The Role of TLR2 in the Pathogenesis of Kawasaki Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

Kawasaki disease (KD) is a childhood vasculitis with a predilection for the coronary arteries (CA). The etiology of KD is unknown; however, superantigens (SAg) have been implicated. SAg-activated T cells undergo massive proliferation followed by apoptosis; conversely, in KD these T cells may persist and target the CAs. Enhanced costimulation can rescue SAg-activated T cells from apoptosis, and Toll-like receptor 2 (TLR2) enhances costimulation. In a murine model of KD, TLR2-deficient mice are disease resistant, and evidence suggests preferential expression of TLR2 at the CA. Results from this study demonstrate that TLR2 is rapidly expressed in the heart following disease induction, and that TLR2 is expressed differentially in various arteries. The aorta, from which the CAs branch off, expressed the highest TLR2 levels. A microvascular endothelial cell line was shown to function as an APC following TLR2 stimulation, supporting the proliferation of SAg-activated T cells and their rescue from apoptosis.
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List of Abbreviations

7-AAD – 7-amino-actinomycin
Ab – antibody
Ach – acetylcholine
Ao – aortic/aorta
AoR – aortic root
APC – allophycocyanin
APC – antigen presenting cell (s)
Bel – B cell lymphoma
BLP – bacterial lipoprotein
BMC – bone marrow chimera
bp – base pairs
BP – blood pressure
BrdU – bromodeoxyuridine
C/EBPs – CCAAT/enhancer binding proteins
Ca\(^{2+}\) – calcium ion
CAAs – coronary artery aneurysms
CALs – coronary artery lesions
cAMP – cyclic adenosine monophosphate
CAs – Coronary Arteries/arteritis
CD – cluster of differentiation
cDNA – complimentary DNA
Ci – Curie
CRP – C reactive protein
CTLA4 – cytotoxic T-lymphocyte antigen 4
DAG – diacylglycerol
DAMPs – damage-associated-molecular patterns
DCs – dendritic cells
DMEM – Dubucco’s Modified Eagles Medium
DNA- deoxyribonucleic acid
DPM – disintegrations per minute
E-selectin – endothelial selectin
ECs – endothelial cells
ERK – extracellular signal related kinase
FADD – Fas-associated protein with death domain
FBS – fetal bovine serum
FITC – fluorescein isothiocyanate
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
h – human
HAECs – human aortic endothelial cells
HCAECs – human coronary artery endothelial cells
HEK – human embryonic kidney cells
HLA – human leukocyte antigen
HPAECs – human pulmonary artery endothelial cells
HSP – heat shock protein
HUVECs – human umbilical vein endothelial cells
ICAM-1 – intracellular adhesion molecule-1
IFNγ – interferon γ
Ig – Immunoglobulin
IHC – immunohistochemistry
IKK – inhibitor of NF-κB kinase
IL – interleukin
ip – intraperitoneal
IP3 – 1,4,5-triphosphate
IRAK – IL-1R-associated kinase
IRF – interferon regulatory factor
ISRE – IFN-stimulated response elements
ITAMs – immunoreceptor tyrosine-based activation motif
ITPKC – 1,4,5-triphosphate-3-kinase C
IVIG - intravenous gamma globulin
JPS – juvenile polyarteritis syndrome
KD – Kawasaki Disease
KO – knock out
L – ligand(s)
L-selectin – leukocyte selectin
L. casei – Lactobacillus casei
LAT – linker of activated T cells
Lck – lymphocyte specific protein tyrosine kinase
LCM – laser capture microdissection
LCWE – Lactobacillus casei cell wall extract
LPS – lipopolysaccharide
LRR – Leucine rich repeat
LTA – lipoteichoic acid
mAb – monoclonal antibody
MAECs – murin aortic endothelial cells
MAL – MyD88-adaptor-like
MAPK – mitogen-activated protein kinase
MBL – mannose-binding lectin
MCP-1 – monocyte chemotactic protein-1
MHC – major histocompatibility complex
MKK – MAP kinase kinase
Mls – minor lymphocyte stimulating antigen
MMTV – mouse mammary tumor virus
mRNA – message RNA
MVECs – microvascular endothelial cells
MyD88 – myeloid differentiation factor 88
NEMO – NF-κB-essential modulator complex
NF-κB – nuclear factor-kappa B
NFAT – nuclear factor of activated T cells
P-selectin – platelet selectin
Pam3Cys – Pam3Cys-Ser-(lys)SEB , Hydrochloride
PAMPS – pathogen-associated molecular patterns
PBMCs – peripheral blood mononuclear cells
PBS – phosphate buffered saline
PCR – polymerase chain reactions
PD-1 – programmed death-1
PE – phycoerythrin
PECAM-1 – platelet endothelial cell adhesion molecule-1
PG – peptidoglycan
PGEs – E-type prostaglandins
PI – propidium iodide
PIP2 – phosphatidylinositol-4,5-bisphosphate
PKC – protein kinase C
PLC – phospholipase C
PRRs – pathogen recognition receptors
PS – phosphatidyl serine
qRT-PCR – quantitative real-time polymerase chain reaction
RAG – recombination activating gene
RNA – ribonucleic acid
rpm – revolutions per minute
SAg – Superantigen/ antigenic activity
SEB – *Staphylococcus* enterotoxin B
SLP-76 – SH2 domain containing leukocyte protein of 76 kDa
SMCs – smooth muscle cells
SNP – single nucleotide polymorphism
SOS – Son of sevenless
Src – sarcoma
STAT – signal transducers and activators of transcription
TAB – TAK-associated binding proteins
TAK1 – TGFβ-activated kinase 1
TCR – T cell receptor
Tg – transgenic
TICAM1 – TIR-domain-containing molecule 1
TIR – Toll/IL-1-receptor homology domain
TIRAP – TIR-associated protein
TLR – toll like receptor
TNFRI – tumor necrosis factor receptor I
TNFα – tumor necrosis factor α
TRAF – TNF receptor-associated factor
TRAM – TRIF-related adaptor molecule
TRIF – TIR-domain-containing adaptor protein-inducing IFNβ
TSST – toxic shock syndrome toxin
UV – ultra violet
V – variable
VCAM-1 – vascular cell adhesion molecule-1
VeCad – vascular endothelial cadherin
VEGF – vascular endothelial growth factor
VSMCs – vascular smooth muscle cells
vWF – von Willebrand factor
WBC – white blood cell
WPb – Weibel-Palade bodies
WT – wild type
1 INTRODUCTION

1.1 Kawasaki Disease

1.1.1 Clinical Manifestations

In Japan in 1967, Dr. Tomisaku Kawasaki studied fifty infants who presented with similar symptoms, representing a new and curious syndrome (1). Initially described as acute febrile mucocutaneous lymph node syndrome, Kawasaki disease (KD) was thought to be a self-limiting inflammatory syndrome, which at times was misdiagnosed as either being scarlet fever, infantile periarteritis nodosa, or Stevens-Johnson syndrome (2). However, the long-term effects of the disease were discovered following the sudden deaths of several infants. The cause of death in these infants was due to cardiac failure associated with formation of coronary artery aneurysms (CAAs) and thrombosis (2). Although the acute signs of inflammation resolve, it is now known that persistent coronary arteritis leading to aneurysm formation can occur, and as a result Kawasaki disease is now acknowledged to be the leading cause of acquired cardiac disease for children in the developed world (3).

Kawasaki disease is an acute, systemic vasculitis that predominantly affects infants and young children (3). Due to a lack of diagnostic tests, a set of clinical criteria based on the physical features of the disease are used as guidelines to identify children with KD (4). Typical characteristics include a persistent fever in duration of 5 or more days, as well as a minimum of four of the five following signs and symptoms: a polymorphous skin rash, erythema of the oral mucosa and lips, extremity changes affecting the hands and feet, bilateral nonexudative conjunctivitis, and cervical lymphadenopathy (3). Laboratory findings for KD patients, which are also typical for inflammation include high C-reactive protein (CRP) levels, elevated sedimentation rate, an elevated white blood cell (WBC) count and an elevated platelet count (5).

1.1.2 Epidemiology

Kawasaki disease demonstrates an age bias, as approximately 80% of cases have occurred in children under the age of 5 (5), with a peak incidence occurring in children 2 years of age or younger. Familial incidence for this disease is approximately 2% (6) with recurring cases in 2 to 4% of patients, respectively. Interestingly, Kawasaki disease affects young males more frequently than young females, with cases occurring 1.5 times more in males than in females (7).
Kawasaki disease affects children from all racial backgrounds, however children of Asian ancestry between 0-4 years of age continue to have the highest annual incidence (7, 8). This is exemplified by children in Japan having an annual incidence of 188.1/100,000 children, 105/100,000 children in Korea, 69/100,000 children in Taiwan, 55.1/100,000 children in China, and 39/100,000 children in Hong Kong (9, 10). In the United States, overall incidence for children younger than 5 years of age is reported as 17.1 per 100,000 children (10, 11), with children of Asian background predominating as many of the diagnosed cases are represented by Asian Americans. Interestingly, the overall incidence of KD for Ontario has been reported as 26.2 cases per 100,000 children indicating that the Ontario region may have one of the highest reported incidences of KD outside of Asia (10). As new cases are diagnosed in North America, a trend has emerged whereby larger numbers of cases occur in the late winter to early spring months, with the highest caseload being reported as 14-31% higher than average from the months of November to March for Ontario.
1.2 Coronary Arteries

It is now known that the coronary arteries are targeted for end organ damage in Kawasaki disease (12), and as such, untreated children may suffer from ischemic heart disease, myocardial infarctions, or sudden death (13). In cases regarding Kawasaki disease where death does occur, it is typically due to ischemic heart disease initiated by a thrombosed coronary artery aneurysm following coronary arteritis (CA) (14). As coronary artery aneurysms represent a fatal complication of KD, cases of ruptured coronary artery aneurysms have been reported, which can result in death (14-16).

1.2.1 Structure and cellular components

The coronary arteries branch off the ascending aorta (17) and encircle the heart so as to provide the myocardium with oxygen and nutrients. The coronary arteries are of special consideration because they experience particularly high pressure ranging from 0 or negative pressure when the aortic valve is closed to very high (systolic BP) with ejection of blood from the heart into the aorta and CAs. These vessels are able to withstand high pressure blood flow due to their cell wall composition, which consists of three distinct layers, or tunics: the tunica interna (intima), the tunica media, and the tunica externa (Figure 1).

The tunica interna, or intima, forms the inner lining of a blood vessel, which is in direct contact with blood as it passes through the arterial lumen. Within the intima is a layer of simple squamous epithelium known as the endothelium. The monolayer of vascular endothelial cells actively participate in vessel-related activities, which include secretion of local chemical mediators capable of influencing the contractile state of the vessel, physically influencing blood flow, and assisting with capillary permeability.

The middle layer of arteries, known as the tunica media, is a muscular and connective tissue layer comprised mainly of smooth muscle cells (SMCs) alongside substantial amounts of elastic fibers. The primary role of the SMCs is to regulate the diameter of the artery lumen so as to control blood flow and pressure; however, SMCs are also involved in producing the elastic fibers found within the tunica media that enable the vessels to stretch and recoil under the applied pressure of flowing blood.

The tunica externa provides the outer covering of a blood vessel, and is comprised of numerous elastic and collagen fibers. The primary role of the tunica externa is to supply its own
vessel wall with oxygen and nutrients as well as nerves; however the *tunica externa* also functions to anchor a vessel to surrounding tissues.

![Diagram of the Artery wall](image)

**Figure 1: Structure of the Artery wall.** The innermost layer, the *tunica interna*, comes into direct contact with flowing blood and is comprised of endothelial cells and subendothelial connective tissue. The surrounding layer, the *tunica media*, is much thicker and consists of smooth muscle cells that help to regulate blood flow and pressure. The outermost layer providing the outer covering of the vessel, the *tunica externa*, is made of elastic and collagen fibers and helps to anchor the vessel to surrounding tissues. Image replicated with permission, © Copyright 2009 John Wiley & Sons Inc. (Principles of Anatomy and Physiology 12th edition).

### 1.2.2 Endothelial cells

The endothelial cells (ECs) that form the lining of the blood vessel lumen provide an interface between blood and the surrounding tissues. Endothelial cells from large vessels *in situ* demonstrate a characteristic squamous morphology, while under tissue culture conditions ECs typically grow in a monolayer while displaying a polygonal cobblestone appearance.
Endothelial cells possess membrane-bound storage granules known as Weibel-Palade bodies (WPb). The two principle molecules typically stored and released from WPBs include von Willebrand factor (vWF) and P-selectin, indicating a dual role for ECs in hemostasis and inflammation. P-selectin, as well as other adhesion molecules (Section 1.2.3) recruit leukocytes to inflamed endothelial tissue, therefore allowing them to extravasate to the site of infection or injury.

Within KD and the LCWE animal model (refer to section 1.4), the vascular endothelium has been found to play an important role in mediating inflammation via recruitment of leukocytes into affected tissue (18, 19). Extravasation of leukocytes across the vascular endothelium requires the appropriate interactions between the leukocytes and the endothelial cells, and is a necessary step in an inflammatory process (5). This migration is mediated by various cell adhesion molecules that can be found to be elevated in soluble form in diseases associated with endothelial cell activation and inflammation.

1.2.3 Kawasaki Disease and Endothelial Activation

Under normal conditions, endothelial cells are in a quiescent state in which one of their functions includes regulating leukocyte migration from the bloodstream into tissues. Under inflammatory conditions one of the initial steps in the immune response is cytokine-mediated activation of ECs (20, 21). There are various factors that can induce EC activation, including TNFα, IL-1β, IL-6, IL-8, CRP as well as costimulatory interactions by CD40-CD40L (21-23). Activation of the endothelium is indicated by five core changes: expression of leukocyte adhesion molecules including E-selectin, VCAM1 and ICAM-1 (see below) (20-23), loss of vascular integrity, phenotypic changes from anti-thrombotic to pro-thrombotic, production of cytokines, and upregulation of HLA molecules (23). Importantly, activated endothelium can upregulate class II HLA molecules, thus allowing them to function as antigen presenting cells (APCs) (24). Following activation, ECs also release P-selectin from the Weibel-Palade bodies (20-23). Activation of endothelial cells appears to be a common pathogenic mechanism, for this process can be initiated by viruses and bacteria, as well as by pro-inflammatory cytokines, physical stress and anti-endothelial cell antibodies that can be found in various autoimmune diseases including the vasculitides (23, 25).

The vascular endothelium is a critical component in Kawasaki disease as it is responsible for regulating arterial function, and is the final target of inflammatory infiltrates in patients who develop coronary artery aneurysms. Lymphocytes are able to infiltrate tissues and initiate
inflammation due to their interactions with specific adhesion molecules and costimulatory ligands expressed on the endothelial cell surface (26, 27). The adhesion molecules involved in extravasation of lymphocytes into affected tissues belong to two families: (i) selectins, including endothelial (E)-, platelet (P)- and leukocyte (L)- selectins, which are involved in the initial contact of lymphocytes with ECs; (ii) integrins, including intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1); platelet endothelial cell adhesion molecule-1 (PECAM-1), which mediate the transmigration of lymphocytes through tight adhesions (28). PECAM-1 is constitutively expressed on ECs, however both VCAM-1 and ICAM-1 are upregulated upon stimulation with various pro-inflammatory cytokines, including TNFα, IFNγ, and IL-1. Increased expression of adhesion molecules have been found in inflamed vessels in patients with vasculitis, indicating that the ECs may have been activated. During the acute phase of KD, circulating levels of ICAM-1 were increased while E-selectin and ICAM-1 adhesion molecule expression was detected on ECs from biopsy samples belonging to patients who suffered from acute KD, indicating that the endothelium was in an activated state (29). It has also been documented that during the acute phase of KD, patients have increased serum levels of soluble P- and E-selectin, which can be shed from the surface following activation with various cytokines (26). As soluble E-selectin is typically considered to be a marker of endothelial activation, this may be an indication of endothelial cell damage in KD (26).

Within the animal model of coronary arteritis (refer to section 1.4), ICAM-1, VCAM-1, and E-selectin adhesion molecule expression is upregulated in cardiac tissue following LCWE injection, indicating that ECs play a role in coronary disease (30). Aside from recruiting lymphocytes utilizing adhesion molecules, endothelial cells also express various costimulatory molecules including CD80 and CD86, which are required in the process of interacting with T cells. Studies that assessed CD80/86 expression on ECs demonstrated that ECs could induce T cell proliferation, while alternate studies that used monoclonal antibodies against CD28 demonstrated a decreased T cell proliferative response, thus indicating the importance of costimulation on ECs in the process of mediating T cell activation (27, 31-33). Activated ECs appear to play important roles in KD as well as in the murine model of disease, indicated by the upregulation of various adhesion molecules that are responsible for recruiting lymphocytes to sites of inflammation. Since activated endothelial cells express both MHC class II and co-stimulatory molecules, they may also function as effective APCs.
1.2.4 Endothelial cells as APCs

Activation of T cells is accomplished by signaling via specialized cells known as antigen presenting cells (34). APCs provide the two distinct signals that optimally activate T cells, mainly the expression of major histocompatibility complex II (MHC) molecules as well as costimulatory ligands (34, 35). The first signal comes from a peptide in the context of the MHC II molecule on the APC to the T cell receptor of the T cell. Although some cells such as macrophages constitutively express MHC class II, other cells may require activation or stimulation to induce expression or upregulation of MHC II before they can develop antigen-presenting capabilities (35). The second signal is triggered by the interaction of costimulatory molecules on the APC with their corresponding receptors on the T cell. APCs typically express a spectrum of costimulatory ligands, including the well known CD80 and CD86 molecules. The second costimulatory signal is thought to be crucial for the expression and regulation of cytokines expressed by activated T cells, including the growth promoting cytokine, IL-2 (34, 35). Having both signal one and signal two delivered to the T cell from the same cell allows for the most efficient T cell activation, therefore expression of both MHC molecules and costimulatory ligands are important for a cell to be an effective APC.

In the early stages of KD, vasculitis in the small and medium-sized vessels is accompanied by edema of the endothelial layer and leukocyte infiltration of the arterial wall. The inflammatory infiltrates are initially neutrophils (36, 37); however as KD progresses this infiltrate is predominantly T lymphocytes and macrophages, as was illustrated by frozen myocardial and coronary artery specimens (38). It is known that immune cells including B lymphocytes, activated T lymphocytes, and antigen presenting cells express MHC class II; alternatively non-classical APCs such as endothelial cells can also express MHC class II molecules (Ia/HLA-DR) (27, 31-33, 39, 40).

Expression of MHC class II is important for the regulation of immune responses and for the functioning of APCs (33, 41). There is much evidence indicating the importance of MHC II on ECs and how these cells may function as APCs (27, 31-33, 40). A study by Seino et al. demonstrated that vascular ECs, specifically human umbilical vein endothelial cells (HUVECs) and microvascular endothelial cells (MVECs), constitutively expressed CD86, MHC class I, and ICAM-1, but that treatment with IFNγ was required to induce expression of MHC class II and also upregulated expression of ICAM-1. Furthermore, CD4+ T cells proliferated in response to the IFNγ-treated ECs, an effect that could be blocked by using monoclonal antibodies (mAb)
against MHC II, ICAM-1 or CD86 (40). Further evidence that ECs can function as APCs was demonstrated by a study that also induced MHC class II on vascular ECs following IFNγ treatment. A bacterial SAg, namely SEB, was added to IFNγ-treated EC cultures and stimulated T cells to proliferate and secrete TNFα and IFNγ (27). Interestingly, these effects could be inhibited through the use of mAb for the costimulatory receptor CD28 and ICAM-1. These studies demonstrate that through the expression of both MHC class II and costimulatory molecules, vascular ECs can serve as functional APCs.

As stated above the endothelium is activated in KD, indicated by the upregulation of various adhesion molecules. Therefore it is noteworthy that Terai et al. demonstrated that MHC class II could be found on coronary artery endothelium from a patient who suffered from Kawasaki disease (37). It was also suggested that the expression of MHC class II on the CA endothelium could have been induced by any of the potent cytokines produced by activated T lymphocytes during the course of disease, therefore priming the vascular endothelium to function as APCs and indicating the important role the endothelium may play in development of CA lesions (37).

1.2.5 Endothelial Dysfunction

The endothelium is located such that it has the ability to sense changes in blood-borne signals as well as in hemodynamic forces and thus respond appropriately via the release of numerous autocrine and paracrine agents. A balanced release of these agents, such as IL-1 or nitric oxide, are important for maintaining vascular homeostasis within an individual (42). Endothelial cell dysfunction, including the expression of proinflammatory adhesion molecules as well as the dysfunctional release of messenger molecules (43), disrupts the balance that allows for vascular homeostasis. This disruption predisposes vessels to vasoconstriction, platelet activation, leukocyte adherence, thrombosis as well as vascular inflammation.

Although there is debate regarding the long term presence of endothelial dysfunction in patients who suffered from KD (44), several studies have indicated that the intense systemic inflammation that occurs during the acute phase of KD may actually lead to persistent endothelial dysfunction in patients in whom the clinical resolution of KD was already observed (36, 45-47). This was demonstrated when adults who had a history of KD were analyzed and demonstrated a reduced percent flow-mediated dilatation (48), indicating that endothelial cells may play a strong role during disease. An alternate study analyzing patients as late as 18 years post-KD resolution observed increased intima media thickness of the carotid artery as well as
arterial stiffness when compared to control patients (49). Furthermore, a study performed by Furuyama et al. verified a significant reduction in myocardial flow reserve, regardless of whether the patient had coronary lesions late after KD (50). These authors concluded that microcirculation may be impaired in these patients due to diffuse coronary vasculitis caused by Kawasaki disease. They further examined endothelial function in the coronary vessels and observed significant impairment when compared to controls. Thus they concluded that their results indicated impaired function of the CA endothelium, regardless of a patient having developed CA lesions late after KD.

Other evidence exists demonstrating the involvement of the endothelium in disease, and how it is affected long after a patient’s recovery. Fujiwara et al. stated that intimal hypertrophy was observed in coronary vessels of patients who had suffered from KD, even in the absence of CA lesions (51), whereas a study performed by Suzuki et al. reported that active remodeling of the CAs persists, indicated by intimal proliferation, for several years following the onset of KD (52). Thus the vascular endothelium may play a pertinent role in KD. As previously mentioned, the vascular endothelium is demonstrated to be activated through the upregulation of adhesion molecules, allowing for recruitment of lymphocytes, and may also serve as an APC to induce the proliferation of effector cells. Furthermore, endothelial cells may continue to be affected long after disease resolution, indicated by persistent endothelial dysfunction in some cases, further implying the depth of their involvement in disease.

As the endothelium has been demonstrated to play an important role in KD, being able to assess EC function may provide further understanding of how ECs are contributing to disease. For assessment of vasodilatation in the CAs, quantitative coronary angiography is used to measure how the diameter of vascular vessels are modified in response to infusions of endothelium-dependent vasodilators such as acetylcholine (Ach). Healthy CAs experience dilatation in response to Ach, while diseased vessels are induced to constrict (42, 43). Coronary microvascular endothelial function can be assessed via intracoronary Doppler techniques, which measure changes in coronary blood flow in response to physiological or pharmacological stimuli. With regards to peripheral circulation, endothelial function can be assessed by utilizing brachial artery ultrasound to monitor vasodilatation (flow-mediated vasodilatation) (42). Lastly, inflammatory markers such as CRP have proven to be powerful predictors for cardiovascular events, and studies have demonstrated that CRP is a predictor as well as a mediator of lesion formation. Preliminary studies have also suggested that endothelium-dependent vasodilatation was impaired in KD patients who had elevated CRP levels. As such, CRP has been regarded as
an indirect but important tool to measure endothelial function (42, 53-55). Currently, no ideal test for EC function exists as some methods are quite expensive and invasive; however, as endothelial cell dysfunction is thought to be one of the earlier events in lesion development, measuring EC function may serve as a useful tool for CA disease. The degree of coronary endothelial dysfunction may be important for prognosis, as it has been suggested that impaired endothelium-dependent CA vasodilatation is associated with an increased risk of subsequent vascular events (56, 57).

1.2.6 Aneurysm Development

The major cause of mortality for Kawasaki disease is the formation of CAA. Formation of aneurysms can typically be detected from one to four weeks following the onset of fever (5). An intense immune response is associated with the acute phase of KD, typically involving and causing damage to the vascular endothelium (58). The vasculitis that occurs early in disease progression is seen initially in the microvessels, with edema of the endothelial cells (36, 45, 59). In the early stages of KD neutrophils can be detected, which rapidly give way to mononuclear cells, lymphocytes and plasma cells (36). Following this infiltration, destruction of the internal elastic lamina occurs, involving all layers of the vessel wall.

Not every child who acquires Kawasaki disease will develop coronary artery aneurysms, as the incidence and degree of severity varies widely in this population. Approximately 25% of untreated patients will develop CAAs within two weeks following the onset of disease (20, (60). Even with standard therapy of high-dose IVIG combined with aspirin during the acute phase of Kawasaki disease, approximately 5% of affected children still develop coronary artery aneurysms (61). This incidence of CAA development further increases in affected children to 20-30% when vessel size is adjusted for body surface area (21). In some cases multiple aneurysms may form, however it is often the case that the proximal region in comparison to the distal region of both the right and left coronary arteries is involved in aneurysm formation (36, 62, 63).
1.3 Disease Etiology

Since the discovery of KD, numerous pathogens including various viruses and bacteria have been linked to patients with KD (Table 1), however no one agent has been specifically identified as the inciting agent (64, 65). Several factors associated with KD, including the predilection for children, epidemics occurring every two-three years, and the seasonal predominance in the winter and early spring months, are all suggestive of an infectious trigger for disease (66). Interestingly, a study done found that 33% of the patients diagnosed with KD also had concurrent infections of both bacterial and viral nature (42).

Although the etiology of the disease remains elusive, there is agreement in the literature that Kawasaki disease displays a strong immune activation during the acute phase (12). It is the mechanism of immune activation that is in debate, focusing on whether the etiologic agent is a single conventional antigen or whether it is from a group of pathogens that have superantigenic (SAg) activities (Section 1.5) (44, 41). Oligoclonal IgA antibodies have been isolated from some patients who suffered from KD, which can either provide evidence for the conventional antigen theory (67) or may confirm that KD has an infectious etiology and the possible route of entry of the pathogen is via the lungs or the gastrointestinal tract. However, a number of infectious pathogens containing superantigenic activity have been isolated from patients with KD, ranging from Cytomegalovirus to Staphylococcus aureus (50-53, 72-73), lending support to the superantigen theory of KD. Recently, a study completed by Suenaga et al. demonstrated that SAg genes could be detected in 70% of the stool samples tested from patients with KD (68). Also in support of the SAg theory is the observation of increased expression of certain Vβ+ T cells in patients with KD (69).
<table>
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Table 1: Various infectious agents implicated in KD

Although the topic of etiology in KD remains controversial, a hypothesis suggests cooperation between the different mechanisms that results in a final common immune-activated pathway (18). Underlying my study is the hypothesis that Kawasaki disease may be initiated by a pathogen containing superantigenic activity, leading to massive activation of the immune system. A subset of pathogenic T cells are presented with a conventional peptide antigen of either self origin or foreign origin that may be a mimic of self, from an antigen-presenting cell. These T cells also receive costimulatory signals from an APC, which may be the endothelial cells themselves, therefore rescuing these T cells from apoptosis and allowing them to target the self-antigen tissue, being CA endothelium in KD (Figure 2).
Figure 2: Hypothesized Disease Model
1.4 Animal Models of Kawasaki Disease

Obtaining patient samples from KD children is very difficult ethically and practically, therefore to better understand the mechanisms that initiate the inflammatory response, which lead to vascular damage and aneurysm formation, the use of an animal model is required. There are currently various animal models being used to investigate KD, including rabbit, swine, canine, and murine models.

The rabbit model used to study Kawasaki disease relies on the infusion of horse serum to induce serum sickness in these animals (108). Upon injection, it was found that only weanling rabbits developed coronary artery aneurysms. Although vasculitis and myocarditis develop in this model, the inflammation is not severe nor specific to the coronary arteries, and both young and mature rabbits are affected.

A serum sickness swine model also exists, where piglets are injected with horse serum to induce vasculitis (109). The piglets develop skin rashes and coronary artery dilatations similar to children with KD, however vasculitis also develops in other systemic arteries including the ascending aorta, renal artery, iliac artery, femoral artery and subclavian artery.

The canine pain syndrome model, also known as juvenile polyarteritis syndrome (JPS), is a necrotizing vasculitis model used to study KD as there are pathologic, laboratory and clinical similarities between the two diseases (110). Although JPS develops spontaneously in the canines, it is characterized by a systemic vasculitis of the small and medium-sized vessels, targeting the coronary arteries and the meningeal arteries. The dogs also become febrile and are non-responsive to antibiotics.

Various murine models are also used to further understand KD. One murine model provided 4 intravenous injections of peptidoglycan isolated from the cell wall of Streptococcus pyogenes, which induced histological changes similar to what is seen in children with KD (111). Another model provided multiple intraperitoneal (ip) injections of an extract of Candida albicans, which was isolated from a patient with KD (112). These mice developed coronary arteritis, also with similar histopathological changes to children with KD, including edema and lymphocytes infiltrating the CAs. These murine models would be more representative of KD if multiple boost injections were not required for a sustained immune response.
1.4.1 *Lactobacillus casei* cell wall extract model of Kawasaki Disease

In 1985, another murine model of Kawasaki disease was discovered by Lehman *et al* when they provided mice with a single ip injection of cell wall components from *Lactobacillus casei* (*L. casei*), inducing coronary arteritis (113). *L. casei* is a gram positive bacterium that is typically considered to be non-pathogenic, however it is has been implicated in human infections (114). *L. casei* is a common bacterium found within the normal enteric flora of both humans and rodents (115). Interestingly, *L. casei* shares some common characteristics with group A *Streptococcus pyogenes*, which induces a rheumatic febrile-like illness in mice with the development of myocarditis and coronary arteritis in some cases (116). Both bacteria are resistant to degradation by lysozymes, which could be due to the fact that they both have cell walls rich in rhamnose (117).

In this murine model of disease, inbred mice receive a single ip injection of *Lactobacillus casei* cell wall extract (LCWE), which induces a vasculitis similar to children with KD. Within the animal model, there is a hierarchy of susceptible mouse strains. The DQ6 transgenic (Tg) mice possess human CD4+ T cells and human HLA-DQ6 but does not express murine CD4+ or CD8+ T cells. These mice reacted strongest in response to LCWE, suggesting that the human immune response is more sensitive than mice in responding to SAg. Furthermore, BALB/c mice elicited a stronger response to LCWE than C57BL/6 mice (118). The time course in mice for coronary arteritis development, histological changes, as well as response to IVIG therapy are all consistent with the corresponding disease development in children (113, 118-120), illustrating the therapeutic benefits for studying this model. Following administration of LCWE, a systemic self-limited vasculitis develops in the periphery, which then localizes to the coronary arteries. Once in the coronary arteries, a focal, asymmetric inflammatory lesion consisting primarily of lymphocytes develops, which invades all layers of the vessel wall (113). Upon inspection, Lehman *et al* did not observe any disease development in the spleen, liver or kidneys (120). Infiltrate can be observed invading the adventitia of the coronary arteries by days 3 to 7 post administration of LCWE, and maximizes at day 28 (113). By day 42 post injection, elastin breakdown of the vessel wall begins, indicative of aneurysm formation. This corresponds to the sub-acute phase of KD in children (121), as aneurysm development in children is most evident 4-8 weeks following the onset of disease. Also of interest is that only young mice are susceptible to LCWE-induced coronary arteritis, similarly to disease only developing in young children, as mice older than 6-weeks old do not develop disease (118). Therefore, as this model most closely
mimics KD in children, with respect to time course, age of susceptible mice, and histopathological changes of disease as well as only requiring one injection, this murine model has proved valuable to dissect KD pathogenesis.

Lehman et al injected LCWE into various mouse strains and determined that C57BL/6, A/J, and BALB/c mice were susceptible to coronary arteritis, whereas C3H/HeJ mice consistently failed to develop any myocarditis or coronary arteritis (113). Interestingly, C3H/HeJ mice do not have a fully functional innate immune system, as their macrophages are defective in responding to LPS and hence have poor production of TNFα and IL-1. From this observation Lehman et al hypothesized that the non-responsiveness to LPS by macrophages may indicate an important role for macrophages in disease development (120). However the non-responsiveness of C3H/HeJ mice to LPS was actually due to a mutation in the TLR4 gene (122), but TLR4 was later shown not to be necessary for disease as TLR4 knock out mice still developed coronary arteritis (123). Alternately, the more probable reason as to why C3H/HeJ mice do not develop coronary arteritis is because they delete their TCRVβ14+ T cells (an LCWE-reactive TCRVβ family) at birth due to the presence of an exogenous MMTV SAg (124). Further analyzing innate immune function in the LCWE model, Rosenkranz et al. tested TLR2- and MyD88-deficient mice for their ability to develop coronary arteritis (123). Wild type (WT) mice developed disease but both TLR2- and MyD88-deficient mice were not susceptible to disease, indicating the importance of TLR2 in the development of coronary arteritis. Coinciding with this observation, recent data from our laboratory has identified the presence of a TLR2 ligand present within LCWE (125), and that TLR2 upregulation of costimulatory molecules directly rescued SAg-activated T cells from apoptosis, lending further insight into the possible role TLR2 may play in the LCWE model.

In further dissecting the mechanisms leading to coronary arteritis in the LCWE model, our laboratory has identified superantigenic activity within the crude extract, as all of the characteristic SAg-mediated responses are present: immense activation of naïve T lymphocytes followed by clonal apoptosis of the Vβ-reactive families, the requirement for MHC II presentation but not Ag processing, non-classical MHC restriction, and Vβ-skewing of T cells. Following injection with LCWE, the specific Vβ families that are activated include Vβ-2, 4, 6, and 14 (118).

Following activation with a SAg there is a massive production of proinflammatory cytokines, which further contribute to disease pathogenesis. Two cytokines known to be involved in Kawasaki disease are IFNγ and TNFα. Following administration of LCWE, IFNγ was
maximally expressed in cardiac tissue 28 days post injection. Although expression of IFNγ corresponds with maximal infiltration of lymphocytes at the CAs, IFNγ is not required for induction of disease as IFNγ-deficient mice remain susceptible to coronary arteritis (126). TNFα follows the same expression pattern as IFNγ, being most highly expressed at the CAs 28 days post LCWE administration, however unlike IFNγ, TNFα is necessary for disease development as TNFR1-knock out mice do not develop coronary arteritis post LCWE injection (30).
1.5 Kawasaki Disease and Superantigens

KD is of an inflammatory nature with the occurrence of an intense immune reaction during the acute phase. As there is no specific diagnostic test, other inflammatory diseases may be mistaken for KD. There are numerous similarities between KD and Staphylococcal and Streptococcal toxic syndromes, as well as Scarlet fever (97, 127-130). There is also some clinical similarities between KD and Toxic shock syndrome (131). Although differences exist between these inflammatory diseases, there is speculation that the common factor initiating the immune reactions is a superantigen (72).

There are two general groupings for SAgs - foreign SAgs and self-SAgs (132). Foreign SAgs are soluble, intermediate-sized proteins that are secreted by a number of bacteria, including *Streptococcus* and *Staphylococcus*, with *Staphylococcus* enterotoxin B (SEB) being the best studied and prototypic bacterial SAg (97). Self-SAgs, including the minor lymphocyte stimulating (Mls) antigens are endogenous SAgs commonly found in mice (133). These endogenous SAgs are a product of the mouse mammary tumor viruses (MMTV), resulting from a germ line integration (133).

A conventional antigen undergoes processing by an APC, which then presents the peptide in the context of a MHC II molecule on its surface (127). This is a highly specific interaction because only the T cell bearing the specific Vβ region of the T cell receptor (TCR) will respond, thus only approximately one in a million T cells are activated via a conventional antigen (87). Superantigens elicit a much stronger response in comparison to a conventional antigen for a number of reasons. SAgs are mitogenic proteins that do not require antigen processing during APC presentation, and they are not classically restricted by self MHC molecules (118, 134-136). Due to their ability to bind outside of the peptide-binding groove and to the variable region of the TCR Vβ chain (41), SAgs are able to potently activate up to 30% of the T cell repertoire when compared to conventional antigens (118, 132, 134) (Figure 3). This specific TCR Vβ-activation leads to a typical TCR Vβ profile known as Vβ skewing, and is a hallmark of SAg activation (69, 137). SAgs also have the ability to activate naïve T cells to elicit a rapid and massive activation of the immune system, which includes the induction of a cytokine storm. Interestingly, a number of proinflammatory cytokines that are produced during this cytokine storm have been identified in the acute phase from KD patients (68, 69, 138), including TNFα, IFNγ, IL-1, IL-2, and IL-6 (30, 68, 126, 138). Of the cytokines involved in KD, TNFα is important as it is a potent inducer of inflammation, it has been shown to be necessary for the induction of coronary arteritis in the
murine model of disease (118), and it is thought to be important in the development of vascular endothelial damage during the acute phase of KD (69). Furthermore, KD patients who have been unresponsive to conventional treatments have received TNFα blocking agents as salvage therapy (139): administration of etanercept, a soluble TNF-receptor, completely abrogated coronary arteritis in mice (30). In support of a superantigen being involved in disease, numerous studies have reported on these previously mentioned ‘hallmarks’ being present in KD patients, namely Vβ-skewing in combination with massive production of proinflammatory cytokines (69, 127, 137, 140-142).

A conventional SAg-mediated immune response is typically followed by apoptosis of the SAg-reactive T cells post-activation (143-145). This effect of apoptosis following SAg activation of the T cells is best demonstrated in mice using a prototypic SAg. In mice, SEB specifically activates Vβ-3, -7, -8, and -17 families. Administration of SEB in mice induces an initial proliferation of Vβ specific cells, which are dominated by the Vβ-8 family as they make up approximately 20% of the T cell repertoire, which peak shortly after administration of the SAg and which are followed by clonal deletion of the SEB-reactive population (143, 146-148). Interestingly, this hallmark of SAg activation followed by clonal deletion can be seen in the LCWE animal model, as LCWE contains a SAg (Animal Models in section 1.4). LCWE was shown to preferentially expand T lymphocytes from TCR Vβ-2, -4, -6 and -14 families (118). To assess the fate of the T cells following LCWE exposure, human MHC class II (DQ6) transgenic mice, which express human CD4 and human HLA-DQ6 but not murine CD4 or CD8, were injected with LCWE and the percentages of splenic CD4+ T cells that expressed Vβ-2, -4 and -6 were monitored. Consistent with the SAg-pattern, the CD4+ TCR Vβ-6 reactive population expanded by day 3, and by day 5 post injection the population began decreasing in size to less than 1% by day 10. Thus the classical deletion pattern for SAg-reactive T cells is observed in vivo following LCWE administration.

Superantigen responses are characterized by this initial intense activation of the immune system as well as proliferation of specific Vβ+ T cells (118). The expansion of these specific Vβ+ T cells occurs over a short period of time, peaking approximately three days following the initial stimulation (149). This SAg-mediated immune response can last approximately between 10 to 14 days depending on the SAg involved (41), since as mentioned the Vβ+ reactive T cells undergo apoptosis shortly following SAg stimulation (148). This SAg-induced apoptosis results in lower circulating levels of Vβ+ T cells than before SAg activation (148-150). The clinical characteristics of KD correlate well with a SAg response and are just as dramatic, demonstrated
by the rapid onset of disease in combination with a prolonged fever, polymorphous rash and overall a very ill child (41).

**Figure 3: T cell activation by conventional antigens versus superantigens.** (A) Conventional antigens are processed and presented to the TCR in the context of an MHC molecule, and therefore only activate a small percentage of T cells (1x10⁴ to 1x10⁶). (B) SAgs do not require antigen processing and bind outside of the peptide groove to the variable region of the TCR and MHC molecule thus activating a larger percentage of T cells (approximately 1 in 3). Adapted from (41).
1.6 Immunological Characteristics of Kawasaki Disease

There are two main branches the immune system utilizes to defend a host from a pathogen – innate immunity, which is the host’s first line of defense, and adaptive immunity, which is a more specific immune response. The immune system depends on both branches to communicate effectively in order to identify foreign invaders and eliminate them. The immune response seen in the pathogenesis of KD is wide-ranging, and both branches of the immune system have been implicated to function cooperatively in KD, with APCs providing costimulatory signals to SAg-reactive T cells thus indicating a potential link for the innate and adaptive systems working together (12, 150).

Within the innate population of immune cells, monocytes/macrophages have been implicated in disease pathogenesis. Following activation, monocytes/macrophages secrete various factors including TNFα, IL-1, IL-6 and VEGF, all of which display elevated levels in Kawasaki patients (151-154). Importantly, during the acute stages of KD monocyte/macrophages increase in number (155), and some studies have demonstrated their infiltration into affected skin and cardiac tissue (156, 157). Furthermore, a chemokine responsible for recruiting monocytes to sites of inflammation and tissue injury, namely MCP-1, exhibits elevated levels in KD and has also been found to colocalize with mononuclear cells in cardiac tissue from patients who suffered from fatal KD (158). Dendritic cells belonging to the myeloid lineage have also been reported in coronary lesions in patients with KD (159).

Of the cells involved from the adaptive branch of the immune system, T cells have been shown to play a predominant role (Section 1.6.1). This is evidenced by the presence of infiltrating T cells that were found in heart tissue from patients who suffered from fatal KD. These infiltrating T cells were potentially activated, as it was found that some of the T cells expressed the IL-2 receptor (156). Notably, staining revealed that CD3+ T cells were found to be colocalized with dendritic cells at the CAs (159). Upon examination of skin biopsies from patients with acute KD, T cells were also identified, specifically belonging to the CD4+ lineage. Previous studies have also identified elevated levels of Vβ2 and Vβ8.1 T cells in KD patients when compared to healthy and febrile controls (69). This TCR Vβ-skewing pattern (refer to section 1.5) has also been observed in various other studies pertaining to KD patients (127, 140-142, 160).
1.6.1  Kawasaki Disease and T lymphocytes

KD has been suggested to be a T cell-mediated disease, as considerable evidence exists supporting the significant role T cells play in disease pathogenesis. Various studies have identified Vβ skewing of specific T cells in KD (69, 127, 137, 140-142), and have also identified infiltrating T cells within coronary arteries, specifically in coronary arterial lesions (156, 157). Furthermore, employing the use of animal models has provided very strong evidence for the dominant role T cells play in the development of coronary arteritis (118, 161). RAG-1/- mice do not possess T or B lymphocytes, and thus are a useful tool for analyzing the role the adaptive branch may play in disease. Following challenge with LCWE, RAG-1/- mice do not develop coronary arteritis, indicating the necessity of lymphocytes for disease (118, 161). To confirm that the lymphocytes involved were indeed T cells, Schulte et al. injected B cell null mice with LCWE and observed that majority of the mice (70%) developed coronary lesions (161), eliminating the possibility that B cells are required for disease. Lastly, they performed IHC staining for CD3+ T cells in LCWE-injected wildtype C57BL/6 mice and observed a significant increase of CD3+ T cells within proximity to aortic and coronary arterial lesions. All of these findings support the importance of T cells in the development of disease.

As T lymphocytes have such a prevalent role in disease progression, a study by Onouchi et al looked at genetic variations with respect to T cells. The group identified a functional single nucleotide polymorphism (SNP) in the inositol 1,4,5-triphosphate 3-kinase C (ITPKC) gene, which is significantly associated with disease susceptibility and an increased risk of developing coronary artery lesions (162). When the TCR complex is stimulated 1,4,5-triphosphate (IP3) is released, which leads to an increase in endoplasmic reticular Ca2+, and therefore a Ca2+ influx across the plasma membrane, allowing translocation of nuclear factor of activated T cells (NFAT) to the nucleus (Figure 4, section 1.6.1.1), initiating the transcription of IL-2, among other cytokines. This study identified ITPKC as a negative regulator of T cells, as over expression of ITPKC resulted in significant reduction in NFAT-mediated T cell activation. They hypothesized that this reduction in NFAT activity may be through the regulation of IP3 levels. It was also shown that the C allele of ITPKC reduces splicing efficiency, and therefore may contribute to the hyper-reactive T cell phenotype that is seen in KD (162).

Recently, other genes related to T cell activation and survival have subsequently been found to be associated with KD. Further investigation by Onouchi et al. studying Japanese and European-American children identified multiple variants in caspase-3 that were reported to be
significantly associated with KD (163). Caspase-3 belongs to a family of proteins that once activated, are responsible for executing the apoptotic pathway (Section 1.6.2.2). Activation of caspases occurs as a cascade that utilizes both initiator and effector enzymes, and once effector caspases, including caspase-3 have been activated they are responsible for majority of the downstream cleavage events that are seen during apoptosis. Further supporting the importance of T cells in KD, one of the commonly associated SNPs in caspase-3 that confers susceptibility to KD was found to affect the binding of NFAT, a transcription factor involved in T cell activation (163).

1.6.1.1 T cell signaling

When the TCR of a T cell is engaged by a conventional peptide-MHC complex, a cascade of events occur that culminates in T cell activation (Figure 4). Following ligation of MHC with the TCR, the intracellular domain of the TCR co-receptor (either CD4 or CD8) that is associated with the Src family tyrosine-kinases Lck or Fyn, induces phosphorylation of the ITAMs on the ζ chain of the CD3 molecule. Phosphorylated ITAMs can recruit ZAP-70, which is then itself phosphorylated and activated by Lck. The phosphorylation of ZAP-70 enables a number of signaling pathways to begin, which induce the translocation of NF-κB, NFAT and ERK1/2 transcription factors into the nucleus where they interact to drive cellular proliferation and differentiation (164).

Engagement of the SAg-MHC complex to activate T cells has been assumed to be similar to TCR signaling via conventional antigens (165); however, differences in the downstream signaling pathway have been observed (Figure 5). Although the tyrosine-kinase Lck is known to be important for signaling via conventional antigens, it has been suggested that SAgs can bypass the need for Lck-dependent TCR signaling by activating Gα11 proteins (a G protein). This leads to the activation of phospholipase C-β as well as protein kinase C, which initiate signaling cascades that culminate in the activation and translocation of NF-κB and NFAT (166, 167).
**Figure 4: T cell signaling following conventional antigen stimulation.** Ligation of MHC with the TCR induces a cascade that results in T cell activation, which begins with the T cell co-receptor (CD4 or CD8) associated Src-family kinase – Lck, phosphorylating the ITAMs of the CD3 complex of the TCR. The cytosolic tyrosine kinase ZAP-70 is recruited to the phosphorylated ITAM on the ζ chain of the CD3 complex, which is then subsequently phosphorylated by Lck. Activated ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76, which in turn leads to the recruitment of SOS (guanine-nucleotide exchange factor); SOS then leads to the activation of the MAP kinase pathway which activates ERK1/2. Alternately, activated LAT and SLP-76 lead to the membrane recruitment of PLCγ, which cleaves PIP₂ to produce DAG and IP₃. Following cleavage, IP₃ induces an increase in intracellular calcium ions that activate the phosphatase, calcineurin, which then leads to the activation of NFAT (transcription factor). The PKC pathway becomes activated by DAG along with the intracellular calcium ions, which induces activation of NF-κB (transcription factor). Together, ERK1/2, NFAT and NF-κB promote T cell differentiation and proliferation. Adapted from (164).
Figure 5: T cell signaling following SAg stimulation. Following activation of the TCR via a SAg, an alternate signaling pathway to the canonical pathway activated by a conventional Ag is seen. The conventional events of ZAP 70 and LAT phosphorylation by Lck are not seen in this pathway. Rather, heterotrimeric Gα11 proteins become activated by a currently unknown mechanism. Activation of the Gα11 proteins leads to the activation of PLCβ, which then induces an influx of calcium ions (Ca^{2+}), activation of PKC and ERK1/2, as well as activation of the transcription factors NFAT and NF-κB. Adapted from (166, 167).

1.6.2 Survival of SAg-activated T cells

1.6.2.1 Superantigen activation

Interactions between SAg and T cells in mice have been well documented based on studies of the prototypic SAg - Staphylococcal enterotoxin B (SEB) molecule, which is secreted from the gram positive bacterium Staphylococcus aureus. As previously mentioned, SEB induces activation of TCR Vβ3, 7, 8 and 17 families in mice that demonstrate specific kinetics,
of which have been determined following staining of one or more of these specific subsets; however SEB induces predominantly Vβ8+ T cells, thus a majority of studies focus on this family (143, 145). One study revealed that when BALB/c mice were challenged with SEB, the Vβ8+ T cells underwent expansion, with a five-fold increase in T cell numbers. This T cell expansion was short-lived and peaked within two days following injections. The massive proliferation of Vβ8+ T cells was sustained for four days, which was then followed by a dramatic decline in T cell numbers by day 7 post injection (144). This rapid expansion in T cell numbers accompanied by deletion is a typical hallmark of SAg activation (143-145, 149, 168). Due to the fact that numerous features of SEB and its effects are well documented in the literature, this SAg was used in this study.

1.6.2.2 T cell outcomes

Following T cell activation a T cell may undergo one of three different fates, which include anergy, apoptosis or survival. Of these various fates SAg stimulation induces T cells to undergo apoptosis or become anergic, while activation of T cells with a conventional Ag and the appropriate amount of costimulation promotes T cell survival.

Anergy is the state where a cell is non-responsive to an antigen, and is one mechanism the immune system utilizes to induce peripheral tolerance (164). Two methods that induce anergy in T cells include interaction with a low affinity ligand in the presence of costimulation, or a TCR receiving a strong signal in the absence of costimulation. Anergy can typically be observed in cells that have previously been activated. Under conditions where a cell has been restimulated, an anergic reaction is characterized by a diminished IL-2 and proliferative response (168, 169).

Apoptosis, also known as programmed cell death, occurs following a cascade of biochemical events inducing the cell to die. Cells undergoing apoptosis experience numerous morphological changes including a decrease in cellular size, nuclear condensation, DNA fragmentation, externalization of phosphatidyl serine (PS), and proteolytic cleavage of certain intracellular substrates (170). Two distinct signaling pathways exist are responsible for inducing apoptosis: the extrinsic and intrinsic pathways (Figure 6). The extrinsic pathway is induced in response to external death signals that function through death receptors of the TNFR superfamily, alongside other members including the death receptor, Fas (CD95). Upregulation of the Fas death receptor on activated T cells initiates the extrinsic pathway. The apoptotic cascade
is induced once Fas ligates with its ligand, Fas ligand (FasL), also located on activated T cells. This ligation leads to the trimerization of the Fas receptor (FasR), allowing for recruitment of downstream adaptor molecules, such as FADD. Effector enzymes, including caspase 8, are then recruited and subsequently activated, which in turn activate caspase 3. The catalysis of the caspase cascade leads to condensation of the nuclei and cell death.

The intrinsic pathway, which utilizes the mitochondria rather than death receptors, is induced when a cell receives a stress signal such as irradiation or withdrawal of growth factors. Upon stressing the cell, the mitochondria releases Cytochrome c, which in turn activates caspase 3, leading to cell death (171). Mitochondrial changes are mediated by the Bcl-2 family of molecules, which are comprised of both pro- and anti-apoptotic members (172). It is the balance of either pro- or anti-apoptotic factors that determines whether a cell will undergo apoptosis or survive. Pro-apoptotic Bcl-2 members, including Bim and Bid, induce Cytochrome c release from the mitochondrial inner membrane, while anti-apoptotic members such as Bcl-2 and Bcl-xL, work to prevent this event through the binding and neutralization of pro-apoptotic members (170, 173).
Figure 6: **Intrinsic and extrinsic pathways involved in apoptosis.** The extrinsic pathway is initiated when FasL binds with its death receptor, Fas, which activates procaspase 8. Following this activation, a cascade of events begins that culminates in the activation of procaspase 9 and then apoptosis. The intrinsic pathway is initiated when various stresses, including DNA or UV damage occur. Following the stress signal, Bim activates pro-apoptotic factors, including Bax and Bak and prevents activation of anti-apoptotic factors such as Bcl-2 and Bcl-XL. Pro-apoptotic factors (Bax/Bak) cause the mitochondria to be leaky, thus allowing the release of Cytochrome c. Following its release, Cytochrome c binds to Apaf-1 to form the Apoptosome, which then activates procaspase 9, leading to apoptosis. Also when released from the mitochondria, Smac/Diablo inhibit IAPs, which serve to negatively regulate caspases. Adapted from (170, 171).
Using SEB as the prototypic SAg, mechanisms of apoptosis following SAg stimulation can be studied; however whether a SAg induces the extrinsic or intrinsic pathway is controversial as evidence exists for both pathways following SAg stimulation. In analyzing the extrinsic pathway one study demonstrated the importance of Fas by stimulating Fas-deficient mice with SEB and reported that these mice demonstrated an impaired ability to induce apoptosis in T cells (174), suggesting that Fas is needed to induce apoptosis. Furthermore, in vitro studies reported that proliferating Vβ8+ T cells can undergo Fas-mediated apoptosis when presented by a Fas receptor cross-linking Ab (175). Conversely, SAg stimulation seems to induce apoptosis in Fas-TNFRI-TNFRII triple knock out mice (176), implying that Fas may not be required for apoptosis, and suggesting a role for the intrinsic pathway. For example, Vβ8+ T cells from mice overexpressing Bcl-2 have been shown to survive following SAg stimulation (176). Whether the extrinsic or intrinsic pathway is induced following SAg-activation is currently unclear. It has been suggested that differences in the initial stimulation due to differences in experimental design could explain why both pathways have been observed. This is demonstrated by experiments which used repeated stimulation and showed a role for Fas in apoptosis. Alternatively, experiments where a single dose of SAg was administered demonstrated Fas-independent apoptosis (176). Thus as evidence exists for the involvement of the extrinsic and intrinsic pathways, either or both pathways may be induced following SAg-stimulation.

1.6.2.3 The rescue phenomenon

In a typical SAg-mediated immune response there is a marked expansion of the SAg-reactive T cells, which are deleted shortly after stimulation. In KD as well as in the LCWE animal model, a population of pathogenic T cells seem to persist in the heart and induce damage to the coronary arteries, apparently contradicting the typical fate of a T cell following SAg activation. Interestingly, a study conducted by McCormack et al. demonstrated that when T cells were stimulated with both a peptide Ag for which the T cells were specific and a SAg, the number of SAg-reactive T cells being deleted was reduced. Furthermore, these investigators also reported that the dually responsive T cells proliferated in response to the specific Ag. From these observations, McCormack et al. concluded that providing an appropriate secondary signal in the form of costimulation to a SAg-reactive population can rescue them from apoptosis (147).

As the persistence of a population of T cells within the animal model following SAg stimulation is at odds with the typical fate of a SAg-mediated response, work conducted in our laboratory was aimed at characterizing the population of T cells localized in the heart following
administration of LCWE in mice. The data was surprising because the SAg-activated T cells were not deleted, and the T cells found within the heart were characterized as an oligoclonal T cell population belonging to the LCWE SAg-reactive Vβ6 family (T. Duong, unpublished observations). Furthermore, in accordance with the data demonstrated by McCormack, previous work from our laboratory has demonstrated that dual stimulation with LCWE (SAg) and a peptide from a microvascular endothelial cell line can rescue T cells from apoptosis. Specifically, a reduction in apoptosis was observed in the LCWE-reactive Vβ6 family of T cells, which was not observed in the non-reactive Vβ5 T cells. Also strengthening the evidence for endothelial involvement in the development of coronary arteritis, this cell survival appears to be specific to the endothelial peptide, as rescue was not observed when a smooth muscle cell line was used (125).

Evidence within the literature as well as from our laboratory seems to imply that the SAg-reactive T cells that persist in KD as well as in the animal model may be rescued from apoptosis (125, 177-179). According to McCormack et al., this rescue occurs when a T cell is stimulated with a SAg but also receives a secondary signal from a peptide antigen. As previously mentioned, optimal presentation of a peptide Ag to a T cell requires a professional APC that can also provide secondary costimulatory signals (refer to section 1.6.3). Professional APCs express high levels of costimulatory molecules, thus it has been suggested that costimulation may be one mechanism by which SAg-activated T cells can survive and lead to persistence, contributing to disease pathogenesis thereby (147, 177).

Vascular endothelial cells have been shown to express costimulatory ligands and can function as APCs, as has previously been mentioned (27, 31-33, 40, 180). In KD, the persistent inflammation is observed to target the CAs, and as an endothelial peptide has been suggested to ‘rescue’ SAg-stimulated T cells, it is possible for the vascular endothelial cells of the coronary arteries to serve as effective APCs and rescue the SAg-activated T cells in the LCWE model of coronary arteritis.

1.6.3 Optimal T cell activation and Co-stimulation

A T cell requires two distinct signals for proliferation and cytokine production to be activated, a process known as the ‘two signal theory’ (181). The first signal is mediated by the TCR and the second signal is provided by costimulation. CD28 is the best characterized prototypic T cell costimulatory molecule and a member of the immunoglobulin superfamily (182). It is expressed on more than 95% of human CD4+ T cells and approximately 50% of
human CD8+ T cells, and is constitutively expressed on almost all murine T cells, with slightly lower expression on murine CD8+ T cells (183). CD80 and CD86 are the natural ligands for the costimulatory receptor CD28. CD80 and CD86 are genetically and structurally related membrane-bound molecules that are expressed on APCs, and have also been shown to have largely overlapping functions (184, 185). CD86 is constitutively expressed on naïve APCs and its expression can be further upregulated following stimulation. In contrast, CD80 is not expressed on naïve APCs but its expression can be induced following stimulation, albeit with slower kinetics compared to CD86 (181). The importance of the CD28-CD80-CD86 costimulatory pathway has been demonstrated through numerous approaches, demonstrating that when CD28 is blocked from receiving a signal, Ag-specific T cell responses are inhibited and T cell-dependent immunoglobulin (IgG) secretion from B cells is impaired (182, 186).

Classical APCs, which are considered to be immune cells include macrophages and dendritic cells, however endothelial cells may also act as APCs. Endothelial cells serve various functions, of which many have been shown to be important for immunological events. As previously mentioned, during an inflammatory response ECs participate in recruiting leukocytes to the interstitium of the tissue via increased expression of adhesion and integrin molecules. In assessing their role as APCs, Seino et al. determined that HUVECs and MVECs constitutively expressed CD86 costimulatory molecules (40). Furthermore, IFNγ-treated HUVEC and MVEC endothelial cells were able to stimulate proliferation of isolated CD4+ T cells. Hancock et al. also demonstrated that cardiac allograft endothelial cells expressed CD86 and CD80 costimulatory ligands, although with differing kinetics (187).

The upregulation of costimulatory molecules, including the CD28 ligands CD80 and CD86, can be induced by multiple stimuli including pro-inflammatory cytokines such as TNFα (184, 188), and various pathogen-associated molecular patterns (PAMPs). TNFα is an important cytokine in the LCWE model, for when TNFα signaling is absent, mice do not develop disease (30). TNFα induces NF-κB signaling, which leads to a plethora of effects, including activation of vascular endothelium (19, 189), upregulation of CD80 and CD86 molecules (184) and TLR2 (190), clearly depicting its instrumental role in disease pathogenesis. PAMPs are molecules responsible for stimulating innate pathogen recognition receptors, including the family of toll-like receptors (refer to section 1.7). Specifically, TLR2 stimulation is capable of upregulating both CD80 and CD86 expression on murine splenocytes (125) (refer to section 1.8). The rescue phenomenon of SAg-reactive T cells via enhanced costimulation can be demonstrated with the use of the prototypic SAg, SEB. SAg activation of splenocytes with SEB results in the activation
of Vβ8+ T cells, which typically undergo apoptosis following stimulation; however when these splenocytes are co-cultured with SEB and αCD28 stimulatory antibody, rescue occurs indicated by the reduction in apoptosis of the reactive Vβ8+ T cells (177). Interestingly, these data can be recapitulated as demonstrated by a proof-of-principle experiment that used SEB and a TLR2 agonist, Pam3Cys showing that TLR2 signaling upregulated costimulatory ligand expression and a reduction in the SAg-reactive T cell population was observed (125). Therefore, interactions with enhanced costimulatory molecules allows for persistent survival such that these pathogenic T cells may take part in an autoimmune reaction targeting the coronary arteries in KD.

1.6.3.1 KD and Costimulation

Optimal T cell activation requires TCR engagement and a second costimulatory signal. It has been shown in the LCWE model that enhanced costimulation can rescue SAg-reactive T cells from undergoing apoptosis (177). It is noteworthy that costimulatory molecules, which provide the second signal involved in regulating optimal T cell activation, have arisen as candidate genes that may confer susceptibility to Kawasaki disease as well as predisposition to poor coronary outcomes. Costimulatory molecules, including B7, CD40L and 4-1BB, have also been shown to play crucial roles in the LCWE-induced coronary arteritis model (125, 177-179). Polymorphisms associated with the programmed death-1 (PD-1) gene, a negative regulator of T cell activation, have been shown to be associated with KD as its dysregulation may be involved in a persistent inflammatory response following an infectious trigger (191). Alternately, CD40 ligand (CD40L), a costimulatory molecule involved in T cell activation, has been correlated with CA lesion development in KD, and notably CD40L expression was found to be upregulated on CD4+ T cells from KD patients (192-194). Interestingly, CD40L has also been reported to be indirectly involved in enhancing the survival of SAg-reactive T cells in the murine model of disease (179). Both PD-1 and CD40L belong to the TNF superfamily of costimulatory molecules, which include the B7 ligands that have been shown to be important in the persistence of T cells in the pathogenesis of KD in the animal model. 4-1BB is another costimulatory molecule that also belongs to the TNF superfamily, with similar roles to CD28 including enhancing IL-2 secretion and T cell proliferation (195). Studies performed in mice using agonistic antibodies against 4-1BB have been shown to rescue SAg-reactive T cells from apoptosis (196). Furthermore, recent data demonstrated that agonistic anti-41BB Abs exacerbate disease in the LCWE model of coronary arteritis in mice (177, 178), and it is interesting to note that 4-1BB induces downstream signalling through TRAF1, which has been strongly implicated
as a gene conferring risk for autoimmunity (197, 198). Thus in providing the second signal to T cells for full activation, costimulatory molecules may also play an important role in perpetuating disease by leading to enhanced survival of SAg-reactive T cells.

1.6.4 **Kawasaki Disease and Innate Immunity**

As KD is believed to be caused by an infectious trigger, some groups have focused on the innate immune system and potential factors that may be contributing to disease pathogenesis. Mannose-binding lectin (MBL) is an innate immune receptor responsible for protection against invading pathogens (199). Children who suffer from KD have increased polymorphisms in the MBL-gene as well as lowered serum concentrations of MBLs, therefore predisposing these children to increased viral and bacterial infections (199). CD14 is a co-receptor for the pathogen recognition receptor (PRR) TLR2, and a CD14 polymorphism has been associated with the development of coronary artery lesions and coronary outcome in Kawasaki disease (200). Notably, an elevation in CD14+ neutrophils, monocytes/macrophages and soluble CD14 has been found in KD, and may contribute to the amplified inflammatory response seen in this disease (200). As previously mentioned, TLR2 signaling can induce upregulation of CD80 and CD86 costimulatory ligands, which play a role in the survival of pathogenic T cells in the animal model (Section 1.7.2 TLR2 signaling). More importantly, a study by Rosenkranz et al. discovered that mice deficient for the TLR2 receptor were protected from developing coronary arteritis following challenge with LCWE, indicating that TLR2 plays an important role in disease pathogenesis in mice (123).

As both TLR2 and costimulatory molecules have been shown to have pertinent roles in the disease model, it is important to understand how they work together within the disease model and how they contribute to the rescue phenomenon. TLR2 signaling can lead to enhanced costimulatory molecule expression on APCs, which when presented to SAg-reactive T cells can enhance their survival, an important concept which links both the innate and adaptive immune responses. Thus the link between TLR2 signaling and enhanced costimulation will be further assessed in order to elucidate the mechanism involved in rescue.
1.7 Toll Like Receptors

The host’s first line of defense against the invasion of a pathogen is to activate the innate immune system, thereby stimulating immune cells such as dendritic cells (DCs) and macrophages to remove the pathogen from the host. The innate immune system uses germ-line encoded pattern-recognition receptors, which are capable of recognizing a plethora of pathogens (201). TLRs are a type of PRR that recognize microbial elements known as pathogen-associated molecular patterns (PAMPs). In Drosophila, the Toll gene was initially identified as being essential in embryonic development, and was later shown to have a critical role in anti-fungal responses (202). Toll-like receptors are the human homologue of Toll, and have been shown to be central for inducing the innate immune response (202). Currently, 11 human and 13 mouse TLRs have been identified and cloned (203).

Upon TLR activation, several mechanisms are initiated that allow for regulation of the innate immune response, as well as stimulation of the adaptive immune system. One such mechanism includes production of proinflammatory cytokines, including IL-1, IL-6, IL-8, chemokines and antimicrobial elements (202). TLR stimulation also leads to the maturation of APCs, which can either phagocytose the invading pathogen or alternately stimulate a T cell response through the presentation of the processed pathogen as a peptide in the context of an MHC II molecule. Although TLRs are mainly associated with the innate immune system, their activation is important for sustaining the adaptive immune response as they can induce the expression of costimulatory molecules such as CD80 and CD86 on APCs (201, 203), and more recently TLRs have been shown to directly activate T cells, also allowing for a persistent adaptive immune response (204-208).

Expression of TLRs can be found on both immune and non-immune cells, including dendritic cells, macrophages, activated T cells, B cells, and endothelial cells (201). Although TLRs are expressed on a variety of tissue types, their expression levels are not constant and TLR expression can be further induced via cytokines, in response to pathogens and due to environmental stresses (201). TLRs can further be divided into groups based on their cellular location and which type of PAMPs they recognize (Table 2): TLRs 1, 2, 4, 5, and 6 are expressed on the extracellular surface, while TLRs 3, 7, 8, and 9 are expressed in intracellular compartments (201) (Figure 7). TLRs 3, 7, 8 and 9 are known as the antiviral TLRs and recognize nucleic acids. TLRs 1, 2, 4, and 6 recognize lipopeptides, with TLR4 recognizing LPS (201, 203). Evidence is also building to indicate that some TLRs, specifically TLR2 and TLR4,
are capable of recognizing host-derived molecules or ‘danger signals’, such as heat shock protein (HSP) 60 (203, 209).

TLRs belong to the type I integral membrane family of glycoproteins (210). The extracellular domain of TLRs possess various numbers of leucine-rich-repeat (LRR) motifs, while their cytoplasmic domain, termed the Toll/IL-1 receptor homology (TIR) domain due to the homology to the IL-1 receptor, is responsible for cell signaling (210). This homology allows for TLRs to activate similar signaling pathways used by the IL-1R, by recruiting adaptor molecules that posses TIR domains (211).

Figure 7: Toll like receptor compartments. Adapted from (164).
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<td>Fibrinogen</td>
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**Table 2: Toll like receptors and their ligands.** Adapted from (201).
1.7.1 **Toll-like receptor Signaling Pathways**

Upon engagement of TLRs with their ligand, signaling cascades are initiated, which begin with dimerization of the receptor. TLR dimerization allows for the receptor to undergo conformational changes, allowing for the recruitment of TIR-domain-containing adaptor molecules to the TLR’s cytoplasmic domain (201). There are a total of four adaptor molecules, which all contain TIR domains: myeloid differentiation factor 88 (MyD88), TIR-associated protein (TIRAP)/MyD88-adaptor-like (MAL), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF)/TIR-domain-containing molecule 1(TICAM 1), and TRIF-related adaptor molecule (TRAM) (201, 203). The activation of a signaling pathway following TLR stimulation is largely dependent on which adaptor molecule is used. Two main signaling pathways exist for TLRs – the MyD88-dependent pathway, and the MyD88-independent pathway. All TLRs except TLR3 are capable of signaling via the MyD88-dependent pathway, whereas TLR4 can signal through both pathways (203).

1.7.2 **MyD88-dependent and Independent Pathways**

Upon TLR-ligand binding, the receptors dimerize, which recruits MyD88 to the cytoplasmic domain; both TLR2 and TLR4 require the presence of adaptor molecules TIRAP/MAL in order to signal MyD88 (Figure 8) (212). Association with MyD88 then recruits IL-1R-associated kinase 4 (IRAK-4) and IRAK-1 to this complex. Activated IRAK-4 phosphorylates IRAK-1, allowing for association with TNFR-associated factor 6 (TRAF-6), a protein ubiquitin ligase (E3) (213). TRAF6 then catalyzes the ubiquitination of itself as well as of inhibitor of NF-κB kinase (IKK)-γ/ nuclear factor-kappa B (NF-κB)-essential modulator (NEMO) complex, which consists of IKK-γ, IKK-α and IKK-β (214). A complex is then recruited to TRAF-6, composed of TGF-β-activated kinase 1 (TAK1) and its associated binding proteins, TAB1, TAB2, and TAB3, which phosphorylates IKK-β as well as MAP kinase kinase (MKK)-6. This allows for the activation of MAP kinase pathways, but also of NF-κB, which translocates to the nucleus and induces the transcription of numerous proinflammatory cytokines and chemokines (215). This activated TLR pathway also leads to the activation of the transcription factor, interferon regulatory factor (IRF)-5. Upon nuclear translocation, IRF-5 binds IFN-stimulated response elements (ISRE), inducing the expression of various cytokine genes (216).
Figure 8: TLR2 Signaling Pathways. Adapted from (201).
1.8 Toll Like Receptor-2

Amongst all of the TLRs, TLR2 is unique as it heterodimerizes with either TLR1 or TLR6, and does not form a homodimer with itself. For this reason, it is considered to be the most promiscuous of the TLRs as it is capable of recognizing a vast array of ligands, including various lipoproteins, glycoproteins, glycolipids and polysaccharides (217). Specifically, the TLR2/1 heterodimer recognizes triacylated lipopeptides, and the TLR2/6 heterodimer recognizes diacylated lipopeptides (Table 3). TLR2 is also capable of recognizing components from gram-positive bacteria (201). TLR2 plays an important role in defending the host from these pathogens, as a polymorphism in the human TLR2 gene has been associated with a decreased response to various bacterial lipoproteins and septic shock following a gram-positive bacterial infection (218). Interestingly, the extract used to induce disease in our animal model – LCWE – originates from a gram positive bacterium, and we have demonstrated that it possesses a TLR2 ligand. TLR2 associates with various co-receptors, which are thought to amplify TLR2 signaling. CD14 can associate with both TLR2 heterodimers, TLR2/1 and TLR2/6; however CD36 only associates with the TLR2/6 heterodimer (219, 220).

Currently, there is debate focusing on the legitimacy of specific TLR2 ligands as the field is evolving and it is suggested that certain preparations possessed contaminants that were actually responsible for the TLR2 signaling. Two examples include peptidoglycan (PG) and an endogenous lignad, HSP60. Initially, PG was thought to induce TLR2 signaling; however it has been demonstrated that the preparations were contaminated as the signaling was actually due to the presence of TLR2 ligands including LTA or other lipoproteins from the cell walls of gram-positive or negative bacteria, as purified PG does not elicit a TLR2 response (221). Recently, it has been proposed that host-derived molecules may serve as endogenous ligands for TLR2. These molecules, also known as damage-associated-molecular patterns (DAMPs), are considered to be mediators of inflammation as they are released upon or in response to tissue damage and are thought to be involved in positive feedback loops initiating more inflammation (222, 223). One of these DAMPs includes the molecular chaperone protein, HSP60 (see below) (209), however there is conflicting data surrounding the preparation and downstream signaling of this molecule.
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**Table 3: Toll like receptor 2 ligands.** Adapted from (201).

Within the TLR family, both TLR2 and TLR4 stand out as TLRs that are typically associated with the pathogenesis of cardiovascular disease (203), as it has been shown that both of these receptors can be found on cardiovascular tissues including endothelial and smooth muscle cells (203, 224, 225). Currently, HSPs have emerged as potential proteins involved in the development of cardiovascular disease as altered hemodynamic conditions similar to those in atherosclerosis-damaged vessels can induce the production of HSPs (226, 227). Interestingly, microbial pathogens, such as *Chlamydia pneumoniae*, has not only SAg and TLR2 activity, but can also induce the production of bacterial and endogenous HSPs (71-74). A study performed by Kol *et al* demonstrated that hHSP60 could activate both human endothelial and smooth muscle cells, both of which express TLR2, and therefore may provide a potential link for vascular disease and HSPs (228).

As it has been proposed that HSP60 may serve as an endogenous ligand for TLR2, de Graaf *et al*. investigated the ability of hHSP60 to induce proliferation of vascular smooth muscle cells via TLR2 (229). They stimulated TLR2-transfected HEK cells with 20ug/mL of hHSP60 for 48 hours and observed an approximate 2-fold increase in total cell number when compared to
the non-transfected cells. Furthermore, when VSMCs were incubated with an anti-TLR2 antibody prior to HSP60 stimulation, the VSMC proliferation otherwise seen without the Ab was attenuated (229).

Contrary to the past literature, recent findings have indicated that in fact, HSPs may not be immunostimulatory, but that all reported effects are actually due to the molecules chaperoned by HSPs (230). In order for HSPs to function as chaperones for folding proteins, their binding sites are of a broad specificity. The binding of HSP to a protein is facilitated by hydrophobic interactions (231, 232), therefore it could be argued that an HSP could bind to a non-protein molecule if it were to have an exposed hydrophobic region, including lipid-based TLR ligands such as LPS (233). The controversy in the literature evolves around the question of whether or not the HSP preparation being used is truly purified, or whether the activity induced is due to a contaminant, as it is curious that HSPs of various molecular masses are reported to share similar cytokine effects. It is also interesting to note that the reported HSP effects are quite similar to the effects seen downstream from PAMP stimulation, including bacterial lipopeptides and LPS (230, 234). Studies performed by Wallin et al. demonstrated that it was only when HSPs were contaminated with LPS did they have any stimulatory abilities, and that highly purified HSPs did not induce any inflammatory cytokine effects (235). Interestingly, studies performed by Habich et al. illustrated that HSP60 is capable of tightly binding to LPS, and in doing so can induce the observed cytokine effects reported from HSP60 preparations, and that HSP60 itself does not induce the production of inflammatory cytokines (233). Furthermore, they reported that when HSP60 bound to LPS, the observed cytokine effect was more potent that when LPS was used alone. Thus due to the failure to use highly purified preparations of HSP60, and to recognize LPS as a potential contaminant or to consider other contaminants, HSP60 does not appear to have the reported stimulatory effects, which are most likely due to chaperoned contaminants.

1.8.1 Differential Expression of TLR2

Various immune cells express TLR2, including monocytes, macrophages and DCs, as well as naïve B cells and activated T cells (236, 237). Other non-immune cell types are also capable of expressing TLR2, as differential expression of TLR2 has been reported on endothelial cells. Various endothelial cell lines have been shown to express TLR2 and studies conducted by Erridge et al. have demonstrated that a human coronary artery endothelial cell line (HCAEC) expresses abundant amounts of TLR2 in comparison to other EC lines (238). This group stimulated HCAECs and HUVECs with Pam3CSK4 – a synthetic TLR2 peptide – and
demonstrated that HCAECs were responsive to Pam while the HUVECs were not. Following stimulation, the HCAECs demonstrated increased expression of E-selectin, as well as increased adhesion to monocytic cells (238). This group also demonstrated that while both HUVEC and HCAEC cell lines expressed TLR4 at the message and protein level, only HCAECs expressed TLR2. The TLR2 present on this cell line was proven to be functional as blocking TLR2 on HCAECs blunted the response to stimulation with non-enterobacterial LPSs as well as the Pam peptide, demonstrated by a statistically significant reduction in E-selectin expression (238). A study conducted by Chen et al. further investigated the expression of TLR2 on endothelial cell lines (239). They analyzed murine aortic endothelial cells (MAECs) and determined that the MAECs expressed robust TLR2 mRNA, which was responsive to TLR2 stimulation as marked by secretion of IL-6 and MCP-1 (239). This group also analyzed human aortic endothelial cells (HAECs), human pulmonary artery endothelial cells (HPAECs), and HUVECs for TLR2 expression and discovered that robust TLR2 protein could be detected intracellularly, but that MAECs expressed TLR2 on the cell surface (239). They were also able to detect TLR1 and TLR6 mRNA and protein in the human EC lines, and determined that intracellular TLR2 could translocate to the cell surface following activation with IFNγ (239). Interestingly, another study conducted by Dunzendorfer et al. showed that resting HCAECs expressed surface TLR2 protein, and that following stimulation with either LPS-IFNγ or TNFα-IFNγ 92-98% of HCAECs stained positive for cell surface TLR2 (240). It can be concluded from the literature that stimulation with various proinflammatory cytokines can induce externalization of TLR2 on the surface of endothelial cells, and that cell-surface TLR2 is functional. Thus TLR2 on the surface of coronary arterial endothelial cells may play an important role in the initial inflammatory stages, with stimulation further inducing endothelial TLR2 expression and increasing the probability for inflammation to be targeted to the CAs.

Expression of TLR2 significantly increases following vascular injury (224), and after ischemia/reperfusion injury it was shown that endogenous TLR2 within the myocardium exacerbated coronary endothelial dysfunction. Arterial curvature, branch points – such as the origins of the coronary arteries - and bifurcations induce alterations in blood flow, including increased turbulence (224, 241). An increase in turbulent flow at these specific arterial locations has been associated with adhesion molecule expression, endothelial activation and increased monocyte migration (242). It is also believed that development of lesions in various arteries can be defined by the shear stress of blood flow. Another study conducted by Dunzendorfer et al. looked at the responsiveness of HCAECs to TLR2 agonists in an in vitro model of chronic
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laminar versus disturbed blood flow (243). HCAECs were exposed to either laminar or disturbed flow conditions and TLR2 expression was determined by fluorescence microscopy. They discovered that under laminar flow conditions, TLR2 protein expression was much less and the HCAECs were hyporesponsive to TLR2 stimulation; however under conditions of disturbed blood flow TLR2 protein was detected in much higher amounts and could be enhanced in response to TNFα stimulation (243). It has previously been mentioned that the coronary arteries are the most common site for aneurysm development in children with KD. Interestingly, the coronary arteries represent branch points and thus experience disturbed blood flow. Therefore, as the CAs are branch points that experience turbulent flow, TLR2 expression may be increased and thus lead to enhanced downstream inflammation, making the CAs more susceptible for aneurysm formation.

1.8.2 Regulation of TLR2

Regulation of TLR2 expression can occur at both the transcriptional level, as well as at the cell surface level, and various factors have been shown to upregulate TLR2. Currently, there is little information available regarding the regulation of TLR2 at the protein level, and alternate mechanisms asides from transcriptional regulation of TLR2 have been suggested that may contribute to TLR2 protein expression, such as externalization of pre-formed TLR2 as well as protein shedding (244). Within the literature, evidence indicates that TLR2 protein can be detected on the cell surface of human monocytes (244). A study conducted by Flo et al. however demonstrated that this expression was not greatly modified following stimulation, indicating that cell surface TLR2 may be regulated at the transcriptional level in human monocytes (244). The murine TLR2 gene contains one cAMP response element-binding protein, one STAT consensus sequence, two CCAAT/enhancer binding proteins (C/EBPs), and 2 NF-κB consensus sequences located within the 5’-upstream region, indicating the importance of TLR2 regulation by inflammatory molecules (190). Various immunological factors are responsible for regulating each of these transcription factors, implying their importance in inflammatory responses. It has been shown that TCR ligation induces a rapid accumulation of cAMP. Furthermore, pharmacologic agents including E-type prostaglandins (PGEs) and inflammatory mediators such as histamine have been shown to induce cAMP levels (245). Cytokines have numerous roles in controlling immune responses, and various cytokines can induce the activation of STAT transcription factors, including IL-6 and IFNγ, both of which are involved in KD and the animal model. Certain viruses, including EBV have also been noted to induce STAT signaling, and
indirectly TNFα, a major pro-inflammatory cytokine involved in KD, can induce STAT activation as there is cross-talk between the NF-κB and STAT signaling pathways (246). Proinflammatory factors, including LPS, IL-1, IL-6 and TNFα, the latter of which are involved in disease pathogenesis, can modulate CCAAT/enhancer binding proteins (C/EBPs), factors that are involved in perpetuating the immune response in KD and therefore indicates their strong role in mediating the inflammatory response (247). The NF-κB transcription factor is activated in response to a vast range of stimuli, including pathogens (which are sensed by TLRs), stress signals, proinflammatory cytokines including IL-1 and TNFα, as well as by the combined effect of TCR ligation and costimulation via CD28 (248). TLR2 signaling can also activate NF-κB, which leads to the maturation of APCs via upregulation of costimulatory molecules, and triggers the release of proinflammatory cytokines, including TNFα.

It has been demonstrated that the 2 NF-κB sites within the TLR2 gene are essential for a transcriptional response by TNFα as deletion of both sites completely abrogated any response by this cytokine (190). TNFα is a key cytokine necessary for the development of inflammatory damage seen within the LCWE model (30), and it has been shown to activate endothelial cells and increase cell surface expression of TLR2, therefore increasing the responsiveness of ECs to TLR2 ligands (249, 250).

1.8.3 TLR2 and Superantigens

Bacterial SAgs are gram-positive exotoxins, with the majority being secreted by *Staphylococcus aureus* and *Streptococcus pyogenes*. SAgs are involved in the pathogenesis of various diseases, including septic shock and have been implicated in KD (251). In the murine model of disease, LCWE possesses a SAg in conjunction with a TLR2 ligand, and as TLR2 signaling can upregulate CD80/86 molecules and is suggested to be preferentially expressed at the CA endothelium, TLR2 has a pivotal role in the enhanced survival of the SAg-activated T cells. Within this model, TLR2 ligand on its own is not sufficient to induce coronary arteritis, however the synergistic activities of both a SAg and TLR2 ligand can induce disease (252). This need for both SAg and TLR2 activity is further demonstrated by a study conducted by Kearney *et al.* who demonstrated that the TLR2 ligand, bacterial lipoprotein (BLP) works synergistically with SAg to enhance the inflammatory response. In vitro data revealed that when PBMCs were primed with SEB then treated with BLP, there was a significant increase in the amount of TNFα and IL-6 released when compared to PBMCs that were primed with SEB alone. They also found that when mice were treated with SAg in combination with BLP in vivo, 100% mortality was
observed within 24 hours (251). BLP is the most abundant protein in the outer membrane of both Gram-positive and Gram-negative bacteria, and as TLR2 is able to recognize various other components from bacteria, viruses and fungi, it is possible that TLR2 may have a direct role in modulating KD for numerous pathogens have been isolated from patients that contained both a SAg as well as TLR2 ligand (Table 4).

<table>
<thead>
<tr>
<th>PATHOGEN:</th>
<th>SAg ACTIVITY:</th>
<th>TLR2 ACTIVITY:</th>
<th>REFERENCES:</th>
</tr>
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<tbody>
<tr>
<td>Human Coronavirus</td>
<td>Unknown</td>
<td>Unknown</td>
<td>(70)</td>
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<tr>
<td>Chlamydia pneumoniae</td>
<td>Yes</td>
<td>Yes</td>
<td>(71-74)</td>
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<td>Cytomegalovirus</td>
<td>Yes</td>
<td>Yes</td>
<td>(75-78)</td>
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<tr>
<td>Epstein-Barr virus</td>
<td>Yes</td>
<td>Yes</td>
<td>(50)(79-81)</td>
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<tr>
<td>Heat shock protein 60</td>
<td>Unknown</td>
<td>Potentially</td>
<td></td>
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<tr>
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<td>Yes</td>
<td>(82-84)</td>
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<tr>
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<td>Yes</td>
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</tr>
<tr>
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<tr>
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<td>Unknown</td>
<td>Unknown</td>
<td>(91, 92)</td>
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<tr>
<td>Propionibacterium acnes</td>
<td>Yes</td>
<td>Yes</td>
<td>(93, 94)</td>
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<td>Rickettsia-like agent</td>
<td>Unknown</td>
<td>Yes</td>
<td>(95, 96)</td>
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<tr>
<td>Staphylococcus aureus</td>
<td>Yes</td>
<td>Yes</td>
<td>(97, 98)</td>
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<tr>
<td>Streptococcus pyogenes</td>
<td>Yes</td>
<td>Yes</td>
<td>(97, 99-101)</td>
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<tr>
<td>Streptococcus sanguis</td>
<td>Yes</td>
<td>Yes</td>
<td>(99, 102, 103)</td>
</tr>
<tr>
<td>Yersinia pseudotuberculosis</td>
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<td>Yes</td>
<td>(97, 104-107)</td>
</tr>
</tbody>
</table>

Table 4: Various infectious agents implicated in Kawasaki Disease.

1.8.4 TLR2 and costimulation

It is believed that TLRs can influence Ag specific responses in a number of ways, among which is regulating costimulatory ligand expression (253). Through this process, it is thought that activation of TLRs can couple activation of both the innate and adaptive immune response. As it has previously been mentioned, ligation of TLR2 with either of its co-receptors, TLR1 or TLR6, leads to activation of NF-κB, which translocates to the nucleus and binds to DNA inducing the upregulation of CD80 and CD86 costimulatory molecules, as well as the production of inflammatory cytokines including TNFα, IL-12 and IL-6 (254). One of the defining characteristics of a professional APC is the ability to express costimulatory molecules, such as
CD80 and CD86, and as previously mentioned, various studies have shown that vascular endothelial cells, which express TLR2, can serve as functional APCs (27, 31-33, 40).

Previous work conducted in our laboratory has demonstrated that SAg-activated splenocytes can be rescued from apoptosis, which is due to enhanced costimulation (125). We have also shown that TLR2 stimulation as well as LCWE administration can directly upregulate CD80 and CD86 expression on splenocytes, and that similar to the experiments utilizing SEB and anti-CD28, simultaneous addition of SEB and Pam to cultures can also rescue SAg-reactive splenocytes from apoptosis (125). When splenocytes were stimulated with SEB and Pam but costimulation was blocked using the fusion protein CTLA4-Ig, which blocks CD28 interactions with either CD80 or CD86, cell viability decreased, demonstrating that TLR2-stimulated expression of costimulatory molecules played a role in rescuing these cells. It has also been shown that when splenocytes from either TLR2-/− or WT mice are cultured with LCWE, and apoptosis is measured by annexin-V staining, that in the absence of TLR2 there was increased cell death compared to the WT cells (K. Little, unpublished data). As TLR2 can enhance costimulatory molecule expression, and it has been shown that vascular endothelial cells can express TLR2 and costimulatory molecules, TLR2 as well as the endothelium may play important roles in the murine model of disease, which will further be explored in this thesis.
2 Rationale

KD is a childhood vasculitis characterized by localized and persistent inflammation at the coronary arteries. In KD and in the LCWE animal model, superantigens have been implicated in disease initiation. Typically, SAg-reactive T cells undergo massive expansion followed by subsequent deletion; however these SAg-activated T cells persist and target the CAs in KD and the animal model. Enhanced costimulation via CD80/86 ligand expression can rescue SAg-reactive T cells from apoptosis. TLR2 is an important mediator in this model as a TLR2 ligand is present in the LCWE extract that can upregulate CD80/86 molecules, further enhancing costimulation, and TLR2-deficient mice are protected from disease. TLR2 is also preferentially expressed in the coronary artery, where it may serve to transform the CA endothelium into an APC capable of presenting enhanced costimulation to SAg-reactive T cells.

3 Hypothesis

TLR2 expression in the vasculature regulates local inflammation in a mouse model of KD.

4 Project Objectives

This project consisted of two main objectives:

1) To delineate the various factors that affect the expression of TLR2 in a mouse model of KD. We will aim to:
   - Determine expression of TLR2 during disease progression
   - Determine if expression of TLR2 is age-dependent
   - Examine if there is differential expression of TLR2 in various tissues
   - To study the contribution of potential endogenous TLR2 ligands in disease pathogenesis, specifically the role of HSP60

2) To determine the functional relevance of TLR2 on endothelial cells in a mouse model of KD. We will aim to:
   - Determine if endothelial cells can serve as APCs, so as to elucidate whether the coronary artery endothelium can serve as a functional APC for the pathogenic T cells in our model
5 Materials and Methods

5.1 Experimental Mice

C57BL/6 wildtype (WT) mice were purchased from the Toronto Centre for Phenogenomics (TCP). TLR2 knock out (KO) mice were ordered from Jackson Laboratory (Bar Harbor, ME). The animals were housed at TCP under specific pathogen-free conditions. All animal procedures were in accordance with and approved by the Animal Care Committee at the Toronto Centre for Phenogenomics.

5.2 LCWE Production

LCWE was prepared as described by Lehman et al (113). Lactobacillus casei (ATCC 11578), which was obtained from the American Type Culture Collection (Rockville, MD) was cultured in Lactobacillus MRS broth (Difco, Detroit, MI) on a shaking platform for approximately 18 hours at 37°C. Cultures were harvested while still in the log phase of growth by centrifugation (10,000 RPM x 40 minutes) and washed repeatedly in PBS (pH 7.2). Bacteria were lysed by an overnight incubation with 4% SDS (EM Science, Gibbstown, NJ) at room temperature on a shaking platform. The pellets were then washed 10 times with PBS, followed by sequential incubation as well as PBS washes with DNAse I, RNAse and 20mg/mL of Trypsin (Sigma Chemicals, St. Louis, MO) in order to remove adherent material from the cell wall. The cell wall was then fragmented via sonication (550 Sonic Dismembrator with a ½” tapped horn and tapered micropipet; 1/8” diameter, tuned to vibrate at a fixed frequency of 20kHz; Fisher Scientific, Napean, Canada) in a dry ice and ethanol bath for 2 hours, at a pulse setting of 5.0 (10 second pulses followed by 5 second pauses). Using a phenol-sulfuric colorimetric determination assay, the rhamnose concentration was measured to provide the concentration of the resulting preparation, which was expressed in mg/mL final concentration in PBS. The Bio-Rad Protein Assay (Bio-Rad Laboratories) was used to determine the total protein concentration within the extract, as per the manufacturer's instructions.

5.3 In-vivo studies

WT or TLR2 KO mice, age 4-5 weeks old were used. Mice were injected with 0.5mL sterile PBS or 1mg LCWE in PBS. To determine the peak expression of TLR2 during disease, hearts were obtained at 12 hours, and 1, 2, 3, 14 and 28 days post injections. Heart bases, as defined as being the superior portion of the heart where the aorta branches out, were obtained at
12 hours post LCWE and PBS injections from 4, 8, 12 and 16-week old mice to analyze age-dependence on TLR2 in the animal model. Various organs and arteries were harvested from LCWE or PBS-injected mice 12 hours following administration to examine differential expression of TLR2. All tissues were analyzed by quantitative real-time PCR (qRT-PCR). Organs and vessels were snap frozen in liquid nitrogen and stored at -80°C until use.

5.4 RNA extraction

Organs and vessels were removed from -80°C storage and homogenized in Trizol reagent (Gibco, Grand Island NY, USA) with the PT1200 homogenizer (Kinematica, Bohemia NY, USA). The organ-Trizol suspension was centrifuged at 13000 rpm for 10 minutes at 4°C and the supernatant was collected. Two chloroform (Sigma, Oakville ON, Canada) extractions were performed and the RNA was precipitated from the organs using isopropanol (Sigma), whereas the vessels required a combination of isopropanol, glycogen and sodium acetate (Fermentas, Burlington ON, Canada). Following centrifugation (13000 rpm), the supernatants were discarded and the RNA pellets were washed with 70% ethanol, air-dried and re-suspended in 50uL (organs) or 7uL (vessels) of 0.01% diethylpyrocarbonate (Sigma) water. RNA concentrations were determined by spectrophotometric analysis (Fisher Scientific, Ottawa ON, Canada).

5.5 Laser Capture Microdissection

C57BL/6 mice, age 4-5 weeks, were administered LCWE or PBS and sacrificed 12 hours post-injection. The heart, kidney, lungs and brain were harvested and snap frozen in liquid nitrogen. The renal, proximal and distal coronary arteries and an artery of the brain were isolated using laser capture microdissection (LCM) performed by a pathologist at the Centre for Modeling Human Disease, Pathology Core at TCP. LCM operates by placing a piece of film with an attached cap over the desired tissue on a slide. Upon activation of the laser, the film melts around the tissue. Once the cap is removed from the slide, the designated tissue remains adhered to the cap. Multiple caps were obtained for each tissue and pooled into a single sample for that tissue. RNA was isolated using the Picopure RNA isolation kit (Arcturus, Applied Biosystems, Carlsbad California, USA) and amplified two times using RiboAmp plus (Arcturus), according to the manufacturer’s protocol. Amplified RNA was converted to complementary DNA (cDNA) using the Superscript II kit (Invitrogen, Grand Island NY, USA), following the manufacturer’s protocol. Samples were analyzed by qRT-PCR.
5.6 **Quantitative real-time reverse-transcriptase polymerase chain reaction**

Complimentary DNA (cDNA) was synthesized from various organs (heart, kidney, brain) and vessels (aortic arch, brachial, femoral) using the GeneAmp RNA PCR kit and murine leukemia virus reverse transcriptase (Applied Biosystems). Converted cDNA was analyzed in duplicates via qRT-PCR using primers and probes for GAPDH (Applied Biosystems), PECAM-1 (Assays-on-Demand, Applied Biosystems) as well as for TLR2 (Assays-on-Demand, Applied Biosystems). A standard curve for TLR2 was generated using cDNA obtained from the spleen of a WT mouse. To determine the relative amounts of gene products, they were compared with a standard curve and expressed as a ratio against the housekeeping gene GAPDH (organs) or PECAM-1 (vessels).

5.7 **Transfection Assays**

Human embryonic kidney 293T (HEK293T) cells were purchased from ATCC and grown in Dubucco’s Modified Eagles Medium (DMEM) supplemented with 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen), 0.1mM non-essential amino acids (Invitrogen), 10 mM HEPES (Invitrogen), and 10% or 1% fetal bovine serum (FBS) (Sigma-Aldrich). The human TLR2 (hTLR2), β-galactosidase, NF-κB reporter Igκ-luciferase and pcDNA3 constructs were all generous gifts from D. Philpott (University of Toronto, Toronto ON, Canada). HEK293T cells were cultured in 10% or 1% DMEM at a density of 7 x 10^4 cells per well in a 24-well plate. After 24 hours, transfection was performed using Fugene6 (Roche, Laval QC, Canada) with 100ng of β-galactosidase and NF-κB reporter Igκ-luciferase construct per well. Additionally, half the wells also received 100ng of the hTLR2 plasmid and the other half received 100ng of pcDNA3 as a control to balance the DNA content. This assay was utilized to study TLR2 responsiveness to LCWE, the endogenous protein hHSP60 and a purified peptide from hHSP60 – p277. Three days post-plating cells were stimulated with various agonists, depending on the experiment: TNFα (10ng/mL) (BD Biosciences, Mississauga ON, Canada) as an internal control for the assay, Pam3Cys (2ug/mL) (EMD Biosciences, Cincinnati Ohio, USA) as a positive control for TLR2 signaling, LCWE (5ug/mL), rhHSP60 (20ug/mL) (Stressgen, catalogue number ESP-540D, Victoria BC, Canada), or various concentrations of the hHSP60 derived peptide – p277 (VLLGGEALLRCIPALDSLTPNED) (Biomatik, Cambridge ON, Canada). All conditions were stimulated for 4 hours, except for conditions where the purified
peptide was used. In testing the peptide, kinetic analysis was performed and cells were stimulated for 4, 24, 48 or 72 hours. Following stimulation, media was aspirated from the wells and replaced with lysis buffer (25mM Tris, 8mM MgCl₂, 1% Triton, 15% Glycerol, 1uM DTT). Luminescence of the supernatants were measured and normalized to their β-galactosidase activities. Samples were run in duplicate and where indicated, error bars represent standard error.

5.8 Cell culture – EOMA and T-cells

To determine if an endothelial cell line – EOMA (ATCC, catalogue number CRL-2586) expressed TLR2, EOMA were cultured overnight in DMEM supplemented as mentioned in section 5.6 with 10% FBS at 2.5 x 10⁵ cells per well in 6-well plates. Refer to section 5.11 for information regarding all antibodies used. Cells were left untreated or stimulated with 20ng/mL of recombinant TNFα (eBioscience, San Diego California, USA) for 6, 12 or 24 hours and TLR2 expression was measured by flow cytometry. For measurement of costimulatory molecule expression, EOMA were cultured as stated above and were either left untreated or stimulated with the TLR2 agonist Pam3Cys (10ug/mL). Costimulatory molecule expression (CD80 and CD86) was determined by flow cytometric analysis 24 and 48 hours following stimulation. For survival assays (Annexin-V apoptotic and BrdU proliferative assays), EOMA were cultured overnight in DMEM supplemented as mentioned in section 5.6 with 10% FBS at 2.5 x 10⁵ cells per well in 6-well plates. Half the EOMA were treated with Pam3Cys (10ug/mL) and the other half were left untreated. The following day, T cells were isolated from WT spleens as mentioned in section 5.8 and 1 x 10⁶ T cells were added to each well containing EOMA in Iscoves media supplemented as mentioned in section 5.8. To determine the purity of T cell isolates, T cells were plated alone. To ensure that T cells functioned appropriately post-isolation, T cells were plated with isolated APC (1 x 10⁶ T cells in combination with 2.33 x 10⁶ APC). Splenocytes from WT mice were also plated at 4 x 10⁶ cells per well, as per standard protocol. Cultures were either stimulated with 0.3ug/mL of SEB or left unstimulated. Cultures were harvested on days 3, 5 and 7 post-SEB addition and analyzed via flow cytometry.

5.9 Proliferation assay – ³H-Thymidine incorporation

EOMA were plated at a concentration of 2.5 x 10⁵ cells per well in 6-well plates. The following day, cells were treated with 10ug/mL Pam3Cys to upregulate CD80 costimulatory molecule expression. Two days post Pam3Cys treatment, EOMA cells were fixed in 1% paraformaldehyde (Canemco Inc, St Laurent QC, Canada) for 20 minutes and quenched in
100mM glycine (Bishop Canada Inc). T cells were isolated from WT spleens using the Pan T-Isolation Kit II (Miltenyi Biotec, Germany) according to the manufacturer’s protocol, and 1 x 10^6 T cells were added to each well containing fixed EOMA and Iscoves media supplemented with 10% heat-inactivated FBS, 1mM sodium pyruvate, 2mM L-glutamine, 0.1mM non-essential amino acids, 10mM HEPES, and 50uM β-mercaptoethanol (ME). Cells were either left untreated or stimulated with 0.3ug/mL of the SAg Staphylococcus aureus enterotoxin B (SEB) (Toxin Technology, Sarasota Florida, USA). To determine the effect of blocking CD28-B7 costimulation, CTLA4-Ig was added to cultures day 4 post-T cell addition, at a concentration of 50ug/mL. To determine if WT APCs contaminated the isolated T cell cultures, isolated T cells were plated with or without SEB. To ensure the measured proliferative response was from the T cells, some EOMA were cultured without T cells. 1uCi of 3H-Thymidine was added to each well and following a 18 hour incubation, the supernatants containing the T cells were centrifuged at 8000rpm for 10 minutes. Pellets were resuspended in 200uL Iscoves, added into a 96-well plate and analyzed for thymidine incorporation using a scintillation counter on days 2, 3 and 4 post SEB addition.

5.10 Proliferation assay- BrdU incorporation

EOMA and T cells were combined in culture as stated in section 5.7. To allow for incorporation of bromodeoxyuridine (BrdU) into the cells’ DNA, BrdU (BD Biosciences, kit catalogue number 552598) was added to wells at a final concentration of 10uM per well the day before harvesting. Cells were harvested on days 3, 5 and 7. counted with trypan blue and surface stained with Vβ8-biotin (clone F23.1), streptavidin –PE and the fixable viability dye efluor450 (ebioscience). Fixation, permeabilization, and intracellular staining were performed using Cytofix Cytoperm from the kit (BD Biosciences) along with the APC-conjugated anti-BrdU antibody. Analyses were performed using flow cytometry.

5.11 Apoptosis assay

EOMA and T cells were combined in culture as stated in section 5.7. Cultures were harvested on day 3, 5 and 7 post-SEB stimulation and surface stained for Vβ8-FITC (clone F23.1). Cell death was measured using 7-amino-actinomycin (7-AAD) and Annexin-V-PE (AnnV) exclusion, according to the manufacturer’s protocol (Annexin-V FITC kit, BD Biosciences). Absolute number of live, SEB-specific T cells undergoing low levels of apoptosis were calculated as follows:
Absolute # live cells = (trypan blue live cell count) X (% 7-AAD<sup>-</sup>, AnnV<sup>lo</sup>, Vβ<sup>+</sup> cells)

5.12 Flow cytometry

For TLR2 (refer to sections 6.9 and 6.10) and costimulatory molecule expression (refer to section 6.11) experiments, analysis was performed on live cells by propidium iodine (PI) exclusion. The following antibodies were used to stain for TLR2, CD80 and CD86 expression on EOMA: TLR2-biotin (clone 6C2, eBioscience), streptavidin-APC (BD Biosciences), CD80-FITC (clone 16-10A1, BD Biosciences), and CD86-FITC (clone GL-1, BD Biosciences). For survival assays (refer to sections 6.13 and 6.14), analysis was performed on live cells by 7AAD exclusion or eFluor450 exclusion. For apoptotic assays, cells were stained with three fluorochromes to determine the percentage of live T cells specific for SEB that were undergoing low levels of apoptosis: Vβ8-FITC (clone F23.1), the viability dye 7-AAD, and Annexin-V-PE. For BrdU-proliferative assays, cells were surface stained with Vβ8-biotin (clone F23.1), streptavidin-PE and the fixable viability dye Efluor450. Cells were then fixed, permeabilized and stained intracellularly for BrdU using the APC BrdU Flow kit (BD Biosciences) according to the manufacturer’s protocol. The samples from all experiments were acquired on the FACSCanto II (BD Biosciences) flow cytometer using FACSDiva and analyzed by FloJo software, (version 9.1, Tree Star, Ashland, OR) respectively.

5.13 Statistical analysis

Where applicable, statistical significance was calculated using unpaired, two-tailed, Student’s t-tests. A P value of <0.05 was considered to be significant. Unless otherwise stated, error bars represent standard deviation (SD).
6 Results

6.1 Stimulation with LCWE rapidly induces TLR2 expression in the heart

It has been previously shown that TLR2-deficient mice are protected from LCWE-induced disease (123), indicating that TLR2 is necessary for disease induction in mice. Thus to determine kinetics of TLR2 expression in disease pathogenesis, heart bases were harvested at various time points following PBS- or LCWE- administration and analyzed by qRT-PCR. TLR2 transcript levels were normalized to expression of a housekeeping gene, GAPDH. The results indicated that TLR2 mRNA is rapidly expressed in affected heart tissue by 12-hours post-LCWE injection, and expression decreases rapidly returning to baseline by day 3. Therefore, as the innate immune system serves as the first line of defense and TLR2 functions as an innate immune receptor, this data supports the observation that TLR2 is important in the early stages of disease in the animal model.
Figure 9: TLR2 mRNA is rapidly upregulated in the heart following LCWE administration. C57BL/6 mice, 4-5 weeks old, were injected with PBS or LCWE (1mg), and heart bases were harvested at various time points. RNA was isolated from the heart base tissue, converted to cDNA and analyzed by qRT-PCR to determine TLR2 mRNA expression. The data indicates that TLR2 upregulation is most marked shortly after challenge with LCWE, suggesting that TLR2 is required in the initial massive immune response leading to disease. Error bars represent standard deviation **12 hours, p = 0.0200. *24 hours, p = 0.0248. (Figure is representative of one experiment with n=3 for each time point with 3 repeats of the experiment).
6.2 LCWE upregulates TLR2 expression in the heart, independent of age in the murine model

Age is an important risk factor contributing to disease susceptibility, as both young children and young mice are the at risk populations for disease development and KD is rarely observed in older humans and older mice are more resistant to disease (10, 65, 143). To determine if the age-dependence seen in disease correlated with increased TLR2 expression, heart bases were isolated from 4-, 8-, 16-, and 20-week old mice and analyzed via qRT-PCR for TLR2 (Figure 10A). Basal TLR2 mRNA expression did not change with age, as comparable levels of TLR2 transcripts were detected from mice of all four age groups. Therefore we investigated if there was a difference in induced TLR2 expression amongst the various aged mice (Figure 10C). Heart bases were harvested from mice of various ages 12 hours post-PBS or LCWE injection and analyzed for TLR2 transcript levels via qRT-PCR. Post stimulation, LCWE induced TLR2 upregulation in each group tested, and the fold induction remained uniform in the different age groups. Thus age does not appear to be a factor in regulating TLR2 expression in the animal model at baseline or post-LCWE stimulation, but LCWE stimulation can lead to marked upregulation of TLR2 and as previously mentioned TLR2 KO mice are protected from disease, further supporting the important role TLR2 plays in disease pathogenesis. As such, basal differences in TLR2 localization were assessed.
Figure 10: TLR2 mRNA expression is unaffected by age in mice. C57BL/6 mice were uninjected (A) or injected with 1mg of LCWE (C), sacrificed at various ages, and the heart bases were harvested. Following RNA isolation and cDNA conversion, qRT-PCR was performed to determine TLR2 transcript levels. (B) Fold induction of LCWE over non-injected TLR2 expression levels. The data indicates that although age is not a determining factor in upregulation of TLR2, LCWE stimulation is important for regulating levels of TLR2 expression in the animal model, respectively. Error bars represent standard deviation (n=10 for un-injected mice; n=3 for LCWE-injected mice).
6.3 The heart expresses higher levels of LCWE-induced TLR2 RNA

Kawasaki disease is characterized by massive systemic inflammation that can be seen to localize to and persist at the coronary arteries (252). Thus in pursuing various factors that may regulate TLR2 expression in the animal model, we looked at differential expression of TLR2 in various organs that may be affected by inflammation during the course of disease. The heart base, heart apex, brain and kidneys were isolated from PBS- or LCWE-injected mice at 12 hours post administration. Samples were analyzed via qRT-PCR for TLR2 transcript levels. Analysis revealed that differential expression of TLR2 can be detected, as the heart base demonstrated the greatest difference between basal and induced levels of TLR2, with a fold induction of 3.23; the kidney did express high amounts of TLR2, although to a lesser extent with a 2.6 fold induction, and minimal expression could be detected in the brain (Figure 11A). Of all the organs studied, the heart expressed the highest levels of induced TLR2 overall (Figure 11B). TLR2 is constitutively expressed on renal endothelial and tubular cells, and expression can be upregulated following TLR2 ligand stimulation (255-257), thus explaining the observed results in the kidney. Higher TLR2 expression within the heart correlates with KD and what is seen in the animal model, as the coronary arteries are the site for end organ damage in this disease, and the heart base is where the CAs are located.
Figure 11: TLR2 is preferentially expressed in the heart following LCWE administration. Various organs were harvested from C57BL/6 mice at 12 hours following PBS or LCWE injections (1mg). qRT-PCR was performed on the cDNA obtained to determine transcriptional levels of TLR2 mRNA (A, B) Fold induction compared to PBS controls for HB (3.23x), HA (3.22x), and KDY (2.6x). The brain did not upregulate TLR2 transcript levels (B). Renal cells constitutively express TLR2, explaining the observed expression. Notably, as the CAs are affected by persistent inflammation in the animal model and branch off of the heart base, which expresses the highest levels of TLR2, TLR2 may have a role in directing the specificity of the inflammatory response to the coronary arteries. HB = heart base; HA = heart apex; KDY = kidney; BRN = brain. (n=3 for HB, HA, KDY; n=1 for BRN; experiment was repeated 2 times).
6.4 The aorta is rich in basal TLR2 RNA

In KD, various vessels are affected by inflammation in the acute phase, but persistent inflammation centers around the root of the aorta and the coronary arteries (3). Aortic root (AoR) dilatation is one type of cardiovascular sequelae associated with KD, and affects approximately 15% of treated patients (258). As TLR2 expression was already shown to be preferentially expressed in the heart, we wanted to specifically assess if differential expression of TLR2 could be detected in the vessels, for the CAs are the site for persistent inflammation and are located at the heart base. Thus utilizing a dissecting microscope, the ascending aorta/aortic arch (Ao. Arch), brachial and femoral arteries were isolated from PBS- or LCWE-injected mice at 12 hours post injection, and TLR2 mRNA expression was determined via qRT-PCR (Figure 12A). It has previously been shown that at sites of disturbed blood flow, such as branch points or curved morphology similar to the aortic arch, TLR2 expression is increased (243). This correlates with our data, as the aortic arch clearly expresses statistically significant more basal TLR2 than the brachial and femoral arteries. Within the model, varied amounts of injected LCWE can have differing effects, such that an increased volume of LCWE leads to panarteritis. Thus to analyze TLR2 expression more thoroughly within the aorta, the aorta was sectioned into ascending aorta/aortic arch, thoracic and abdominal aorta (Figure 12B). The ascending aorta/aortic arch, thoracic and abdominal aorta expressed comparable levels of basal TLR2; however following stimulation with LCWE, each segment of the aorta upregulated TLR2. The ascending aorta/aortic arch expressed 5.79 times more TLR2 in the disease condition when compared to the PBS control, the thoracic aorta expressed 7.96 times more TLR2 and the abdominal aorta expressed 3.15 times more TLR2 when compared to basal levels. Thus the aorta, being a large vessel that is affected by vasculitis in disease and is the location where the CAs originate, expresses high amounts of TLR2, which can be further upregulated following treatment with a TLR2 ligand. This data lends further support to the hypothesis that TLR2 is important in directing site specific inflammation to the CAs.
Figure 12: Differential expression of TLR2 is detected amongst various arteries with high basal TLR2 levels in the aorta. (A, B) Various vessels were harvested from C57BL/6 mice under a dissecting microscope at 12 hours following PBS or LCWE injections. Isolated RNA was converted into cDNA for analysis by qRT-PCR to determine expression TLR2 transcript levels. Localization plays an important role in regulating TLR2 expression in the animal model, as differential expression of TLR2 could be detected. The aortic arch expressed marked basal levels of TLR2, implying that TLR2 has an important role in inducing site-specific inflammation. Ao – aortic. (**p = 0.0193, *p = 0.0243, #p = 0.0017). Error bars represent standard deviation (A: n=3 for PBS; n=4 for LCWE; B: n = 4 for PBS and LCWE; each graph is representative of 2 repeats).
6.5 TLR2 may be differentially expressed in murine coronary arteries

In approximately 25% of untreated cases, the systemic inflammation that affects children with KD may localize to the coronary arteries of the heart (252, 259). It was previously demonstrated that the heart upregulates more TLR2 mRNA following LCWE administration than other organs, and the aortic arch basally expresses more TLR2, thus we wanted to specifically analyze TLR2 expression at the coronary arteries to determine if it has a role in guiding site-specific inflammation. Isolating the coronary arteries from mice is technically challenging due to their extremely small size, therefore we employed the use of laser capture microdissection (LCM). Mice were injected with either PBS or LCWE and sacrificed at 12 hours. The heart, brain and kidneys were harvested and LCM was used to isolate the necessary arteries (Figure 13A). The proximal and distal coronary arteries, as well as the brain and renal vessels were analyzed for TLR2 mRNA expression via qRT-PCR. All samples were normalized to PECAM, an endothelial cell marker. Preliminary data had revealed that there was a striking preferential expression of TLR2 at the proximal coronary artery in comparison to the controls and other vessels tested (Figure 13B). However, pursuing this experiment further proved to be technically challenging. We have not been able to replicate the data from panel B thus it is currently preliminary data. However, the data is promising, showing that TLR2 may have a role in directing inflammation to the CAs. Therefore it would be worthwhile to pursue this experiment further in the future.
Figure 13: The CAs may preferentially express TLR2, however no detectable differences could be detected in the various vessels tested. The proximal coronary artery, distal coronary artery, artery of the brain and renal artery were isolated at 12 hours from PBS or LCWE injected mice (C57BL/6) (A) Isolated RNA was amplified for two rounds prior to cDNA conversion and analyzed via qRT-PCR for expression of TLR2 mRNA (n=1 for each condition; experiment was repeated with different normalizing genes and the same pattern of expression was achieved). Yellow circles indicate the targeted vessel to be removed, and black circles represent the location of the isolated vessel. (B) Preliminary data indicated that the proximal CAs preferentially express TLR2, suggesting that TLR2 plays a role in guiding inflammation to the CAs in the model. Attempts to pursue this data proved to be technically challenging, and the results could not be repeated under our experimental conditions, respectively.
TLR2 is important for the development of disease in the murine model of coronary-arteritis. As such, we investigated some factors that may play a role in regulating TLR2 expression in this model to try and elucidate the specific role of TLR2 in this inflammatory disease. Localization proved to be an important factor in regulating TLR2, as its expression was found to be differentially expressed in mice. In support of the animal model and KD, the heart and more specifically the aortic arch express high amounts of TLR2. This suggests that more inflammation can be induced in these tissues as TLR2 expression was the highest. Although age was not concluded to be a factor in regulating basal TLR2 expression in this model, the bacterial agent LCWE induced expression of TLR2 in each age group. LCWE contains a SAg that is required to induce disease; however as many pathogens implicated in KD possess superantigenic activity and also contain a TLR2 ligand (Table 4), we were interested in determining if this was the case for LCWE. Additionally, as various endogenous and exogenous ligands can regulate TLR2 activity, we were interested in discovering if other potential endogenous and exogenous ligands could play a role in regulating TLR2 expression during disease.
6.6 The bacterial agent LCWE possesses a TLR2 ligand

The cell walls of gram-positive bacterium are rich in TLR2 ligands (260, 261). As LCWE is isolated from the cell wall of a gram positive bacterium, it was highly likely that TLR2 ligands such as lipoproteins would be present. A TLR2 ligand was confirmed to be present within the LCWE extract using a transfection assay. The HEK293T cell line is naturally deficient for TLR2; therefore we either dually transfected the cells with an NF-κB luciferase reporter construct in combination with a hTLR2 construct, or with the NF-κB luciferase reporter construct in combination with the pcDNA3 vector (empty vector). For this assay, all samples were normalized to β-galactosidase. TNFα is a potent inducer of the NF-κB pathway and does not require stimulation via TLR2 to induce activity and thus serves as an internal positive control for the assay, clearly indicating that transfection of HEK293T cells was successful. Pam3Cys is a synthetic TLR2 ligand, which induced strong NF-κB activity in the dually transfected cell line and not in the single transfected cell line. Stimulation with LCWE induced comparable levels of TLR2 activity, providing evidence for the existence of a TLR2 ligand. As LCWE contains superantigenic activity, and a SAg has been shown to be necessary for disease in the animal model, it is probable that the SAg works in combination with the TLR2 ligand to induce disease in this model.
Figure 14: TLR2 activity is detected in LCWE. HEK293T cells were transfected overnight with either an NF-κB luciferase reporter construct alone, or in combination with a human TLR2 construct. Post-transfection, HEK cells were stimulated with TNFα (10ng/mL), Pam3Cys (2ug/mL) or LCWE (5ug/mL) for four hours. While the singly transfected cells did not respond to Pam3Cys or LCWE, cells that were dually transfected were responsive to Pam3Cys, a known TLR2 ligand as well as to LCWE indicating the specificity and presence of a TLR2 ligand within LCWE. Results were normalized to β-galactosidase activity.
6.7 TLR2 activity cannot be detected with human Hsp60

As an exogenous ligand was found to induce TLR2 activity, we wanted to determine if an endogenous ligand existed that could also signal via TLR2 as well as amplify the immune response. HSP60 seemed to be a good candidate as evidence in the literature suggests, although controversial, that HSP60 may serve as an endogenous ligand for TLR2 (209). HSPs are immunogenic molecules, and when cells are exposed to unfavourable and stressful conditions, including infection, high temperatures and cytokine stimulation, cells will produce increased levels of HSPs (232). Due to the fact that HSPs chaperone other proteins, the possibility for observed activity due to contamination is increased. Therefore a peptide (p277) derived from HSP60 was also analyzed as it has been suggested to signal via TLR2 (262). We thus hypothesized that HSP60 may be involved in an endogenous positive feedback loop, signaling via TLR2 to induce increased inflammation. We therefore wanted to determine if under our experimental conditions, HSP60 could serve as an endogenous ligand capable of inducing TLR2 activity. The same transfection assay mentioned in 6.6 was utilized to analyze if hHSP60 could induce TLR2 signaling following 48 hours of stimulation, as this was the predominant timepoint used in the literature. Pam3Cys induced strong NF-κB activity in the dually transfected cell line and not in the singly transfected cell line. This activation was not seen in either of the transfected cell lines when hHSP60 was used, suggesting that under our experimental conditions, hHSP60 may not signal as an endogenous TLR2 ligand.
Figure 15: hHSP60 does not have TLR2 ligand activity. The TLR2-deficient cell line, HEK293T, was either singly transfected with an NF-κB luciferase reporter construct, or dually transfected with a hTLR2 construct as well. The cells were transfected overnight and then stimulated for 48 hours with either Pam3Cys (2μg/mL) or hHSP60 (20μg/mL). A response was detected in the dually transfected cells following stimulation with Pam3Cys; however hHSP60 did not induce a response in either cell line indicating that hHSP60 may not signal via TLR2, and that previous observed reports of TLR2 activity were actually due to contaminants being chaperoned by the HSP. Results are representative of three experiments. Results were normalized to β-galactosidase activity.
6.8 Peptide p277 from human Hsp60 does not appear to induce TLR2 activity in TLR2-transfected HEK cells

In the literature, there is some controversy surrounding the use of Hsp60, as it has been suggested that contaminants from chaperoned molecules may be involved in signaling (262); therefore a 24 amino acid long peptide purified from HSP60 – p277 (VLGGGCALLRCIPALDSLTPANED) – may be used to eliminate any chance of contamination. It has been observed in the literature that p277 can induce TLR2 signaling, and may therefore serve as an endogenous ligand for regulating TLR2 activity (262). To confirm whether p277 could induce TLR2 signaling, the same transfection assay as mentioned above in 6.6 was used. Data from the literature suggested that serum present in media can sequester the peptide from binding a receptor, thus assays were performed using various concentrations of FBS in the media, including 10%, 1% and 0%. When FBS was not provided in the media the HEK cells did not adhere to the wells and therefore the assay could not be completed, thus it was determined that 1% FBS provided the best condition to grow the HEK cells and also stimulate them with the peptide. Kinetics were also performed in combination with using various concentrations of the p277 peptide to determine the optimal conditions for observed TLR2 activity. Cells were stimulated for either 4- (Figure 16A), 24- (Figure 16B), 48- (Figure 16C), or 72 hours (Figure 16D) following an overnight transfection. At this time, it is inconclusive if p277 can signal via TLR2 and therefore function as an endogenous ligand.
Figure 16: The hHSP60 – derived peptide, p277 does not elicit TLR2 signaling. HEK293T cells were transfected with an NF-κB construct alone, or in combination with a hTLR2 construct overnight. The cell lines were then stimulated with Pam3Cys (1μg/mL), and various concentrations of p277 (0.1μg/mL, 10μg/mL) or left unstimulated for 4- (A), 24- (B), 48- (C), or 72 hours (D). TLR2 activity induced by Pam3Cys in the dually transfected cell line indicates proper functioning of the assay; however activity could not be observed for the TLR2 transfected cells following stimulation with the peptide. Currently, as numerous conditions were tested to assess TLR2 activity following p277 stimulation, it appears that p277 does not signal via TLR2 under these experimental conditions. Experiments were repeated 2-4 times for each time point. Results were normalized to β-galactosidase activity.
In the animal model, superantigenic activity has been shown to be necessary for disease induction (118), which is also the case for TLR2 as mice lacking this receptor do not develop coronary arteritis (123). Importantly, the bacterial agent used to induce disease in the animal model contains both superantigenic activity as well as a TLR2 ligand. In searching for other potential TLR2 ligands that may play a role in upregulating TLR2 expression, it was stated in the literature that HSP60, which is released from inflamed and damaged tissues, can function as an endogenous TLR2 ligand. Although some of the literature supports hHSP60 as a TLR2 ligand, under our experimental conditions we were not able to observe any TLR2 activity following stimulation with hHSP60 or a derived peptide, p277. TLR2 plays an important role in this disease model, and it is known that TLR2 can upregulate costimulatory molecule expression. Enhanced costimulation is the proposed mechanism responsible for rescuing the pathogenic T cells, thus we wanted to determine the functional relevance of TLR2 within this model, specifically on endothelial cells to assess if ECs can rescue SAg-activated T cells, as it is hypothesized that this ‘rescue’ occurs at the CA endothelium.
6.9 EOMA expresses high basal levels of TLR2

Inflammation in KD as well as in the animal model is localized to the coronary arteries. The coronary arteries are also the site for end organ damage, thus various factors could be involved in homing the pathogenic T cells to the CAs. T cell activation requires interaction with an APC, where enhanced costimulation is presented to the T cells, allowing them to persist; in KD these APCs may be endothelial cells. Therefore we wanted to determine if endothelial cells could function as APCs capable of interacting with T cells. Isolating CA endothelial tissue is technically challenging, and as no mouse CA cell line exists to date, an EOMA cell line was used. EOMA cells are originally derived from a mouse hemangioendothelioma and serve as a general model to study microvascular endothelial cells (263) so were used as proof of principle. TLR2 signaling upregulates costimulatory molecule expression, and a functional APC must be able to provide these costimulatory signals. To determine if EOMA express TLR2, cells were cultured to confluency, then trypsonized and stained for TLR2 antibody for flow cytometric analysis. The data was first gated on live cells (Figure 17A), then assessed for TLR2 expression (Figure 17B). Our data indicated that all of the EOMA cells are positive for TLR2 protein.
Figure 17: TLR2 is highly expressed on EOMA cells. EOMA cells were cultured until confluency was reached, and expression of TLR2 was measured by flow cytometry. Analysis was performed on live cells, indicated by PI exclusion. All of the ECs were positive for TLR2 expression, which further supports the hypothesis that TLR2 is important for localization of inflammation to the CA endothelium. Data is representative of multiple experiments (n=3).
6.10 TNFα can further upregulate TLR2 protein expression on EOMA cells

TNFα is an important pro-inflammatory cytokine for our disease model, and it is also necessary for disease development as TNFRI knock out mice are not susceptible to coronary arteritis (30). To determine if TNFα could induce increased expression of TLR2 on these cells, EOMA cells were grown to confluency, and then cultured with 20ng/mL of recombinant TNFα or left unstimulated for 6 (Figure 18A), 12 (Figure 18B), or 24 hours (Figure 18C). Titration of TNFα was performed to determine a suitable concentration. EOMA were stimulated with 2, 20, 50 and 100ng/mL of TNFα. Stimulation with 20ng/mL proved to be the optimal concentration, as 50 and 100ng/mL proved to have a cytotoxic effect, killing majority of the EOMA cells (data not shown). Analysis via flow cytometry revealed that at 6 hours, the mean fluorescent intensity (MFI) for TLR2 expression following TNFα stimulation was 55.6 compared to 53.6 for the unstimulated condition. The MFI for TLR2 expression increased to 72 for the TNFα stimulated condition versus 36.8 for the control at 12 hours and was maximal at 24 hours, being 101 in the stimulated condition versus 51.2 for the unstimulated condition. Thus TLR2 could be further upregulated on EOMA up to 24 hours post-stimulation.
Figure 18: TNFα stimulation enhances TLR2 expression on EOMA cells. EOMA cells were grown in culture and either left untreated, or stimulated with 20ng/mL of recombinant TNFα. Expression of TLR2 was measured via FACS analysis at 6- (A), 12- (B), and 24-hours (C) following stimulation. Analysis was determined from live cells, as measured by PI exclusion. TLR2 expression on EOMA strongly supports the hypothesis that localized inflammation to the CA endothelium is in part due to high TLR2 expression. Interestingly, TNFα, which is produced in high quantities in the animal model and is one of the cytokines responsible for the massive inflammation observed in disease, can further upregulate TLR2 expression on ECs. This suggests that within the model, TNFα may work with TLR2 to further enhance inflammation by upregulating TLR2.
6.11 CD80 expression on EOMA is enhanced following stimulation with a TLR2 ligand

It has previously been shown in the lab that signaling via TLR2 can upregulate costimulatory molecule expression on splenocytes, enhanced expression of these costimulatory molecules can rescue SAg-activated T cells from apoptosis, and that TLR2 is also capable of rescuing SAg-activated T cells from programmed cell death (125). As EOMA cells express high quantities of TLR2, we wanted to determine if signaling via TLR2 could enhance expression of either CD80 or CD86 costimulatory molecules on EOMA – as expression of costimulatory molecules is one requirement for a cell to function as an effective APC (264). Initial experiments conducted to determine the appropriate dose of the TLR2 ligand, Pam3Cys demonstrated optimal expression using 10μg/mL (data not shown). Kinetics were performed to determine the optimal time point of expression (data not shown). EOMA cells were grown to confluency and cultured with Pam3Cys for 24 and 48 hours and analyzed via flow cytometry. Low levels of CD80 could be detected basally at 24 hours (MFI of 426); however stimulation with Pam3Cys for 24 hours induced upregulation of CD80 (MFI of 971). Basal expression of CD80 was also detected at 48 hours, as indicated by the histogram shift (MFI of 460), and expression could be further upregulated following stimulation with Pam3Cys (MFI of 777). Thus CD80 could be upregulated on EOMA up to 48 hours following stimulation, but appears to be maximal at 24 hours (Figure 19A). Our data indicated that although CD86 could not be detected on this cell line (Figure 19B), CD80 expression was enhanced beyond basal expression at 24 hours and 48 hours following stimulation, suggesting that EOMA have the potential to serve as functional APCs.
Figure 19: EOMA upregulate CD80 expression following TLR2 stimulation. EOMA were grown to confluency and either left unstimulated, or were cultured with 10µg/mL of Pam3Cys for 24 and 48 hours. Flow cytometric analysis was performed to determine expression levels of CD80 and CD86. Analysis was performed on live cells, measured by PI exclusion. At 24 hours, baseline MFI for CD80 was 426, which increased to 971 following stimulation. At 48 hours, baseline MFI for CD80 was 460, which increased to 777 following stimulation. Signaling via TLR2 leads to enhanced expression of CD80 on ECs, thus TLR2 stimulation may transform EOMA cells into functional APCs. Data is representative of three repeat experiments.
6.12 TLR2 stimulated EOMA can support proliferation of SAg-stimulated T cells

A functional APC is able to provide costimulatory signals to a T cell to support proliferation and survival (265). It was demonstrated that TLR2-primed EOMA can enhance the expression of CD80, thus we wanted to determine whether EOMA could serve as functional APCs and support T cell proliferation in response to SAg. EOMA were grown in 6-well plates overnight and primed the following day with Pam3Cys to allow for CD80 upregulation (under these experimental conditions, all EOMA were primed with TLR2 agonist). On day 2, EOMA were fixed with 1% paraformaldehyde to ensure that the proliferative response was solely due to the T cells. T cells were also isolated from WT spleens and added to the fixed EOMA cultures in the presence or absence of the prototypic SAg – SEB. Proliferation of T cells was measured on days 2, 3, and 4 post-mixing. The data revealed that T cells proliferated following 4 days in culture with TLR2-primed EOMA and SEB. This proliferation is evidenced by the increase in the disintegrations per minute (DPM) radioactive count, from 2760.65 DPM at day 2 to 39,973.32 DPM at day 4. The observed T cell proliferative response was due to T cells interacting with the TLR2-primed EOMA, for when EOMA or T cells were cultured on their own, minimal T cell proliferation can be detected (Figure 20A). To dissect if the observed proliferative response was in part due to the expression of CD80 on EOMA, CTLA4-Ig - a fusion protein that prevents costimulatory signaling via CD28 and CD80/86 molecules, was added to cultures, which were harvested on day 4 (Figure 20B). Preliminary data illustrated a trend, suggesting that when costimulatory signaling via CD28 on T cells to CD80/86 molecules on the TLR2-primed EOMA is blocked, the proliferative ability of T cells is decreased. Therefore, TLR2-primed ECs can express enhanced costimulatory signals and function as APCs that support T cell activation following SAg stimulation.
Figure 20: EOMA pre-treated with TLR2 ligand can support T cell proliferation following SAg stimulation. EOMA cells plated overnight were stimulated the following day with 10ug/mL Pam3Cys. T cells from WT mice were isolated on day 2 post-stimulation and added to EOMA cultures with or without SEB (0.3ug/mL). Thymidine was added to cultures 18 hours prior to proliferative measurements, respectively. Proliferation of T cells was analyzed on day 2, 3 and 4 following addition to EOMA cultures (A) CTLA4-Ig (50ug/mL), a fusion protein that prevents the interaction between CD28 on T cells with B7 molecules on APCs, was added to cultures and the proliferative response was analyzed at day 4 (B) ECs are transformed into functional APCs following treatment with a TLR2 ligand, as CD80 expression is enhanced. TLR2-primed ECs are functional APCs that support the proliferation of SAg-stimulated T cells, an effect that may be mediated by the CD28-CD80/86 interaction, for when this pathway is blocked, T cell proliferative responses are decreased. Results are representative of 3 experiments.
6.13 TLR2 stimulation transforms endothelial cells into APCs capable of enhancing survival of SAg-activated T cells

It has previously been shown that superantigenic activity is required for the development of coronary arteritis in the murine model of disease (118). Previous studies have also demonstrated that numerous mononuclear cells, including T cells, infiltrate the walls of the coronary lesions found in KD (45, 266). Although the clinical features of the disease are well studied (5), there are only a limited number of studies that examine possible mechanisms of SAg-mediated vascular damage. Studies from our lab have provided evidence supporting the notion that enhanced costimulation can increase the survival of SAg-activated T cells (125, 177-179) and it has been shown that EOMA can upregulate CD80 expression following TLR2 stimulation. Thus we hypothesized that TLR2 stimulation upregulates costimulatory ligands on EOMA, transforming these cells into functional APCs that can interact with and lead to the survival of SAg-reactive T cells.

In order to test this hypothesis, isolated T cells were added to cultures of TLR2-primed or non-TLR2-primed EOMA with or without the addition of SEB. T cell survival was quantitated via flow cytometric analysis. Enhanced survival was determined by gating on 7-AAD negative, Annexin-V low Vβ8+ cells (Figure 21A) on days 3, 5 and 7 post-SEB stimulation. As indicated in figure 21B, a higher percentage of live, Annexin-V low, Vβ8+ T cells are found in the condition with TLR2-primed EOMA plus SEB than when compared to the condition with non-TLR2-primed EOMA plus SEB. An increase in the absolute number of live, Annexin-V low, Vβ8+ cells is also observed when T cells are cultured with TLR2-primed EOMA and was calculated by multiplying the absolute number of live cells (determined by trypan blue exclusion) by the percentage of live, Annexin-V low, Vβ8+ T cells for each condition on days 3, 5 and 7 (Figure 21C). Figures 21B and C support that TLR2 priming of EOMA, which upregulates CD80 costimulatory molecules, can enhance the number of T cells that survive following SAg stimulation.
Figure 21: TLR2-primed EOMA can function as APCs to enhance survival of SAg-activated T cells: EOMA cells were plated overnight and half the cultures were primed the following day with 10μg/mL Pam3Cys. T cells from WT mice were isolated on day 1 post-priming and added to EOMA cultures with or without SEB (0.3μg/mL). Cultures were harvested and analyzed on days 3, 5 and 7 post-SEB stimulation. (A) Gates were placed around Annexin-V low, Vβ8+ events based on fluorescence minus one (FMO) and unstained controls, respectively, from left to right: T cells that were stained with 7-AAD alone, with Annexin-V plus 7-AAD, or Vβ8 plus 7-AAD. (B) A higher percentage of live, Annexin-V low, Vβ8+ T cells can be observed in the TLR2-primed EOMA condition (left panel) in comparison to the condition with non-TLR2-primed EOMA (right panel). (C) Summary of 3 independent experiments indicating the absolute number of live, Annexin-V low, Vβ8+ T cells when cultured as mentioned above for days 3, 5 and 7. Data is representative of 3 independent experiments. Statistics were calculated using a student’s paired T-test.

From the above data, it can thus be concluded that the upregulated CD80 on EOMA is functional as enhanced survival and rescue from apoptosis of SAg-reactive T cells was observed. The data supports that signaling via TLR2 can transform endothelial cells into functional antigen presenting cells that are able to interact with and provide enhanced costimulation to SAg-activated T cells, allowing them to survive and proliferate and likely stay localized to the coronary artery leading to damage.
6.14 T cells that are activated by SEB in the presence of TLR2-stimulated ECs, which function as APCs, exhibit enhanced survival and proliferation

In the previous experiment it was demonstrated that TLR2-primed endothelial cells can lead to the enhanced survival of SAg-activated T cells, as indicated by an increase in the absolute number of live cells in the Vβ8+ T cell population. To analyze the population of rescued T cells following SAg stimulation, BrdU staining was performed to measure proliferation of the T cell population. Cells were cultured as mentioned above, however BrdU was added to cultures on days 2, 4, and 6 to allow for incorporation. Cells were stained on days 3, 5, and 7 with αBrdU to identify proliferating populations as well as with Vβ8 to specifically identify the SEB-reactive population of T cells, and analyzed via flow cytometry. Figure 22 A summarizes the results from figure 21 C, and depicts the fold increase in the absolute number of live cells following stimulation with SEB. The fold increase in the absolute number of live Vβ+ T cells from the TLR2-primed condition compared to the non-TLR2 primed condition was 1.84 times for day 3, 2.29 times for day 5 and 2.69 times for day 7. In accordance with previous data from our laboratory, day 7 demonstrated the greatest amount of T cell survival post-SAg stimulation, which was 1.46 times greater when compared to day 3. Further supporting this enhanced survival, it was observed that SEB-reactive Vβ8+ T cells proliferated to a greater extent when combined with TLR2-primed EOMA than when they were combined with non-TLR2-primed EOMA, as indicated by a 9.5 fold increase in the MFI and the histogram shift observed in Figure 22B. Therefore, TLR2 signaling can transform endothelial cells into functional APCs that not only lead to the enhanced survival of SAg-reactive T cells, but also supports their proliferation following SAg stimulation.
Figure 22: T cells that interact with TLR2-primed EOMA and are stimulated with SEB proliferate to a greater extent when compared to T cells interacting with non-TLR2-primed EOMA. EOMA cells were plated overnight and half the cultures were primed the following day with 10μg/mL Pam3Cys. T cells from WT mice were isolated on day 1 post-priming and added to EOMA cultures with or without SEB (0.3μg/mL). BrdU was added to half the cultures on days 2, 4 and 6. Cultures were harvested and analyzed on days 3, 5 and 7 post-SEB stimulation. (A) Data from 3 independent experiments represented as the fold increase in absolute numbers of T cells that were cultured with TLR2-primed EOMA as compared to T cells cultured with non-TLR2-primed EOMA. The black dotted line represents the point at which no difference exists between EOMA cultures primed with a TLR2 agonist. (B) BrdU was added to cultures 18 hours prior to harvesting. The viability dye Efluor450 was utilized to exclude dead cells and Vβ8 PE was used to identify specific Vβ8 populations. Overlay of histograms indicates that Vβ8+ T cells mixed with TLR2-primed EOMA (blue histogram, MFI of 1022) incorporate more BrdU and proliferate more so than Vβ8+ T cells combined with non-TLR2-primed EOMA (black histogram, MFI of 108). SAg-activated T cells can be rescued from apoptosis when combined with a functional APC, being the TLR2-primed endothelium in this case. Furthermore, these ‘rescued’ cells are responsive post-SAg stimulation, as the increased number of live cells observed correlates with the increased proliferative capacity of the Vβ8 + T cells. Data is representative of 3 independent experiments.
7 Discussion

The events leading to the development of KD continue to be elucidated. An important question remaining to be answered is why the coronary arteries are the site for target organ damage. While various hypotheses regarding KD exist, the expression and regulation of TLR2 may be one of the contributing factors as to why inflammation is localized to the coronary arteries in this disease. Within this disease model, TLR2 has been shown to play an essential role as evidence indicates that mice lacking this innate immune receptor are not susceptible to coronary arteritis (123). Superantigenic activity has also been shown to be necessary for disease induction (118), and the proposed mechanism for rescue of the SAg-activated T cells is through enhanced costimulation. Interestingly in completing this thesis, our lab may have uncovered the mechanism that TLR2 stimulation can upregulate costimulatory molecules on endothelial cells, and in combination with SAg, may lead to decreased apoptosis of the specific SAg-activated T cells (K. Little, published thesis).

In this thesis, the role of TLR2 in the LCWE model was further elucidated. The evidence indicates that there is differential expression of TLR2 amongst various vessels, with the aorta expressing the highest levels and also being the vessel from where the CAs originate. The data also implicates that TLR2 plays a functional role on endothelial cells, as TLR2 signaling can upregulate CD80 on ECs, changing them into APCs that provide a costimulatory signal to the SAg-activated T cells, allowing them to persist and potentially target the coronary arteries.

Currently, the etiology of KD remains to be discovered however, animal models have provided some insight into this disease, indicating that SAg activity is required to induce coronary arteritis in mice. This is evidenced by administering SAg-containing LCWE to mice, who demonstrate maximal infiltrate at the coronary arteries preceding elastin breakdown and aneurysm formation at day 28 post-injection. Interestingly, LCWE shares similar qualities with other pathogens isolated from children with KD, for example *Staphylococcus* aureus and *Streptococcus* pyogenes (Table 4), as SAg activity can be detected in combination with TLR2 ligand in these preparations, hinting that there may be a role for TLR2 in SAg-mediated responses. Clearly identifying a role for TLR2 in disease, Rosenkranz et al demonstrated that TLR2 knock out mice were protected from coronary arteritis following LCWE injection, while KO mice for another common extracellular innate immune receptor, TL4, still developed disease (123). Initially, a mutation within the TLR4 gene was thought to be responsible for the inability
of C3H/HeJ mice to develop coronary arteritis (120); however as just mentioned TLR4 KO mice are still susceptible to disease. It was later discovered that C3H/HeJ mice are infected with a retrovirus following their birth that produces a Vβ14-specific SAg (124). Consequentially, these mice delete their T cells bearing the TCRVβ14 chain, which is an LCWE-Vβ responsive family, implying that these mice are less responsive to LCWE and less likely to develop disease. Thus a defect in the T cell repertoire and not TLR4 is a more probable cause as to why C3H/HeJ mice are protected from CA, also supporting a role for T cells in coronary disease. The importance of T cells in disease is further evidenced in previous studies as coronary arteritis in mice was shown to be a T cell-mediated process (Section 1.6.1); however, as a role for TLR2 in disease pathogenesis became apparent, we sought to elucidate the specific role TLR2 may be playing in disease, beginning with understanding what factors affect TLR2 expression.

In response to infection, PRRs expressed on innate immune cells— including TLR2—sense the invading pathogen, and activate the immune cells, such as macrophages and neutrophils, to initiate an inflammatory response (261). KD is thought to be induced by an infectious pathogen, and as the innate immune receptor TLR2 has clearly been shown to be necessary for disease in mice, understanding how TLR2 functions in pathogenesis could prove to be useful in delineating downstream processes involved in coronary arteritis. Following administration of LCWE, a marked upregulation of TLR2 was observed shortly after injection, suggesting that TLR2 is involved in the early inflammatory processes of disease pathogenesis. As the innate immune response occurs earlier than the adaptive immune response, this result is in agreement with the literature, as TLR2 sensing of a pathogen should lead to the activation of innate immune cells involved in the initial response, which induce inflammation as part of a process to clear the pathogen.

There are many intriguing characteristics of KD. For example, younger children are predominantly affected by KD, implicating a pivotal role for age in disease development. This interesting characteristic can be similarly observed in the animal model, as coronary arteritis is inducible much easier in young mice compared to older mice, for when older mice were injected with LCWE, they did not develop coronary disease. Therefore, as age may be a key component for disease in children, we studied whether age may be one factor that regulates TLR2 expression in the disease model. Specifically, it was postulated that as younger mice are susceptible to disease, and TLR2 is necessary for disease, then younger mice may express higher quantities of TLR2 when compared to older mice, who are protected from coronary arteritis. However it was found that basal and stimulated TLR2 expression is independent of age in this model, as
comparable levels of TLR2 were observed regardless of the age of the mice. Although age was not deemed to affect expression of TLR2 in this model, it was interesting to note that LCWE induced marked upregulation of TLR2 in the heart, as this provides further evidence for the involvement of TLR2 in disease pathogenesis, and suggests that TLR2 may be involved in targeting inflammation to the heart and surrounding vessels.

KD is thought to be initiated by an infectious trigger, however the coronary vessels are specifically targeted in some patients, with aneurysm formation occurring in approximately 25% of untreated cases (3), which is more suggestive of an autoimmune-type reaction. Currently, it is unknown why there is site-specific inflammation at the coronary arteries, and although numerous factors may contribute to this localized inflammation, differential expression of TLR2 may be a key mechanism involved in increasing inflammation at the CAs. KD is a systemic vasculitis, thus multiple organs may be affected by inflammation. In an effort to determine if TLR2 can be differentially expressed and thus have a role in inducing higher amounts of localized inflammation, we analyzed TLR2 expression in the heart base, heart apex, kidney and brain following administration of LCWE. Our data indicated that the overall increase in fold expression of TLR2 was induced to a greater extent in the heart in comparison to the kidney, which also upregulated TLR2; however minimal levels of TLR2 were detected in the brain. Within the kidney, renal endothelial cells have been reported to express endogenous levels of TLR2 (255-257), therefore explaining the observed expression. The kidneys can be affected in KD, as sterile pyuria (WBC in the urine) is a common yet underappreciated finding in this disease (267-269). From these findings, it was concluded that TLR2 is differentially expressed following challenge with LCWE as higher amounts of TLR2 were detected within the heart. This data further strengthens the hypothesis that TLR2 plays a role in localizing inflammation to the coronary arteries, which are located on the heart base. Although there is a relevant trend observed, more replicates are needed to detect statistical significance.

In continuing to elucidate the role of TLR2 and how differential expression of the receptor may be involved in increasing site-specific inflammation at the CAs, we sought to determine specific expression of TLR2 within various large and medium-sized vessels that are potentially affected by inflammation in the model. Thus we initially assessed TLR2 expression in the aorta and other medium sized vessels and interestingly, the ascending aorta/aortic arch expressed statistically higher levels of basal TLR2 RNA in comparison to the brachial and femoral arteries. Further indicating the importance of TLR2 expression in vessels, LCWE stimulation greatly upregulated TLR2 expression in each segment of the aorta. Thus it is possible
that the inflammation observed in the aorta in KD may be attributed to a high TLR2 content. The importance of TLR2 localization in the aorta specifically, can be exemplified by previous studies analyzing TLR2 expression on aortic endothelium in atherosclerosis (243). Earlier studies have demonstrated that laminar flow in the vessels is atheroprotective and reduces expression of TLR2. Interestingly, in atherosclerosis TLR2 can be detected on endothelial cells at sites of disturbed blood flow, and is also associated with atherosclerotic plaque formation and increased accumulation of intimal leukocytes (241, 243). Vessel branch points are common sites for atherosclerosis, and are also typical locations for observing disturbed blood flow. Various regions of the aorta can experience disturbed blood flow and the aorta is also where the CAs originate from, which are the common location for inflammation and aneurysm formation in KD. The CAs also experience the greatest pulse pressure when compared to all of the vessels within the body, making them unique such that due to their origin from the aorta and ability to experience high pressure and disturbed blood flow, it is likely that they upregulate TLR2 expression, similarly to the high basal expression levels within the aorta.

The findings thus far have strongly suggested that differential expression of TLR2 is a factor in guiding site-specific inflammation to the CAs. Therefore specifically studying the coronary arteries to determine their expression levels of TLR2 was necessary for further understanding the mechanism leading to site-specific inflammation in this model. Murine coronary vessels from young mice however, due to their small size, are difficult to visualize even with the aid of a dissecting microscope. Therefore we employed the use of a novel technique, laser capture microdissection, to isolate the coronary arteries alongside other medium sized vessels from saline-injected and LCWE-injected mice. Our initial data demonstrated an upregulation of TLR2 in the proximal CA compared to the other vessels, strongly implying that TLR2 may be one factor that induces the site-specific inflammation observed in disease. Pursuing this exciting preliminary data, however proved to be technically challenging thus the data has not been confirmed.

In studying KD, numerous pathogens have been detected in patients (Table 4), which further supports the hypothesis that the disease is initiated by an infectious trigger. However, no single agent has been repeatedly isolated to indicate which pathogen may be inducing disease. Interestingly, these pathogens share a common characteristic, which is the presence of superantigenic activity. Superantigens cause an exacerbated inflammatory immune response that can be observed in KD and have also been shown to be necessary for disease induction in mice (118, 127, 266). Furthermore, a number of these isolated pathogens also possess TLR2 activity.
Similarly to these other pathogens, LCWE, the agent used to induce coronary arteritis, has also been shown to possess superantigenic activity and contains a TLR2 ligand. This data may suggest that while a SAg may initiate the massive immune response seen in disease, TLR2 signaling may be one factor leading to the sustained inflammatory response. As the previous data has demonstrated that LCWE can also upregulate TLR2 expression (as indicated by upregulation in the heart and vessels), we were intrigued to search for other potential exogenous or endogenous ligands that may augment the immune response in disease. HSP60 seemed to be a good candidate as this endogenous danger signal can be released from inflamed tissues to induce further inflammation (230, 270) and was also suggested to signal via TLR2 (209, 229, 271), as was a peptide of HSP60 – p277 (262). However, through rigorous studies analyzing various time points and concentrations of the peptide p277 (Figure 16), as well as assessing HSP60 signaling, it was determined that HSP60 and p277 do not signal via TLR2 under our experimental conditions. This is in accordance with very recent literature that provides evidence that past observed TLR2 signaling was due to enterotoxin contaminantns in the HSP preparations (Section 1.8) (230).

Evidence is increasing that suggests a strong role for vessel inflammation during the initiation and maintenance of vascular diseases (28, 272, 273). Other findings indicate that vascular inflammation within the coronary arteries and other medium-sized vessels during the acute stages of KD can lead to endothelial dysfunction in patients suffering from chronic KD (48, 224). Furthermore, the endothelium has been reported to be activated in KD as well as in the animal model, as evidenced by upregulation of adhesion molecules, indicating that the endothelium may play an important role during the inflammatory process of disease. Interestingly it has also been found that following vascular injury, TLR2 expression was markedly increased, but that in TLR2-deficient mice the production of the pro-inflammatory cytokines, namely TNFα, IL-1β, IL-6 and MCP-1 was depressed (224). In approximately 25% of untreated cases of Kawasaki disease, the observed systemic inflammation targets the coronary vessels, where all layers including the endothelium undergo damage (14). Although various studies exist that examine the etiology and treatment of KD, an important yet unanswered question remains to be why the coronary vessels are the target of end organ damage in this disease. Although numerous factors must play a role in answering this question, one possible contributing factor may be TLR2 expression on the endothelium, since numerous vascular endothelial cell lines are rich in TLR2 expression, including human coronary arterial cells lines (224, 238, 239, 241, 243, 249, 250). In these studies we revealed an interesting feature of
differential expression of TLR2 in the animal model, thus strengthening the evidence that TLR2 is important for localized inflammation.

During a typical immune reaction the initial response involves recruiting inflammatory mediators to the endothelium, which has been shown to be activated during disease pathogenesis. Although numerous studies utilizing human endothelial cell lines have demonstrated that TLR2 expression can be detected intracellularly and expressed on the surface following stimulation, it is possible that a functional role exists for TLR2 on ECs, such that TLR2 may be one factor responsible for activating these cells by upregulating adhesion molecules and costimulatory ligands. Evidence of EC dysfunction in KD patients as well as activated endothelium in mice suggests that ECs are important for the development of disease, and as TLR2 KO mice are protected from coronary arteritis TLR2 is also important for this model. Thus we wanted to delineate the functional significance linking these two mediators of disease together. Thus we hypothesized that TLR2 signaling can transform endothelial cells into functional APCs via the upregulation of costimulatory molecules. As the proposed mechanism for rescue of the SAg-reactive T cells from apoptosis is enhanced costimulation, it is possible that the activated endothelium serves as the APC responsible for interacting with these SAg-activated T cells, leading to their survival. The ideal conditions would involve isolating coronary artery endothelium, however this is technically very difficult and as no murine coronary artery endothelial cell line exists to date, a microvascular endothelial cell line (EOMA) was used in these studies. All of the EOMA cells studied expressed high basal levels of TLR2, further providing evidence for TLR2’s involvement in localizing inflammation to the CA endothelium. TNFα is an important pro-inflammatory cytokine that has been shown to be necessary for disease as mice lacking TNFα signaling are protected from disease, and high quantities can be found to be localized to the CAs (30). TNFα may be involved in regulating local expression of TLR2 in this disease model, as the TLR2 promoter region contains an NF-κB binding sequence (190) and TNFα can lead to activation of NF-κB. TNFα was found to upregulate TLR2 expression on EOMA, suggesting that TNFα and TLR2 may work together within the model to enhance the inflammation at the CAs. Thus a microvascular endothelial cell line expresses high levels of TLR2, which can be further upregulated by the pro-inflammatory cytokine TNFα, indicating a potential positive feedback loop for TLR2 regulation in the LCWE murine model. Furthermore, these results demonstrate that endothelial cells express TLR2, and thus probably play a strong role in localizing inflammation to the CA endothelium.
The rescue phenomenon demonstrates that when SAg-activated T cells also receive stimulation by a peptide specific for those T cells, not all the T cells undergo apoptosis (147). Alternatively, studies from our lab as well as others have shown that SAg-activated T cells can be rescued from apoptosis as T cell proliferative responses were increased following enhanced costimulatory signaling via CD28 (177, 264, 274-276). A key component in optimal T cell activation is the expression of costimulatory ligands. Thus in further delineating the hypothesis that EOMA can serve as functional APCs, expression of costimulatory molecules on this endothelial cell line were assessed. TLR2 signaling can upregulate CD80/86 costimulatory ligands on splenocytes and other immune cells, as has been shown by our lab (125) as well as others (253, 277, 278). The CD28/CD80/86 costimulatory pathway is well studied in the literature, and studies from our lab utilizing this pathway and splenocytes have demonstrated the ‘rescue’ phenomenon, thus we were interested in analyzing CD80 and/or CD86 expression on EOMA. Therefore we cultured EOMA with or without the agonistic TLR2 ligand, Pam3Cys, and interestingly it was found that some basal CD80 expression could be detected, which was further upregulated following TLR2 stimulation; however CD86 expression was not detected either basally or after culture with the TLR2 ligand. It is known that CD80 and CD86 have largely overlapping functions (185), thus it is possible that only CD80 expression is needed for costimulatory signaling on this cell line. Therefore, TLR2 signaling activates EOMA by upregulating CD80 costimulatory molecule expression, potentially transforming these endothelial cells into functional APCs.

Since EOMA express high amounts of TLR2, signaling via TLR2 enhances the expression of CD80 costimulatory molecule expression and our studies have demonstrated that enhanced costimulation can lead to the survival of SAg-activated T cells, we hypothesized that TLR2-primed EOMA could function as APCs and provide enhanced costimulatory ligands to the SAg-reactive T cells, thereby allowing them to persist and target the CA. To test this hypothesis, various co-culture experiments were performed that allowed for the combination of isolated T cells and TLR2-primed or non-TLR2-primed EOMA cells. T cell proliferation could be detected maximally by day 4 post-mixing, a response which was SAg-specific as the EOMA/T cultures that did not receive SEB did not proliferate to the same capacity. These effects also required the presence of APCs as T cells cultured with SEB alone did not proliferate. Thus TLR2-primed EOMA can support the proliferation of SAg-activated T cells, an effect which requires the presence of both a SAg and an APC, and may be mediated via CD28-CD80 interactions for blocking this pathway decreased T cell proliferation. It is interesting to note that using CTLA4-
Ig to block the CD28/CD80 pathway did not completely abrogate T cell proliferation, suggesting that other costimulatory ligands may be expressed on this cell line and may have compensatory roles.

As TLR2-primed EOMA were shown to support the proliferation of SAg-activated T cells, we wanted to confirm whether or not EOMA’s ability to function as an APC extended to the rescue phenomenon such that co-culturing TLR2-primed EOMA with SAg-activated T cells would lead to enhanced survival. Thus as a proof-of-principle to demonstrate that ECs can function as APCs, cultured EOMA were either primed with a TLR2 ligand (Pam3Cys) or left unprimed prior to having isolated T cells added to culture in the presence or absence of SEB. SEB was used for these studies because it is a prototypic SAg, and as LCWE contains superantigenic activity, the same principles can thus be applied. In this survival assay and according to our method for measuring survival, an increased number of live SAg-reactive T cells were detected when cultured with TLR2-primed EOMA versus the control cultures utilizing non-TLR2-primed EOMA. This enhanced survival could be observed as early as 3 days post-SEB stimulation and was maximal at day 7, in accordance with others’ observations using splenocytes from the laboratory. This finding was also confirmed by utilizing the BrdU proliferative assay, which indicated that Vβ8+ T cells cultured with TLR2-primed EOMA and SEB proliferated to a greater capacity than when combined with non-TLR2-primed EOMA, with maximal proliferation also occurring on day 7, although this was not statistically significant. Therefore TLR2 signaling leads to upregulation of CD80 costimulatory ligand expression on EOMA, which transforms the EOMA cells into functional APCs capable of enhancing the survival of SAg-reactive T cells. Thus via this proposed mechanism, it is possible that localized TLR2 plays a large role in the rescue phenomenon allowing for pathogenic T cells to persist and initiate further inflammatory damage at the CA endothelium.
Limitations of Study and Future Directions

This project assessed the role of TLR2 in the pathogenesis of LCWE-induced coronary arteritis, and analyzed various factors that can regulate TLR2; however various limitations for some studies existed. The transfection assays concerning hHSP60 and its peptide, p277, posed many difficulties. Reports in the literature suggested that serum present in the media could potentially sequester HSP60 from binding to its necessary receptors, therefore cells should be serum-starved as a preventive measure; however we found that if HEK cells were cultured in anything less than 1% FBS DMEM, the cells would no longer adhere to the flask and apoptose. Culturing the cells in 1% FBS DMEM proved to be difficult at times, as the cells were very easily detached from the flasks. Furthermore, as the peptide from HSP60 was able to induce NF-kB activity within 48 hours following stimulation, it is possible that there are other receptors on HEK cells that lead to downstream activation of this transcription factor. Under our experimental conditions, and in accordance with recent literature, the endogenous ligand HSP60 does not signal via TLR2. Thus as TLR2 clearly plays an important role in pathogenesis, alternate exogenous and endogenous ligands for TLR2 should be investigated regarding their abilities to regulate TLR2 in disease.

In utilizing LCM, many limitations of study existed including the astronomical cost of the technique and the reagents required to perform the RNA extractions, amplifications, and cDNA conversions. As the vessels themselves are very small, numerous vessel sections were obtained and pooled together for each artery; however the RNA quantities being utilized were still quite minute and posed many challenges for performing qRT-PCR successfully. Therefore, to rectify this difficulty in the future, if LCM becomes more cost effective, more sections from each vessel should be obtained to ensure the quantity of isolated RNA is sufficient to perform the appropriate techniques. As the larger vessels tested were rich in TLR2 mRNA, future studies can also be performed to determine protein levels of TLR2 by performing en face immunostaining on specific tissue sections and analyzing expression via confocal microscopy. En face immunostaining allows for the entire endothelium of a vessel to be exposed and stained so large surfaces can be examined for protein expression. Staining the endothelium for both TLR2 and PECAM-1, an endothelial cell marker, would potentially demonstrate at the protein level where specifically on the endothelium TLR2 can be seen to localize.

Conducting the \(^3\)H-Thymidine proliferative assay required much optimization and presented some technical challenges as the initial culture conditions required 6-well plates and
thymidine proliferation assays utilize 96-well plates. The issues were resolved by centrifuging the supernatants prior to harvesting and transferring them to 96-well plates; however multiple steps were added to this assay therefore increasing the manipulation of the samples and possibly the chances of contamination. In the future, this assay should be repeated for later time points that include days 5 and 7, as day 7 is when there is maximal enhanced survival and proliferation of these specific cells following SAg stimulation.

The typical rescue assay performed in our laboratory utilizes splenocytes, which can be easily obtained in high numbers. However, modifying this assay to culture EOMA and T cells provided initial technical challenges for it proved to be difficult to obtain enough live T cells to continue with the experiment. This issue was resolved by culturing multiple plates for each time point and assay and pooling the wells for one condition together, which increased the live cell count, enabling the experiment to proceed forward to flow cytometric analysis. In the future, to confirm that EOMA require expression of costimulatory molecules to function as professional APCs, rescue assays can be performed where CTLA4-Ig is added to cultures and analyses can be compared between conditions with enhanced CD80 expression and those with dampened CD80 expression. As a result, addition of CTLA4-Ig should lead to reduced T cell survival as any CD80-CD28 interactions would be blocked. Furthermore, supernatants from the rescue assays can be harvested and utilized for ELISAs to look for various pro-inflammatory cytokines, including TNFα to analyze if the rescued T cells are functional.

Although the data presented provide a better understanding for TLR2 involvement in disease, the findings are limited as majority of the data arose from in vitro experiments. However through the use of bone marrow chimeras and site-specific knock out mice, new and exciting routes can be explored to further understand this disease using in vivo studies. To assess which compartment – endothelial or immune cells – is important in mediating disease, bone marrow chimeras (BMC) can be utilized. Irradiated WT mice would be reconstituted with TLR2 ko immune cells, therefore these mice would only express TLR2 on the endothelium. Contrarily, irradiated TLR2 ko mice would be reconstituted with WT immune cells, thus these mice would only express TLR2 on their immune cells. Each mouse would be challenged with LCWE and observed for development of coronary arteritis. This experiment would aim to identify which compartment, endothelial versus immune cell, is the major contributor to disease development. If the endothelial cell compartment is responsible for providing the TLR2 involved in disease, we would hypothesize that the mice reconstituted with TLR2 ko immune cells would develop coronary inflammation. An alternate in vivo study would be to use a site specific knockout
mouse that lacked TLR2 signaling on its endothelium and inject these mice with LCWE to see if they develop disease. Vascular endothelial cadherin (VeCad) is a calcium-dependent cell-cell adhesion glycoprotein expressed in vascular endothelial cells, that has been shown to be required for proper vascular development and integrity (279). The adaptor protein, MyD88, is downstream of and required for TLR2 signaling. Therefore, these two proteins and the CreLox system can be utilized to specifically delete MyD88 from all cells expressing VeCad (endothelial cells), providing a situation where TLR2 cannot signal on ECs. Cre is a site-specific DNA recombinase that catalyzes site-specific recombination of a DNA sequence located between two loxP sites. The LoxP recognition element is a 34 base pair sequence that is comprised of two 13 base pair palindromic repeats flanking an eight base pair spacer region that determines directionality. When the DNA sequence between the two loxP sites is in the same orientation, the DNA sequence (or gene of interest) is excised in circular form from the DNA. Under our experimental conditions, two transgenic mice would be used: VeCad cre/cre mice will express cre under the control of the VeCad promoter and MyD88 flox/flox mice will have the MyD88 gene floxed by two loxP sites. Breeding these mice will allow for Cre to delete MyD88 from endothelial cells. It would be hypothesized that if endothelial TLR2 is involved in disease, then the VeCad-cre/MyD88-loxP mouse would be protected from disease. Thus utilizing this system and injecting these mice with LCWE to induce disease would allow for the in vivo approach of determining the functional relevance of TLR2 on the coronary artery endothelium.
9 Conclusion and Disease model

It has previously been shown that TLR2 is necessary for the development of coronary arteritis, as a lack of TLR2 completely prevented disease in mice. LCWE possesses a TLR2 ligand, which is similar to many other pathogens that have been implicated in KD that have a SAg as well as a TLR2 ligand (Tables 1 and 4). Furthermore, human and murine vascular endothelial cell lines are rich in TLR2. Taken together this provides supportive evidence that TLR2 may have a role in directing site-specific inflammation to the coronary endothelium in KD.

In this thesis, we further characterized factors that regulate TLR2, and the functional role TLR2 may play in disease. It was shown that TLR2 is highly upregulated early in disease pathogenesis, implying that TLR2 is involved in the inciting inflammatory reactions. In addition, TLR2 is preferentially expressed in the heart and in the aorta, lending support to the fact that TLR2 is involved in directing inflammation to the aorta and CAs in disease. Lastly, microvascular ECs that are enriched with TLR2, were activated with a TLR2 agonist, which lead to the upregulation of CD80 costimulatory ligand and thus transformed the ECs into functional APCs. Not only did TLR2 signaling change the ECs into APCs, but they were functional and capable of supporting proliferation of SEB-reactive T cells, which were also shown to be rescued from cell death, thus allowing these SAg-reactive T cells to persist as indicated by the disease model (Figure 23). Therefore, localized TLR2 expression transforms ECs into functional APCs and can lead to site specific inflammation at the CAs.

Kawasaki disease from a clinical perspective has been well documented; however the question at large is why is there specific end organ damage – a question that can be applied to other organ-specific autoimmune diseases. Although there are a plethora of factors that may contribute to the site-specific inflammation seen in KD, the answer may involve TLR2 acting as a localization factor, as much evidence indicates its importance in this inflammatory disease. This knowledge can be applied to other autoimmune and inflammatory diseases, as TLR2 has been implicated in rheumatoid arthritis, type I diabetes, inflammatory bowel disease and psoriasis. Therefore, understanding that differential expression of an innate immune receptor (TLR2) can be one factor leading to organ-specific inflammation, indicating how both the innate and adaptive immune systems can be activated in one disease, can be very beneficial for numerous pathologies involving TLR2.
Figure 23: Disease Model. Superantigenic activity present within LCWE induces a substantial immune response leading to the production of proinflammatory cytokines, including TNFα, as well as massive proliferation of specific Vβ T cells. LCWE contains a TLR2 ligand, which can bind to TLR2 receptors present on the CA endothelium and lead to upregulation of B7.1 ligands on this tissue. TNFα signaling can also upregulate TLR2 on the endothelium, providing more chances for downstream signaling via TLR2. Signaling via TLR2 transforms the CA endothelium into antigen presenting cells that provide enhanced costimulatory signals to the SAg-reactive T cells rescuing them from undergoing apoptosis. This rescued population of T cells targets the CA inducing persistent inflammation and elastin breakdown leading to the formation of CA aneurysms, similar to what is seen in children who suffer from Kawasaki disease.
Contributions

Figure 5: Experiments performed in conjunction with Ken Little.
References


