Costimulation-Mediated Rescue of Superantigen-Activated T cells in an Animal Model of Kawasaki Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Abstract

*Lactobacillus casei* cell wall extract (LCWE)- induced coronary arteritis in mice models Kawasaki disease (KD). LCWE injections consist of T-cell dependent factors that expand superantigen (SAg)-activated T-cell receptor (TCR) $\beta^6$ cells, and T-cell independent factors (i.e. TLR2 activity) that localize and sustain the immune response. TLR2 can upregulate costimulatory molecules to rescue SAg-activated T-cells from apoptosis. Accordingly, SAg-activated costimulation-rescued TCR$\beta^6$ cells are predicted to express activation markers, produce cytokines and be able to induce coronary arteritis. MAM was identified as a SAg able to activate TCR$\beta^6$ cells in a manner similar to LCWE; however a combination of MAM and TLR2 agonist Pam3Cys could not induce coronary arteritis. As another marker of disease, leukocyte recruitment molecule expression in the hearts of MAM+Pam3Cys- injected mice was found to be lower than in LCWE- injected mice. Therefore, LCWE contains unique features beyond TCR$\beta^6$ stimulation and TLR2 activity that are important for disease induction.
Acknowledgments

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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Bad</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>Caspases</td>
<td>Cysteine proteases that cleave proteins immediately after aspartic acid residues</td>
</tr>
<tr>
<td>Cbl-b</td>
<td>Casitas B-lineage lymphoma B</td>
</tr>
<tr>
<td>CCL3</td>
<td>Chemokine ligand 3</td>
</tr>
<tr>
<td>CCL5</td>
<td>Chemokine ligand 5</td>
</tr>
<tr>
<td>CD28RE</td>
<td>CD28-responsive element</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity determining region</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CLIP</td>
<td>MHC class II-associated invariant chain peptide</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EVG</td>
<td>Elastic-van Gieson</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>Foxp3</td>
<td>Forkhead box p3</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronic acid</td>
</tr>
<tr>
<td>HEV</td>
<td>High endothelial venules</td>
</tr>
<tr>
<td>IAPs</td>
<td>Inhibitors of apoptosis proteins</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>ITAMs</td>
<td>Immunoreceptor-based tyrosine activation motifs</td>
</tr>
<tr>
<td>iTreg</td>
<td>Inducible T regulatory cell</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IVlg</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>JPS</td>
<td>Juvenile polyarteritis syndrome</td>
</tr>
<tr>
<td>KD</td>
<td>Kawasaki disease</td>
</tr>
<tr>
<td>LCWE</td>
<td>Lactobacillus casei cell wall extract</td>
</tr>
<tr>
<td>LRR</td>
<td>Leucine-rich-repeat</td>
</tr>
<tr>
<td>MAM</td>
<td>Mycoplasma arthritidis mitogen</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>Mls</td>
<td>Minor lymphocyte stimulating</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Matrix metalloproteinase-9</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mammary tumor virus</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>nTreg</td>
<td>Naturally occurring T regulatory cell</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>Pam3Cys-Ser-(Lys)4, Hydrochloride</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pcytC</td>
<td>Pigeon cytochrome c peptide</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon Activation, Normal T-cell Expressed, and Secreted</td>
</tr>
<tr>
<td>RORα</td>
<td>Retinoid-related orphan receptor α</td>
</tr>
<tr>
<td>RORγt</td>
<td>Retinoid-related orphan receptor γt</td>
</tr>
<tr>
<td>SAg</td>
<td>Superantigen</td>
</tr>
<tr>
<td>SE</td>
<td>Staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>Smac/Diablo</td>
<td>Second mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>Sme</td>
<td>Streptococcal mitogenic exotoxin</td>
</tr>
<tr>
<td>Spe</td>
<td>Streptococcal pyogenic exotoxin</td>
</tr>
<tr>
<td>SSA</td>
<td>Streptococcal superantigen</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-β</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T helper 2</td>
</tr>
<tr>
<td>Th17</td>
<td>T helper 17</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNF-β</td>
<td>Tumor necrosis factor-β (also known as lymphotoxin)</td>
</tr>
<tr>
<td>TNFL</td>
<td>Tumor necrosis factor ligand</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic shock syndrome toxin-1</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>Zap-70</td>
<td>ζ- associated protein of 70kDa</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Kawasaki Disease

1.1.1 History of Kawasaki Disease

In Japan in 1967, Dr. Tomisaku Kawasaki identified an acute condition that presented with fever, swollen cervical lymph nodes, rash and changes in the mucous membrane of the mouth and lips in young children (1). Although Dr. Kawasaki named this disease mucocutaneous lymph node syndrome, it was later named after him as Kawasaki disease (KD) (1). Dr. Kawasaki followed up his initial report of the disease with a description of clinical and epidemiological characteristics including autopsy findings of coronary arteritis, coronary thrombosis and coronary aneurysms from patients who died suddenly of cardiac failure (2). Deaths occurred in approximately 1-2% of KD patients. Similar cardiac changes were observed in some surviving KD patients.

1.1.2 Epidemiological and Clinical Features of Kawasaki Disease

Vasculitis, particularly inflammation of the small- and medium-sized blood vessels in the body, is the main feature of KD. This inflammation is acute and self-resolves; however, persistent inflammation and end organ damage may be observed in the coronary arteries. Coronary artery aneurysms may appear in about 5% of treated patients and may be complicated by myocardial infarction, ischemic heart disease or sudden death (3). When adjusted for body surface area, coronary artery lesions are found to develop in up to 20-30% of treated patients (3). Thus, KD has become the leading cause of acquired heart disease in children of the developed world (4). In Canada, the annual incidence of KD is on an increasing trend and estimated to be 26.6 per 100 000 children under the age of 5 (5). In contrast, the annual incidence of KD in Japan— which is also on an increasing trend— is 216.7 cases per 100 000 children aged 0-4 years (6). Since the etiology is unknown, KD continues to be diagnosed clinically by a prolonged fever (for 5 or
more days) along with 4 out of the following 5 criteria: polymorphous skin rash, nonpurulent conjunctivitis, oral mucosal changes, extremity changes and cervical lymphadenopathy (7). KD patients normally receive a combined treatment of high-dose aspirin and IVIg (intravenous immunoglobulin); however, the mechanism of action of these drugs is still unknown (7-9).

1.1.3 Etiology of Kawasaki Disease

The etiology of KD is still unknown; however, there are many features of KD that point to the participation of an infectious agent. For example, there is a baseline rate of disease with outbreaks every 2-3 years and most cases occur during late winter/early spring. Furthermore, cases that have similar clinical presentation tend to occur close in time together. Not surprisingly, many of the symptoms experienced by KD patients resemble those of patients with bacterial or viral infections, including signs of inflammation (such as heat, swelling, redness and pain). There have been a range of viral and bacterial factors that have been linked to KD but efforts to identify a single factor that can be definitely linked to the cause of this disease have not been promising. An etiological theory that could explain the variety of bacterial and viral agents that have been isolated from KD patients is a superantigen (SAg) mediated one. This theory is particularly convincing because superantigenic activity is a property that can be found in many different infectious agents (10).

1.2 Superantigens

SAgs can be divided into two main groups: foreign SAgs and self SAgs (11). Self SAgs are known as minor lymphocyte stimulating (Mls) antigens and are encoded by mouse mammary tumor virus (MMTV) genes that have been integrated into the genome of infected mice (12). On the other hand, foreign SAgs are exotoxins that are produced and secreted by various microbes such as gram positive and gram negative bacteria, viruses and mycoplasma (13).
1.2.1 Properties of Superantigens

SAgs differ significantly from conventional peptide antigens (Ags) (Figure 1). Conventional Ags are normally processed by the Ag presenting cell (APC) into peptide fragments that are then loaded into the peptide binding groove of a major histocompatibility complex (MHC) class II molecule and presented to the T cell. Presentation of a conventional Ag is classically restricted to self MHC and is a very specific interaction between an APC and a T cell—only a small proportion (about 1 in $10^4$ to 1 in $10^6$) of the host repertoire of T cells will be activated by a conventional Ag (14).

SAgs on the other hand are unique in their ability to interact as an intact protein with the MHC class II molecule on the APC and the Vβ domain of the T cell receptor (TCR) (13, 15). This interaction takes place outside of the peptide binding groove (16). Presentation of a SAg by the APC is not classically restricted to self MHC and there is a hierarchy in the efficiency of different MHC molecules to present SAgs (16, 17). Another property that results from this unique interaction is that SAgs have the ability to cause marked proliferation of naïve T cells (activating up to 1 in 5 host T cells) (14). SAgs can elicit such a strong proliferative response because there is a higher precursor frequency of responding T cells. This is due to the fact that mammals only have a total of about 20-50 TCR Vβ families and a single SAg may have the ability to interact with a number of different Vβ families (14). The repertoire of T cells that are exposed to a SAg display a characteristic TCR Vβ skewing whereby T cells containing responsive TCR Vβ families become overrepresented due to their activation and subsequent proliferation (13). Shortly following activation, the responding T cells can become apoptotic and a rapid disappearance of T cells containing reactive TCR Vβ families is observed (18, 19).
1.2.2 Bacterial Superantigens

The most well characterized SAgs are secreted by the bacteria *Staphylococcus aureus* and *Streptococcus pyogenes*. These bacteria have been implicated in many human diseases including food poisoning, toxic shock syndrome, necrotizing fasciitis (flesh-eating disease) and KD.

1.2.3 Bacterial Superantigens and Kawasaki Disease

The ability of SAgs to induce massive immune activation is hypothesized to contribute to the development of KD. Consistent with this hypothesis, there are several lines of evidence that implicate SAgs in the pathogenesis of KD. Firstly, investigators have found SAg-producing bacteria in KD patients. Clonally derived TSST producing strains of *Staphylococcus aureus* and

![Figure 1. Presentation by an APC to a T cell of a conventional Ag compared to a SAg.](image-url)

A conventional peptide Ag (left) is contained within the peptide-binding groove of an MHC molecule of an APC. The conventional Ag is recognized by the peptide binding groove that is formed by the hypervariable regions (in the variable regions (V) of both α and β chains) of the TCR. A SAg (right) usually binds outside of the peptide-binding groove to the variable region of the β chain on T cells.
SPEB/SPEC producing strains of *Streptococcal pyogenes* were isolated from a significantly higher percentage of cultures from untreated KD patients than from febrile patient controls (20). Secondly, investigators have extracted total DNA from stool samples and performed polymerase chain reaction (PCR) for multiple bacterial SAg genes. A significantly higher percentage of stool samples from KD patients (when compared to febrile patient controls and healthy children controls) contained more than 1 or more than 2 SAg genes (21). Thirdly, significantly elevated levels of TCRVβ2⁺ T cells have been repeatedly detected in the peripheral blood of KD patients compared to control groups (22-25). In addition, selective expansion of TCRVβ2⁺ T cells was found in the myocardium and coronary artery of one patient that had died of acute KD (26). Consistent with superantigenic activity, expansion of TCRVβ2⁺ T cells in the peripheral blood and the heart was polyclonal (23, 24, 26). Lastly, studies have found serum levels of antibodies against various SAgs of *Staphylococcus aureus* and *Streptococcal pyogenes* to be higher in KD patients compared to control groups (25, 27).

### 1.2.4 Superantigens from *Staphylococcus aureus* and *Streptococcus pyogenes*

*Staphylococcus aureus* and *Streptococcus pyogenes* exotoxins share common phylogeny, structure, function and sequence homology. They are generally intermediate sized, basic proteins that range from 20-30 kilodaltons (kd). The enterotoxins secreted by *Staphylococcus aureus* include staphylococcal enterotoxin (SE) A, B, C1, C2, C3, D and E. These SEs can be further divided into two main groups based on their structural homology. The first group consists of SEB, SEC1 and SEC3, and the second group consists of SEA, SED and SEE. Toxic shock syndrome toxin (TSST) is another enterotoxin secreted by *Staphylococcus aureus* but is the least related to the other SEs. The pyrogenic toxins secreted by the Group A Streptococci include streptococcal pyrogenic exotoxin (SPE) A, B, C and D. SPEA has been shown to be
phylogenetically most similar to the group of staphylococcal toxins SEB and SEC. On the other hand, SPEC is more similar to the group of staphylococcal toxins SEA, SED and SEE.

Different SAgs vary in the way that they interact with MHC molecules on the APC. The MHC molecule contains two sites that can bind to SAgs. One is the Zn$^{+}$ dependent, high affinity binding site located on MHC β chain and the other is a low affinity binding site located on the MHC α chain. In addition to binding to the MHC molecule in different ways, some SAgs may also interact with the bound peptide in the antigen groove of the MHC molecule. For example, SEA, SEB, SEC3 and TSST-1 have all been shown to bind to the MHC α1 domain; however, only TSST-1 has been shown to interact with the C-terminal of the bound peptide. Some SAgs, such as SEA, have been shown to interact with binding sites on both the α and β chains of the MHC molecule leading to the dimerization of MHC molecules. SEA has been shown to interact through its COOH terminal with the β chain of the MHC molecule and through its NH$_2$ terminal with the α chain of the MHC molecule (28). Furthermore, it has been shown that the cross-linking of two MHC molecules by one molecule of SEA is necessary for the gene expression of inflammatory cytokines such as interleukin 1β (IL-1β) and tumor necrosis factor-α (TNF-α) from APCs (in this case, a human monocytic cell line) (29, 30). It was shown that mutant SEA that could not bind to the α or the β chains of the MHC molecule could not induce gene expression of these inflammatory cytokines. However, the addition of F(ab)$_2$ antiSEA mAb, that crosslinked MHC molecule-bound mutant SEA, allowed for the gene expression of these inflammatory cytokines (29).

1.2.5 Superantigen from *Mycoplasma arthritidis*: MAM

*Mycoplasma arthritidis* mitogen (MAM) is a SAg produced by *Mycoplasma arthritidis*. MAM causes a chronic arthritis-like disease in some genetic backgrounds of rodents. It is often used to
model human rheumatoid arthritis (RA). Nucleotide and amino acid sequence analyses demonstrate that MAM shares much less phylogenetic and structural relatedness to the other staphylococcal and pyrogenic SAgs; however, there are some short sequences of homology found between MAM and other SAgs. These similarities between MAM and other SAgs may reflect common SAg functional domains that are required for binding to the TCR or to MHC class II molecules. MAM has many of the features of a classical SAg such as the ability to activate large proportions of T cells, TCR Vβ restricted expansion of T cells and requirement of APC presentation but not processing (31).

Similar to the SAgs from *Staphylococcus aureus* and *Streptococcus pyogenes*, MAM is proposed to bind to the MHC α chain, to the bound peptide and to a smaller degree the MHC β chain (32). It was demonstrated that the N terminus of MAM mediates interactions with human MHC class II molecules and that this interaction could be inhibited by the presence of the MHC class II-associated invariant chain peptide (CLIP) in the peptide binding groove of the MHC class II molecule. Inhibition of MAM/MHC class II interactions could be reverted by the expression of HLA-DM molecules which suggests that the MAM/MHC class II interaction may be influenced by the identity of the bound peptide or the stability of the MHC class II molecule (33). Using wild-type and truncated forms of MAM, it was demonstrated that the C terminus of MAM is required for T cell activation. MAM interacts mainly with the β chain of the TCR and studies suggest that both the C and N terminals of MAM make contact with the TCR (32, 33).

In addition, like SEA, it has been shown that MAM can dimerize MHC molecules. An asymmetrical MAM homodimer may bring together two TCR and two MHC molecules to form a TCR$_2$MAM$_2$MHC$_2$ complex (32). Dimerization of two MAM molecules involves interactions between the C terminal of one MAM molecule and both the C and N terminal of the second
MAM molecule; this dimerization may depend on the presence of a zinc ion (32). In a slightly different study, it was suggested that dimerization of MAM and MHC molecules may be mediated through a MHC molecule dimer instead. The study looked at the interaction between MAM, and the class II MHC molecule HLA-DR1 complexed with a hemagglutinin peptide (HA). It was demonstrated that Zn\(^{2+}\) was important for the formation of HLA-DR1/HA dimers, but was not important for the interaction between MAM and HLA-DR1/HA nor the formation of MAM dimers (34). These results suggest that the formation of MAM-HLA-DR1/HA dimers was mediated by Zn\(^{2+}\) dependent formation of HLA-DR1/HA dimers (35). In addition, it was found that dimerization of HLA-DR1 facilitated binding to MAM and that there was cooperative binding of MAM to the HLA-DR1 dimer (35).

On the other hand, MAM differs from classical SAgs because MAM not only binds to the V\(\beta\) region but also with the CDR3 (Complementarity determining region 3) of the \(\beta\) chain of the TCR (36). Interaction with CDR3 is a characteristic feature of a peptide antigen that is bound inside the antigen groove of the MHC molecule and not of a SAg. Therefore, MAM may belong to a family of proteins that is different from both conventional peptide Ags and from SAgs. Furthermore, MAM has a novel fold that consists of two \(\alpha\)-helical domains. This is in contrast to pyrogenic toxins that share a very similar three-dimensional structure consisting of a \(\beta\)-grasped motif and a \(\beta\) barrel; however, despite the structural differences between MAM and the pyrogenic toxins, it is interesting to note that the sites on the MHC class II molecule that interact with MAM are very similar to the ones that interact with the pyrogenic toxins (in particular TSST-1). Lastly, MAM has been reported to have TLR2 activity (refer to section 1.8.1).
1.2.6 TCR V\(\beta\) Expansion by Superantigens

Each SAg has a characteristic TCR V\(\beta\) profile that it can activate. Among the mentioned SAgs, there are some that are better characterized and studied than others. Some of the SAgs that are the best characterized and relevant to this study include TSST-1, SEB and MAM. TSST-1 is reactive with TCR V\(\beta\) 3, 15 and 17 in mice, and TCR V\(\beta\) 2 in humans. MAM possesses the ability to skew TCR V\(\beta\)5.1, 6 and 8 in mice, and TCR V\(\beta\)3, 14 and 17 in humans. SEB is the best-studied prototypical SAg. In mice, SEB is reactive with TCR V\(\beta\)3, 7, 8.1, 8.2, 8.3 and 17 (37, 38). In humans, SEB is reactive with TCR V\(\beta\)3, 12, 14 and 17 (39).

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Organism</th>
<th>V(\beta) specificity</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
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<td>Mouse</td>
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<tr>
<td>SEA</td>
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</tr>
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</tr>
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<tr>
<td>MAM</td>
<td><em>M. arthritidis</em></td>
<td>3, 14, 17</td>
<td>5.1, 6, 8.1, 8.2, 8.3</td>
</tr>
</tbody>
</table>

Table 1. TCRV\(\beta\) specificity of SAgs that have been characterized so far in mice and humans
1.3 Animal Models of Kawasaki Disease

Animal models provide an opportunity for investigators to study the pathogenesis of KD and to develop different therapeutic strategies. Animal models are particularly useful because human samples are not readily available and difficult to obtain for ethical reasons. Various animal models have been developed to study KD including canine, swine, rabbit and mouse models.

Felsburg et al. describes a spontaneous and naturally occurring canine disease which models KD (45). Canine juvenile polyarteritis syndrome (JPS) is a systemic necrotizing vasculitis that primarily affects the small and medium-sized arteries particularly the coronary arteries (45, 46). The immunological abnormalities found in this disease are similar to those seen in patients during the acute stage of KD (45). A rabbit model of KD was developed by Onouchi et al. whereby rabbits received intravenous (IV) injections of horse serum to induce serum sickness. Following the injections, both weanling and mature rabbits developed coronary arteritis as evidenced by histological changes that resemble those seen in KD. However, coronary artery aneurysm formation was only found in weanling rabbits (47). In another model, repeated injections of horse serum into piglets have also been shown to induce several symptoms consistent with KD including skin rashes and coronary arteritis (48).

Several coronary arteritis mouse models have been developed to study KD. In one such model, mice were injected intraperitoneal (IP) multiple times with Candida albicans extract resulting in the development of a localized coronary arteritis (49, 50). The extract was prepared from Candida albicans yeast cells of the MCLS-2 strain which were isolated from the feces of KD patients (49). In an alternate mouse model of KD, coronary arteritis was induced by repeated IV injections of cell wall PGL from Streptococcus pyogenes. Diffuse cellular infiltrate in the vascular wall and perivascular space were observed as well as fragmentation and degeneration of
elastic fibers (51, 52). Lastly, IP injections of \textit{Lactobacillus casei} cell wall extract (LCWE) has been shown to induce coronary arteritis in mice.

1.3.1 LCWE- Induced Coronary Arteritis as a Model of Kawasaki Disease

When Group B LCWE is injected IP into inbred mouse strains, the development of coronary arteritis can be observed (Figure 2). This mouse model was first developed in 1985 by Lehman \textit{et al.} (53). \textit{Lactobacillus casei} is part of the normal gut flora in both humans and rodents, and shares some similarities to Group A \textit{Streptococcal pyogenes}. These similarities include high amounts of rhamnose in the cell wall and resistance to breakdown by lysozymes. The disease that is induced in mice resembles KD in many ways including selective development in young mice, occurrence of a persistent immune response at the coronary arteries and effectiveness of IVIg treatment (53, 54). An accumulation of mononuclear inflammatory infiltrate in the adventitia can be observed as early as 3 days post injection of LCWE. A predominantly mononuclear, focal and asymmetric invasion of the vessel wall develops by day 14 which progresses to a circumferential and maximal infiltration by day 28. Marked proliferation of the intima and media along with narrowing or complete obstruction of the vascular lumen can also be observed by day 28 (53, 55). With elastic-van Gieson (EVG) staining of the coronary arteries, degradation and loss of elastin—which is a precursor of aneurysm formation—can be detected by day 42 (55). Interestingly, when LCWE is injected into rats, the development of arthritis is observed and not coronary arteritis (56, 57).

LCWE has been shown to possess superantigenic activity which has been shown to be critical for disease induction. LCWE exhibits many of the hallmark properties of a SAg. First, LCWE induces massive dose-dependent activation of naïve T cells. Similar to the kinetic response of T
cells to a SAg, the massive response of T cells to LCWE is followed by a rapid decline to below baseline levels. Secondly, the kinetics of cytokine production (such as the production of TNF-\(\alpha\)) induced by LCWE is reflective of that induced by a prototypical SAg like SEB. Both SEB and LCWE illicit a biphasic pattern of TNF-\(\alpha\) production in mouse T cells, with peak production at 1.5h and 18h post-stimulation. In addition, there is a hierarchy in the ability of different MHC class II molecules to present LCWE. Ag presentation but not processing by the APC is required for LCWE-mediated responses and this response is non-classically MHC class II restricted. Furthermore, different MHC class II isotypes have different efficiencies in presenting SAg to T cells. The hierarchy in presentation of LCWE is demonstrated by the following: LCWE elicits a stronger proliferative response in splenocytes from DQ6 transgenic mice (which express human MHC class II DQ6 transgenes) compared to splenocytes from B6 mice, and LCWE elicits a stronger proliferative response in splenocytes from B6 mice compared to splenocytes from BALB/C mice. This hierarchy of MHC presentation is consistent with what is known, which is that human MHC is better at presentation than murine I-E (B6 mice) which is better at presentation than murine I-A (BALB/C mice). Requirement of presentation by LCWE was demonstrated by showing that purified T cells alone, irradiated APCs alone, or purified T cells+APCs from MHC class II-deficient mice did not proliferate in response to LCWE; however purified T cells+ irradiated APCs did. This presentation of LCWE did not require processing because purified T cells+ fixed APCs were able to proliferate in response to LCWE. Non-classically MHC class II restricted presentation of LCWE was demonstrated by mixed lymphocyte reactions that occurred in cultures with purified T cells from DQ6 mice and non-autologous APC from BALB/C mice. Lastly, the same TCR V\(\beta\) skewing was observed in T cell populations from different strains of mice exposed to LCWE, with preferential activation of TCR V\(\beta\) 2, 4, 6 and 14. Treatment of mice with LCWE leads to over-representation of the LCWE-
reactive T cell populations followed by a decline back to and then below baseline levels (as the LCWE-reactive populations are deleted). Therefore, LCWE possesses superantigenic activity. More importantly, as mentioned previously, this superantigenic activity has been found to correlate with the ability of different LCWE preparations to induce coronary arteritis in mice (58). Different LCWE preparations have been found to elicit varying degrees of T cell proliferation and cytokine production. For example, LCWE preparations that induce stronger \textit{in vitro} T cell proliferation and higher \textit{in vivo} production levels of TNF-\(\alpha\) were able to induce a greater inflammatory response in the coronary arteries of injected mice (58).

1.3.2 The Role of Cytokines in the LCWE-Induced Coronary Arteritis Model of Kawasaki Disease

Upon activation, T cells are known to produce a variety of proinflammatory cytokines. In KD, proinflammatory cytokines such as interferon-\(\gamma\) (IFN-\(\gamma\)), TNF-\(\alpha\), IL-1 and IL-6 are elevated and infiltrating T cells have been found in coronary artery lesions. As a result, the role of T cells and a variety of proinflammatory cytokines in our animal model has been studied. In our animal model, T cells infiltrating the hearts of diseased mice have been characterized and found to be predominantly TCR V\(\beta 6^+\). TCR V\(\beta 6\) is one of the LCWE–reactive T cell populations. In addition, a variety of proinflammatory cytokines have been examined in our animal model and two in particular have been found to be of interest: TNF-\(\alpha\) and IFN-\(\gamma\).

In the peripheral immune system, TNF-\(\alpha\) levels were increased shortly after disease induction (59). In the heart, TNF-\(\alpha\) levels were highest at day 28 which coincided with the time of maximal infiltrate at the coronary arteries (59). Interestingly, mice treated with a TNF-\(\alpha\)-blocking agent, etanercept, or tumor necrosis factor receptor (TNFR) I knockout mice were resistant to the development of coronary arteritis and elastin breakdown (59). Consistent with
these findings, TNF-α induces vascular smooth muscle cells to produce matrix metalloproteinase-9 (MMP-9) whose activity has been found to be responsible for elastin breakdown during disease development (55). In contrast, although IFN-γ was upregulated in the heart post LCWE injection, it was not necessary for the development of coronary arteritis; IFN-γ-deficient mice still developed coronary arteritis (60).

Figure 2. Timeline of LCWE-induced coronary arteritis in mice. When mice are injected IP with LCWE, systemic immune activation and cytokine release can be detected within hours. Infiltration at the heart can be observed as early as 3 days post-injection and maximizes 28 days post-injection. Inflammation at the heart continues with elastin breakdown observed at 42 days post-injection and infiltrates still detectable at 60 days post-injection.
1.4 T Lymphocytes

1.4.1 T Cell Receptor

The TCR is a disulfide-linked heterodimer composed of an alpha (\(\alpha\)) and a beta (\(\beta\)) chain (61) (Figure 3). Each chain consists of two large extracellular Ig-like domains, one constant domain and one variable domain. The variable domains of the \(\alpha\) and \(\beta\) chains of the TCR consists of segments named V, D and J segments. The variable domain of the \(\alpha\) chain contains V and J segments, and the \(\beta\) chain contains V, D and J segments. Diversity of the TCR is found in the V, D and J gene segments. The variable domain of both the TCR \(\alpha\) and \(\beta\) chains contain three hypervariable regions known as complementarity determining region (CDR) 1, 2 and 3. CDR1 and CDR2 are found in the V region of the variable domain. CDR3 spans the V region, the D region and the J region in the variable domain. As a result, CDR3 is the most variable region.

When a peptide Ag is presented by an APC to a T cell within the peptide binding groove, the peptide Ag must be specific for the CDR3 region (the peptide Ag must be specific for the V, D and J segments); this is a very specific interaction. On the other hand, when a SAg is presented by an APC to a T cell, the SAg binds outside of the peptide bind groove and so needs only to be specific for the V segment on the variable domain of the \(\beta\) chain (the \(\text{V}\beta\) domain).

A group of T cells that share the same V, D and J segments recognize the same peptide Ags and is called oligoclonal. An oligoclonal group of T cells also recognizes the same SAGs but are only a subset of the T cells that recognize those SAGs. A group of T cells that only share the same V segment can be activated by a SAg specific for that V segment and is called polyclonal.
1.4.2 Peptide Antigen Mediated T cell Activation

The complete activation of a T cell requires one signal from the engagement of the TCR with the MHC/Ag and a second costimulatory signal (62, 63). T cells that are activated through the TCR complex (signal one) in the absence of costimulation (signal 2) experience suboptimal proliferation, do not secrete IL-2 and become anergic; subsequent activation through the TCR complex and costimulatory molecules will be unable to elicit proliferation or IL-2 secretion (62-65).

Activity of tyrosine kinases is required for signaling downstream of the TCR (Figure 4). Since the TCR does not contain intrinsic tyrosine kinase activity, non-receptor tyrosine kinases are required for TCR signaling (particularly the Src family tyrosine kinases). Lck and Fyn are two

Figure 3. The T cell Receptor. A) The TCR is a heterodimer of an α and a β polypeptide chain. Each chain has a large extracellular structure that is folded into two Ig-like domains (the variable and the constant domains). An Ag would recognize a site formed by the variable domains of the α and β chains. B) The variable domain of the β chain consists of V, D and J segments (the VDJ junction). A peptide Ag binds within the peptide binding groove and is specific for the V, D and J segments; however, a SAg binds outside of the peptide binding groove and is only specific for the V segment (known as the Vβ region).
protein tyrosine kinases that are part of the Src family and responsible for phosphorylation of tyrosine residues within immunoreceptor-based tyrosine activation motifs (ITAMs) on the TCR.

CD4 and CD8 are co-receptor molecules that are expressed on the surface of T cells and that interact with MHC class II and I molecules, respectively. Co-receptors stabilize the interaction between the T cell and the APC and contribute to early signal transduction events in TCR signaling. Lck is a signaling protein that is noncovalently associated with the cytoplasmic tails of CD4 or CD8. When the TCR binds to a peptide-MHC complex, the concurrent interaction of CD4 or CD8 with the MHC molecule brings the associated Lck in proximity to tyrosine residues in the ITAMs of the TCR complex. ζ- associated protein of 70kDa (Zap70) tyrosine kinase is recruited to the TCR by binding to the tyrosine phosphorylated ITAMs. Bound Zap70 becomes substrate for Lck which phosphorylates specific tyrosine residues on Zap70. This gives Zap70 tyrosine kinase activity so that it can phosphorylate a number of adaptor molecules such as LAT. LAT acts as a scaffold that recruits a number of mediator molecules associated with different intracellular signal transduction pathways. The signal transduction pathways lead to the upregulation of important transcription factors like NF-AT and NF-κB that influence the transcription of different genes involved in proliferation, cytokine secretion, differentiation and survival.

1.4.3 Superantigen Mediated T cell Activation

The signaling pathway activated by bacterial SAgs differs from that activated by conventional Ags. This became apparent when investigators found that bacterial SAgs could activate T cells that lacked Lck and that SAgs—which are usually presented by MHC class II molecules—were able to activate both CD4+ and CD8+ T cells. This indicated that the CD4 coreceptor and Lck were not required for SAgs to activate T cells. Investigators found that Lck-deficient T cells
could activate and translocate important transcription factors such as NF-AT and NF-κB which have a variety of functions including upregulation of IL-2 expression. An alternative pathway was found to be activated following SAg engagement of the TCR. This pathway involved activation of the Gα11 G protein (likely via a G protein-coupled coreceptor on T cells) which led to PLC-β and PKC activation. This resulted in Ca^{2+} influx, activation of NF-AT and NF-κB, and IL-2 production. Induction of this alternate pathway upon SAg activation suggests that SAgs likely bind to T cells through a G protein-coupled coreceptor.

As mentioned previously (see section 1.2.1), after SAg activation, responding T cells become apoptotic (18, 19, 66). The fate of SAg-activated T cells appears to contradict its proposed role in KD where a persistent immune response is observed at the coronary arteries of patients; however, a phenomenon whereby SAg-activated T cells could be rescued from apoptosis may provide an explanation for this apparent contradiction.
Figure 4. T cell signaling induced upon activation of the TCR. When the TCR binds to a peptide-MHC complex, the concurrent interaction of CD4 or CD8 with the MHC molecule brings the associated Lck in proximity to tyrosine residues in the ITAMs of the TCR complex. Zap-70 is recruited to TCR and phosphorylated by Lck leading to phosphorylation of adaptor molecules such as LAT. LAT acts as a scaffold that recruits a number of mediator molecules associated with different intracellular signal transduction pathways that eventually affect transcription of different genes involved in proliferation, cytokine secretion, differentiation and survival.
1.4.4 Fate of Superantigen-Activated T cells: Apoptosis

Apoptosis (also known as programmed cell death) is a regulated and controlled form of cell death that is initiated by the cell in response to a variety of stimuli including the induction of DNA damage by radiation or chemical toxins, activation of the stress response, absence of growth factors, and initiation of specific signaling receptors. Characteristic features of apoptosis include chromatin condensation, nuclear disruption, cytoplasmic contraction, chromosomal DNA fragmentation and membrane blebbing. Eventually, the cell is fragmented into tiny packages called apoptotic bodies that can be engulfed and removed by phagocytic cells. Contents of the apoptotic cell are therefore not released into the surroundings thereby preventing damage to nearby cells and preventing activation of the innate immune system. The process of apoptosis can be contrasted to a form of cell death known as necrosis where there is uncontrolled cell death including lysis of the cell and initiation of an inflammatory response. Apoptosis is mediated by two main pathways; they are the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways (Figure 5).

Both pathways of apoptosis lead to the activation of caspases (cysteine proteases that cleave proteins immediately after aspartic acid residues) which are a group of proteolytic enzymes. Caspases normally exist as inactive pro-enzymes that are activated upon cleavage of a pro-piece that ends in an aspartyl residue. A cascade of caspase activations can ensue as activated caspases go on to activate other caspases in a similar manner. Signals from either the intrinsic or extrinsic pathway of apoptosis would activate “initiator” caspases which in turn cleave and activate “executioner” caspases. The regulation of caspases at the post-translational level allows for rapid activation.
The intrinsic pathway of apoptosis leads to the activation of specific proteins of the Bcl-2 family that alter the mitochondrial membrane integrity. The Bcl-2 family of proteins can be divided into three main groups: anti-apoptotic proteins, pro-apoptotic proteins, and BH3-only proteins that regulate anti-apoptotic proteins. Pro-apoptotic family members include the Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist/killer protein (Bak) which have been shown to be responsible for the formation of pores in the mitochondrial membrane. If mitochondrial membrane integrity is lost, death inducing proteins such as cytochrome c (cyt c) and second mitochondria-derived activator of caspase (Smac/Diablo) can be released into the cytosol. In the cytosol, cyt c binds to Apaf-1 and ATP to form an Apaf-1/cyt c complex known as the apoptosome. The apoptosome can then recruit and induce the autoactivation of the key initiator caspase in the intrinsic pathway of apoptosis, procaspase-9. Caspase-9 can then activate the common death pathway. On the other hand, Smac/Diablo functions by neutralizing the inhibitory actions of inhibitors of apoptosis proteins (IAPs). IAPs can interact with and inhibit activated caspases in the cytosol to keep them in check.

In contrast, anti-apoptotic Bcl-2 family proteins like Bcl-2 and Bcl-XL inhibit pro-apoptotic proteins like Bax and Bak. For example, Bcl-2 has been shown to heterodimerize with Bax (67). When Bcl-2 loses its ability to heterodimerize with Bax but maintains its ability to homodimerize with itself, the anti-apoptotic effects of Bcl-2 are lost (68). This suggests that formation of the Bax/Bcl-2 heterodimer is required for repression of apoptosis by Bcl-2. Hence one of the anti-apoptotic functions of Bcl-2 may be to heterodimerize with Bax and interfere with the function of Bax. Consistent with this, it has been shown that Bcl-2 can inhibit the pore-forming functions of Bax (69).
Lastly, the BH3-only proteins contribute to apoptosis by directly binding and inhibiting anti-apoptotic proteins like Bcl-2. This results in the release and resumption of pro-apoptotic protein (Bax and Bak) functions. Examples of BH3-only proteins include Bcl-2-associated death promoter (Bad), Bid, Bim and PUMA. Some evidence shows that BH3-only proteins (in particular Bim and PUMA) can directly activate pro-apoptotic proteins like Bax and Bak.

The extrinsic pathway of apoptosis is initiated by the activation of cell surface receptors that have been termed “death receptors”. The prototypical death receptor is Fas. Fas contains a protein-protein interaction domain known as a death domain. When FasL binds to Fas, an adaptor protein called Fas-associated death domain (FADD) links the intracellular death domain on Fas to the initiator caspase, procaspase-8; this leads to the activation of procaspase-8. Although the extrinsic pathway is able to induce apoptosis independent of mitochondrial alterations, it is possible for caspase-8 to cleave and activate Bid which in turn disrupts the mitochondrial membrane via activation of Bak and Bax, and inhibition of Bcl-2 and Bcl-XL.

Following the activation of initiator caspases, caspase-9 in the intrinsic pathway and caspase-8 in the extrinsic pathways, activation of a cascade of caspases follows; effector caspases, including caspase-3, -6 and -7 are activated. The downstream effects of caspase activation include all the hallmark characteristics of apoptosis such as chromatin condensation, plasma membrane blebbing and DNA degradation.

One downstream target of caspase activation is the activation of pro-apoptotic substrates to create a positive feedback. An example of a downstream pro-apoptotic target is Bid which, as mentioned above, can be activated by caspase-8. Another downstream effect of caspase activation is the inhibition of proteins that mediate survival and proliferation signals such as phosphatidylinositol 3-kinase (PI3K)/Akt, Raf-1, Bcl-2 and Bcl-XL. Lastly, activated caspases
target different structural and housekeeping proteins to facilitate the dismantling of the cell as it undergoes apoptosis. For example, nuclear lamins (the major cytoskeletal structural component of the nucleus) are cleaved by caspase-6 during apoptosis. In addition, caspases inactivate the enzyme flippase and activate the enzyme scramblase. This results in the externalization of phosphatidylserine (PS) which is normally maintained in the inner leaflet of the plasma membrane.

Some investigators believe that the Fas/FasL interaction plays a dominant role in SAg-mediated apoptosis. These investigators have shown that T cells from mice expressing the lpr or gld mutation (lacking functional Fas or FasL expression, respectively) do not undergo apoptosis following exposure to SAg. In addition, studies have shown that cells constitutively express high levels of Fas and exposure to SEB induces a rapid increase in expression of FasL which is associated with induction of apoptosis.

In contrast, other studies have found that the Bcl-2-mediated (intrinsic) pathway of apoptosis plays the dominant role following SAg activation. These studies have found that SAg activated T cells do die in the absence of Fas. SAg-activated T cells have been found to express lower levels of the anti-apoptotic protein Bcl-2 at the time that they reach their maximal response, which is just before death occurs. Lowered levels of Bcl-2 were shown to be important because transgenic or retroviral expression of Bcl-2 is sufficient to prevent SAg-induced death. In addition, in the absence of Bim, death of activated T cells was prevented both in vitro and in vivo; this suggests that the mechanism of Bcl-2 prevention of apoptosis may be Bim neutralization. Therefore, the balance between Bcl-2 and Bim levels appears to contribute to the fate of SAg-activated T cells.
Figure 5. The intrinsic and extrinsic pathways of apoptosis. Stresses such as DNA damage and UV damage initiate the intrinsic pathway of apoptosis. BH3-only proteins such as Bim activate pro-apoptotic proteins such as Bax and Bak, and inhibit anti-apoptotic proteins such as Bcl-2 and Bcl-XL. Bax/Bak induce mitochondrial leakiness leading to the release of cyt c. Cyt c binds Apaf-1 to form the Apoptosome which can then activate procaspase-9. IAPs are negative regulators of caspases and inhibited by Smac/Diablo which is also released from the mitochondria. In the extrinsic pathway of apoptosis, death receptor ligation leads to activation of procaspase-8. A cascade of activations leading to apoptosis follows both the intrinsic and extrinsic pathways.
1.5 Rescue of Superantigen-Activated T cells by Co-Presentation of a Peptide Antigen

It has been observed that under certain conditions, SAg-activated T cells could evade their apoptotic fate. In a study by McCormack et al., deletion of T cells mediated by the SAg SEA was blocked in the presence of cotreatment by a specific Ag, pigeon cytochrome c peptide (pcytC) in vivo (70). SEA and pcytC share similar TCR Vβ3 specificity (66, 71). When mice were treated with both SEA and pcytc, all SEA-reactive (TCR Vβ3+) T cells were deleted except for a subpopulation of T cells that were also reactive to pcytc (70). This subpopulation was not only rescued from deletion but proliferated in response to the specific peptide Ag, pcytC (70).

1.5.1 TCR Vβ6+ T cells in LCWE-Induced Coronary Arteritis

Interestingly, characterization data of T cells at the hearts of LCWE-injected mice suggests that a peptide Ag may play a role in disease. Sequence analyses from our lab of the infiltrating T cells in LCWE-injected mice were found to be predominantly TCR Vβ6+ and oligoclonal. TCR Vβ6+ T cells are among the T cells that are reactive to LCWE; LCWE-reactive T cells also include TCR Vβ2, 4 and 14. The oligoclonal nature of the LCWE-reactive TCR Vβ6+ T cells at the heart suggests that this family of T cells could be responding to both the SAg in LCWE and to a peptide Ag. TCR Vβ6+ T cells may be the pathogenic population of T cells that are activated in response to LCWE, rescued from apoptosis by a peptide Ag and therefore responsible for the persistent immune response that is seen at the coronary arteries. The mechanism underlying this rescue phenomenon, which was first observed by McCormack et al., is poorly understood; however, costimulation was proposed to play a role. It has been proposed that SAg activation may occur in the absence of a second costimulatory signal and this leads to T cell apoptosis.
1.6 Costimulation of T Lymphocytes

1.6.1 Two Signal Model of T Cell Activation

For T cells to become fully activated with the ability to proliferate, secrete cytokines and differentiate, two signals must be received. Signal 1 is TCR stimulation and signal 2 is costimulation. When T cells receive only stimulation through the TCR, they undergo apoptosis or become anergic. The CD28 molecule on the T cell binding to the B7 (B7.1/B7.2) molecule on the APC is one of the most dominant and well-characterized costimulatory pathways.

1.6.2 Costimulation via CD28

The CD28 molecule is a glycoprotein and a homodimer with one ligand-binding domain (72). Although CD28 is constitutively expressed on almost all murine T cells, expression levels of CD28 can also increase following activation of T cells (73, 74). Signaling through CD28 alone does not lead to activation; however, in combination with signaling through the TCR, T cell proliferation and cytokine production is enhanced (75).

Costimulation through CD28 promotes the production of extrinsic survival factors such as the cytokines IL-2, IFN-γ, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). This is accomplished through changes in transcriptional and post-transcriptional regulation (73, 76-78). The CD28-responsive element (CD28RE) located in the IL-2 promoter is required for CD28-induced IL-2 upregulation. In addition, signaling through the TCR complex in association with costimulation through CD28 enhances the intrinsic ability of T cells to resist apoptosis. This signaling augments the expression of Bcl-XL both at the mRNA and protein levels which contribute to T cell survival (79).
There is evidence that suggests there are distinct motifs within the cytoplasmic tail of the CD28 molecule that differentially regulate T cell proliferation (including cytokine production) or T cell survival (such as Bcl-X<sub>L</sub> expression). Mutation of C-terminal proline residues abolished CD28-dependent proliferation and significantly reduced IL-2 production; however, Bcl-X<sub>L</sub> levels were unaffected. On the other hand, the YMNM motif on the cytoplasmic tail of CD28 has been shown to be important for Bcl-X<sub>L</sub> expression. When the YMNM motif was mutated, PI3K could not bind, PI3K remained unactivated and CD28-dependent Bcl-X<sub>L</sub> upregulation was abrogated. CD28-dependent T cell proliferation was only partially reduced due to this mutation (80).

### 1.6.3 Costimulation via CD28: Signaling Pathway

The cytoplasmic domain of CD28 lacks any direct enzymatic activity, therefore it likely signals via the recruitment of cellular enzymes. CD28 signals are mediated by the phosphorylation of tyrosine residues (that are not part of an ITAM) and proline rich motifs on the cytoplasmic tail. A small subset of the molecules involved in the signaling pathway downstream of the TCR have also been found to be important downstream of CD28. Molecules that have been implicated in CD28 signaling include Lck and Fyn which have been shown to phosphorylate the cytoplasmic tail of CD28. PI3K has been found to associate with the cytoplasmic tail of CD28 upon tyrosine phosphorylation leading to PKB (also known as Akt) activation. Akt activation has been linked to increased IL-2 secretion; however the signaling pathway involved remains unclear. Other molecules implicated in CD28 signaling include ITK and TEC which are tyrosine kinases, Casitas B-lineage lymphoma B (Cbl-b), and VAV (81, 82).

Cbl-b plays a critical role in CD28 signaling as a negative regulator. This molecule became of interest when investigators discovered that T cells that were deficient in Cbl-b did not require CD28 signaling for IL-2 production and that T cell antibody responses in CD28<sup>−/−</sup> mice could be
restored when Cbl-b was knocked down as well. Cbl-b does not appear to affect the main TCR signaling pathways (such as Zap70, Lck, PLCγ and Ca^{2+} mobilization) but suppresses TCR induced phosphorylation (activation) of VAV. Cbl-b appears to inhibit VAV-induced activation of the Rac/JNK pathway which normally leads to upregulation of AP-1 (refer to 1.4.2), but not VAV-dependent Ca^{2+} mobilization. CD28 costimulation is required to overcome the inhibitory effect of Cbl-b (81, 83, 84).

### 1.6.4 Other Costimulatory Molecules

Although CD28 acts as the dominant costimulatory molecule, other molecules exist that are involved in costimulation. These molecules belong to the immunoglobulin superfamily or the TNFR/TNF ligand (TNFL) superfamily. These include the following ligand/receptor pairs which are present on the APC and T cell respectively: OX40L/OX40, 4-1BBL/4-1BB, CD40/CD40L and ICOSL/ICOS. OX40 is a secondary costimulatory molecule that is not constitutively expressed on T cells but expressed following activation. Its ligand OX40L is also not constitutively expressed on the APC but induced upon activation (see section 1.10.5 for more details on OX40). 4-1BBL and ICOS, and their ligands are also inducible costimulatory molecules that are only found on activated cells. To study the effects of signaling through 4-1BB on T cell survival, an agonistic anti-4-1BB mAb (clone 3H3) has been used in previous studies in the lab. The agonistic anti-4-1BB mAb would stimulate T cells through the 4-1BB molecule and this would presumably mimic 4-1BBL ligation to 4-1BB.
1.7 Rescue of SAg-Activated T Cells by Increased Costimulation

Since the deletion of SAg-activated T cells often follows significant expansion of responsive T cells, one possible explanation is that the deletion is a natural end result of T cell proliferation. However, in the absence of significant proliferation (due to treatment with very low amounts of SAg) deletion of responsive T cells could still be detected (66). In an alternative explanation, it has been proposed that SAg activation may occur in the absence of a second costimulatory signal, leading to T cell apoptosis. If this were true, supplying missing costimulatory signal should rescue SAg-activated T cells from apoptosis. Providing costimulatory signals (for example through CD28) will enhance the production of extrinsic survival factors like cytokines IL-2, IFN-γ, TNF-α and GM-CSF, as well as intrinsic pro-survival factors like Bcl-XL. All these factors help promote the survival of SAg-activated T cells.

Moolani et al. showed in vitro that increased costimulation (via αCD28 antibody or α4-1BB antibody) could reduce SEB-induced apoptosis in the reactive TCR Vβ8 subset of T cells (Figure 6). Further characterization of the SEB-activated, costimulation rescued T cells showed that they were not anergic. These cells had increased proliferative capacity as well as increased expression of the anti-apoptotic protein, Bcl-XL; the proliferative capacity of cells was assessed by both tritiated thymidine incorporation and bromodeoxyuridine (BrdU) incorporation. CD28 signaling can be blocked with CTLA4-Ig which is homologous to CD28 but binds to B7 molecules with much higher affinity. Blocking costimulatory signals with CTLA4-Ig (a soluble form of the extracellular portion of the CTLA4 receptor) resulted in a dose-dependent inhibition of Bcl-XL (Y. Moolani, submitted manuscript).
1.7.1 Increased Costimulation Exacerbates Disease

The relevance of costimulation in an animal model of KD was assessed. Coronary arteritis was induced in experimental mice with IP injections of LCWE. In addition, anti-4-1BB (3H3) costimulatory mAb was injected IP. By assessing the cellular infiltrate at the heart, both disease severity and incidence was found to be increased when anti-4-1BB mAb was injected in addition to LCWE (Table 2).

Figure 6. Costimulation-mediated rescue of SEB-activated T cells. A) Treatment of mouse splenocytes with enhanced costimulation (via an αCD28 antibody) rescues the reactive TCR Vβ8⁺ T cells from SEB-induced apoptosis, as measured by the apoptosis marker Annexin V. B) The SEB-activated costimulation-rescued T cells have increased proliferative capacity as indicated by BrdU incorporation. C) The SEB-activated costimulation-rescued T cells have increased expression of the anti-apoptotic protein, Bcl-xL. Data collected and compiled by Yasmin Moolani, 2009.
1.8 Rescue of Superantigen-Activated T Cells by TLR2-Mediated Upregulation of Costimulatory Molecules

The Toll-like receptor (TLR) proteins are part of a family of pattern recognition receptors (PRRs). TLRs are type I integral membrane glycoproteins. Their extracellular domains contain a varying number of leucine-rich-repeat (LRR) motifs and their cytoplasmic domains are homologous to the IL-1 receptor. These receptors can be found on innate immune cells and are responsible for detecting invading organisms though the recognition of pathogen associated molecular patterns (PAMPs). TLRs have a wide variety of effects including transcription of

**Table 2. Incidence of coronary artery inflammation in mice injected with LCWE and enhanced costimulation.** Data collected and compiled by Yasmin Moolani, 2009.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCWE</td>
<td>7 / 13</td>
</tr>
<tr>
<td>LCWE + anti-4-1BB</td>
<td>12 / 13 $^{b,c,d}$</td>
</tr>
<tr>
<td>LCWE + Isotype</td>
<td>5 / 10 $^{b,e}$</td>
</tr>
<tr>
<td>4-1BB alone</td>
<td>1 / 6</td>
</tr>
<tr>
<td>Isotype alone</td>
<td>0 / 6</td>
</tr>
</tbody>
</table>

$^a$ Incidence is defined as the number of mice with coronary artery inflammation divided by the total number of mice injected. Statistics calculated using Fisher’s Exact test.

$^b_p<0.05$, compared to Isotype alone group

$^c_p=0.037$, compared to LCWE group

$^d_p=0.035$, compared to LCWE + Isotype group

$^e$ NS, compared to LCWE group
proinflammatory cytokines and chemokines, and the upregulation of costimulatory molecules to influence the adaptive immune response. TLR2 is a member of the TLR family that has a broad specificity. As a result, TLR2 is able to recognize a wide variety of ligands including various glycolipids, polysaccharides, glycoproteins and lipoproteins (85).

Interestingly, TLR2 knockout mice do not get LCWE-induced coronary arteritis (86). In addition, as previously described, TLR2 mediated upregulation of costimulatory molecules was able to rescue SAg-reactive T cells from apoptosis. This principle was demonstrated with the prototypical SAg, SEB, as a proof of principle. When cells cultured with SEB alone were compared to cells cultured with SEB and Pam3Cys (a TLR2 ligand), the cultures containing SEB and Pam3Cys underwent decreased amounts of apoptosis (Figure 7). Therefore, similar to CD28 stimulation, TLR2 stimulation was able rescue SAg-activated T cells from apoptosis. Furthermore, it was found that TLR2 stimulation upregulated the expression of costimulatory molecules on T cells and that CTLA4-Ig treatment (which blocks costimulatory signals) blocked Pam3Cys-mediated rescue of SEB-activated T cells (Figure 7). These results demonstrated that TLR2 mediated upregulation of costimulatory molecules could rescue SAg-activated T cells from apoptosis (K. Little, submitted manuscript).

1.8.1 MAM and TLR2 Activity

MAM has been demonstrated to contain TLR2 activity. Stimulation with MAM led to increased production of inflammatory cytokines and increased surface expression of both TLR2 and TLR4 on macrophages. Interestingly, macrophages from mice that were deficient in TLR4 exhibit increased levels of TLR2 expression and inflammatory cytokine production in response to MAM. Furthermore, when macrophages (that express both TLR2 and TLR4) were treated with an antibody that blocked TLR4 activity, there was an increase in TLR2 expression and Th1
associated cytokine production in response to MAM (when compared to macrophages that were not treated with the antiTLR4 Ab). These results suggest that TLR4 was responsible for suppressing the MAM-induced proinflammatory functions and expression of TLR2 (87). This was consistent with in vivo data whereby TLR4 deficient mice developed a Th1 immune response when they were injected IP with MAM and then rechallenged with MAM. On the other hand, mice that expressed both TLR2 and TLR4 developed a Th2 immune response when they were injected IP with MAM and then rechallenged with MAM (88).

Figure 7. TLR2-mediated upregulation of costimulatory molecules rescues SEB-activated T cells from apoptosis. A) Treatment of mouse splenocytes with a TLR2 ligand (Pam3cys) rescues the reactive TCR Vβ8⁺ T cells from SEB-induced apoptosis, as measured by the apoptosis marker Annexin V. B) Pam3Cys-mediated rescue of TCR Vβ8⁺ T cells from SEB-induced apoptosis is dependent on costimulatory signals because the addition of CTLA4-Ig blocks the rescue (CTLA4-Ig blocks signaling through CD28). Data collected and compiled by Ken Little, 2009.
1.9 T Lymphocyte Subsets

T helper cells are a CD4$^+$ subgroup of lymphocytes that have little or no cytolytic or phagocytic activity. They are instead involved in activating and directing other cells of the immune system. T helper cells can be further divided into different functional subsets including T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 (Th17) cells and regulatory T cells (Tregs). The differentiation of T helper cells into the different subsets depends on a variety of factors including the magnitude and pattern of TCR, costimulatory and cytokine signals encountered.

1.9.1 T Helper 1

Th1 cells are characterized by the synthesis and secretion of IFN-$\gamma$ and tumor necrosis factor-$\beta$ (TNF-$\beta$; also known as lymphotoxin). Th1 cells are involved in cell-mediated immunity (including delayed-type hypersensitivity, DTH, reactions). Th1 responses protect the host against various intracellular pathogens. The transcription factors STAT-4 and T-box transcription factor (T-bet) have been shown to be vital for Th1 differentiation (89); however, the sequence of signaling events involving STAT-4 and T-bet remain unclear. In one proposed model of Th1 differentiation, IL-12 signals through receptors on the T cell leading to an upregulation of the transcription factor, STAT-4. IL-12 is produced by many cells, in particular by macrophages that are responding to different microbial products. STAT-4 induces a transient increase in IFN-$\gamma$ levels that signals back to the cell to upregulate STAT-1 and eventually T-bet. T-bet then drives continued increases in IFN-$\gamma$ levels. In a separate, although not mutually exclusive pathway, TCR and IFN-$\gamma$ signals induce increases in STAT-1 and then T-bet levels. T-bet then promotes the production of IFN-$\gamma$ and the IL-12r$\beta$2 chain of the IL-12R. This allows for the cell to respond to IL-12, which then leads to STAT4 induction followed by more IFN-$\gamma$ production. The functional relationship between STAT-4 and T-bet in the development of Th1 cells is complex,
and both STAT-4 and T-bet have been shown to be necessary for complete Th1 fate determination (90). Diseases that have been described as Th1-dominant include Multiple sclerosis (MS), diabetes and RA.

1.9.2 T Helper 2

Th2 cells are characterized by the synthesis and secretion of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th2 cells are involved in the humoral immune response by helping B cells produce antibodies, and are involved in the host defense against extracellular parasites. Therefore, Th2 cells assist B cells in the production of antibodies (particularly IgE antibodies). IL-4 has been shown to activate STAT-6 leading to an increase in GATA-3 levels. Activation of STAT-6 and GATA-3 is followed by production of more IL-4 and commitment to the Th-2 fate (91). The source of IL-4 that contributes to Th2 differentiation is not completely understood but basophils and mast cells have been implicated (92, 93). Allergy and atrophy are thought to be Th2-dominant conditions.

1.9.3 T Helper 1 and T Helper 2 Balance

The signature cytokines produced by Th1 or Th2 cells not only determine their effector functions but lead to the development and expansion of their respective subsets. For example, IFN-γ produced by Th1 cells promotes further Th1 differentiation while inhibiting the proliferation of Th2 cells (94). On the other hand, IL-4 and IL-10 produced by Th2 cells promote further Th2 development while inhibiting Th1 development (95). In other words, IFN-γ and IL-4 antagonize each other. Consequently, as the immune response begins to develop along one pathway, it becomes increasingly polarized to that pathway.
1.9.4 T Helper 17

Th17 cells are characterized by the synthesis and secretion of IL-17. Th17 cells are potent inducers of tissue inflammation and are involved in host defenses against fungi and extracellular bacteria. Th17 cells have been shown to play an important role in the pathogenesis of various experimental models of autoimmunity, as well as human autoimmune diseases. For example, mice that are deficient in IL-17 are resistant to the development of collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) (96, 97). In humans, IL-17 has been found in patients with RA, MS and systemic lupus erythematos (SLE) (98-100). Other cytokines that Th17 cells are known to secrete include IL-21 and IL-22. Transforming growth factor-β (TGF-β) together with IL-6 induces signaling through STAT3 leading to the activation of the transcription factors retinoid-related orphan receptor (ROR)γt and RORα. These transcription factors are responsible for the differentiation of Th17 cells and IL-17 production.

Studies in mice have shown that IL-21 is similar to IL-6; IL-21 can reportedly induce Th17 differentiation when combined with TGF-β. Since IL-17 does not function as a differentiation or growth factor for Th17 cells, it can not feedback onto Th17 cells and strengthen the commitment to this lineage. Instead it has been proposed that IL-21 may be responsible for the amplification of Th17 cells once they have started to differentiate. Generally, IL-6 plays the more dominant role in Th17 differentiation in vivo. For example, a normal immune response during the acute phase of an infection involves a large production of IL-6 and so together with TGF-β, they are the main drivers of Th17 differentiation. On the other hand, IL-6 levels are limited in the absence of inflammation so IL-21 may become more important in the differentiation of Th17 cells. This differentiation may be important in maintaining the precursor population of Th17 cells.
Further studies in mice have demonstrated that IL-23 is another important cytokine. IL-23 does not contribute to the initial differentiation of Th17 cells; however, it is important for full and continued differentiation. Consistent with this, the receptor for IL-23 (IL-23R) is not expressed on naïve T cells but induced by TGF-β. Either TGF-β and IL-6, or TGF-β and IL-21 induce the expression of IL-23R allowing cells to respond to IL-23 and therefore stabilize to the Th17 lineage.

1.9.5 Regulatory T Cells

Treg cells, as the name implies, function to limit effector T cells responses and therefore maintain tolerance to self-antigens and prevent autoimmunity. There are different types of Treg cells including naturally occurring Treg cells (nTreg) and inducible Treg cells (iTreg). nTreg cells are generated in the thymus while iTreg cells are induced from naïve T cells either in the periphery or in vitro following the addition of antigen and TGF-β. Tregs are recognized by their expression of the transcription factor forkhead box p3 (Foxp3) and the IL-2 receptor alpha chain (CD25).

1.9.6 T Helper 17 and Treg Balance

TGF-β is important for both the differentiation of Treg and Th17 cells as it can induce either RORγt or Foxp3 expression. When RORγt and Foxp3 are both present, Foxp3 has been shown to associate with and inhibit RORγt leading to Treg differentiation (101). Conversely, when IL-6 is also present, expression of Foxp3 is abrogated and Th17 differentiation ensues (102). Therefore, IL-6 plays a critical role in regulating the balance between Treg and Th17 cells. Consistent with the important role that IL-6 plays in Th17 differentiation, IL-6 knockout mice are resistant to the development of EAE and have an immune response that is dominated by Foxp3+ Treg cells. Interestingly, when Tregs are depleted in IL-6 knockout mice, the mice
become susceptible to EAE. IL-21 may be responsible for the EAE observed in Treg-depleted IL-6 knockout mice because IL-21 has the ability to suppress TGF-β-induced Foxp3 expression, and the ability to induce TGF-β-dependent IL-17 production. *In vitro*, it is evident that there is a reciprocal relationship between the development of Th17 and Treg cells; however, whether this is true *in vivo* is still not yet fully understood.

*Figure 8. Differentiation of T helper subsets.* T helper cells are a CD4⁺ subgroup of lymphocytes. The differentiation of T helper cells into the different subsets depends on a variety of factors including the magnitude and pattern of TCR, costimulatory and cytokines signals encountered. T helper cells can be further divided into different functional subsets including T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 (Th17) cells and regulatory T cells (Tregs).
1.10 Activation Markers

Activated and proliferating lymphocytes are known to express a number of molecules on their surface that are found minimally or are absent on resting cells (103, 104). These molecules have been termed activation antigens or markers. Resting or naïve T cells are cells that have not yet encountered an antigen (105). In this unactivated state, the cells remain in the G\textsubscript{0} phase of the cell cycle with densely packed chromatin, few mitochondria, and poorly developed endoplasmic reticulum and Golgi apparatus. These cells are sometimes referred to as small lymphocytes because they are only about 6\(\mu\)m in diameter and are thought to have a short life span. The resting or naïve phenotype of a T cell can be defined as CD25\textsuperscript{−}CD69\textsuperscript{−}CD44\textsuperscript{low}CD62L\textsuperscript{hi}.

Interaction with an antigen allows the cell to progress from G\textsubscript{0} to G\textsubscript{1} and then through subsequent cell cycle stages. Upon activation, cells become enlarged to a diameter of about 15\(\mu\)m, are termed lymphoblasts and have increased organelle complexity. Lymphoblasts proliferate and differentiate into effector cells or memory cells. The activated or memory phenotype of a T cell can be defined as CD25\textsuperscript{+}CD69\textsuperscript{+}CD44\textsuperscript{hi}CD62L\textsuperscript{low}.

1.10.1 CD69

CD69 is a cell surface glycoprotein which functions as a homodimer and is induced upon T cell activation. The extracellular domain of CD69 contains a C-type lectin-binding domain. C-type lectin-binding domains have been shown to be involved in glycoprotein endocytosis, glycoprotein degradation and the innate immune response. CD69 contributes to the innate immune response by facilitating binding to sugar residues found on the cell surface of bacteria, parasites and fungi. Approximately 30-60 minutes after T cell activation, CD69 transcripts can be detected. By 2-3 hours, cell surface expression of CD69 may be observed and this cell surface
expression declines with a half life of about 24 hours if the stimulus is removed. Elevated intracellular levels of Ca$^{2+}$ and sustained PKC activation are required for induction of CD69.

1.10.2 CD62L

CD62L is also known as L-selectin and is a cell adhesion molecule found on leukocytes. CD62L acts as a homing receptor. Ligands that are expressed on the surface of endothelial cells bind to receptors like CD62L to slow leukocyte movement through the blood and therefore facilitate the extravasation of circulating lymphocytes through high endothelial venules (HEVs) into peripheral lymph nodes, mucosal lymphoid tissues and synovium of inflamed joints. CD62L is expressed on naïve T cells that have not yet encountered a specific antigen and hence need to enter secondary lymph nodes to encounter that antigen. Effector/memory T cells do not express CD62L allowing them to circulate in the periphery and perform effector functions.

1.10.3 CD44

CD44 is a cell surface glycoprotein that mediates cell attachment to extracellular matrix components or specific cell surface ligands (106). CD44 has been shown to be the principle cell surface receptor for hyaluronic acid (HA) which can be found as a component of the extracellular matrix (106). In addition, CD44 has been shown to play a role in T cell activation (107, 108). Signaling through CD44 enhances suboptimal CD3, or suboptimal and optimal CD2 receptor induced T cell proliferation (108). CD44-induced augmentation of T cell responses may be a mechanism to heighten activation during cell-cell contact.

Primary adhesion (rolling) of lymphocytes to endothelial cells can be mediated not only by the selectin family of receptors (including CD62L; please refer to section 1.10.2), but also by CD44 on lymphocytes binding to HA (109). In addition, CD44 appears to bind to HA in an activation
dependent manner (110, 111). This suggests that CD44 may selectively be involved in trafficking or extravasation of activated lymphocytes to sites of inflammation (112).

1.10.4 CD25

CD25 is the α chain of the IL-2 receptor. The IL-2 receptor occurs in three forms that exhibit different affinities for IL-2. The complete trimeric form of the receptor has the highest affinity for IL-2 and is composed of the α, β and γ chains. Whereas the γ chain appears to be constitutively expressed on the majority of lymphoid cells, expression of the α and β chains are more restricted. For example, the α chain is only expressed by activated T cells. When a cell receives a signal through both the TCR complex and CD28, transcription of IL-2 and the α chain of the IL-2 receptor is induced (113). Secretion of increased levels of IL-2 followed by binding of IL-2 to the high affinity trimeric IL-2 receptor induces proliferation and differentiation of the T cell. Failure to produce either IL-2 or the IL-2 receptor ceases the T cell immune response (114).

1.10.5 CD134

CD134, also known as OX40, is a member of the TNFR-superfamily of receptors. CD134 is a secondary costimulatory molecule that is upregulated only after activation of T cells (115). Unlike CD28, CD134 is not constitutively expressed on naïve T cells. Similarly, the ligand for CD134, OX40L, is only expressed on APCs following activation (116). Signaling through CD134 has been found to be essential for the long-term survival of T cells. For example, whereas early IL-2 production and proliferation were unaffected, high levels of Bcl-2 and Bcl-xL could not be maintained in CD134−/− T cells (117, 118). Consistent with this, retroviral transduction of Bcl-2 and Bcl-xL into CD134−/− T cells was able to reverse this survival defect (117).
2 Rationale

LCWE-induced coronary arteritis in mice mimics KD in children in several ways. Our lab has shown that LCWE contains a SAg that is reactive with certain TCR Vβ families, including TCR Vβ6. Upon SAg stimulation, T cells expressing responsive TCR Vβ families should undergo massive proliferation followed by apoptosis. The apparent contradiction between the apoptotic fate of SAg-activated T cells and the persistent immune response in KD may be explained by a phenomenon whereby co-presentation of a peptide antigen could rescue SAg-activated T cells from apoptosis. The mechanism of this rescue may be TLR2-mediated upregulation of costimulatory signals that are delivered to SAg-activated T cell when there is co-presentation of a peptide antigen.

Evidence from the lab suggests that this phenomenon may be responsible for the coronary arteritis that is seen in LCWE-injected mice. First, this rescue phenomenon has been demonstrated with a prototypical SAg, SEB. SEB-reactive T cells experienced enhanced survival when treated with increased costimulatory signals or a TLR2 ligand. TLR2 ligand mediated rescue of SEB-activated T cells could be inhibited by CTLA4-Ig (which blocks signaling through CD28). Secondly, mice treated with LCWE, and costimulatory antibodies or a TLR2 ligand exhibit increased disease severity and incidence. Thirdly, when T cells at the hearts of diseased mice were sequenced, they were found to be predominantly TCR Vβ6 and oligoclonal. The presence of TCR Vβ6+ T cells in the heart suggests that they may have a pathogenic role in disease. The oligoclonality of TCR Vβ6+ T cells at the hearts of diseased mice suggests that these T cells may be present because they are being rescued from their LCWE-induced apoptotic fate by the phenomenon described above.
Therefore, there are a number of factors that may contribute to LCWE-induced coronary arteritis. These factors may be divided into T cell dependent factors (such as activation of TCR Vβ6+ T cells) and T cell independent factors (such as TLR2 signaling).

3 Hypothesis

SAg-activated costimulation-rescued TCR Vβ6+ cells express activation markers, produce cytokines and are able to induce coronary arteritis in mice.

4 Objectives

There are three main objectives in this project. The first two objectives involve assessing T cell dependent factors, and the third objective involves assessing the contribution of T cell dependent and T cell independent factors in the pathogenesis of coronary arteritis.

(1) To identify and study bacterial SAgS able to activate TCR Vβ6 expressing T cells.

(2) To determine the phenotype of LCWE-activated costimulation-rescued T cells and compare this to the phenotype of costimulation-rescued T cells that have responded to other TCR Vβ6 stimulating SAgS.

(3) To dissect the contribution of T cell dependent versus T cell independent factors in the pathogenesis of coronary arteritis.
5 Materials and Method

5.1 Experimental Mice

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under specific pathogen-free conditions at The Center for Phenogenomics (TCP, Toronto). All animal procedures were approved by the Animal Care Committee at TCP.

5.2 Panel of Superantigens

The following recombinant bacterial superantigens were generously provided by Dr. John McCormick (University of Western Ontario): Streptococcal pyogenic exotoxin (Spe) A, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, Staphylococcus aureus enterotoxin (SE) K, Streptococcal mitogenic exotoxin, (SmeZ) and Streptococcal superantigen (SSA). Briefly, the protocol followed by Dr. Cormick: DNA primers containing restriction enzyme recognition sequences were designed to amplify the SAg gene of interest. PCR was performed with these primers on the DNA extracted from strains of *Staphylococcus aureus* or *Streptococcus pyogenes*. The amplified gene was digested with the appropriate restriction enzymes and cloned into an expression vector using *Escherichia coli* as the host. *E. coli* were grown and the concentrated crude protein was collected. Recombinant SAg of interest was separated by isoelectric focusing (119).

The superantigen, *Mycoplasma arthritidis*-derived mitogen (MAM), was generously provided by Dr. Walid Mourad (Université de Montréal). Briefly, MAM was purified as follows: DNA primers containing restriction enzyme recognition sequences were designed to amplify the MAM gene. PCR was performed with these primers on the DNA extracted from a strain of *Mycoplasma arthritidis*. The amplified gene was digested with the appropriate restriction enzymes and cloned into a pGEX-6P-1 GST fusion protein expression system (Pharmacia) using *E. coli* as the host,
following the manufacturer’s protocol (33). This vector produces a glutathione S-transferase (GST)-fusion protein that can be purified with glutathione and digested with a manufacturer provided protease to remove the GST tag and produce the recombinant protein.

5.3 Ex vivo Cell Culture Conditions

Splenocytes were cultured in 6-well plates at 8X10^6 cells/well in complete medium alone or in complete medium containing 0.3μg/ml SEB alone (Toxin Technology Inc., Sarasota, FL), a mixture of SEB and 0.5μg/ml of anti-CD28 Ab (Biolegend, San Diego, CA; clone 37.51), LCWE alone (see below for preparation), or a mixture of LCWE and anti-CD28 Ab. Control cultures were grown in complete medium containing anti-CD28 Ab alone, or a mixture containing SEB or LCWE with the appropriate isotype control Ab. All cultures were incubated for the indicated amount of time in 5% CO_2 at 37°C.

5.4 [³H] Thymidine Incorporation

Splenocytes from C57BL/6 mice (8-10 weeks) were cultured in 96-well plates at 5X10^5 cells/200μl of complete medium alone (Iscove’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-glutamine, 1mM sodium pyruvate, 0.1mM non-essential amino acid, 50μM 2β mercaptoethanol and 10mM HEPES) or in complete medium containing the indicated bacterial superantigen. The following superantigens: Spe A, SpeC, SpeG, SpeH, SpeI, SpeJ, SpeK, SpeL, SpeM, SEK, SmeZ, SSA and MAM. All cultures were performed in triplicates and incubated for the indicated amount of time in 5% CO_2 at 37°C. Cultures were pulsed with 1μCi/well of [³H] thymidine (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) during the last 18-20 hours of incubation. Please refer to Figure 9 for more details.
5.5 BrdU Incorporation

For the screening of the panel of bacterial SAgs, splenocytes were cultured in 6-well plates as described above in complete medium alone or in complete medium containing the indicated bacterial superantigen (refer to section 5.4 for the list of tested bacterial superantigens). Cultures were incubated for the indicated amount of time in 5% CO$_2$ at 37°C and pulsed with BrdU (BD Biosciences) for the last 18-20 hours of incubation. Cells were then harvested and stained with Abs against TCR V$\beta$6 (BD Biosciences, clone RR4-7) or TCR V$\beta$8 (BD Biosciences, clone F23.1), the pan-T cell marker Thy 1.2 (BD Biosciences, clone 53-2.1), and BrdU (according to manufacturer’s protocol, BD Biosciences). Control samples were stained similarly but with isotype-matched Abs. Samples were collected using FACSDiva software on a FACSCanto II (BD Biosciences) and analyzed with FlowJo (v 9.1, Tree Star Inc, Ashland, OR). Please refer to Figure 9 for more details.
Figure 9. Materials and Methods: Thymidine and BrdU incorporation.

**Thymidine incorporation.** Mouse splenocytes were cultured in medium containing each SAg from the panel for 3 or 4 days. Cultures were pulsed with thymidine during the last 16-20 hours of incubation. Cells were harvested and thymidine incorporation was measured with a liquid scintillation counter.

**BrdU incorporation.** Mouse splenocytes were cultured in medium containing each SAg from the panel for 3 or 4 days. Cultures were pulsed with BrdU during the last 16-20 hours of incubation. Cells were harvested, stained and analyzed via flow cytometry.
5.6 Activation Marker Expression

Splenocytes were cultured ex vivo in the cell culture conditions described above (refer to section 5.3). Cells were then harvested at the specified time points cells and stained with Abs specific for cell surface antigens (TCR Vβ8 or a TCR Vβ6) and with Abs specific for various activation markers including: CD25 (BD Biosciences, clone 7D4), CD44 (eBioscience, clone IM7), CD62L (eBioscience, clone MEL-14), CD69 (BD Biosciences, clone H1.2F3) and CD134 (eBioscience, clone OX-86). In addition, cells were assessed for early-phase apoptosis using 7AAD and AnnexinV staining, according to the manufacturer’s protocol (BD Biosciences, Mississauga, ON). Control samples were stained similarly but with isotype-matched Abs. After staining, cell samples were collected using FACSDiva software on a FACSCanto II and analyzed with FlowJo. Please refer to Figure 10 for more details.
Figure 10. Materials and Methods: Activation marker expression. Splenocytes were cultured ex vivo in the cell culture conditions described above (refer to section 5.4). Cells were then harvested at the specified time points cells and stained with Abs specific for cell surface antigens (TCR Vβ8 or a TCR Vβ6) and with Abs specific for various activation markers including: CD25, CD44, CD62L, CD69 and CD134. In addition, cells were assessed for early-phase apoptosis using 7AAD and AnnexinV staining. After staining, cell samples were collected using FACSDiva software on a FACSCanto II and analyzed with FlowJo.
5.7 Intracellular Cytokine Expression

Splenocytes were cultured ex vivo in the cell culture conditions described above (refer to 5.3). For the last 4-5 hours of incubation, cell cultures were resuspended in complete medium containing PMA, Ionomycin and GolgiPlug (BD Biosciences). After culture, all cells were first stained with Fixable Viability Dye (eBioscience) and with the following Abs specific for cell surface markers, as indicated: TCR Vβ6 Ab or TCR Vβ8 Ab, and CD4 Ab (BD Biosciences, clone GK1.5). Secondly, cells were fixed and permeabilized with Cytofix/Cytoperm Fixation and Permeabilization Kit according to the manufacturer (BD Biosciences). Thirdly, cells were stained with the following cytokine-specific Abs: IFN-γ (BD Biosciences, clone XMG1.2), TNF-α (BD Biosciences, clone MP6-XT22), IL-4 (BD Biosciences, clone 11B11), IL-10 (BD Biosciences, clone JES5-16E3) or IL-17A (Biolegend, clone TC11-18H10.1). Control samples were stained similarly but with isotype-matched Abs. After staining, cell samples were collected using FACSDiva software on a FACSCanto II and analyzed with FlowJo. Please refer to Figure 11 for more details.
Figure 11. Materials and Methods: Intracellular cytokine expression. Splenocytes were cultured ex vivo in the cell culture conditions described above (refer to section 5.4). For the last 4-5 hours of incubation, cell cultures were resuspended in complete medium containing PMA, Ionomycin and GolgiPlug. After culture, all cells were first stained with Fixable Viability Dye and with the following Abs specific for cell surface markers, as indicated: TCR Vβ6 Ab or TCR Vβ8 Ab, and CD4 Ab. Secondly, cells were fixed and permeabilized. Thirdly, cells were stained with the following cytokine-specific Abs: IFN-γ, TNF-α, IL-4, IL-10 or IL-17. After staining, cell samples were collected using FACSDiva software on a FACSCanto II and analyzed with FlowJo.
5.8 LCWE Preparation

LCWE was prepared as previously described (53). Briefly, *Lactobacillus casei* (ATCC 11578) obtained from American Type Culture Collection (Rockville, MD) was cultured in Lactobacillus MRS broth (Difco, Detroit, MI) on a shaker platform, for 24 hours at 37°C. Bacteria were harvested by centrifugation (at 10,000 RPM for 40 minutes) during the log phase of growth. Bacteria were then washed multiple times with PBS (pH 7.2) and lysed overnight by shaking at room temperature with 4% SDS (EM Science, Gibbstown, NJ). Bacteria were washed again with PBS (10 times) in order to remove cellular debris and SDS. The pellet was then subjected to sequential incubations with (250µg/ml) RNAse, then DNAse, and then trypsin (Sigma Chemicals, St. Louis, MO) to remove adherent material from the cellular walls. Incubations were performed at 37°C for four hours at twice the packing volume. After the RNAse and DNAse treatments, the extract was washed twice in PBS and after the trypsin treatment, the extract was washed four times in PBS. Cell wall fragmentation was then carried out by sonication at 4°C in a continuous dry ice/ethanol bath for 2 hours with a pulse setting of 5.0 (10 second pulse followed by 5 second pause) (550 Sonic dismembrator with a tapped horn and tapered microtip: 1/8” diameter, tuned to vibrate at a fixed frequency of 20kHz, Fisher Scientific, Nepean, ON). The extract was then centrifuged for 1 hour (at 40,000 RPM at 4°C) and the supernatant was harvested to obtain cell wall fragments. The concentration of the cell wall extract was determined by phenol-sulfuric acid determination of the rhamnose sugar content.

5.9 In vivo Studies

For histological analysis, C57BL/6 mice (4-5 weeks old) were injected i.p. with 0.3µg of MAM and 100µg of Pam3Cys-Ser-(Lys)4, Hydrochloride (Pam3Cys, EMD4Biosciences). Control mice were similarly injected with PBS only, MAM only, Pam3Cys only, 100µg of SEB only, SEB and
Pam3Cys, 100μg TSST-1 (Toxin shock syndrome toxin-1, Toxin Technology Inc., Sarasota, FL) only, and TSST-1 and Pam3Cys. Cardiac tissues were removed 28 days post-injection and immediately fixed in Neutral Buffered Formalin 10% (EMD Chemicals).

For quantitative real-time PCR analysis, C57BL/6 mice (4-5 weeks old) were injected i.p. with 5μg of MAM and 100μg of Pam3Cys. Control mice were similarly injected with PBS only, Pam3Cys only or 1mg of LCWE only. Cardiac tissues were removed 24 hours or 3 days post-injection and snap frozen in liquid nitrogen and stored at -80°C until use.

5.10 Histology

Six micron serial sections were prepared and stained with hematoxylin and eosin (H&E) according to a standard protocol by a histologist. Sections were evaluated by a blinded assessor and a score from 0-4 was assigned based on the presence of coronary arteritis. Please refer to Figure 12 for more details.

5.11 RNA Extraction

Organs that were stored at -80°C were thawed at room temperature in Trizol reagent (Gibco) and homogenized using the PT1200 homogenizer (Kinematica). Samples were then centrifuged at 13000 rpm for 10 minutes. Two chloroform (Sigma) extractions were performed with the supernatant. Following, RNA was precipitated with isopropanol (Sigma) and then centrifuged at 13000 rpm. Ethanol (70%) was used to wash the RNA pellet. The pellet was allowed to air-dry and then re-suspended in about 20ul of 0.01% diethylpyrocarbonate (Sigma) water. The concentration of RNA that was obtained was determined by spectrophotometric analysis (Fisher Scientific).
5.12 Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Complementary DNA (cDNA) was converted from isolated RNA using the GeneAmp RNA PCR kit and murine leukemia virus reverse transcriptase (Applied Biosystems). Resulting cDNA was used for quantitative real time RT-PCR (qRT-PCR) using primers and probes for GAPDH (Applied Biosystems) as well as the genes of interest (Assays-on-Demand, Applied Biosystems). Genes of interest include RANTES (Mm01302428_m1), ICAM-1 (Mm00516023_m1) and E-selectin (Mm00441278_m1). A standard curve for RANTES, ICAM-1 and E-selectin was plotted using cDNA obtained from the hearts of an LCWE injected mouse. Using the standard curve and the levels of the housekeeping gene GAPDH, the relative amounts of gene products for the genes of interest were determined and expressed as a ratio (gene of interest to GAPDH). Duplicates of each sample were run using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems).
Figure 12. Materials and Methods: Histological studies. Mice (4-5 weeks old) were injected i.p. with the indicated reagent(s). Cardiac tissues were removed 28 days post-injection and immediately fixed in formalin. Serial sections were prepared and stained with H&E by a histologist. Sections were evaluated by a blinded assessor for the presence of coronary arteritis.
6 Results

6.1 Panel of bacterial superantigens from *Staphylococcus aureus* and *Streptococcus pyogenes* induces a dose dependent proliferative response in mouse splenocytes

The aim of this study was to first assess the T cell dependent factors and then the T cell independent factors that are present in LCWE and that are important for disease induction. The ultimate goal is to treat mice with a combination of the known T cell dependent and T cell independent factors and assess for disease induction.

The first focus is on T cell dependent factors. The T cell dependent factors that are suspected to play a role in disease induction contain superantigenic activity. LCWE has been shown to contain superantigenic activity that correlates with disease induction and has been shown to specifically activate the T cells bearing TCR Vβ2, 4, 6 and 14. In addition, evidence from the lab indicates that in the hearts of diseased mice, the LCWE-reactive TCR Vβ6+ population of T cells is predominantly oligoclonal. This suggests that TCR Vβ6+ T cells may be the subset of LCWE-reactive T cells that does not undergo the usual apoptotic fate and instead is rescued from apoptosis and persists in the hearts of diseased mice.

Since the T cell dependent factor present in LCWE appears to be a SAg that activates pathogenic TCR Vβ6+ T cells, we therefore attempted to identify a pure SAg that is able to activate TCR Vβ6+ T cells in the same way that LCWE can. This SAg along with identified T cell independent factors will then be combined and administered to mice to test for their ability to induce disease.

The mycoplasma-secreted SAg, MAM, is known to activate TCR Vβ6+ T cells and hence a possible candidate SAg to use. There have been reports of mycoplasma infections prior to the development of KD (120, 121). In addition, MAM contains TLR2 activity and TLR2 activity has
been shown to be important for the development of LCWE-induced coronary arteritis. However, MAM is not the most ideal SAg to use in this study because MAM differs from classical SAgs (refer to section 1.2.5). MAM has been reported to interact with both the Vβ region of the TCR and to the highly variable CDR3 of the TCR. Furthermore, TCR Vβ8 is the predominant Vβ family activated by MAM and not TCR Vβ6. Therefore, in search of a more suitable SAg, a panel of novel bacterial SAgs from *Staphylococcus aureus* and *Streptococcus pyogenes* was screened for TCR Vβ6 reactivity along with MAM.

The first step to screening the panel of SAgs from *Staphylococcus aureus* and *Streptococcus pyogenes* for TCR Vβ6 reactivity was to find the optimal SAg dose and culture duration. To determine this, the proliferative response of mouse splenocytes to the panel of SAgs was assessed via \[^3\text{H}\] thymidine incorporation. To determine the dose response and kinetics of proliferation, mouse splenocytes were cultured with each SAg at varying concentrations from 0µg/ml to 10µg/ml (0µg/ml to 2.5µg/ml for MAM) and for varying durations of time from 2 to 6 days (2 to 8 days for MAM) (Figure 13). Cells responding to each SAg from the panel exhibited a proliferative response that was characteristic of known SAgs. Mouse splenocytes differed in their proliferative response to different SAgs in a dose-dependent manner. The dose response did not reach a maximum under the conditions tested because concentrations higher than 10µg/ml were not tested.; this was due to limitations in the availability of reagents. All SAgs induced a dramatic proliferative response that increased with culture duration. This proliferative response reached a maximum at around 3-5 days, after which a rapid decline was observed. The optimal dose and culture duration was determined for use in subsequent experiments.
Figure 13. There is a dose dependent proliferative response of mouse splenocytes to a panel of SAgs from *Staphylococcus aureus*, *Streptococcus pyogenes* and *Mycoplasma arthritidis*. **A) Dose response.** Mouse splenocytes were cultured with 0μ/ml to 10μg/ml of the indicated SAg (0-2.5μg/ml for MAM) for 3 to 4 days. Cultures were pulsed with [3H] for the last 18-20 hours of incubation. Amount of [3H] incorporation was measured by radioactivity as disintegrations per minute (DPM). Splenocytes differed in their proliferative response in a dose-dependent manner. The dose response did not reach a maximum under the conditions tested because concentrations higher than 10μg/ml were not tested. This was due to limitations in the availability of reagents. **B) Kinetics.** Mouse splenocytes were cultured with 10μg/ml of each SAg (2.5μg/ml of MAM) for 2 to 8 days. The amount of thymidine incorporation was assessed on each day. All SAgs induced a dramatic proliferative response that increased with culture duration. This proliferative response reached a maximum at around 3-5 days, after which a rapid decline was observed. Cells responding to each SAg exhibited a proliferative response that is characteristic of known SAgs. The dose and culture duration that induced the greatest proliferative response was determined for use in subsequent experiments. Results are the mean values of DPM of triplicate wells. Error bars represent standard error. Representative of three experiments.
6.2 MAM mediates the expansion of TCR Vβ6+ T cells

With the optimal dose and culture duration, each SAg from the panel was tested for its ability to specifically expand TCR Vβ6+ T cells. To do this, mouse splenocytes were cultured with each SAg at the predetermined concentration and culture duration. The live population of TCR Vβ6+ cells was identified via flow cytometry analysis for propidium iodide (PI), Thy 1.2 (T cell marker) and TCR Vβ6 (Figure 14). SAg cultures where TCR Vβ6+ T cells could be detected were further assessed; these include the cultures with SpeA, SpeC, SpeG, SpeH, SpeJ, SpeK, SpeM, SEK, SmeZ, SSA and MAM. To determine whether the detected population of TCR Vβ6+ T cells was actually expanding in response to the SAg, cells cultured with each of the selected SAgs were assessed for BrdU incorporation along with TCR Vβ6 expression (Figure 15). Although a TCR Vβ6+ population could be detected in the cell cultures with SpeA, SpeC, SpeG, SpeH, SpeJ, SpeK, SpeM, SEK, SmeZ, SSA or MAM (Figure 14), only MAM could mediate the specific expansion of TCR Vβ6+ cells (Figure 15).

Therefore, all 12 novel bacterial SAgs that were screened failed to mediate the expansion of TCR Vβ6+ T cells. MAM remains as the only known SAg that mediates TCR Vβ6+ expansion. The results from these experiments confirm the TCR Vβ6 reactivity of MAM. As expected, MAM also mediated the specific expansion of TCR Vβ8+ T cells. Interestingly, the TCR Vβ8+ population was expanded by MAM to a much greater extent than the TCR Vβ6+ T cells (Figure 16). Although not the most ideal SAg (as discussed in section 6.1), MAM was used in subsequent experiments.
Figure 14 (please refer to next page for figure caption)
**Figure 14. Expansion of TCR Vβ6+ T cell populations.** A) Mouse splenocytes were cultured with the indicated reagent (*blue histograms*) at a concentration and for a culture duration that was previously determined to be optimal for proliferation. Cells were then harvested, stained for PI, Thy1.2 and TCR Vβ6, and analyzed by flow cytometry. Populations shown are gated on live Thy1.2+ cells. *Red histograms* represent the unstained controls. Representative of three experiments. B) Bar graph representing the three replicates from A. Error bars represent standard error. SAgs cultures where TCR Vβ6+ T cells could be detected were further assessed in subsequent experiments to determine whether the detected population of TCR Vβ6+ T cells is actually proliferating in response to each SAg.
Figure 15 (please refer to next page for figure caption)
Figure 15. MAM mediated the specific expansion of TCR Vβ6+ T cells. A) Mouse splenocytes were cultured with select SAgs from the panel at a concentration and for a culture duration that was previously determined to be optimal for proliferation. Only the SAg cultures where TCR Vβ6 could be detected in the previous experiment were tested in this experiment. Cultures were pulsed with BrdU during the last 18-20 hours of incubation. Cells were harvested, stained for BrdU and TCR Vβ6, and analyzed by flow cytometry. MAM was the only culture where TCR Vβ6+BrdU+ cells could be identified and therefore the only SAg that could mediate the expansion of TCR Vβ6+ T cells. Populations shown are gated on live cells based on forward scatter and side scatter properties. Representative of three experiments. B) Bar graph representing the three replicates from A. Error bars represent standard error.
6.3 SEB-activated costimulation-rescued T cells exhibit an activated phenotype

Once again, since the T cell dependent factor present in LCWE appears to be a SAg that activates pathogenic TCR Vβ6+ T cells, we attempted to identify a pure SAg that is able to
activate TCR Vβ6+ T cells in the same way that LCWE can. This SAg along with identified T cell independent factors will then be combined and administered to mice to test for their ability to induce disease. From the previous experiments, MAM was the only tested SAg that could activate TCR Vβ6+ T cells. To evaluate the ability of MAM to activate T cells, MAM will be compared first to a prototypical SAg, SEB and then to the known disease inducing agent, LCWE.

SEB is a prototypical purified recombinant SAg. This is in contrast to LCWE which is a crude extract and MAM which is not a classical SAg (refer to section 1.2.5). SEB will be used to demonstrate what the response of T cells is to a prototypic SAg. Previously in the lab SEB has been used to demonstrate the rescue phenomenon. Mouse splenocytes cultured with SEB and an anti-CD28 Ab undergo less apoptosis compared to mouse splenocytes cultured with an isotype control Ab.

For this experiment, mouse splenocytes were cultured with SEB and an anti-CD28 Ab. On day 7, cells were harvested and stained for flow cytometry analysis. The expression levels of CD25, CD69, CD134, CD44 or CD62L by the rescued cells (identified by TCRVβ8+AnnexinVlow staining) were determined. The TCRVβ8+AnnexinVlow cells express high levels of CD25, CD69, CD134 and CD44, but express low levels of CD62L (Figure 17) compared to medium only. This is consistent with an activated phenotype. These results are representative of the response of T cells to a prototypical SAg and enhanced costimulation. Please refer to section 6.5 for the comparison to MAM-activated costimulation-rescued T cells.
Figure 15 (please refer to next page for figure caption)
6.4 LCWE-activated costimulation-rescued T cells exhibit an activated phenotype

With the activation marker expression in SEB-activated costimulation-rescued T cells determined, the next step was to determine the activation marker expression in LCWE-activated costimulation-rescued T cells. This will allow for the comparison of LCWE and MAM to see if MAM can activate T cells in the same way LCWE does. LCWE is the disease inducing agent and LCWE-activated costimulation-rescued TCR Vβ6+ T cells are suspected to play a pathogenic role in our disease model. Consistent with this hypothesis, LCWE has been shown to contain superantigenic activity which is correlated with the induction of disease. In addition, it has been previously shown in the lab that costimulation can indeed rescue SAg-activated T cells from apoptosis. In vitro, LCWE-reactive TCR Vβ6+ T cells undergo decreased amounts of apoptosis.
and increased amounts of proliferation when cultured with LCWE and an anti-CD28 Ab, compared to cells that are cultured with LCWE and an isotype control Ab.

To determine the activation state of LCWE-activated costimulation-rescued T cells, mouse splenocytes were cultured with LCWE and an anti-CD28 Ab. On day 5, cells were harvested and stained for flow cytometry analysis. The expression levels of CD25, CD69, CD134, CD44 or CD62L by the rescued cells (identified by TCRβ6+AnnexinVlow staining) were determined. The TCRβ6+AnnexinVlow cells express high levels of CD25, CD69, CD134 and CD44, but express low levels of CD62L (Figure 18) compared to medium only. This activation maker profile is consistent with that of an activated phenotype. Therefore, LCWE-activated costimulation-rescued T cells not only experienced decreased levels of apoptosis (as shown previously) but also exhibit an activated phenotype. This is consistent with the hypothesis that these rescued cells are pathogenic and responsible for disease. Since there was no difference detected in the activation profiles of SEB- and LCWE-activated costimulation rescued T cells, we can conclude that the activation marker profile of LCWE-activated costimulation-rescued T cells is generalizable to that of the prototypical SAg, SEB. This suggests that these responses are SAg-mediated. Please refer to section 6.5 for the comparison to MAM-activated costimulation-rescued T cells.
Figure 18 (please refer to next page for figure caption)
**Figure 18.** LCWE-activated costimulation-rescued T cells exhibit an activated phenotype as indicated by cell surface marker expression. Mouse splenocytes were cultured with LCWE and anti-CD28 Ab for 5 days. Cells were harvested and stained for flow cytometry analysis. **A)** Analysis was first gated on TCRVβ6+AnnexinV\text{low} cells. **B)** After gating on TCR Vβ6+AnnexinV\text{low} cells, the expression of CD25, CD44, CD62L, CD69 or CD134 was assessed (blue histogram). The green, red, turquoise and orange histograms represent the unstained, medium only, fluorescence minus one and isotype controls, respectively. The TCRVβ6+AnnexinV\text{low} cells (the rescued cells) express high levels of CD25, CD69, CD134 and CD44, but express low levels of CD62L compared to medium only. This pattern of activation marker expression indicates an activated phenotype and is consistent with the hypothesis that these rescued cells are pathogenic and responsible for disease. These data will be used to compare to MAM-activated costimulation-rescued T cells. Representative of three experiments.

### 6.5 MAM-activated costimulation-rescued T cells exhibit an activated phenotype

With the activation marker expression in SEB-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells determined, the ability of MAM to activate T cells could be evaluated. The expression of various activation markers in MAM-activated costimulation-rescued T cells was assessed, and compared to SEB-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells. These results will be used to determine the suitability of MAM to be injected as a T cell dependent factor along with identified T cell independent factors to induce disease.

To determine the activation state of MAM-activated costimulation-rescued T cells, mouse splenocytes were cultured with MAM and an anti-CD28 Ab. On day 6, cells were harvested and
stained for flow cytometry analysis. The expression levels of CD25, CD69, CD134, CD44 or CD62L by the rescued cells (identified by TCRVβ6⁺AnnexinVlow staining) were determined. The TCRVβ6⁺AnnexinVlow cells express high levels of CD25, CD69, CD134 and CD44, but express low levels of CD62L (Figure 19) compared to medium only.

Therefore, there was no difference detected in the activation profiles of LCWE- and MAM-activated costimulation rescued T cells. This suggests that MAM has T cell activating properties that are similar to that seen in LCWE and is supportive of the hypothesis that MAM may be suitable for injection into mice as a potential disease-inducing T cell dependent factor. Furthermore, since there was no difference detected in the activation profiles of SEB- and MAM-activated costimulation rescued T cells, we can conclude that the activation marker profile of MAM-activated costimulation-rescued T cells was generalizable to that of the prototypical SAg, SEB. This suggests that these responses are SAg-mediated.
Figure 19 (please refer to next page for figure caption)
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Figure 19. MAM-activated costimulation-rescued T cells exhibit an activated phenotype as indicated by cell surface marker expression. Mouse splenocytes were cultured with MAM and anti-CD28 Ab for 6 days. Cells were harvested and stained for flow cytometry analysis. A) Analysis was first gated on TCRβ6⁺AnnexinVlow cells. B) After gating on TCR Vβ6⁺AnnexinVlow cells, the expression of CD25, CD44, CD62L, CD69 or CD134 was assessed (blue histogram). The green, red, turquoise and orange histograms represent the unstained, medium only, fluorescence minus one and isotype controls, respectively. The TCRβ6⁺AnnexinVlow cells (the rescued cells) express high levels of CD25, CD69, CD134 and CD44, but express low levels of CD62L compared to medium only. This pattern of activation marker expression is consistent with an activated phenotype. Similarity between LCWE- and MAM-activated T cells suggests that MAM would be a suitable candidate SAg to test in vivo. Similarity between SEB- and MAM- activated T cells suggests that these responses are SAg-mediated. Representative of two experiments.

6.6 SEB-activated costimulation-rescued T cells secrete IFN-γ and TNF-α

To further evaluate the T cell activating properties of MAM, the production of signature T helper cytokines by MAM-activated costimulation-rescued T cells was compared first to a prototypical SAg, SEB, and then to the disease inducing agent, LCWE.

As before, SEB was used to demonstrate what the response of T cells was to a prototypic SAg. Mouse splenocytes were cultured with SEB and an anti-CD28 Ab for 7 days. For the last 5 hours of culture, cells were recultured with PMA/Ionomycin to stimulate the production of cytokines, and GolgiPlug to maintain detectable levels of cytokines within the cell. The production of IFN-γ, TNF-α, IL-4, IL-10 and IL-17 by the rescued cells was determined via flow cytometry analysis. Cytokine levels were measured in the live (indicated by a fixable viability dye), CD4⁺
and TCRVβ8<sup>+</sup> population of cells. In response to restimulation, the rescued T cells produced IFN-γ and TNF-α. However, there were no detectable levels of IL-4, IL-10 or IL-17 produced (Figure 20). TNF-α is a proinflammatory cytokine that is produced by different T helper subsets including Th1 cells. TNF-α has been shown to be necessary for disease induction in our animal model as TNFR1 knockout mice are resistant to the development of coronary arteritis and coronary artery aneurysms. The production of IFN-γ is consistent with a Th1 phenotype. The cytokine production profile from the rescued cells indicated that they were functional. These results are representative of the response of T cells to a prototypical SAg and enhanced costimulation. Please refer to section 6.8 for the comparison to MAM-activated costimulation-rescued T cells.
Figure 20 (please refer to next page for figure caption)
With the cytokine production pattern in SEB-activated costimulation-rescued T cells determined, the next step was to determine the cytokine production pattern in LCWE-activated costimulation-rescued T cells. This will allow for the comparison of LCWE and MAM to see if MAM can activate T cells in the same way LCWE does. LCWE is the disease inducing agent and LCWE-activated costimulation-rescued TCR Vβ6+ T cells are suspected to play a pathogenic role in our disease model.

To determine the cytokine production pattern of LCWE-activated costimulation-rescued T cells, mouse splenocytes were cultured with LCWE and an anti-CD28 Ab for 5 days. For the last 5 hours of culture, cells were recultured with PMA/Ionomycin to stimulate the production of cytokines, and GolgiPlug to maintain detectable levels of cytokines within the cell. The
production of the following cytokines by the rescued cells was determined via flow cytometry analysis: the Th1 cytokine IFN-γ, TNF-α, the Th2 cytokines IL-4 and IL-10, and the Th17 cytokine IL-17. Cytokine levels were measured in the live (indicated by a fixable viability dye), CD4+ and TCRβ6+ population of cells. In response to restimulation, the rescued T cells produced IFN-γ and TNF-α. However, there were no detectable levels of IL-4, IL-10 or IL-17 produced (Figure 21).

Therefore, LCWE-activated costimulation-rescued T cells not only experience decreased levels of apoptosis (Y. Moolani, submitted manuscript) and increased expression of activation markers (as shown in current study), but also exhibit a functional phenotype (as indicated by cytokine production). This is consistent with the hypothesis that these rescued cells are pathogenic and responsible for disease. Since there was no difference detected in the cytokine production pattern of LCWE- and SEB-activated costimulation rescued T cells, we can conclude that the cytokine production pattern of LCWE-activated costimulation-rescued T cells was generalizable to that of the prototypical SAg, SEB. This suggests that these responses are SAg-mediated. Please refer to section 6.8 for the comparison to MAM-activated costimulation-rescued T cells.
Figure 21 (please refer to next page for figure caption)
With the cytokine production pattern in SEB-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells determined, the ability of MAM to activate T cells could be further evaluated. The cytokine production pattern in MAM-activated costimulation-rescued T cells was assessed and compared to SEB-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells. This will be used to determine the suitability of MAM to be injected as a T cell dependent factor along with identified T cell independent factors to induce disease.

To determine the cytokine production pattern of MAM-activated costimulation-rescued T cells, mouse splenocytes were cultured with MAM and an anti-CD28 Ab for 6 days. For the last 5
hours of culture, cells were recultured with PMA/Ionomycin to stimulate the production of cytokines, and GolgiPlug to maintain detectable levels of cytokines within the cell. The production of IFN-γ, TNF-α, IL-4, IL-10 and IL-17 by the rescued cells was determined via flow cytometry analysis. Cytokine levels were measured in the live (indicated by a fixable viability dye), CD4^+ and TCRVβ6^+ population of cells. In response to restimulation, the rescued T cells produced IFN-γ and TNF-α. However, there were no detectable levels of IL-4, IL-10 or IL-17 produced (Figure 22). This pattern of cytokine production is consistent with a Th1 phenotype.

Therefore, there was no difference detected in the cytokine production pattern of LCWE- and MAM-activated costimulation rescued T cells. MAM-activated costimulation-rescued T cells are functional and able to produce the same pattern of cytokines upon restimulation as LCWE-activated costimulation-rescued T cells. This suggests that MAM has T cell activating properties that are similar to that seen in LCWE and is supportive of the hypothesis that MAM may be suitable for injection into mice as a potential disease-inducing T cell dependent factor. Furthermore, since there was no difference detected in the cytokine production pattern of SEB- and MAM-activated costimulation rescued T cells, we can conclude that the cytokine production pattern of MAM-activated costimulation-rescued T cells was generalizable to that of the prototypical SAg, SEB. This suggests that these responses are SAg-mediated.
Figure 22 (please refer to next page for figure caption)
Figure 22. MAM-activated costimulation-rescued T cells are functional and produce cytokines that are consistent with the Th1 subset. Mouse splenocytes were cultured with MAM and anti-CD28 Ab for 6 days. For the last 5 hours of culture, cells were recultured with PMA/Ionomycin to stimulate the production of cytokines, and GolgiPlug to maintain detectable levels of cytokines within the cell. Cells were then harvested and stained for flow cytometry analysis. A) Analysis was first gated on live CD4$^+$ cells. B) After gating on live CD4$^+$ cells, the expression of TCR Vβ6 along with IFN-γ, TNF-α, IL-17, IL-4 and IL-10 was assessed. In response to restimulation, the rescued T cells produced IFN-γ and TNF-α. However, there were no detectable levels of IL-4, IL-10 or IL-17 produced. This suggests that MAM-activated costimulation-rescued T cells are functional and exhibit a Th1 phenotype. Similarity between LCWE- and MAM- activated T cells suggests that MAM would be a suitable candidate SAg to test in vivo. Similarity between SEB- and MAM- activated T cells suggests that these responses are SAg-mediated. Representative of two experiments.

6.9 The TCR Vβ6-stimulating SAg, MAM, does not induce coronary arteritis in mice

In the following experiments, we aimed to further dissect and understand how T cell dependent and T cell independent factors contribute to disease. Since previous studies in the lab have shown that disease induction from LCWE-injections consists of T cell dependent and T cell independent factors, we predicted that the injection of these known factors would induce coronary arteritis in mice. T cell dependent factors which may be important in our disease model would expand TCR Vβ6$^+$ expressing T cells. MAM was found to be the most ideal T cell dependent factor to be used since it was demonstrated that MAM-activated costimulation-rescued TCR Vβ6$^+$ T cells exhibit an activated phenotype (as indicated by activation marker expression and cytokine production) that is very similar to LCWE-activated costimulation-rescued TCR Vβ6$^+$ T cells. T cell
independent factors which are hypothesized to be important for disease include TLR2 agonists (such as Pam3Cys). Therefore, it is hypothesized that together MAM and Pam3Cys will activate functional and persistent TCR Vβ6+ T cells leading to disease induction; MAM will activate TCR Vβ6+ T cells and TLR2 will mediated the upregulation of costimulatory molecules allowing for rescued of MAM-activated T cells.

In dissecting the contribution of T cell dependent versus T cell independent factors in the pathogenesis of coronary arteritis, the ability of MAM and Pam3Cys injections to induce coronary arteritis was assessed. Control mice were similarly injected with PBS only, Pam3Cys only, MAM only, SEB only, TSST-1 only, SEB and Pam3Cys, and TSST-1 and Pam3Cys. Since MAM activates both TCR Vβ6 and TCR Vβ8, SEB and TSST-1 were used as specificity controls; SEB is a SAg that activates TCR Vβ8 but not TCR Vβ6, and TSST-1 is a SAg that activates neither TCR Vβ6 nor TCR Vβ8. After 28 days, mice were sacrificed and cardiac tissues were removed to assess for the presence of coronary arteritis. Coronary arteritis was not significantly observed in any of the control groups nor observed in any of the mice that were injected with MAM and Pam3Cys (Figure 23). The results are summarized in Table 3. It is interesting to note that although coronary arteritis was initially observed in 2 of the mice injected with TSST-1 and Pam3Cys, these observations were not significant because they could not be seen again in subsequent repeats of the experiment. Out of a total of 17 mice that were injected with TSST-1 and Pam3Cys, coronary arteritis could only be observed in 2 of the mice. The coronary arteritis observed in those 2 mice was likely due to a nonspecific effect of the Pam3Cys injections. Since Pam3Cys is a TLR2 agonist, it sends a generalized danger signal when injected into mice; therefore, it is not surprising that we may see an inflammatory response at the coronary arteries. If we looked at the rest of the heart or at the other organs, we would likely see
inflammation there as well. This nonspecific effect of Pam3Cys has been previously demonstrated in the lab; Pam3Cys injections were shown to not only exacerbate coronary arteritis in the LCWE-induced coronary arteritis model of KD but also to cause increased myocarditis and inflammation in other organs (K. Little, submitted manuscript).
Figure 23. The TCR Vβ6 stimulating SAg, MAM, does not induce coronary arteritis. A) C57BL/6 mice were injected i.p. with MAM and Pam3Cys. Control mice were injected with PBS only, Pam3Cys only, MAM only, SEB with or without Pam3Cys, and TSST-1 with or without Pam3Cys. Hearts were removed 28 days post-injection and H&E sections prepared. Sections are representative of at least 5 injected mice (refer to Table 3). Coronary arteritis (as indicated by lymphocytic infiltrate) was not significantly observed in any of the control groups. Although our T cell activation assessments of MAM indicated that MAM would be a good candidate SAg to induce disease in mice, coronary arteritis was also not observed in any of the mice that were injected with MAM and Pam3Cys. B) C57BL/6 mice that were injected i.p. with PBS or LCWE. H&E sections show coronary arteries 28 days post-injection. B is previously collected data from Hui-Yuen et al (59).
Table 3. Incidence of coronary arteritis in mice injected with MAM and Pam3Cys.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incidence*</th>
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<tbody>
<tr>
<td>MAM</td>
<td>0/8</td>
</tr>
<tr>
<td>MAM+Pam3Cys</td>
<td>0/10</td>
</tr>
<tr>
<td>SEB</td>
<td>0/4</td>
</tr>
<tr>
<td>SEB+Pam3Cys</td>
<td>0/5</td>
</tr>
<tr>
<td>TSST-1</td>
<td>0/8</td>
</tr>
<tr>
<td>TSST-1+Pam3Cys</td>
<td>2/17</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>3/17</td>
</tr>
<tr>
<td>PBS</td>
<td>0/14</td>
</tr>
<tr>
<td>LCWE</td>
<td>30/35**</td>
</tr>
<tr>
<td>PBS</td>
<td>0/22**</td>
</tr>
</tbody>
</table>

*Incidence defined as the number of mice with coronary artery arteritis divided by the total number of mice injected.

**Previously collected data by Hui-Yuen et al (59).

***Pooled with previously collected data by K. Little.

6.10 Unlike LCWE-injected mice, MAM and Pam3Cys-injected mice do not upregulate adhesion and leukocyte recruitment molecules in the heart

It is possible that coronary arteritis was not observed with MAM and Pam3Cys injections despite the same T cell activating properties for MAM and LCWE, and despite the presence of a TLR2
agonist because there are yet additional unidentified factors beyond T cell activation and TLR2 agonistic activity that may be important for disease induction.

It has been previously shown that LCWE injections can upregulate adhesion and leukocyte recruitment molecules such as RANTES (chemokine ligand 5 or CCL5), ICAM-1 (intracellular adhesion molecule-1) and E-selectin (CD62E) in the hearts of mice. In TNFR1 deficient mice, where a localized inflammatory response is not seen at the coronary arteries of LCWE-injected mice, upregulation of these adhesion and leukocyte recruitment molecules is not observed.

To examine why injections of MAM and Pam3Cys did not induce disease despite the activated and functional phenotype of MAM-activated costimulation-rescued TCR Vβ6+ T cells, we looked at the expression of local migratory signals at the hearts of MAM and Pam3Cys injected mice. Real time RT-PCR was performed to determine mRNA expression of RANTES, ICAM-1 and E-selectin in the heart tissues of LCWE-injected, and MAM and Pam3Cys-injected mice at 24h and 3 days post-injection. As expected, LCWE induced upregulation of RANTES, ICAM-1 and E-selectin at both time points (Figure 24). However, expression levels of RANTES, ICAM-1 and E-selectin mRNA were significantly lower in MAM and Pam3Cys-injected mice. Therefore, the impaired upregulation of local migratory signals at the hearts of MAM and Pam3Cys injected mice may indicate that there are yet other unidentified factors that are important for disease induction.
Figure 24 (please refer to next page for figure caption)
Figure 24. There is lower expression of local migratory signals in the hearts of MAM and Pam3cys- injected mice when compared to LCWE-injected mice. Mice were injected with MAM and Pam3cys, LCWE only, Pam3cys only or PBS. Mice were sacrificed and heart tissues removed at 24h. Real time RT-PCR was performed to determine the expression of A) RANTES, B) E-selectin and C) ICAM-1. As expected, LCWE induced upregulation of RANTES, E-selectin and ICAM-1 at 24h post-injection. However, in MAM and Pam3cys-injected mice, expression levels of RANTES (p=0.002), E-selectin (p=0.0016) and ICAM-1 (p=0.0045) mRNA were significantly lower. The lack of upregulated migratory signals at the hearts of injected mice may indicate that there are yet other unidentified factors that are important for disease induction. Error bars represent standard error. Representative of two experiments.
7 Discussion

7.1 LCWE-induced coronary arteritis model of KD

Evidence from both patients and from a murine model has implicated SAgs in the etiology of KD. In KD patients, there is skewing of the peripheral blood T cell repertoire particularly towards TCR Vβ2, increase in SAg- secreting bacteria and seroconversion of IgM antibodies against SAgs of 

*Staphylococcus aureus* and *Streptococcus pyogenes*. In a murine model of KD, the disease inducing agent LCWE has been shown to have superantigenic activity that directly correlates with the ability of LCWE to induce coronary arteritis. LCWE skews T cells towards TCR Vβ2, 4, 6 and 14.

The apparent contradiction between the apoptotic fate of SAg-activated T cells and their possible involvement in the persistent immune response at the coronary arteries in KD patients remains unresolved. One possible explanation is provided in a study by McCormack *et al*, whereby concurrent stimulation with a specific peptide antigen could block SAg-mediated deletion of T cells *in vivo*. When mice were treated with a SAg and a peptide antigen that shared similar TCR Vβ specificity, all SAg- reactive T cells were deleted except for a subpopulation of T cells that were dually reactive to both the SAg and the peptide antigen. This subpopulation of T cells instead proliferated in response to the peptide antigen.

We propose that this rescue phenomenon may be involved in the pathogenesis of KD. It may provide an explanation for how SAg-activated T cells could be involved in a persistent immune response at the coronary arteries. Evidence from our animal model is consistent with this hypothesis. Characterization data of T cells infiltrating the heart of diseased mice suggests that they are predominantly TCR Vβ6+ and oligoclonal. These results are in line with the hypothesis that TCR Vβ6+ T cells may be the LCWE-reactive subpopulation that is rescued by the presence of a peptide antigen and therefore able to evade apoptosis to persist at the heart.
7.2 The T cell dependent factor in LCWE-induced model of KD

In this project, we aimed to dissect the factors that are involved in disease induction in our animal model. Previous studies have shown that there were both T cell dependent and T cell independent factors that are important. One goal of this study was to determine the importance of T cell activation, particularly the pathogenicity of TCR Vβ6⁺ T cells. One of the biggest challenges that face testing this hypothesis is that the peptide in question remains unidentified and as a result, the clonality of the pathogenic subpopulation of TCR Vβ6⁺ T cells remains unknown. With the identity of the peptide unknown, we are limited to testing our hypothesis in conditions where either all TCR Vβ6 clones are deleted or all TCR Vβ6 clones are expanded. We could demonstrate that TCR Vβ6⁺ T cells are necessary for disease induction if we can show that disease does not occur in LCWE- injected mice that are specifically deficient for these cells. On the other hand, we could demonstrate that TCR Vβ6⁺ T cells are sufficient for disease if we can show either that disease occurs in mice where there is specific expansion of these cells, or that disease occurs in mice that receive adoptively transferred TCR Vβ6⁺ T cells.

There exist a few models where TCR Vβ6⁺ T cells can be depleted in mice. For example, exogenous MMTV (SW) can be maternally transmitted via milk leading to the deletion of among others, TCR Vβ6⁺ T cells in neonatal mice. In addition, mice that carry the Mls-1a gene (which is a SAg gene incorporated into the mouse genome from a MMTV) delete all T cells expressing TCR Vβ6, 7, 8.1 and 9 from their peripheral T cell pool. Examples of mice that carry the Mls-1a gene are CBA/J, AKR and DBA/2 mice (122). The role of TCR Vβ6⁺ T cells could be tested in these mice by treating them with the disease inducing agent (LCWE). In the absence of the pathogenic TCR Vβ6⁺ population of T cells, we would not expect to see the development of coronary artery disease. Alternatively, if a SAg that is specific for TCR Vβ6 can be found,
neonatal mice could be injected with an the SAg, resulting in the deletion of all T cell clones that carry TCR Vβ6.

Although depletion of TCR Vβ6 could be explored, it did not present as the best approach for a variety of reasons. With the above methods, other TCR Vβ families are deleted in addition to TCR Vβ6. As a result, the specificity of any differences that are observed could not be attributed solely to the TCR Vβ6 population of T cells. In addition, the mice strains that carry the Mls-1a antigen have a different genetic background from the C57BL/6 mice used in our animal model. LCWE does not induce disease in all genetic backgrounds of mice. For example, LCWE has been shown to induce disease at different rates in C57BL/6, A/J, Balb/c and C3Heb/FeJ mice, and not induce disease in C3H/HeJ mice. As a result, differences observed in disease induction between LCWE-injected C57BL/6 mice and LCWE-injected Mls-1a antigen-carrying mice could not be attributed solely to TCR Vβ6+ T cells. One possibility is to find a strain of C57BL/6 mice that carry exogenous MMTV specific for TCR Vβ6 that is maternally transmitted via milk. However, to the best of our knowledge, that is not commercially available.

Hence, the positive and not the negative route was taken to test the pathogenicity of TCR Vβ6+ T cells. To accomplish this, the TCR Vβ6+ T cells were expanded in mice and the presence of coronary arteritis was evaluated. If expansion of the TCR Vβ6+ T cells lead to coronary arteritis, it would show that these cells are necessary for disease. However, as previously mentioned, disease induction involves more than just T cell activation and therefore other T cell independent factors need to be provided as well (refer to Section 7.4 for a discussion about T cell independent factors).

Firstly, we aimed to identify a T cell dependent factor (a SAg that could activate TCR Vβ6 T cells) that could be used to test for disease induction. To our knowledge, the only known purified
recombinant SAg that activates TCR Vβ6 is MAM and this remains the case even after our screen of a panel of novel bacterial SAgs. Consequently, MAM was tested for its ability to activate T cells and for its ability to induce coronary arteritis.

### 7.3 Assessment of the pathogenic role of TCR Vβ6 T cells

The suitability of MAM to be used *in vivo* to activate the suspected pathogenic TCR Vβ6 expressing T cells was determined by comparing the ability of MAM to activate T cells to that of a prototypical SAg (SEB) and the disease inducing SAg (LCWE).

In this study the pattern of activation marker expression in SEB-activated costimulation-rescued T cells was found to be CD25<sup>hi</sup>CD69<sup>hi</sup>CD134<sup>hi</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> which is indicative of an activated phenotype, not a resting phenotype. Similar to this prototypic SAg, MAM-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells were also found to express the CD25<sup>hi</sup>CD69<sup>hi</sup>CD134<sup>hi</sup>CD44<sup>hi</sup>CD62L<sup>lo</sup> activated phenotype. Since there was no difference detected in the activation profiles of SEB-, LCWE- and MAM-activated costimulation rescued T cells, we can conclude that MAM can activate T cells in a similar manner as the disease inducing SAg (LCWE) and the prototypical SAg (SEB).

The expression of CD25, CD44, CD69 and CD134 is activation dependent. In addition, signaling through these molecules promotes T cell proliferation, T cell differentiation and/or assists in effector T cell functions. For example, CD25 is the α chain of the IL-2 receptor which is required to form the receptor variant that has the highest affinity for IL-2. IL-2 induces proliferation and differentiation of T cells. CD44 mediates attachment to extracellular matrix components (primarily to HA) and so may be involved in extravasation of activated lymphocytes to sites of inflammation. CD69 contains an extracellular domain that facilitates binding to sugar residues found on the cell surface of bacteria, parasites and fungi. CD134 is a secondary costimulatory
molecule that has been found to be essential for the long-term survival of T cells (i.e. maintenance of high levels of Bcl-2 and Bcl-xL). On the other hand, CD62L on naïve T cells facilitates extravasation into secondary lymph nodes for the initial antigen exposure. Effector/memory T cells do not express CD62L allowing them to circulate in the periphery and perform effector functions. Overall, the expression profile of CD25^{hi}CD69^{hi}CD134^{hi}CD44^{hi}CD62L^{lo} is consistent with that of an activated population of cells with the capacity to further differentiate, proliferate and perform T cell effector functions that are required to propagate an immune response, presumably at the coronary arteries.

In further characterizing the SAg-activated costimulation-rescued T cells, the pattern of cytokine release was determined. In this study, the pattern of cytokine release in SEB-activated costimulation-rescued T cells was found to include detectable levels of IFN-γ and TNF-α, but not IL-4, IL-10 or IL-17. These results are consistent with a functional and Th1 phenotype in the rescued cells. Similar to this prototypic SAg, MAM-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells were also found to secrete detectable levels of IFN-γ and TNF-α, but not IL-4, IL-10 or IL-17. Since there was no difference detected in the cytokine production profiles of SEB-, LCWE- and MAM-activated costimulation rescued T cells, we can conclude that MAM can activate T cells in a similar manner as the disease inducing SAg (LCWE) and the prototypical SAg (SEB).

Furthermore, results from this study are supportive of a Th1 phenotype for the rescued T cells; this is consistent with what is found in KD patients as well as in our animal model of KD. Some studies find mRNA levels of both the Th1/Th2 cytokines (IFN-γ and IL-4), and both the Th1/Th2 transcription factors (T-bet and GATA-3) were decreased in the PBMCs of KD patients when compared to healthy controls (123, 124). On the other hand, there are studies that find a decrease
in IFN-γ producing T cells and unchanged levels of IL-4 producing T cells during the acute stage of KD. Although this could suggest a diminished role for Th1 cells in KD, an alternative explanation is that the decrease in IFN-γ producing cells during the acute phase of KD is due to the infiltration of activated T cells and therefore a shift of the IFN-γ producing T cells into the vascular tissue compartment. Supportive of this hypothesis, biopsy specimens from skin lesions of acute KD patients indicate increased TNF-α levels and IFN-γ levels (125, 126). Furthermore, as previously mentioned, studies in our animal model have shown that both TNF-α and IFN-γ are upregulated in the hearts of LCWE-injected mice 28 days post-injection. Time of maximal expression of TNF-α and IFN-γ mRNA in the hearts of LCWE-injected mice coincide with time of maximal coronary artery infiltration (28 days post-injection) (59, 60).

In this study, LCWE-activated costimulation-rescued T cells were characterized by activation marker and cytokine expression. This characterization was important because it was used to evaluate the activation state of MAM-activated costimulation-rescued T cells. In addition, this characterization was important because it has not been shown before that SAg-activated costimulation-rescued T cells are activated and functional. It has been shown previously that SAg-activated costimulation-rescued T cells undergo less apoptosis but the activation state of these persisting T cells and their ability to perform effector functions was undetermined until now.

7.4 T cell independent factors in LCWE-induced model of KD

Previous studies in the lab suggest that disease induction from LCWE-injections consists of both T cell dependent and T cell independent factors. Therefore, we predicted that the injection of known T cell dependent and T cell independent factors would induce coronary arteritis in mice. T cell dependent factors which are hypothesized to be important in our disease model would
expand TCR Vβ6 expressing T cells. MAM was found to be the most ideal T cell dependent factor to be used since it was demonstrated that MAM-activated costimulation-rescued TCR Vβ6+ T cells exhibit an activated phenotype (as indicated by activation marker expression and cytokine production) that is very similar to LCWE-activated costimulation-rescued TCR Vβ6+ T cells. T cell independent factors which are hypothesized to be important for disease include TLR2 agonists (such as Pam3Cys). Evidence of TLR2 involvement in our disease model are as follows: TLR2 knockout mice do not get coronary arteritis, TLR2-mediated upregulation of costimulatory molecules can rescue SAg-activated T cells, injection of mice with a TLR2 ligand along with LCWE results in an increased incidence and severity of coronary disease, and there is some evidence of preferential expression of TLR2 at the coronary arteries. TLR2 appears to play a role in the localization and persistence of the immune response via TLR2-mediated upregulation of costimulatory molecules rescuing SAg-activated T cells from apoptosis. Therefore, it is hypothesized that MAM and Pam3Cys would activate TCR Vβ6+ T cells which would be rescued from apoptosis by Pam3Cys mediated upregulation of costimulatory molecules. Since this population of TCR Vβ6+ T cells would be activated and functional, we postulated that it would be able to mediate coronary artery inflammation. However, when MAM and Pam3Cys were injected into mice, coronary arteritis was not observed. There are several reasons why this may have occurred.

First, MAM causes the expansion of TCR Vβ8+ T cells to a much greater extent. One reason for this may be that the precursor population of TCR Vβ8 T cells (about 15-20%) is much higher than that of TCR Vβ6 (about 5%). Therefore, expansion of TCR Vβ8 T cells by MAM in addition to expansion of TCR Vβ6 T cells results in a disproportionately large increase in TCR Vβ8 T cells in comparison to the modest increase in TCR Vβ6+ T cells. The population of TCR Vβ8 T cells is not LCWE- reactive (LCWE- reactive populations include only TCR Vβ2, 4, 6
and 14) and not suspected to be pathogenic in our animal model. The effect of this massive population on expanded Vβ6+ T cells and their ability to cause damage at the heart is unknown.

Secondly, coronary arteritis may not be observed with MAM and Pam3Cys injection despite the same T cell activating properties for MAM and LCWE, and despite the presence of a TLR2 agonist because there may be as yet additional unidentified factors beyond T cell activation and TLR2 agonistic activity that may be important for disease induction.

One difference found between mice that were injected with LCWE and mice that were injected with MAM+Pam3Cys was the expression of various local migratory signals at the heart, which served as a useful metric preceding inflammation of the heart. Impaired upregulation of chemokines such as RANTES and MIP-1α; and adhesion molecules such as ICAM-1, E-selectin and VCAM-1 (vascular cell adhesion molecule-1) in the hearts of mice has been shown to be associated with the absence of localized inflammation at the coronary arteries (59). Since coronary arteritis could not be seen in our system of MAM and Pam3Cys injected mice, we aimed to determine whether the lack of disease, in this case, was also associated with impaired upregulation of local migratory signals at the heart. Real time RT PCR performed on hearts of injected mice demonstrated that mRNA expression of RANTES, ICAM-1 and E-selectin was significantly upregulated in LCWE injected mice when compared to MAM and Pam3Cys injected mice (24h post-injection). Upregulation was not seen in mice injected with MAM and Pam3Cys at 72h post-injection. This is consistent with previous data whereby maximal expression of RANTES, ICAM-1 and E-selectin was seen in the hearts of LCWE-injected mice 24h post-injection and this expression was diminished by 72h post-injection.

At 24h post-injection, there was a significantly higher expression of RANTES and ICAM-1 in hearts of MAM and Pam3Cys-injected mice compared to PBS-injected control mice. Since the
expression levels of these molecules were not significantly different between the hearts of MAM+Pam3Cys- injected mice and Pam3Cys-injected control mice, the upregulation of these molecules in MAM+Pam3Cys- injected mice (when compared to PBS-injected mice) are likely due to the Pam3Cys. The absence of coronary arteritis in mice injected with Pam3Cys despite the significant upregulation of leukocyte recruitment molecules when compared to PBS-injected mice suggests that there may be a threshold of expression that must be reached before the induction of disease could be observed. Because LCWE is a crude extract, it is not surprising that it contains many factors (besides SAg and TLR2 activity) that are important for disease induction—such as factors important for localizing the immune response to the coronary arteries. These factors are of course missing when we inject a purified recombinant SAg such as MAM and Pam3Cys into mice.

Other variables that could contribute to the lack of disease development include the concentration of reagents and time point of sacrifice. The concentrations and time points used in the in vivo experiments were based on those used in our model of LCWE-induced coronary arteritis and on an established murine model of MAM-induced arthritis. The kinetics of disease in mice injected with MAM and Pam3Cys may be different than those in mice that are injected with LCWE; therefore, it is important to test other experimental conditions before a definitive conclusion can be reached.

7.5 Limitations of study

The initial experiments that demonstrated this rescue phenomenon used a combination of 7AAD and AnnexinV staining to identify the T cells that were undergoing decreased levels of apoptosis and hence were being rescued. In determining the activation marker profile of the rescued population, 7AAD and AnnexinV staining was used, as before, to identify the population of
interest. However, 7AAD and AnnexinV staining could not be used to identify the population of interest when determining the cytokine production profile. This is because the cytokine detection assay involves fixation and permeabilization of cells and both 7AAD and AnnexinV staining depend on an intact cell membrane to identify dead and/or apoptosing cells. With the fixable viability dye, we are looking at cells with intact membranes including those that may already be starting to undergo early apoptosis and that would have previously been excluded in the analysis. Although use of the fixable viability dye does not identify exactly the same population of cells that was previously identified in the rescue phenomenon, due to the constraints imposed by the cytokine detection assay, the fixable viability dye is one of the best and only options available to identify the T cell population of interest.

There was a focus on T helper (CD4\(^+\) T cells) cells in this project and there are a few reasons for that. The main reason is that there is evidence suggesting a role for T helper cells in the pathogenesis of KD. First, the importance of T helper can be demonstrated in DQ6 transgenic mice. These transgenic mice are genetically modified to express human CD4 and human MHC class II DQ6 transgenes in a background lacking endogenous mouse CD4 and CD8. In these transgenic mice, even without CD8\(^+\) T cells, they still get coronary arteritis when injected with LCWE. Secondly, the mononuclear infiltrate present in biopsy specimens from skin lesions of acute KD patients have been shown to consist mostly of CD4\(^+\) T cells and CD13\(^+\) macrophages with very few infiltrating CD8\(^+\) T cells (125, 126).
8 Conclusion

The first objective was to identify TCR Vβ6 stimulating SAgs from a panel of bacterial SAgs. The SAgs that were tested were able to induce a dramatic dose dependent proliferative response in mouse splenocytes, however MAM was the only SAg able to mediate expansion of TCRVβ6+ cells and so was used for subsequent experiments.

The second objective was to evaluate the T cell activating properties of MAM. Firstly, the SAg, SEB, was studied to determine what the prototypical SAg response was. SEB- activated costimulation-rescued T cells were found to express the following activation markers: CD25+CD134+CD69+CD44hiCD62Llow. Upon restimulation, the rescued cells produced IFN-γ and TNF-α, but not detectable levels of IL-4, IL-10 or IL-17. This cytokine profile is consistent with a Th1 phenotype. MAM-activated costimulation-rescued T cells and LCWE-activated costimulation-rescued T cells were both found to express the same activation marker profile and cytokine production pattern as SEB-activated costimulation-rescued T cells. Since MAM was found to activate T cells in a similar manner as the prototypical SAg SEB, the observations made with MAM could be attributed to its superantigenic activity. Furthermore, since MAM was found to activate T cells in a similar manner as the disease inducing SAg LCWE, MAM was a suitable T cell dependent factor to test in vivo for disease induction.

The third objective was to dissect the contribution of T cell dependent versus T cell independent factors in the pathogenesis of coronary arteritis. To accomplish this, we combined the known T cell dependent factor (MAM) and known T cell independent factor (Pam3Cys); we then evaluated the ability of these to induce coronary arteritis in mice. When injected into mice, MAM and Pam3Cys did not induce coronary arteritis at the time point and concentrations tested. The presence of yet other unidentified T cell independent factors in LCWE but not in
MAM+Pam3Cys could explain why MAM+Pam3Cys injections did not induce disease despite the activated and functional phenotype of MAM-activated costimulation-rescued cells. Consistent with this hypothesis, mRNA expression of RANTES, ICAM-1 and E-selectin were significantly lower in MAM and Pam3Cys-injected mice compared to LCWE-injected mice.

We therefore propose that in addition to the known T cell dependent factors (that expand activated and functional TCR Vβ6 T cells) and known T cell independent factors (such as TLR2 agonists) that contribute to the pathogenesis of coronary inflammation in our KD model, there are other yet to be identified factors— some of these unidentified factors may play a role in upregulating leukocyte recruitment molecules in the hearts of diseased mice (refer to Figure 25 for the proposed disease model).
Figure 25. **Disease model.** T cell dependent factors may expand activated and functional TCR Vβ6⁺ T cells, and T cell independent factors (such as TLR2 agonists) may localize and sustain the immune response. TLR2 can mediate the upregulation of costimulatory molecules to rescue SAg-activated T cells from apoptosis. In addition to these known factors, there are other yet to be identified factors in LCWE that contribute to the pathogenesis of coronary inflammation. Some of these unidentified factors may play a role in upregulating leukocyte recruitment molecules in the hearts of diseased mice.
9 Future Directions

It has been previously shown that different preparations of LCWE have varying levels of superantigenic activity. This superantigenic activity has been shown to correlate with the ability of LCWE to induce coronary arteritis. Coronary arteritis can be observed in mice injected with batches of LCWE with relatively high levels of superantigenic activity whereas coronary arteritis can not be observed in mice injected with batches of LCWE with low superantigenic activity.

In this study, injection of the TCR Vβ6-stimulating SAg, MAM, into mice did not induce coronary disease even though MAM has the same T cell activating properties as LCWE. We propose the reason for this may be because LCWE contains other elements that are important for disease induction such as factors that upregulate leukocyte recruitment molecule expression in the hearts of injected mice. We showed in this study that there is indeed an absence of RANTES, E-selectin and ICAM-1 upregulation in mice injected with MAM and Pam3Cys when compared to mice injected with LCWE.

To test the hypothesis that in the LCWE extract, there are factors (in addition to T cell activating factors) that are important for disease, we can inject mice with a batch of LCWE that contains low superantigenic activity along with MAM. Injecting the batch of LCWE with low superantigenic activity should not induce disease (58); however, with the addition of MAM (which has been shown in this study to activate T cells in the same manner as LCWE) we would expect to see the induction of disease. In this case, MAM would activate the pathogenic population of T cells and the batch of LCWE with low superantigenic activity would provide the non-T cell dependent factors that are important for disease induction (such as factors that localize the disease to the coronary arteries).
Contributions

Table 2: Experiments were performed by Yasmin Moolani.

Table 3: Sections were scored by Dr. Rae Yeung.

Table 3: LCWE-injected condition and accompanying PBS-injected control were performed by Joyce Hui-Yuen.

Table 3: Pam3Cys-injected and PBS-injected conditions consist of data pooled with previously collected data by Ken Little.

Figure 6: Experiments were performed by Yasmin Moolani.

Figure 7: Experiments were performed by Ken Little.

Figure 23: Histology was performed by Lilly Morikawa.

Figure 23b: Experiments were performed Joyce Hui-Yuen.


