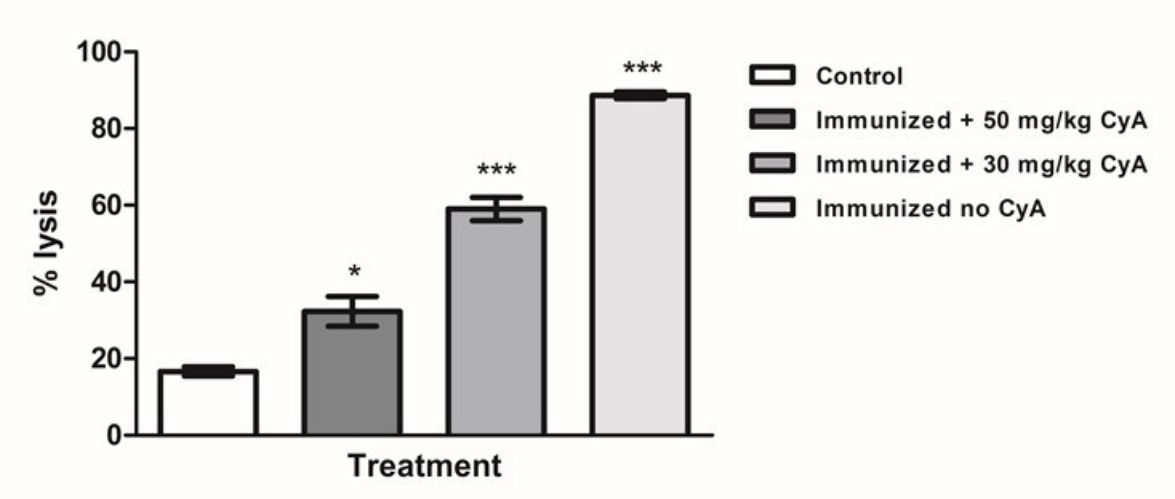


Figure 3.2. *Antiserum raised by alloimmunization is donor-specific.* Donor splenocytes and third party cells were incubated with a 1:16 dilution of serum from unimmunized animals (control), and a 1:16 dilution of antiserum raised with (immunized +30 mg/kg CyA) and without CNI immunosuppression (immunized no CyA). Results shown are mean ± SEM of three experiments (***p<0.001 compared to 3rd party cells).

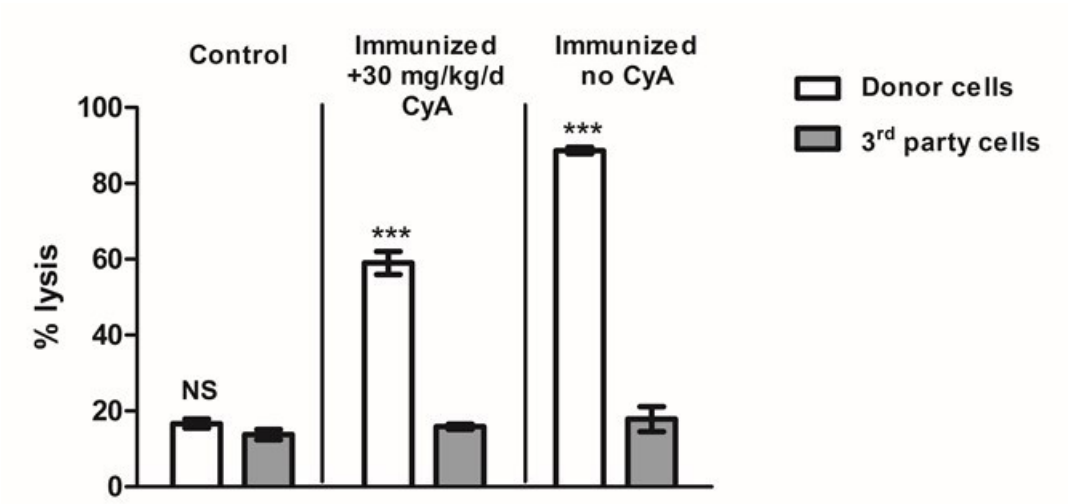


Figure 3.3. *Alloantibody raised with CNI immunosuppression contributes to lesion formation.* C3H grafts transplanted into: (a) B6 RAG1$^{-/-}$ recipients with passive transfer of alloantibody raised with CyA (AlloAb); or (b) B6 RAG1$^{-/-}$ recipients with no alloantibody transferred (No Ab, control). Internal elastic lamina (IEL) are marked with an arrow. (c) Mean intimal area per aortic section (mean ± SEM). Lesion area calculated using aortic sections from 3 individual sections per graft (*$p<0.05$). (d) SMC numbers from animals treated with alloantibody were compared to those untreated (control). Mean counts of medial SMC were calculated using 3 individual sections per graft (***$p<0.001$). Results are shown as mean ± SEM.

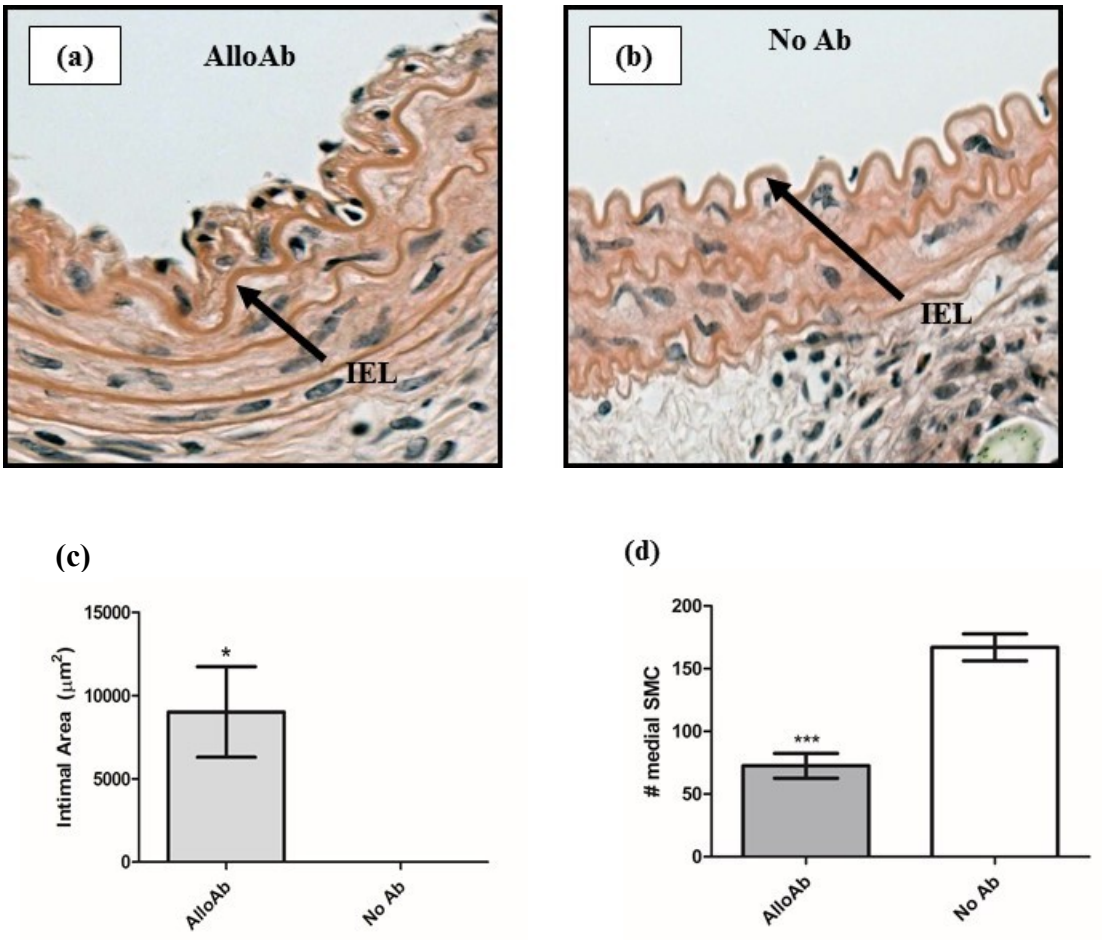


Figure 3.4. *Lesions induced by alloantibody raised with CNI immunosuppression are alpha-actin positive.* C3H grafts transplanted into: (a) B6.RAG1$^{-/-}$ recipients with passive transfer of alloantibody raised with CyA (AlloAb); or (b) B6.RAG1$^{-/-}$ recipients with no alloantibody transferred (No Ab, control). Sections shown are representative of the control and experimental groups (n=4 per group). Internal elastic lamina (IEL) are marked with an arrow.

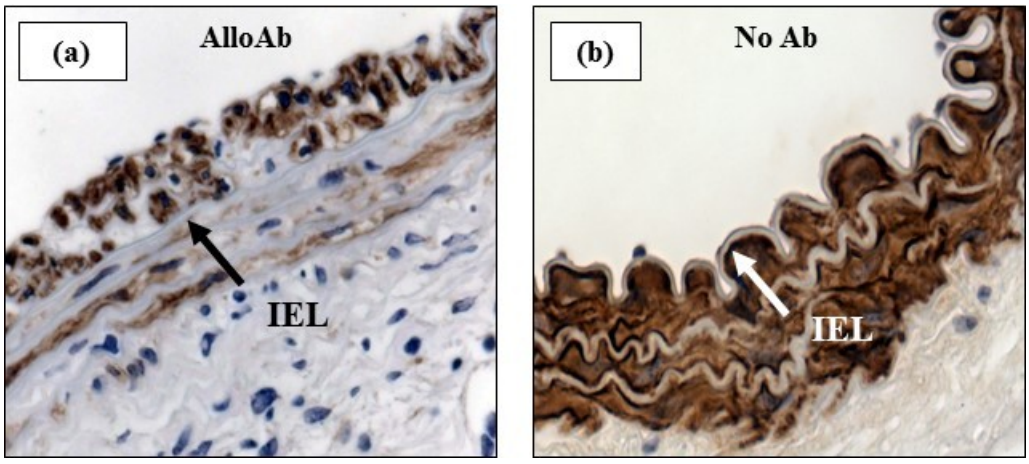


Figure 3.5. *Serum from transplant recipients contains complement-fixing alloantibody capable of mediating donor cell lysis.* Donor splenocytes were incubated with a 1:16 dilution of pooled serum from: animals with no transplant (control); transplant recipients given CyA (50 mg/kg/d); splenocyte-immunized mice given CyA (50 mg/kg/d). Serum-facilitated percent lysis was compared between the two groups. Data shown are the result of three independent experiments; results shown are mean ± SEM (**p<0.01).

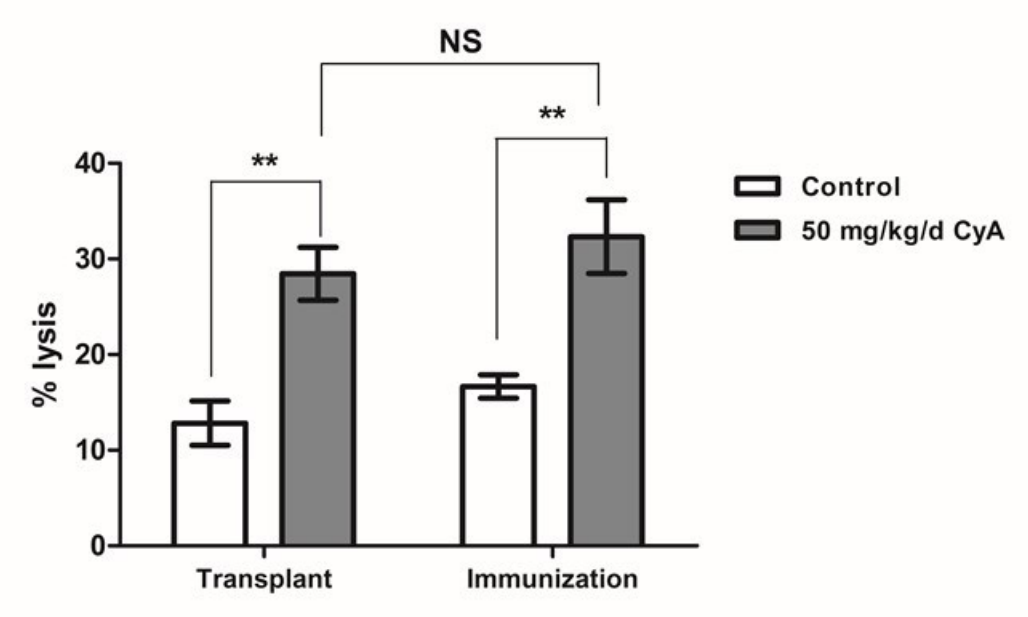
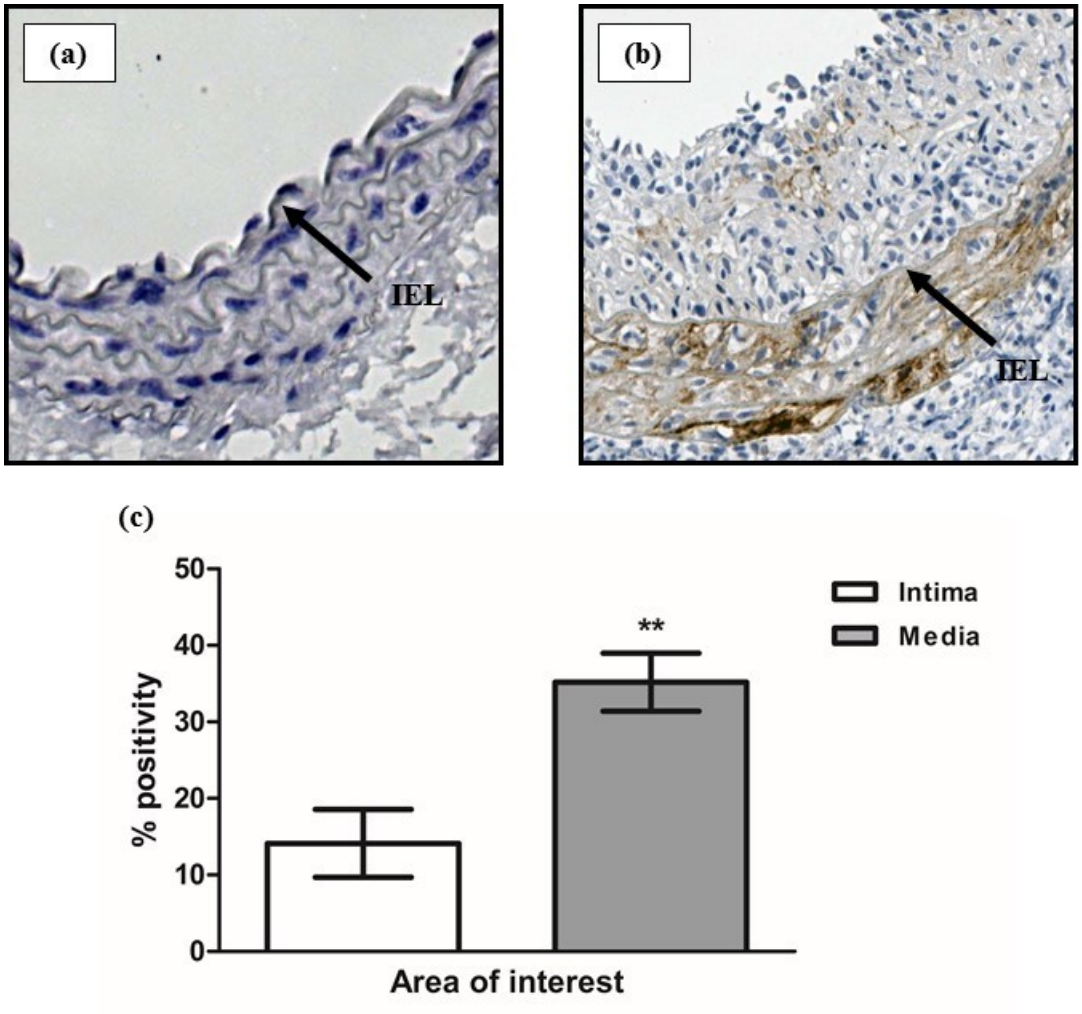


Figure 3.6 *C4d is located in media of grafts.* C4d deposition in (a) native aorta and (b) in fully MHC-mismatched grafts from animals treated with 50 mg/kg/d CyA. Quantification of C4d deposition shows greater localization within the media of grafts compared to the intima (**p<0.01).



3.3 Discussion

CNI immunosuppression remains the mainstay of cardiac transplant immunosuppressive therapy. Attempts to withdraw CNI-based therapy and substitute other immunosuppressive agents have resulted in increased episodes of early acute rejection, increased number of infections, and more adverse events.[89,91,92,186] For the foreseeable future, the immunosuppressive regimen prescribed for cardiac transplant patients will continue to include CNI therapy.

CNI immunosuppression is effective at preventing acute rejection but fails to prevent late graft rejection. One possible reason for this is the fact that CNI immunosuppression may not be effective at blocking AMR. Transplant recipients receiving CNI therapy routinely show anti-HLA antibody,[187] and this been suggested to be associated with late graft rejection.[112,119,123,188–190] We found this counterintuitive since we have previously demonstrated, in our animal model, that CNI limits T helper cell activity, a pre-requisite of alloantibody formation. The clinical data would suggest that certain elements of T helper activity are more resistant to CNI immunosuppression. To examine this, we established an animal model to assess antibody formation and function in a transplant setting that included CNI immunosuppression. We included doses of CyA to approximate the range of circulating levels in patients 2 hours post-dosing (C2) found in the early post-operative period (up to 6 months) after cardiac transplantation (600-1400 ng/ml) before reduction to maintenance levels of 400-600 ng/ml.[191]

We have previously shown, using the mouse model described here, that CyA therapy at 50 mg/kg/d results in debilitated $CD4^+$ T cell cell-mediated function.[131] impairing the ability of these cells to induce either acute rejection or AV. In this study,

we investigated if CyA would have a similar negative effect on the ability of $CD4^+$ T cells to activate B cells and support their differentiation into IgG-producing plasma cells. There are some limitations to the interpretation of mouse models that use single immunosuppressive moieties. This is characteristic of mouse models that are more sensitive to immunosuppression than human transplants. Nevertheless, we demonstrate here that even in the presence of 50 mg/kg/d CyA (1100 ng/ml C2 in mice), we could raise primed alloantiserum capable of lysis of donor cells via the classical pathway of complement activation. The antiserum was allospecific in that third party cells were not damaged. Antibody titres in primed CyA treated animals were lower than in animals in the absence of immunosuppression. Other studies have shown that CyA-treated animals demonstrated a reduced number of activated splenic-derived $CD4^+$ T cells,[192] reduced production of immunoglobulin,[193] and lower expression of co-stimulatory ligands by $CD4^+$ T cells.[193] Combining these findings with our own, we believe that CyA may be reducing the number of T cells available to activate B cells, and suppressing the ability of $CD4^+$ T cells to provide stimulatory signals required for their activation. Our data, combined with previous evidence, indicates that the T cell-mediated assistance required for alloantibody production is reduced, but not ablated, in the presence of CyA. In the clinical setting, this could partially account for the low-grade AMR that has been postulated to be associated with cardiac allograft vasculopathy.

The presence of complement-fixing alloantibody, even in the context of CNI immunosuppression, suggests a role for this antibody in AV generation. We tested this directly by passively transferring alloantibody, raised in the presence of CNI immunosuppression, into B6.$RAG1^{-/-}$ recipients of C3H grafts. These immunodeficient

mice do not acutely reject their grafts, or develop AV without immune reconstitution in some manner. In this study, we demonstrated that alloantibody, raised in the context of CyA, could induce limited, but demonstrable, neointimal lesions, in addition to medial SMC loss. In murine transplants, this medial remodeling, coupled with intimal remodeling demonstrated by the development of quantifiable neointimal lesions, contributes to the pathologic sequalae of AV. The presence of myofibroblasts within the neointimal lesions, as evidenced by alpha-actin staining, is also characteristic of AV. These data are consistent with our hypothesis that alloantibody leads to medial SMC destruction and that this damage creates a response to injury repair process, leading to the formation of a neointimal lesion.[141]

We further demonstrate that a MHC-mismatched aortic transplant, in the presence of CNI immunosuppression, leads to the production of alloantibody. Although there was a significant inhibition of antibody production compared to animals immunized in the absence of CNI, the antibody produced in response to transplantation retained the capacity to facilitate complement-mediated lysis of donor cells *in vitro*. Over time, we believe that even reduced levels of antibody would be sufficient to induce a late rejection response in the graft.

These grafts also exhibit deposition of the stable complement split product C4d. C4d has become a widely accepted surrogate marker for antibody-mediated rejection in cardiac transplants,[82] and has been associated with worse graft prognosis[194] and reduced allograft function.[195] In cardiac grafts, endomyocardial biopsy specimens have exhibited C4d deposition,[184,196] which has been associated with subsequent development of AV.[197] We examined localization of C4d in aortic grafts to identify where alloantibody was

binding and initiating activation of the complement cascade. In our grafts, C4d deposition was predominantly localized to the media of the graft, implying that this is the area of complement cascade activation. This observation is consistent with our previous theory that antibody is targeting medial SMC for destruction by the classical pathway of complement activation.

Taken together, these data confirm that therapeutic levels of CNI immunosuppression reduce, but do not ablate, the generation of alloantibody. In this model, the alloantibody produced has the capacity to induce limited, but detectable, allograft vasculopathy. The fact that medial SMC loss and neointimal lesion formation are both seen in this model further implicates an important relationship between these two phenomena in the etiology of cardiac AV. Furthermore, the presence of the complement split product C4d in the media suggests a role for medial SMC loss in antibody-mediated AV lesion development.

CHAPTER 4

ECTOPIC LYMPHOID STRUCTURES ARE PRESENT IN THE CORONARY ARTERIES OF CARDIAC TRANSPLANT RECIPIENTS AND CONTAIN MEMORY B CELLS AND ACTIVE ANTIBODY PRODUCTION

Alison J. Gareau*, Gregory M. Hirsch, Timothy D.G. Lee

*All experimental studies were completed by the candidate unless otherwise indicated and manuscript was prepared by candidate under editorial guidance of supervisor Tim Lee

4.1 Introduction

Cardiac transplantation remains the favoured therapy for patients with end-stage heart failure. Although early outcomes of cardiac transplantation are excellent, the long-term survival of heart transplants has not improved over the last three decades.[1] A major contributor to this poor survival is progressive cardiac allograft vasculopathy (CAV).[2] The etiology of CAV has not been fully elucidated but it is clear that, like in acute rejection, the T cell component of the adaptive immune system plays a major role. Thus, for many years research into the causes of CAV focused on the contribution of T cells. Likewise, treatment strategies focused on ablation of T cell activities. More recently, however, the humoral component of the immune response, comprised of B cells and antibody, has emerged as another important limitation to long-term graft survival.[77,125,141,198] Antibodies targeting donor HLA molecules as well as autoantigens such as vimentin, myosin, and MICA, have been shown to be detrimental to long-term graft survival.[160] We have demonstrated, in an animal model of allograft vasculopathy, that anti-donor antibody can contribute to lesion formation in the absence of direct T cell activity. We implicated activation of the classical complement cascade as a means of inducing initiation of vasculopathy.[199]

The exact contribution of humoral immunity to CAV remains unclear, as does the origin and activation pathways of the B cells responsible. Recently, we have observed ectopic lymphoid structures (ELS) in the adventitia of human coronary arteries developing CAV. ELS containing B cells have been found in patients suffering from a number of autoimmune diseases linked to humoral dysregulation.[156,161] It is still undetermined what role these ELS play in autoimmune diseases, but they are often

associated with chronic inflammation. Chronic inflammation is a shared characteristic between solid organ transplantation and autoimmune disease. This suggests that such ELS, in the transplant setting, may contribute to chronic rejection. Indeed, evidence of these ELS has been found in animal models of chronic graft rejection,[160,167] as well as in human grafts undergoing chronic rejection.[157,168,171,200]

We hypothesize here that the ELS found in transplanted epicardial coronary tissue are active groupings of antibody-producing plasma cells that can lead to the formation of graft-damaging antibodies. Although a previous study into transplant ELS identified B cells within the structures, information as to the phenotype and function of the B cells is lacking. A link between transplant rejection and the presence of ELS within a graft has been established but these studies[159] concentrated on the T cell compartment; the B cell compartment has not been thoroughly explored to date.

In the context of transplantation, antibody has the potential to target donor antigens, specifically HLA, and result in graft damage through activation of the classical complement cascade or the induction of antibody-dependent cellular cytotoxicity, culminating in antibody-mediated rejection (AMR). It is therefore important to characterize the functional capacity of B cells in the ELS in the adventitia of coronary vessels post-transplant. It is particularly important to identify the presence of memory cells in this compartment. In this manuscript, we describe the presence of ELS in the epicardial coronary arteries of cardiac transplant patients. These ELS were immunologically active and contained $CD4^+$ and $CD8^+$ T cells, $CD20^+$ B cells, plasma cells, and IgM and IgG. The ELS were in close proximity to the medial area of grafts, leading us to hypothesize that the antibody being produced in these ELS may be targeting

medial smooth muscle cells, as we have previously demonstrated in our animal model of allograft vasculopathy. We also demonstrated the presence of $CD20^{+}CD27^{+}$ memory B cells in ELS from long-term cardiac graft recipients.

4.2 Results

4.2.1 Ectopic Lymphoid Structures are Present in Patients at Later Timepoints but not Earlier Timepoints Post-Transplant

Epicardial coronary artery tissue was collected at autopsy from 8 patients who died 1 day to 8 years after cardiac transplantation. The tissue was processed for general histology. Figure 1a demonstrates that, at earlier times post-transplant (less than one year), there were no ectopic lymphoid structures present in the adventitia, whereas coronary vessels collected from hearts obtained at later times post-transplant (1-5 years and greater than 5 years; Fig 4.1b & 4.1c), showed distinct ectopic lymphoid structures. These ELS were present exclusively in the adventitia of the coronary vessels, and found in 5 of the 8 patients examined.

To determine the relative frequency of ectopic lymphoid structures, we assessed the number of clusters per histological section, using more than 2 histological sections for each of the coronary arteries available to us. Figure 4.1d demonstrates that vessels harvested less than 1 year post-transplant showed no ectopic lymphoid structures, while coronary vessels harvested from hearts obtained 1-5 years post-transplant showed a mean of 3.80 (±1.625) ectopic lymphoid structures per section. In addition, coronary vessels harvested from hearts obtained more than 5 years post-transplant showed a mean of 4.25 (±1.013) ectopic lymphoid structures per section. The number of clusters did not differ significantly ($p>0.05$) between the latter two groups (Figure 4.1d).

The distance of these ectopic lymphoid structures from the external elastic lamina, which demarcates the medial layer from the adventitia, was also quantified. In the group

of vessels harvested at 1-5 years post-transplant, we found that the ELS were located at a mean of 628.6 μm (±78.5 μm) away from the medial layer (Figure 4.2). In vessels harvested from 5-10 years post-transplant, ectopic lymphoid structures were located at a mean of 637.5 μm (±81.6 μm) from the media (Figure 4.2). There was no statistically significant difference between these two groups ($p>0.05$), indicating that the location of the ELS does not change over time.

4.2.2 Ectopic Lymphoid Structures Contain B Cells

Based on the mounting evidence that B cells play a significant role in transplant rejection, coronary tissue was stained for $CD20^+$ B cells. At earlier time points post-transplant, B cells were found dispersed throughout the tissue and were not organized into ELS (Fig 4.3a). In contrast, staining of tissue derived from patients who died later post-transplant resulted in positivity for B cells organized in the ectopic lymphoid structures (Fig 4.3b).

4.2.3 Ectopic Lymphoid Structures are Sites of Active Antibody Production

Since B cells require $CD4^+$ T cells to become fully activated and develop into either memory B cells or antibody-producing plasma cells, we stained our tissue for the presence of $CD4^+$ T helper cells. We found significant numbers of T helper cells present in the ectopic lymphoid structures (Appendix 1, Figure 1.1), an indication that there is ample assistance available for the B cells in the clusters to become antibody-producing plasma cells. We confirmed this by staining for the plasma cell marker syndecan-1. The

data shown in Fig 4.4a revealed that plasma cells were present in the ELS. These plasma cells were actively producing IgM (Figure 4.4b) and IgG (Figure 4.4c), the major antibody subclasses responsible for activation of the classical complement cascade, which has previously been shown to contribute to graft damage.

4.2.4 Ectopic Lymphoid Structures Contain Memory B Cells

If B cells play an important role in graft rejection, it is likely that B memory cells contribute significantly to the late graft rejection response. Memory B cells are rapidly reactivated following exposure to their cognate antigen and can produce 8 to 10 times more antibody-secreting plasma cells during the secondary response than during the primary immune response. In the epicardial coronaries, this late rejection response is classified as allograft vasculopathy. The ectopic lymphoid structures are ideally situated to impact this pathology. For this reason, we examined the ectopic lymphoid structures for presence of memory B cells. Using CD20 and CD27 as markers, we performed immunofluorescent staining of the ectopic lymphoid structures and found that in 60% (3 out o5) of patients who had ectopic lymphoid structures in their adventitial tissue, the clusters contained memory B cells (Figure 4.5).

Figure 4.1 *Epicardial coronary arteries harvested at later timepoints post-transplant contain ectopic lymphoid structures*. Sections of epicardial coronary arteries from patients at: (a) 10 days post-transplant, (b) 2.5 years post-transplant, and (c) 10 years post-transplant. (d) Graph illustrating the mean number of ectopic lymphoid structures per coronary vessel at various times post-transplant. Only ELS associated with a vessel (visible within one field of view at 5x magnification) were quantified. Bars represent mean ± SEM.

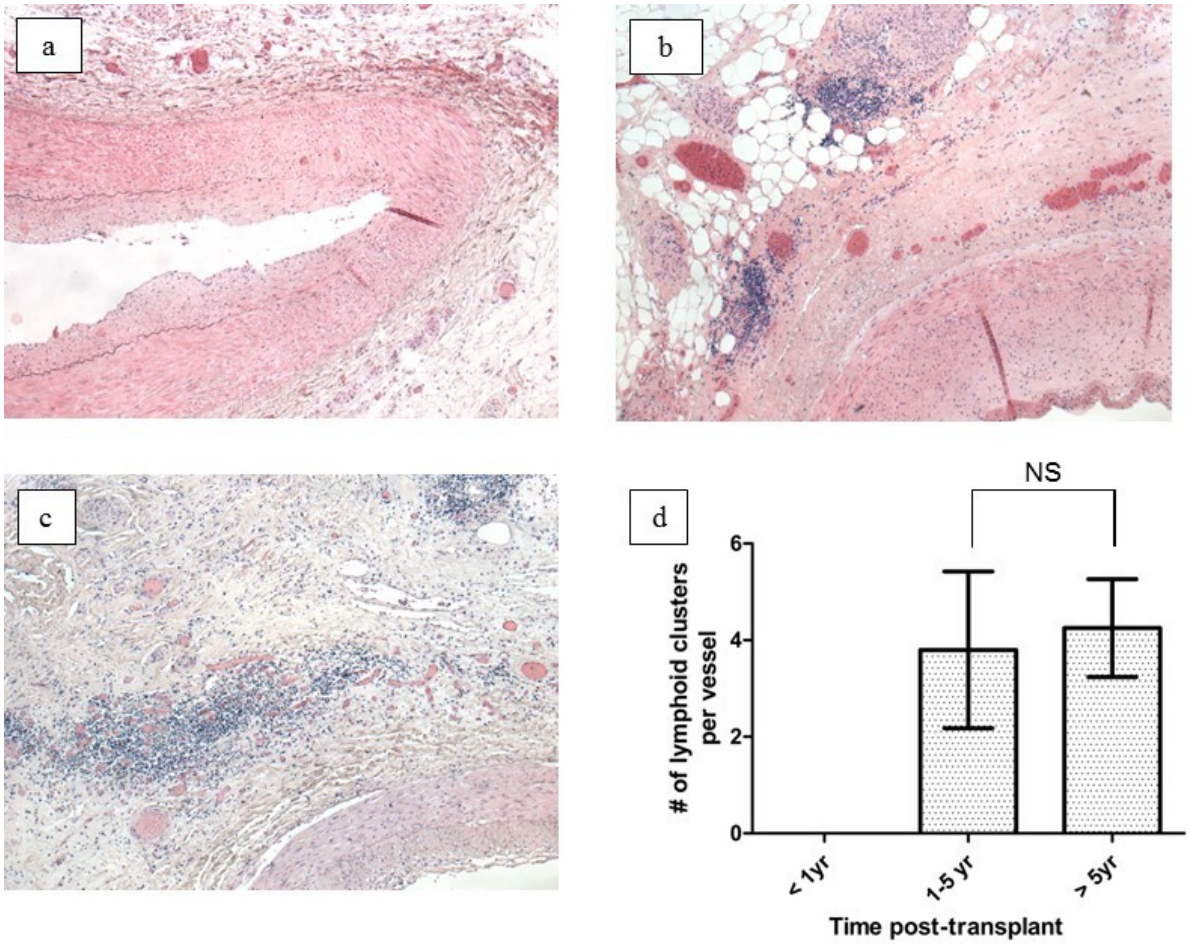


Figure 4.2 *Distance of ectopic lymphoid structures from medial layer does not change over time.* This graph depicts the mean distance of the ectopic lymphoid structures from the external elastic lamina demarcating the medial layer of the epicardial coronary vessels. Bars represent mean ± SEM.

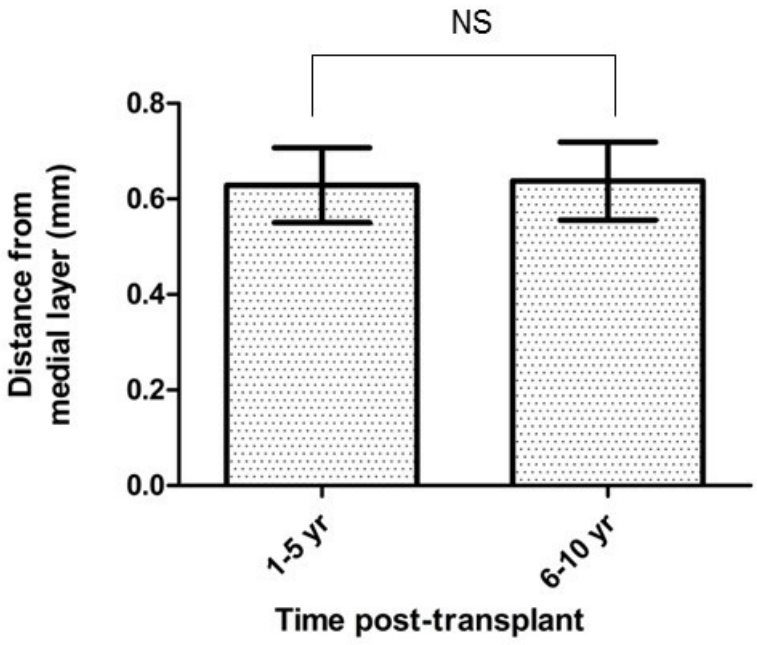


Figure 4.3 *B cells are present in transplanted tissue, but are distributed differently depending on time post-transplant*. (a) Typical pattern of dispersed B cells found in tissue from early timepoints following transplantation, with this tissue harvested at 10 days post-transplant (20x magnification). (b) Typical ELS found in patients in later timepoints following transplantation. This tissue was obtained at 8 years post-transplant (10x magnification).

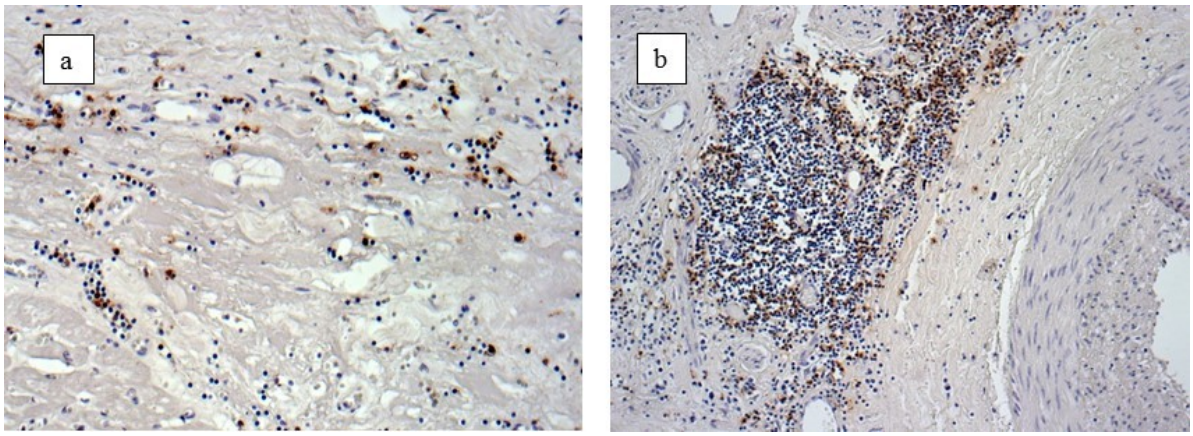


Figure 4.4 *Ectopic lymphoid structures are sites of active antibody production.* (a) Epicardial coronary artery tissue from a patient obtained at 10 yr post-transplant exhibiting the presence of plasma cells in an ELS lymphoid cluster (40x magnification), (b) Epicardial coronary artery tissue from a patient obtained at 2.5 yr post-transplant exhibiting the presence of IgM in an ELS (40x magnification), (c) Epicardial coronary artery tissue from a patient obtained at 2.5 yr post-transplant exhibiting the presence of IgG in an ELS (20x magnification).

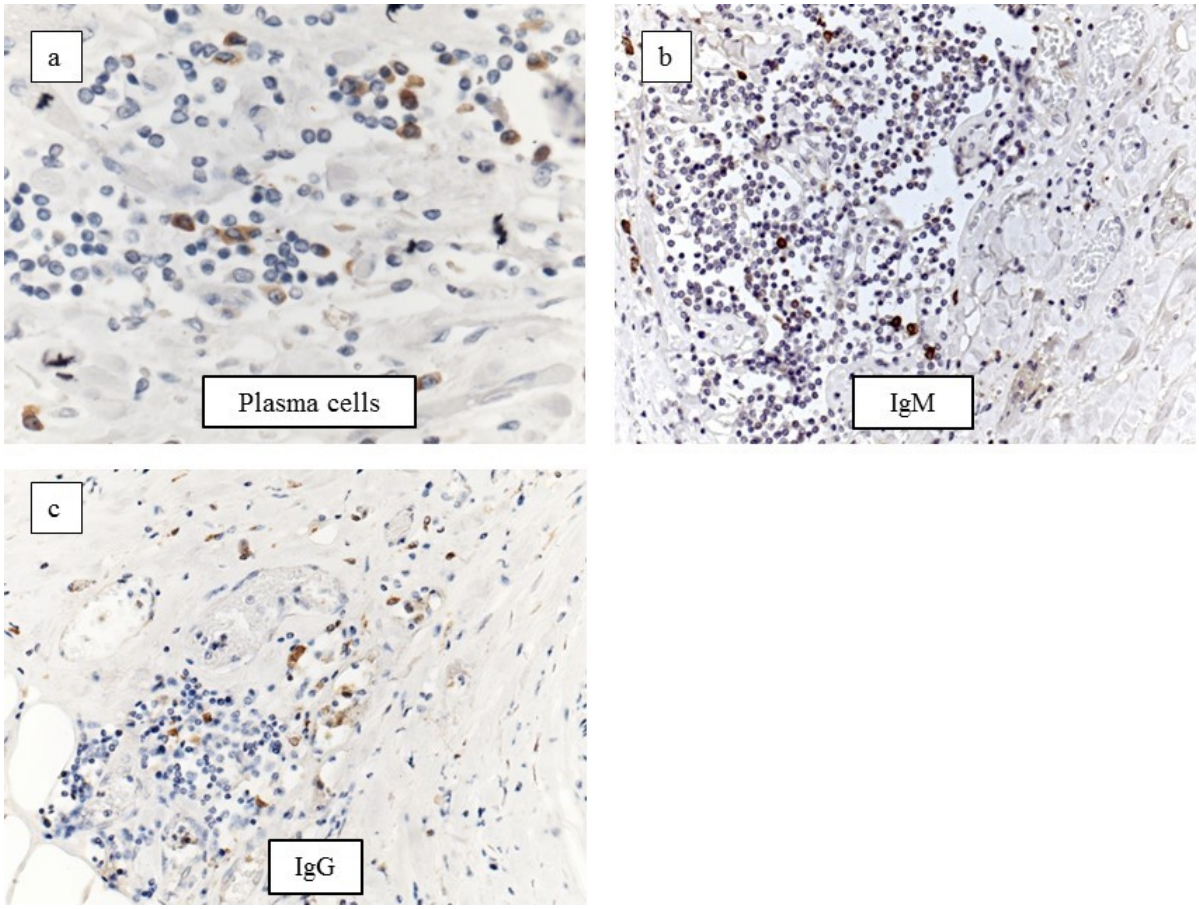
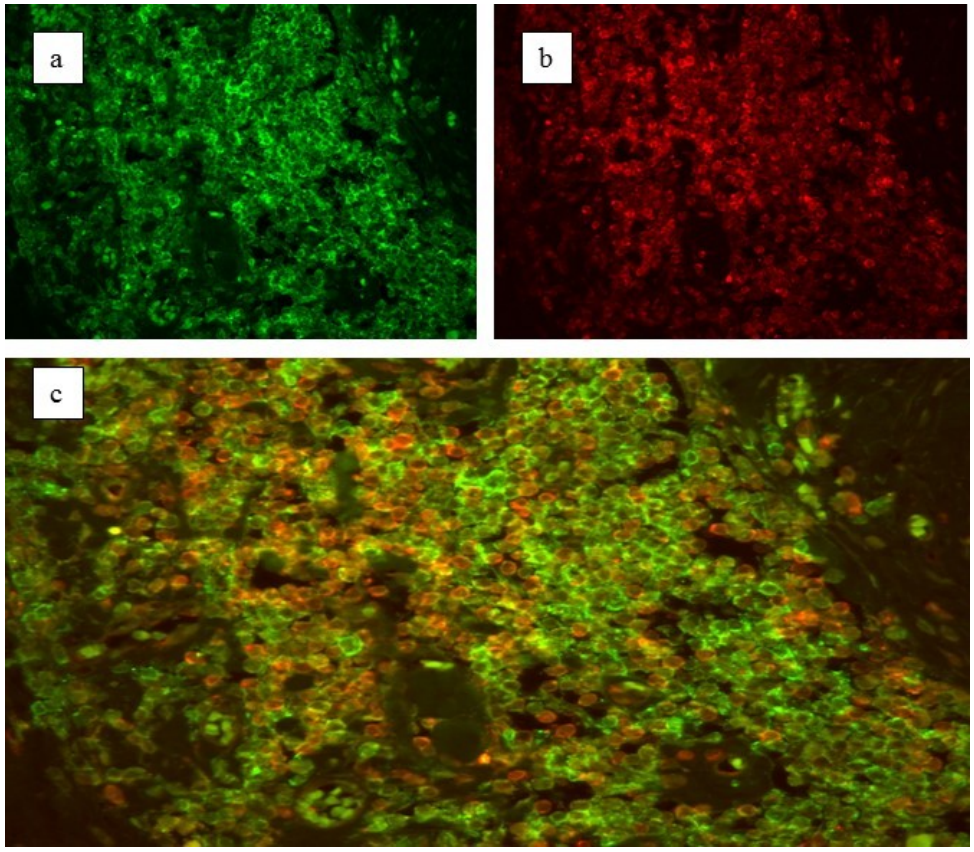


Figure 4.5 *$CD20^{+}CD27^{+}$ memory B cells are present in ectopic lymphoid structures*. Immunofluorescent staining of a typical ELS at 40x magnification demonstrates cells expressing: (a) CD20, (b) CD27, with (c) showing the merged image with memory B cells expressing both immune markers.



4.3 Discussion

The aim of the second part of this thesis was to investigate the potential link between ELS in the adventitia of coronary arteries and CAV. In particular, we have proposed that active B cells in the ectopic lymphoid structures could contribute to lesion formation.

Adventitial ELS were not present associated with coronary vessels in normal (non-transplanted) hearts or in the immediate aftermath of transplantation. This observation suggests that the ELS were associated with transplant rejection in some way. At later times post-transplant (greater than 2.5 years), ELS could be easily identified and occurred in the majority of the histological sections examined at late times post-transplant. Given that each of these histological sections is, in essence, a thin (5μm thick) random sample of the epicardial coronary artery examined, it is remarkable that so many of these ELS were seen. This means that the number of ELS in the major coronary arteries must be very high.

Tertiary lymphoid organs (TLO) develop outside the conventional locations of secondary lymphoid organs, such as the spleen and lymph nodes [201]. These TLO exhibit similarities in structure and morphology to the traditional lymphoid tissues.[159] Although the ELS we observed possessed some characteristics of TLO, including the presence of both T and B cells, and the presence of $CD21^+$ follicular dendritic cells (Appendix 1, Figure 1.2), the clusters we observed lacked several other structural elements that characterize TLO. Even though both T and B cells were found in our ELS, the T and B cells were not organized into discrete compartments as they have been demonstrated in other TLO. Other features that were lacking in the ELS we observed were the presence of

high endothelial venules and distinct germinal centers.[167,202] These structures are believed to be ideal sites for local T and B cell activation.[160]

It is important to distinguish the ELS we observe from Quilty lesions. Quilty lesions are also characterized by organization of lymphoid cells. In contrast to our observed ELS, Quilty lesions typically contain a central aggregate of B cells, surrounded by T cells with capillary-sized blood vessels throughout the lesion.[203] Based on the structural appearance and location of the clusters observed by the pathologists on our team, it was determined that the clusters are not Quilty lesions. Moreover, Quilty lesions are almost exclusively found in the endomyocardium,[204] although one report has observed a Quilty lesion within the neointimal lesion of a patient with allograft vasculopathy.[205] There is still much uncertainty in the transplant literature regarding any role that Quilty lesions may play in transplant rejection.

Previous studies examining ectopic lymphoid structures in organ transplants have suggested a relationship between chronic graft rejection and presence of TLO.[167,200,206] The proximity of the ELS we observed to vessels with early or late stages of vasculopathy likewise suggests a role for the ELS in the development of allograft vasculopathy. Work performed previously in our laboratory supports this suggestion. We have previously demonstrated that in our immunodeficient animal model of allograft vasculopathy, passive transfer of antibody alone can lead to medial SMC loss.[141] We hypothesized that this was due to binding of antibody to the medial SMC, leading to activation of the classical complement cascade. We stained our grafts for C4d, a surrogate marker of antibody-mediated rejection, and found that there was C4d present primarily in the medial area of our allografts.[199] This indicated to us that the antibody was responsible

for activation of the complement cascade in that region, which ultimately led to medial cell destruction. Based on the proximity of the ectopic lymphoid structures in our human patients to the medial areas of the vessels examined, we believe that the antibody being produced in these immunologically active ectopic lymphoid structures could have the same potential.

In support of this hypothesis we note that Wehner and colleagues have previously demonstrated that transcripts of immunoglobulins were increased in arteries dissected from heart transplants with vasculopathy.[79] This group followed this work with a study of tissue from cardiac transplant recipients with advanced chronic rejection. In their patient population, they found that B cells and plasma cells were present in 14 out of the 16 samples tested, but were distributed in three distinct patterns. This study reported B cells dispersed through the adventitia, in adventitial clusters, and dispersed throughout the neointimal lesion.[168] In contrast to their findings, although we found B cells in 7 out of 8 patients, we did not find any B cells or plasma cells in the neointimal areas of the tissue we sampled. This may be due to a smaller patient sample size, which limited the number of transplants we were able to examine, or differences in timepoints post-transplant. They did not examine the time post-transplant to determine if there were differences in distribution patterns based on how long the grafts were implanted, as they focused particularly on patients diagnosed with AV who had their grafts for longer periods post-transplant.

In addition to the presence of B cells and antibody subclasses, the presence of memory B cells that we report in the ectopic lymphoid structures has serious clinical ramifications. Rituximab, which targets $CD20^+$ B cells and is used to treat patients with

antibody-mediated rejection, has been shown to be ineffective in depleting B cells from secondary lymphoid organs in patients following kidney transplantation, which is where memory B cells traditionally reside.[100] Because these ectopic lymphoid structures resemble tertiary lymphoid organs, which, in turn, resembles the structure of germinal centers in secondary lymphoid organs, it would be interesting to investigate whether treatment with rituximab removes memory B cells from these structures. In this study, treatment with rituximab also resulted in a higher percentage of switched memory B cells ($IgD^{-}CD27^{+}$).[100] Given the fact that memory B cells become rapidly activated following re-exposure to antigen, this creates a greater number of cells primed to produce detrimental antibodies.

Taken together, the data we present here combined with previous findings, demonstrates that the ectopic lymphoid structures we observe in epicardial tissue from long-term transplant recipients are sites of B cells (particularly, memory B cells) and active antibody production. This is the first report of memory B cells within these clusters in a transplant setting and in close proximity to vessels developing CAV. Furthermore, the presence of antibody has been shown to be detrimental to long term graft survival.[122,124] Specifically targeting memory B cells in transplant patients could have significant impact on graft survival, and potential therapeutic options to target this cell type should be explored further.

CHAPTER 5

DISCUSSION

5.1 Discussion

Almost fifty years have elapsed since the first successful cardiac transplant.[207] Advances in immunosuppressive therapies have bolstered the short-term survival rates of grafts, but long-term survival still remains poor, demonstrating the inefficiency of the modern immunosuppressive armamentarium. Immunosuppressive therapies currently in use have primarily focused on eliminating T cell effector function.[189] The inability of this strategy to reduce long-term rejection rates[2] has caused attention to shift to potentially controlling other elements of the immune response. One such element is humoral immunity, involving B cells and antibody.

This thesis addresses the post-transplant pathology in the coronary vascular tree called allograft vasculopathy (AV). AV is considered by the ISHLT as the "Achilles heel" of cardiac transplantation because it is thought to represent a primary limitation to long-term cardiac graft survival. Early studies investigating the role of B cells in AV employed animal models lacking B cells or with induced B cell deficiencies. Conflicting data arose from this research[140,142,208] with the majority of studies demonstrating that antibody is not required for AV. Prior to conducting the research described in this thesis, I investigated this issue using two different models of B cell deficiency in recipients of abdominal aortic transplants. This research demonstrated that in both models, B cells were not required for the development of cardiac allograft vasculopathy.[141] This experimentation solidified the for us the conclusion that, although antibody may contribute to the formation of a neointimal lesion, these lesions can develop in the absence of humoral immunity.[140–142]

Having demonstrated that B cells and antibody were not required for AV, we next investigated whether B cells and antibody could induce AV in the absence of direct T cell effector function. To accomplish this we injected antibody directly into immunodeficient mice and treated them with clinically relevant immunosuppression (CyA). We found that antibody alone can induce the neointimal lesions characteristic of AV. This suggested to us that although antibody is not required for AV development it is sufficient.

The major weakness of this previous study was that the antibody passively transferred into the recipients was raised in normal healthy mice that did not receive immunosuppressive treatment. In the clinical setting, antibody formation would be occurring in the face of powerful immunosuppressive drugs including CyA. This is of significant importance since CyA and other CNI immunosuppressive drugs exert their maximum impact on $CD4^+$ T cells and these cells provide the T cell help required for B cell activation, differentiation and class switching of antibody.

Therefore the first objective of this thesis was to investigate the production of antibody under cover of clinical immunosuppression. Previously published evidence suggests that administration of CyA substantially decreases the amount of anti-donor antibody produced,[193] so it was important to demonstrate that complement-fixing anti-donor antibody can be raised in the presence of CyA. In the first set of experiments, we used the alloantibody production protocol where WT B6 mice were immunized with donor splenocytes, and we introduced CyA treatment at a dose of either 50 mg/kg/d or 30 mg/kg/d. In the presence of both doses of CyA, complement-fixing alloantibody was present, although at lower levels than in serum from animals immunized in the absence of CyA. As the dose of CyA decreased, the titre of the complement-fixing alloantibody

increased. We believe that this is because as CyA dosage increases, there are fewer $CD4^+$ T cells proliferating and available to activate B cells to produce antibody. Of note, even at doses of CyA that are clinically relevant and chosen to mimic the C2 levels of CyA in human transplant recipients (50 mg/kg), alloimmunization still resulted in the presence of complement-fixing alloantibody. We also tested the antiserum to ensure that it did not non-specifically target third party cells, and it was shown to be specific to only the donor cell type. Taken together, this data corroborates clinical research, which has shown that antibody targeting donor HLA is present in immunosuppressed patients at early and late timepoints post-transplant.[26,122,125,189]

Levels of CyA in patients undergoing treatment following heart transplantation are measured using C2 levels, which are taken 2 hours post-dosage.[191] These experiments were done in the presence of levels of CyA that, in our model, prevent acute rejection but allow for the development of chronic rejection,[11,131] which is approximately 1200 ng/mL.[131] This level approximates that upper range of CyA clinically detected in patients.[192] According to published data, CyA levels early post-transplant can range from 800-1100 ng/mL.[192] At this dose of CyA, $CD4^+$ T cells cannot facilitate acute rejection. In the study included in this thesis, antibody was produced in the animal model even at the highest CyA dose. This suggests that even at the highest dose of CyA, some $CD4^+$ T cell effector functions (or effector cells) escape. Otherwise complement fixing antibody to T dependent alloantigen could not occur.

The practice of slowly weaning patients off CNI over time has become widespread in the kidney transplantation field and is being considered in cardiac transplantation.[92] For this reason we tested for the production of antibody at C2 levels approximating the range

found in humans with longer-term grafts (400-600 ng/mL). C2 levels in mice given 30 mg/kg/d CyA resulted in C2 levels around 700 ng/mL.[131] As noted above, this resulted in increased alloantibody levels and would likely have the same result in patients.

One weakness of the experiments discussed above is that the alloantibody used for passive transfer was produced through immunization with donor splenocytes. In the clinical setting, the graft itself would act as the primary antigenic challenge. We therefore went on to confirm that fully MHC-mismatched aortic transplants, under the influence of CyA immunosuppression, can likewise lead to the production of complement-fixing alloantibody.

Because tacrolimus has now taken over as the preferred CNI and is routinely used in clinical practice,[1] it would be relevant to repeat these experiments in the presence of this immunosuppressant. The general mechanism of action of these two drugs is similar, in that Tac also prevents transcription of IL-2.[209] For this reason one would expect that if antibody production is a result of escape of some effector T cells from CNI inhibition (in other words, some T cell proliferation does occur) one would predict that similar results would be obtained, in that the proliferative capacity of $CD4^+$ T cells would be impaired, and therefore fewer $CD4^+$ T cells would be available to activate B cells. It is also possible that CyA affects some effector function more than others, for example, proliferation more than cytokine secretion. Since Tac acts as a CNI by a slightly different mechanism, it is possible that the use of Tac might answer the question as to whether the escape of function is a quantitative or qualitative phenomenon.

Evidence to support this consideration comes from Heidt and colleagues who demonstrated that CyA and Tac both decreased production of IL-2 to a similar extent,

while cyclosporine did not significantly decrease the amount of IL-4 and IL-5, cytokines that are implicated in the production of antibody.[193]

In any event, once it was established that complement-fixing alloantibody could be produced in the presence of CyA, we investigated whether this antibody could induce the development of AV. To accomplish this we passively transferred this antibody into B6.RAG1$^{-/-}$ graft recipients. The results showed that alloantibody was capable of inducing lesion formation but that the vascular lesions that developed were smaller than the lesions induced by the administration of alloantibody raised in the absence of CyA. The lesions were phenotypically similar to those formed in WT recipients. The grafts exhibited loss of medial smooth muscle cells when compared to native aortic sections, consistent with previous findings and with our hypothesis that alloantibody targets the medial SMC and leads to their destruction. Myofibroblasts were also present within the neointimal lesion as evidenced by the presence of alpha-actin staining in the lesion.

Our overall hypothesis for lesion formation in this animal model is that medial SMC death resulting from the alloimmune response, initiates a repair mechanism that is expressed as a neointimal lesion. For that reason, although we know that the alloantibody produced in the presence of CyA is capable of mediating complement-dependent cytotoxicity of donor splenocytes *in vitro*, in future experiments we need to confirm that this alloantibody was capable of mediating death of murine aortic SMC. This would either support or refute our hypothesis that medial damage is the driving event propelling the development of vasculopathy in the presence of antibody.

To begin to address this issue whether complement fixation could be demonstrated in the SMC rich media of the transplanted grafts undergoing AV. In these grafts, where

recipients were treated with 30 mg/kg and 50 mg/kg CyA, we indeed saw evidence of C4d deposition in the media. C4d deposition is a routinely used, widely accepted surrogate marker of AMR in heart transplantation and its presence is frequently associated with poorer graft survival. When we examined C4d staining in our grafts we found that there was C4d deposited in both the media and the neointimal lesion. Perhaps the most interesting aspect of this finding was the fact that the C4d was predominantly localized to the medial area of the grafts, indicating that the media is where the antibody deposition was occurring and the complement cascade was being activated. This data is also consistent with our theory that the antibody produced as a result of transplantation is, in our model, targeting medial SMC for destruction.

As alluded to above, a particularly compelling aspect of this thesis research is why the T cell help escapes CyA treatment and allows for production of alloantibody, given that the immunodominant antigens in transplantation are Class I and Class II MHC, as well as a variety of minor antigens. All of these are T cell-dependent antigens and T cell help is required for specific alloantibody generation. This T cell help is likely to come from $CD4^+$ T cells since $CD8^+$ T cells have not been previously described to provide such B cell assistance. This is not to say that $CD8^+$ T cells are not capable of this help since our knowledge of the spectrum of activities of $CD8^+$ T cells is constantly increasing. Given our previous findings that CyA ablates the ability of $CD4^+$ T cells to induce allograft vasculopathy, we postulated that either CyA has a differential impact on the variety of aspects of $CD4^+$ T cell effector function, or that $CD8^+$ T cells must be providing help for B cell activation, proliferation, differentiation and antibody production. To investigate this theory, I attempted to raise antibody in mice lacking $CD4^+$ T cells. If $CD8^+$ T cells

could provide help to B cells, then I should have been able to raise complement-fixing alloantibody in these mice. The fact that I was not able to raise antibody in this experiment (Figure 5.1) supports the primary role for $CD4^+$ T cells in B cell help and suggests that CyA reduces, but does not ablate, this function.

Finally, this research needs to address the question of why, if there is sufficient $CD4^+$ T cell activity to allow for alloantibody production, and that alloantibody induces AV, no AV is seen in $CD8^+$ deficient mice in the presence of CyA.[131] Moreover, passive transfer of $CD4^+$ T cells into B6 $RAG1^{-/-}$ recipients of allografts, in the presence of CyA, does not induce AV.[131] In the latter experiment, there are no $CD8^+$ T cells present, indicating that AV cannot occur in the absence of $CD8^+$ T cells. This presents a conundrum since the data above, and the interpretation given to it, does not completely agree with this finding. It is possible, albeit unlikely, that $CD4^+$ T cells are required for alloantibody production in the experiments in this thesis because they support the activities of $CD8^+$ T cells. There are two potential ways to explain the production of antibody in an immunosuppressed animal with limited $CD4^+$ T cell function. The first explanation is that not all of the $CD4^+$ T cells are affected by the presence of CyA, or that there are a small enough number of $CD4^+$ T cells able to proliferate to provide B cell help. Another possible explanation is that there are other innate cells that are capable of providing B cell help not affected by the presence of CyA. Splenic neutrophils can provide helper signals to B cells in the marginal zone of the spleen through expression of CD40L,[210] and invariant natural killer T (iNKT) cells are also distributed throughout the spleen at rest and can be found in germinal centers. These iNKT cells can express typical $CD4^+$ T helper cell molecules and cytokines, including IL-21 and CD40L.[211] Based on the fact that these innate immune

cells typically mediate B cell activation to TI antigens, including blood-borne bacterial antigens and antigens derived from glycolipids,[211,212] and the antigens in our model are cell-derived proteins, it is more likely that a small number of $CD4^+$ T cells is providing help to B cells.

The second objective of this thesis addresses AV in human cardiac transplant recipients. Upon initial examination of histological sections of epicardial coronary arteries from these patients, we observed the presence of ectopic lymphoid structures in the adventitia of the tissue from longer-term transplants (2.5 yr to 10 yr). These ectopic lymphoid structures were found in close proximity to vessels that were developing AV and not in association with normal coronary arteries. This demonstrates that the presence of ELS is associated with late transplant rejection and possibly with AV.

In an attempt to elucidate the phenotype of the cells present in the ELS, we stained for the presence of $CD20^+$ B cells, $CD4^+$ T cells and $CD8^+$ T cells. These immune cells have been seen before in cardiac allografts explanted because of AV development.[168] B cells and both types of T cells were present in 7 out of 8 patients, but in the 2 patients that were short-term graft recipients (10d and 24d), the T and B cells were found in a dispersed pattern and not in an organized ELS, suggesting that the organization of immune effector cells into ELS may have a temporal relationship to the time post-transplant. An extension to this current study would employ a much larger sample size to confirm or refute this hypothesis.

Based on our interest in the antibody response post-transplant, we also stained the tissue sections for IgM and for IgG; 3 of the 5 samples with ELS stained positively for IgG, and 4 of the 5 samples with ELS stained positively for IgM. With respect to memory

B cells, 3 out of the 5 samples examined contained memory B cells in the ELS. Plasma cells were also present exclusively in the ELS, and distributed randomly throughout the ELS.

The presence of memory B cells has not previously been demonstrated in ELS from experimental models, or from ELS found in kidney or cardiac transplants. These memory B cells are capable of rapid recall responses and can differentiate into plasma cells much more quickly than their naïve counterparts. The presence of memory B cells in the ELS has not previously been shown in human cardiac graft recipients, although effector and memory T cells have been demonstrated in experimental models of ELS.[159] The primary treatment for the depletion of $CD20^+$ B cells, rituximab, is ineffective in the depletion of CD20-expressing memory B cells from secondary lymphoid organs in patients following kidney transplantation.[100] Presence of memory B cells and their resiliency to conventional treatment may present another obstacle in the long-term survival of cardiac grafts.

The ELS we saw in the adventitial areas and in the epicardial fat of the coronary in this thesis show some characteristics of TLO, although they do not fulfill all of the criteria to be classified as TLO. Of note, the ELS found in our tissue lack the presence of high endothelial venules, which are traditionally characterized through the presence of peripheral node addressin (PNAd). Staining for PNAd showed negative results in all samples of artery studied, indicating that HEV were absent in the ELS. CD21 staining was present to a small extent in 4 of the 5 ELS (Appendix 1, Figure 1.2), indicative of the presence of follicular dendritic cells, an important cell type in antigen presentation and the functioning of germinal centers. However, we did not find that the T and B cells in

our ELS were organized into distinct areas representative of germinal centers. Taken together, we these observations suggest an attempt by the lymphoid cells in the transplanted tissue to organize into a tertiary lymphoid organ.

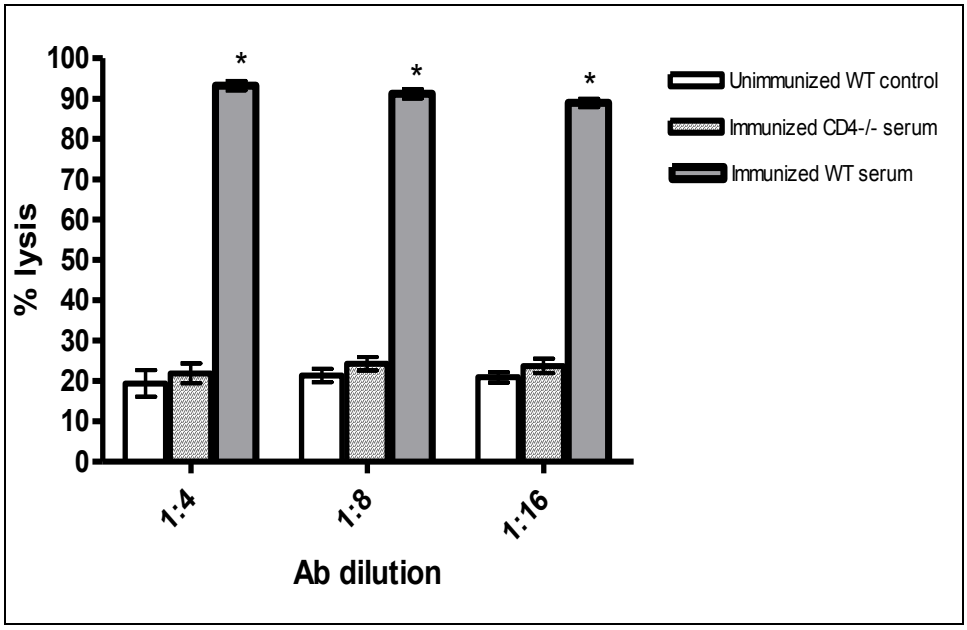
One thing lacking in this study was the anti-HLA antibody status of the patients examined. Although allospecific antibody responses can be readily detected in animal models, these antibodies are often detected only in small quantities in the clinical scenario. Moreover, their detection in the later months and years following transplant varies.[213] However, the presence of antibody post-transplant has repeatedly been shown to be harmful to long-term graft survival.[122,124] Based on our findings in this study, which suggest that the ELS are sites of active antibody production close to vessels developing AV, we can hypothesize that the patients with IgM or IgG positive staining would be producing some form of detectable antibody. Whether this antibody would be anti-HLA in nature could be determined if the HLA type of the donor was known.

In this case, the graft itself appears to not only be the target of the immune response, but also a site where an immune response actually develops. This may be an attempt to localize the communication between the coronary artery and the immune cells that are orchestrating the response.[153] TLO has also been shown to provide survival signals to B cells which allows them to escape rituximab-induced apoptosis, limiting the therapeutic efficiency of this drug,[214] which is routinely used to treat episodes of antibody-mediated rejection.[215]

In summary, we have shown that alloantibody can be raised in the presence of clinical immunosuppression in the form of CyA. This antibody is capable of mediating donor cell destruction via a complement-dependent pathway, and can also induce lesion

formation when passively transferred to immunodeficient aortic graft recipients. Furthermore, allogeneic transplants are a sufficient stimulus to induce anti-donor antibody production. As more research focuses on the role played by antibody in the development of AV, it is evident that alloantibody must be identified a potential therapeutic target in the prevention and treatment of long-term graft rejection. In addition, we have demonstrated that ELS in the epicardial coronary arteries of cardiac transplant patients contain B cells, antibody subclasses and plasma cells, immune elements that are indicative of active antibody production. These ELS also contained $CD20^{+}CD27^{+}$ memory B cells and we believe that the antibody produced has a local impact on the development of AV in these vessels.

Figure 5.1 *Serum from CD4$^+$ knockout mice does not have the ability to mediate lysis of allotargets.* Allosplenocytes were incubated with doubling dilutions of antiserum from: unimmunized B6 WT mice (control), immunized mice lacking CD4$^+$ T cells (CD4$^{-/-}$), and immunized WT mice. In the presence of complement, there was no overall statistical difference between the ability of serum from mice lacking CD4$^+$ T cells and control serum to mediate cytotoxicity in the presence of complement ($p>0.05$). Values represent the mean ± SEM for 3 independent experiments. (* denotes statistical significance from control).



Appendix 1: Supplemental Figures

Figure 1.1 *$CD4^+$ T cells are present in ectopic lymphoid structures*.

Immunohistochemical staining demonstrated the presence of $CD4^+$ T helper cells in ectopic lymphoid structures (20x magnification).

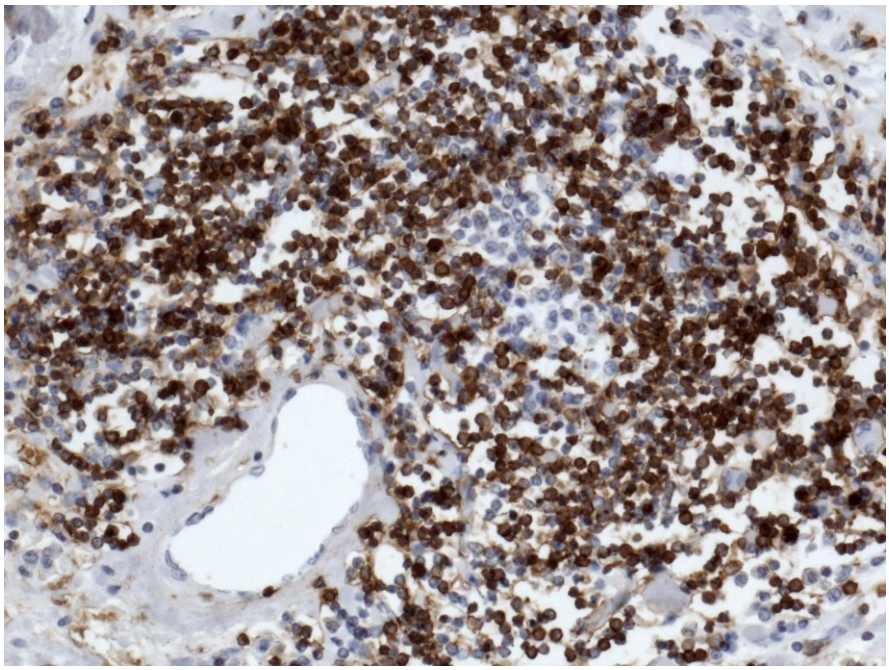
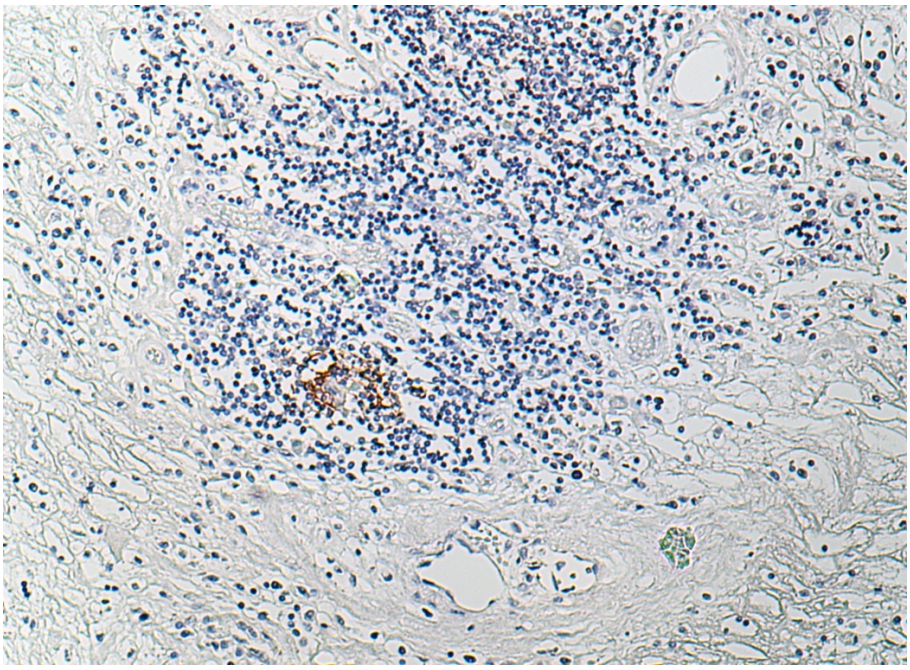


Figure 1.2 *$CD21^+$ follicular dendritic cells are present in ectopic lymphoid structures.* Some of the ectopic lymphoid structures found in the epicardial coronary arteries from cardiac transplant recipients contained $CD21^+$ FDC. FDC are present in TLO and we hypothesize that in our tissue, presence of $CD21^+$ FDC represents an attempt to form TLO (10x magnification).



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