The Role of 4-1BB in Kawasaki Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Immunology
University of Toronto

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Department of Immunology
University of Toronto
2011

Abstract

Kawasaki disease (KD) is a multisystem vasculitis with predilection for the coronary arteries. Although the cause of KD remains elusive, there is evidence to suggest a superantigenic trigger. When T-cells are activated by a superantigen (SAg) they undergo massive proliferation but eventually apoptose; however, in KD, we hypothesize that these T-cells persist and infiltrate the coronary arteries. Previous studies have shown that enhanced costimulation through CD28 or 4-1BB rescues T-cells from apoptosis and exacerbates disease in a mouse model of KD. Our results suggest that this signal needs to be initiated close in timing to that of the SAg. In addition, the two molecules can act independently of one another, but are not additive. Also, stimulation of the 4-1BB pathway in the presence of a SAg elicits a Th1 phenotype. Lastly, TRAF1 regulates this enhanced survival downstream of 4-1BB. Thus, these results provide new insights into the effects of costimulation in SAg-mediated disease, and suggest that these pathways need to be targeted early to abrogate the enhanced survival of SAg-activated T-cells.
Acknowledgments

There have been several people without whom this project would not have been possible, and I would like to take this opportunity to voice my gratitude.

Firstly, I would like to thank my supervisor, Dr. Rae Yeung for taking me under her wings and giving me the chance to work on this project. Without her guidance, support and unwavering optimism, this project would not have been as enjoyable as it has been.

I would also like to give a heart-felt thanks to all the past and present members of the Yeung lab. I have learnt a lot about science and life in general from all of you. The memories of the past two years will be cherished and not soon forgotten. I definitely don’t think I’ve ever gotten so many nicknames in such a short period of time! (Almy, Fifi, Fifs, FAFA).

Suzanne/Susan, my lab bench partner - thank you for always being there to help me around the lab and whenever I had computer problems…which was often. Trang, my other lab bench partner - thank you for all your helpful input in planning out my experiments. I don’t think I’ve ever met anyone on whom it has been so hard to play a prank as you. You’re amazing!

My committee members, Dr. Tania Watts and Dr. Pamela Ohashi have been instrumental in shaping my project. Thank you both for all the time you have put into listening to my presentations and for the advice you have given me along the way. A special thanks to Dr. Watts and her lab members for being so generous with mice and reagents, without which this project would not exist!

Last, but not least, I would like to thank my family for their constant love and support.
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<tbody>
<tr>
<td>AP-1</td>
<td>Activating protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>C</td>
<td>Constant</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>MAF</td>
<td>musculoaponeurotic fibrosarcoma oncogene homolog</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T-lymphocyte Antigen 4</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal related kinase</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated protein with death domain</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Gads</td>
<td>Grb2-related adaptor downstream of shc</td>
</tr>
<tr>
<td>Grb-2</td>
<td>Growth factor receptor bound protein 2</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse-radish peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITK</td>
<td>IL-2-inducible T-cell kinase</td>
</tr>
<tr>
<td>ITPKC</td>
<td>Inositol 1,4,5,-triphosphate 3-kinase C</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KD</td>
<td>Kawasaki disease</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker of activated T-cells</td>
</tr>
<tr>
<td>Lck</td>
<td>Lymphocyte specific protein tyrosine kinase</td>
</tr>
<tr>
<td>LCWE</td>
<td><em>Lactobacillus casei</em> cell wall extract</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic-activated cell sorting</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kappa-B</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI 3-kinase</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>ROR</td>
<td>RAR-related orphan receptor</td>
</tr>
<tr>
<td>SAg</td>
<td>Superantigen</td>
</tr>
<tr>
<td>SAPK</td>
<td>Stress-activated protein kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SE</td>
<td>Staphylococcal enterotoxin</td>
</tr>
<tr>
<td>SLP-76</td>
<td>SH2 domain containing leukocyte protein of 76 kDa</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>Spe</td>
<td>Streptococcal pyrogenic exotoxin</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</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</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T-regulatory</td>
</tr>
<tr>
<td>TSST-1</td>
<td>Toxic shock syndrome toxin-1</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>V</td>
<td>Variable</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>YMXXM</td>
<td>Tyrosine, methionine, any amino acid, methionine</td>
</tr>
<tr>
<td>ZAP-70</td>
<td>Zeta-associated protein of 70 kDa</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Kawasaki Disease

1.1.1 Clinical, pathological and immunological characteristics

Kawasaki Disease (KD) is an acute febrile disorder characterized by multisystem vasculitis. As there is no laboratory diagnostic test in existence for KD, a diagnosis of KD is made when fever persisting for five or more days and four of the five following principal symptoms are present: oral mucosal changes such as the characteristic strawberry tongue and/or red cracked lips, polymorphous skin rash, conjunctival injection, swelling of the hands and feet and cervical lymphadenopathy \([1]\). The major feature of this disease is the coronary artery aneurysms that are found in \(~25\%\) of untreated patients and \(\sim5\%\) of treated patients and may result in ischemic heart disease, myocardial infarction or sudden death \([2, 3]\).

Symptoms not included in the diagnostic criteria also exist. These include gastrointestinal manifestations such as abdominal pain, vomiting and diarrhea \([4]\) and neurological symptoms such as irritability and facial palsy \([5]\). Arthritis of the hands, knees, ankles, and sometimes hips is also seen in patients in the first few weeks of disease \([6]\).

The disease is self-limiting in nature with the acute inflammatory phase lasting from 4-6 weeks. During the course of disease, pathological changes are seen in the blood vessels and organs. The vascular changes can be divided into three stages corresponding to the length of illness: the acute stage which lasts 1-2 weeks, the subacute phase which occurs 2-4 weeks after disease onset, and finally the convalescent phase \([7]\).

The immune system of KD patients also undergoes changes. Characteristic of an inflammatory response, patients with KD have elevated levels of C-reactive protein and increased erythrocyte sedimentation rates. Furthermore, the platelet count, although normal during the first week of illness, quickly increases during the subacute phase of disease \([8]\). As well, the cytokines IL-1, IL-6, TNF-α and IL-8 are increased during the acute phase \([9, 10]\). IFN-γ levels increase in the acute phase and then decrease in the subacute phase \([11]\).
Several innate immune cells are also implicated as having a role in the pathogenesis of KD. Among these are activated monocytes/macrophages which are thought to be responsible for secreting TNF-α, IL-1, IL-6 [9, 10, 12] and VEGF [13], which are all elevated in patients with KD. Furthermore, monocytes/macrophages increase in number during the acute stage of disease [14] and studies have shown infiltration of these cells into affected cardiac and skin tissue [15, 16]. As well, MCP-1, which attracts monocytes to sites of inflammation and is elevated in KD, has been shown to colocalize with mononuclear cells in cardiac tissues from patients with fatal KD [17]. Lastly, dendritic cells, specifically those of the myeloid lineage, have also been found in coronary artery lesions in KD patients [18].

Cells of the adaptive arm of immunity also play a role, specifically T-cells. Analysis of heart tissue from fatal KD patients revealed the presence of infiltrating T cells within the heart. Furthermore, some of these cells were positive for the expression of the IL-2 receptor, suggesting that these T-cells were activated [15]. More importantly, staining of the coronary arteries revealed the presence of CD3+ T-cells colocalizing with dendritic cells [18]. Similarly, examination of skin biopsies from acute KD patients revealed the presence of T-cells, specifically those of the CD4+ lineage, within the skin lesions [16]. Within the CD4+ lineage, Th17 cells appear to increase during the acute phase and Treg cells appear to decrease. Also, patients resistant to disease treatment tend to have higher Th17 levels than those who are sensitive to treatment. Following a similar pattern to these subsets, the corresponding cytokines for Th17 cells (IL-17, IL-6 and IL-23) are upregulated during the acute phase of disease and that of Tregs, TGF-β, is decreased [19].

1.1.2 Treatment

Aspirin and intravenous immunoglobulin (IVIG) are the first line of treatment for patients with KD. Ideally, treatment should be started within the first ten days of disease [20]. Treatment with aspirin reduces inflammation but does not appear to reduce the incidence of coronary artery abnormalities; however, IVIG treatment has been correlated with reduced frequency of coronary artery lesions [3, 21] although the mechanism is still unknown.

As current treatments are far from perfect, recent studies have focused on finding better therapies to treat this disease. One such study looked at the effect of TNF-α in the animal model.
of KD and found that in the absence of the effects of this cytokine, accomplished either through use of the soluble TNF-α receptor, etanercept, or TNFRI−/− mice, inflammation as well as elastin breakdown at the coronary arteries was abrogated [22]. Interestingly, increased levels of serum TNF-α have been correlated to increased vascular damage in KD patients [23]. These findings have led to the current TNF trials that are aimed at looking at the effect of etanercept and infliximab (a monoclonal antibody against TNF-α) [24, 25].

1.1.3 Epidemiology

Japan has had the highest number of reported cases, and therefore most of the epidemiological studies have come out of surveys done in this country. From these studies, it has been determined that KD is predominantly found in children with the peak incidence of disease occurring between 9 and 11 months [1]. Males are slightly more susceptible to disease than females, with a male:female ratio of 1.5:1. As well, about 0.7-1.4% of individuals with KD also have an affected sibling [1]. Additionally, although cases of KD have been reported in all ethnicities, studies in the United States have shown that those of Asian descent, specifically Japanese and Korean, appear to have the highest rates, while those of Caucasian ancestry have the lowest rates [26]. Lastly, KD is now considered to be the leading cause of acquired heart disease in the developed world [1].

1.1.4 Etiology

Although the symptoms and pathology of KD have been well established and described, the cause of this disease is still unknown. Of all the theories put forth, an infectious trigger of this disease appears to have the most supporting evidence. Firstly, there are several features of the disease that share similarities to those of infectious diseases: 1. the number of cases of KD spikes during the winter and spring months 2. there are periodic occurrences of epidemics 3. there exists temporal and geographical clustering of cases and 4. those affected tend to be younger than 5 years of age [27-30]. Furthermore, the symptoms of KD resemble those of diseases caused by infectious agents and indeed several other infectious diseases have been mistaken for KD including measles, scarlet fever and staphylococcal scalded skin syndrome [31]. Interestingly enough, 33% of patients with KD have one or more infections at the time of
diagnosis [32] and several microorganisms have been isolated from patients with KD. These include viruses such as cytomegalovirus and Epstein Barr virus and bacteria such as *Staphylococcus aureus* and *Streptococcus pyogenes* [33-36].

Within the infectious trigger theory, there exists a controversy as to whether the immune response is initiated by a conventional antigen or a superantigen. Those who believe that a conventional antigen is responsible for disease point to the fact that the IgA plasma cells found at the vascular wall of 3 patients with fatal KD secreted antibodies that were oligoclonal in nature suggesting an antigen-driven response [37]. However, many different pathogens have been isolated from patients with KD and none of the pathogens isolated thus far share any common conventional peptide antigens.

One commonality shared among a lot of the pathogens isolated from patients with KD is the presence of superantigenic activity [38-41]. Most superantigens (SAgs) activate T-cells by binding to the variable (V) region of the β chain of the T-cell receptor (TCR). In this way, a SAg can stimulate all the T-cells of the specific Vβ family for which the SAg is specific [42]. Indeed, blood taken from patients with KD has been found to possess a Vβ skewing of T-cells, most notably of the Vβ2 family [43, 44]. Superantigens that create this Vβ2 skew include toxic shock syndrome toxin-1 (TSST-1) from *Staphylococcus aureus* and streptococcal pyrogenic exotoxin B (Spe-B) and Spe-C from *Streptococcus pyogenes* [38, 41]. Both staphylococci and streptococci strains have been found in KD patients [34, 35]. As well, when the peripheral blood T-cells taken from KD patients were activated with Spe-C, a lower proliferative and IL-2 response was obtained as when compared to healthy controls, indicative of T-cells that have been previously activated by a SAg and are anergic [45]. Furthermore, Vβ2 expressing T-cells were found to be present in the coronary artery of a patient who died of KD [46]. A Vβ6 and Vβ8 skew has also been found in patients [47, 48]. Lastly, IgM antibodies against superantigens from *Staphylococcus aureus* and *Streptococcus pyogenes* have been detected in the serum of KD patients [49].

Although the superantigen theory accounts for the presence of multiple organisms isolated from patients with KD, it does not speak to the coronary artery specificity of this disease. Therefore, our lab has proposed a final common pathway that unifies the superantigen
with the peptide-antigen theory in postulating that while a superantigen initiates the immune response, a self-peptide at the heart may be responsible for the specificity.

The fact that not all patients infected with the particular pathogens implicated in KD get the disease, that KD is more predominant in Japanese children [26] and that those with siblings with KD have a higher chance of getting KD themselves [1] suggests that genetics might also play a role in disease susceptibility. As such, polymorphisms in several genes involved in regulating T-cell activation and survival have been linked to the emergence of KD [50-53]. One such gene is the inositol 1,4,5-triphosphate 3-kinase C (ITPKC) gene whose gene product is a negative regulator of signal transduction downstream of the T-cell receptor. A single nucleotide polymorphism (SNP) in intron 1 of ITPKC was found to be associated with an increased risk of KD and coronary artery aneurysm formation in children in both the United States and Japan. Reduced activity of this gene could lead to increased T-cell activation and thus a stronger inflammatory response [50]. Furthermore, a SNP in the casp3 gene was also found to be associated with disease in both Japanese and American children. The gene product of casp3, caspase 3, is involved in the apoptotic pathway of cells [53]. Reduced expression of caspase 3 or ITPKC could lead to immune cells evading cell death or proliferating to a greater degree, respectively, and thus could be a mechanism of perpetuating the immune response to cause the coronary artery pathology seen in KD.

1.2 Animal models of Kawasaki Disease

Samples from patients are limited to peripheral blood, skin biopsy specimens or tissues from autopsies. Therefore, in order to gain a better understanding of KD, several animal models which use dogs, piglets, rabbits, or mice have been designed to study this disease [54-61]. The canine model involves the spontaneous generation of polyarteritis in young dogs. This canine juvenile polyarteritis syndrome was first described and put forth as a KD model in 1992. The symptoms that the young dogs develop, which include an increase in serum IgA and monocyte/macrophage activation, are similar to those seen in KD patients during the acute phase [55]. Although the symptoms may resemble those that are seen in KD patients, the fact that this is a spontaneous autoimmune disease and that KD is thought to be triggered by a microbial agent, makes utilization of this model impractical. The next model involves the injection of horse serum into
rabbits to induce serum sickness. Although coronary arteritis is seen in the rabbits, histologically, the disease is similar in both the young and adult, unlike what is found in KD [56]. The third and most recent model utilizes pigs to study KD. It was first established in 2004 and involves the generation of immune complex vasculitis by injecting piglets with multiple infusions of horse serum. Platelet and leukocyte numbers mimic that which is seen in KD patients, and the piglets also develop skin rashes and show coronary artery dilation [57]. However, the fact that more than one injection of horse serum is needed to induce disease and the fact that the pathology seen in KD is not thought to be immune complex mediated makes this model inaccurate. Furthermore, from an economical and practical point of view, the use of dogs, rabbits and pigs is not very feasible. A model that utilizes smaller, inbred animals like mice is more practical and economical in a laboratory setting.

Three mice models currently exist. The first of these involves repeatedly injecting mice intraperitoneally (i.p) with a fragment of peptidoglycan from group A streptococcus. Mice in this model show cellular infiltration in the vascular wall and perivascular space and breakdown of the elastic fibers [61]. The second model involves injecting mice i.p. with repeated doses of extracts from the yeast, *Candida albicans*. Elevations of the cytokines IL-1, IL-6 and TNF-α are seen within 24 hours of injection. Furthermore, the infiltrate at the coronary arteries are macrophages, lymphocytes, plasma cells and neutrophils, similar to what is seen in humans with KD. However, not all mice strains are sensitive, and in those that are sensitive, only 70% of mice develop coronary arteritis disease [59, 60]. Furthermore, this model, as well as the streptococcus model, needs repeated injections to sustain disease; however, there is no evidence of repeated infections of the inciting agent in KD.

1.2.1 *Lactobacillus caseii* cell wall extract mouse model

The third mouse model was developed in 1985 by T.J. Lehman and involves one i.p. injection of group B *Lactobacillus caseii* cell wall extract (LCWE) into mice [58]. When injected into rats, chronic polyarthritis is induced [62]; however, when this same extract from this gram positive bacteria is injected into mice, a focal, inflammatory coronary arteritis develops. In order to obtain the extract, the cell wall of *L. caseii* is disrupted with the detergent SDS, incubated with
DNase, RNase and trypsin to get rid of the cytoplasmic elements and then finally sonicated [58]. This cell wall has a high rhamnose content and is resistant to degradation by lysozyme [62, 63].

Histologically, the coronary arteries of these mice resemble those that are seen in humans in that the infiltrate consists of lymphocytes, polymorphonuclear cells and monocytes [64]. Furthermore, inflammation at the coronary arteries is seen as early as day 3 and maximizes by day 28 and is still present at day 56 [58], which is consistent with the time course of disease seen in children. As well, these mice also respond to IVIG treatment, similar to humans with KD [65].

Further studies with this extract have shown it to possess superantigenic activity. Not only does the extract activate T-cells in a Vβ restricted fashion by expanding the Vβ2, 4 and 6 families which subsequently delete, but it also does not require antigen presentation and binds to different MHC molecules with differing affinities [66] (see section 1.3.2 for more on superantigens and T-cell activation). Interestingly, the superantigen-specific cells found at the heart were found to be oligoclonal in nature (T. Duong, unpublished observation), supporting the hypothesis that while a SAg triggers the immune response in KD, a peptide at the heart drives the specificity of the disease. As well, cytokine analysis has shown that both TNF-α and IFN-γ are upregulated in the mice [22, 67]. Lastly, toll-like receptor (TLR)-2 deficient mice are resistant to disease, suggesting that the LCWE extract has a TLR2 ligand and that this ligand is important for disease induction in mice [68].

1.3 Superantigens

1.3.1 Types of Superantigens

Bacterial or exogenous superantigens (SAgs) are a class of exotoxins secreted by bacteria (gram-positive and gram-negative), viruses and mycoplasma [69]. Although several microorganisms have been shown to produce SAgs, including cytomegalovirus and Yersinia pseudotuberculosis [70, 71], those produced by Staphylococcus aureus and Streptococcus pyogenes are the most well-described of the SAgs. Together, these two organisms produce numerous different SAgs including staphylococcal enterotoxin (SE-) A-M and TSST-1 for S. aureus and SPE-A1-4, C, G, H, I, J, L, and M, streptococcal mitogenic exotoxin 2-24 and SSA
for *S. pyogenes* [42, 69, 72-74]. It is important to note that while each of these bacteria produces quite a few different SAgs, each strain only produces a subset of the exotoxins [42, 75].

The aforementioned describes exogenous superantigens; there also exists endogenous superantigens. These are superantigens whose genes are carried within the genome of mice. As such, mice strains that carry these genes lack the Vβ families of the T-cells for which the superantigen is specific. Although first discovered in 1974 as proteins expressed on the surface of thymic stromal cells, they are now known to have originated from the mouse mammary tumor virus whose provirus genome can integrate into the genome of mice [69, 76, 77].

**1.3.2 T-cell activation**

In order for a conventional antigen to cause T-cell activation, the antigen has to first be processed by an antigen presenting cell (B-cell, macrophage or dendritic cell) into smaller peptides. These peptides then become associated with either the major histocompatibility complex class I (MHC I) moiety or MHC II depending on whether the pathogen is extracellular or intracellular, respectively. Next, the T-cell has to be able to recognize this self MHC-peptide complex, a process known as MHC-restriction (Figure 1A). Lastly, T-cells expressing the accessory molecule CD4 can usually only be activated by MHC II-peptide complexes and CD8-expressing T-cells can usually only be activated by MHC I-peptide complexes [78].

Superantigens, on the other hand, bypass the need for antigen processing by APCs and directly attach to the MHC molecule, outside the peptide binding groove (Figure 1B). Furthermore, it has been shown that they only bind to MHC II and not MHC I molecules [79-82]. They can bind to the MHC II molecule in three different ways and thus can be divided into three different groups depending on their binding strategy. The first group, which includes SEB, binds to a single α chain of the MHC molecule. The second group binds to a single β chain and includes SEH as a group member. The last group of SAgs binds to two MHC molecules, either two α chains, two β chains or an α and β chain. SEA belongs to this last group of SAgs. As well, in order to achieve optimal binding to the MHC molecule, a lot of SAgs also require a zinc ion [42].
Although SAgs bind to MHCII, they are not confined to activating only CD4+ T-cells like conventional antigens; as such, they can activate both CD4+ and CD8+ T-cells [83]. Interestingly, however, SAgs cannot activate all T-cells. As alluded to earlier, they are restricted to activating T-cells whose TCR contains the Vβ chain for which the superantigen is specific. Furthermore, superantigens stimulate different Vβ families of similar structure in different organisms. For example, the superantigen TSST-1 activates human T-cells bearing the Vβ2 chain and the structurally similar Vβ15 chain of murine T-cells [81, 82, 84]. Therefore, since humans and mice have about 46 and 21 functional Vβ genes, respectively, and since several T-cells share the same Vβ chain [85], superantigens can stimulate between 1/20 - 1/4 of the T-cells while conventional antigens can only stimulate between 1/10⁴ – 1/10⁶ [81].

Additionally, SAg activation of T-cells is not MHC-restricted; that is, T-cells do not have to recognize the MHC-peptide complex to which the SAg has bound and the MHC molecule can be allogeneic in nature [79]. However, although there is no MHC-restriction, SAgs bind to MHC molecules of differing isotypes with different affinities. For example, in general the SAgs produced by *S. aureus* bind with greater affinity to mouse I-E than I-A and to human HLA-DR than to HLA-DQ or HLA-DP [81, 82, 86, 87]. Consequently, different MHC isotypes produce SAg responses of varying strengths.
Figure 1. Conventional antigen versus superantigen stimulation of T-cells. A. Conventional antigens are first processed into smaller peptides by antigen presenting cells. Then, after association with an MHC molecule, it binds to T-cell receptors that are specific for the MHC-peptide complex [78]. B. Superantigens are not processed into smaller peptides, but rather bind directly to the MHCII molecule and usually to the variable region of the β chain of the T-cell receptor. As a result, the T-cell receptor does not have to be specific for the MHC-peptide complex [78-82]. (V), variable; (C), constant.
As organisms increase in age, their immune system changes as well. As such, SAg activation of T-cells has been shown to be different in neonatal, young and old mice. Neonatal mice give a robust T-cell response followed by complete and prolonged deletion of virtually all mature T-cells bearing the SAg-specific Vβ TCRs [82]. On the other hand, beyond this neonatal period, although mice still exhibit massive proliferation upon SAg activation, the deletion following the expansion is not permanent [88]. As mice get older in age, the cells still proliferate and then undergo temporary deletion; however, this proliferative response is weaker than what is seen with young or neonatal mice. This reduction in response to SAg stimulation has been attributed to reduced efficiency of SAg presentation by APCs and age-related changes in cytokine profiles [89-91]. Consequently, those really young in age would be protected from disease due to deletion of T-cells while those older in age would be protected from disease due to reduced SAg-activation of T-cells. Interestingly, this is consistent with the epidemiological findings of KD which suggests that the peak incidence of disease is beyond the neonatal period at 9-11 months and this disease is almost completely absent in adulthood [1].

Among the different superantigens that have been and are currently actively being explored, the Staphyloccocal enterotoxin B (SEB) molecule from S. aureus has been the most well-characterized, and a lot has been learnt about superantigenic interaction with T-cells by studying this prototypical superantigen and its effect on mice. In mice, SEB preferentially activates the Vβ3, 7, 8, and 17 families [82, 92]. By staining for one or more of these specific subsets, the kinetics of the T-cell response has been determined. The T-cells in the spleen of BALB/c mice injected with SEB were shown to undergo a short lived massive proliferation where both the CD4+ and CD8+ subset of T-cells increased five-fold in numbers. This expansion peaked early (about 2 days after injection) and was sustained for four days, after which the numbers declined dramatically. Furthermore, CD4+ and CD8+ T-cells were activated with differing kinetics. While greater numbers of CD4+ T-cells were seen after expansion with SEB, the numbers dropped more rapidly than those of CD8+ T-cells and even plateaued at a lower level than the CD8+ population [88]. In fact, this rapid decline in numbers with deletion or induction of anergy in SAg reactive T-cells is characteristic of a SAg response and has been shown in multiple studies [82, 93]. Because of the fact that a lot of the features of SEB and its effect on the immune system have been well documented, this SAg was employed in this study.
1.3.3 T-cell receptor signaling

A number of signaling events take place within a T-cell once the TCR has been engaged. First, the Src family tyrosine-kinases Lck or Fyn, which are associated with the TCR co-receptor (either CD4 or CD8), phosphorylates the ITAMS of the CD3 complex chains. This leads to the binding of the ZAP-70 molecule to the ITAM on the ζ chain of the CD3 molecule. Being in close proximity to Lck, ZAP-70 itself is then phosphorylated and activated by Lck. Once ZAP-70 is phosphorylated a number of signaling cascades ensue (Figure 2) which lead to the activation of the transcription factors NFκB and NFAT, and ERK1/2 which work together to cause cellular proliferation and differentiation [78].

Studies suggest that when the T-cell receptor (TCR) is engaged by a conventional antigen-MHC complex the aforementioned signaling cascade ensues; however, a different signaling pathway appears to be used by the TCR when it is engaged by a superantigen-MHC complex. A study by Bueno et. al. determined that while the signaling molecule Lck is important for conventional peptide signaling, superantigens bypass the need for this molecule. Instead, heterotrimeric Gα11 proteins are activated. Activation of these proteins then lead to the activation of phospholipase C-β and protein kinase C, which in turn initiate signaling cascades that activate NFκB and NFAT (Figure 3) [94]. Interestingly, Lck appears to have a negative regulatory role in SAg-mediated T-cell signaling [95].
Figure 2. T-cell signaling events after conventional antigen stimulation. After TCR activation, the Src-family protein kinase Lck, which is associated with the T-cell co-receptor (CD4 or CD8), phosphorylates the ITAMS of the CD3 complex associated with the TCR. ZAP-70 then binds to a phosphorylated ITAM on the ζ-chain of the CD3 complex and subsequently becomes phosphorylated by Lck. Phosphorylated ZAP-70 then phosphorylates LAT and SLP-76. This then leads to the recruitment of the guanine-nucleotide exchange factor SOS which leads to the activation of ERK1/2 through the MAP kinase pathway. Activated SLP-76 and LAT also lead to the recruitment and activation of PLC-γ which cleaves PIP₂ to produce DAG and IP₃. IP₃ leads to an increase in intracellular calcium ions which activate the phosphatase calcineurin. Calcineurin then leads to the activation of the transcription factor, NFAT. The intracellular calcium ions along with DAG lead to the activation of the PKC pathway which leads to the activation of the transcription factor NFκB. ERK1/2, NFAT and NFκB work together to promote T-cell differentiation and proliferation [78].
Figure 3. T-cell signaling events after superantigen stimulation. After the TCR has been activated by a superantigen, the conventional phosphorylation events of Zap70 and LAT initiated by Lck is not seen. Instead, heterotrimeric Gα11 proteins are activated by a yet to be discovered mechanism. This then leads to the activation of PLC-β which in turn leads to the activation of PKC and ERK1/2, an influx of calcium ions, and activation of the transcription factors NFκB and NFAT [94].
1.3.4 T cell lineages, KD and the LCWE mouse model of disease

When CD4+ or CD8+ T-cells are activated, they evolve from their naïve state into cells with effector function. Activated CD8+ T-cells gain cytotoxic capabilities while CD4+ T-cells take on a helper phenotype [78]. These helper T-cells can further be divided into several other subsets, of which the Th1, Th2, and Th17 (Figure 4) subsets will be discussed.

Th2 cells secrete IL-4, IL-5, IL-10 and IL-13. This cocktail of antibodies leads to strong antibody responses including IgE isotype switching by B-cells and favors eosinophil differentiation and activation. As such, these cells have been implicated in the allergy response. IL-4, along with IL-10 and IL-13, inhibit macrophage function and thus dampen the Th1 response [96-98]. No increase in IL-4 mRNA was detected at the heart in mice injected with LCWE (J. Hui-Yuen, unpublished observation), suggesting that the Th2 subset of cells may not play as big a role as the Th1 and Th17 subsets. Consequently, more emphasis was placed in determining the effect of superantigen stimulation on the differentiation of the Th1 and Th17 subsets in this thesis.

Th1 cells produce the cytokines IFN-γ, IL-2, TNF-α and TNF-β. These secreted cytokines give Th1 cells the ability to increase the production of opsonizing and complement-fixing antibodies by B-cells, and to activate macrophages. The activated macrophages in turn produce reactive oxygen species such as nitric oxide and TNF-α. Essentially, Th1 cells are important for cell-mediated immunity and therefore lead to a predominantly phagocytic inflammation. This subset of cells is employed by the immune system to kill intracellular parasites and is implicated in many autoimmune diseases [78, 96-98].

The presence of IL-12 and IL-18 in the cytokine milieu is instrumental in the differentiation of CD4+ naïve T-cells into Th1 cells [96, 99-101]. IL-12 is produced by dendritic cells and phagocytes mainly after exogenous stimulation by pathogens through toll-like receptors. Together with IL-18, IL-12 binds to its receptor on activated T-cells and works through the transcription factors STAT4 and NFκB to induce transcription of the IFN-γ gene. NFAT, downstream of the TCR, also works to induce transcription of the IFN-γ gene. The transcription factor T-bet has also been shown to be important in Th1 cell differentiation and is thought to lead to remodeling of the IFN-γ locus, thus increasing its access to the aforementioned
transcription factors. T-bet expression is induced through the STAT1 transcription factor which is activated by IFN-γ signaling [101].

In addition to the specific cytokines secreted by this group of cells, specific cell-surface markers are preferentially expressed on Th1 cells. These include CXCR3 and CCR5. Being chemokine receptors, these molecules probably play a role in the migration and homing of Th1 cells to sites of inflammation [96]. Furthermore, the cytokine TGF-β, along with the Th2-promoting cytokine IL-4, leads to the downregulation of the transcription factor T-bet, thus favoring other T-helper arms [101].

Recently, a new subset of T-helper cells, Th17, has been described. These cells are characterized by the production of IL-17, although they can also produce IL-21. Although the role of these cells are still being elucidated, they are thought to play a role in clearing certain types of pathogens that need a large inflammatory response and are not effectively cleared by either Th1 or Th2 responses. These pathogens are diverse and span from the gram-positive *Propionibacterium acnes* to the yeast *Candida albicans*, which are interestingly, both implicated in KD [102, 103]. Unfortunately, because of the potency of these cells, they have been implicated as being the inciting agents in a number of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis [104].

Although it was originally thought that the cytokine IL-23 was important for the differentiation of Th17, it is now known that IL-6 along with TGF-β lead to the creation of Th17 cells from naïve CD4+ T-cells in both mice and humans. IL-23, rather, plays a role in the survival of these cells. Like Th1 and Th2 cells, the action of these cytokines is mediated through various transcription factors. IL-6 and TGF-β activate the nuclear receptors RORγt and RORA through STAT3 [105]. STAT3 can also directly bind to the promoters of IL-17 to induce transcription of these genes. IRF4 has also been shown to be important in the induction of Th17 cells as mice deficient in this transcription factor were not able to develop a Th17 response [104].

While certain cytokines promote Th17 differentiation, others inhibit it. One such cytokine is IL-27 which is a member of the IL-12 family of cytokines. This effect is mediated through the transcription factor STAT1 [104]. IFN-γ also negatively regulates Th17 lineage determination
through STAT1. IL-2 has also been shown to have a negative effect on Th17 cells through the action of STAT5 [105]. Lastly, IL-4 inhibits expression of several Th17-family genes, an effect shown to be mediated by STAT6 [106].

Th1 and Th17 cells have been implicated in KD and its mouse model, with more emphasis being placed on the Th17 subset recently. Both IFN-γ and IL-17 are found to increase in the serum of patients with KD [11, 19]. Interestingly, IFN-γ is also upregulated in mice injected with LCWE [67]. Furthermore, IL-6, which is important for the Th17 lineage is also found to increase in those with KD [10]. Lastly, in the mouse model, in the absence of IL-6, mice still had inflammation, but no elastin breakdown, suggesting that Th17 cells could be responsible for the coronary artery damage seen in this disease [107].

**Figure 4. Th0 differentiation after T-cell activation.** Upon activation, Th0 cells can undergo several differentiation fates. The differentiation into Th1, Th2 and Th17 are three such fates. Different cytokine milieu promote different cellular fates, and each subset expresses characteristic transcription factors and secretes distinct cytokines [78, 96-101, 104, 105].
1.3.5 T cell fate

After a T-cell has been activated, it can undergo one of three different fates: anergy, apoptosis or survival. While stimulation with a SAg promotes anergy or apoptosis, T-cell activation with a peptide and appropriate costimulation leads to survival of the T-cells.

Anergy is one of the mechanisms used by the immune system to induce peripheral tolerance. Although the cell is still alive, it tends to be functionally inactive. Anergy can be induced in T-cells either by a strong TCR signal in the absence of costimulation (for more on costimulation see section 1.4) or by a low affinity ligand in the presence of costimulation. Furthermore, it is mostly observed in previously activated cells and is characterized by a diminished proliferative and IL-2 response upon restimulation [88, 108]. Although the signaling pathways important for clonal anergy are not yet fully elucidated, the calcium/calmodulin/calcineurin pathway appears to be important as blockage of this pathway with cyclosporine A blocks anergy. Furthermore, cells lacking NFAT1 are resistant to clonal anergy when activated by an anti-CD3 antibody while cells overexpressing NFAT1 show reduced IL-2 secretion when activated by anti-CD3 and an antibody against the costimulatory receptor CD28 [108]. A study by Heissmeyer et. al. in 2004 dissected this pathway further to show that enhanced signaling through the calcineurin pathway leads to an upregulation in the expression of the E3 ubiquitin ligases Itch, Cbl-b and GRAIL. These proteins were shown to promote lysosomal degradation of PLC-γ and PKC, two proteins important in signaling downstream of the TcR [109]. The MAPK pathway, however, does not appear to be important as cells treated with the drug SB203580, an inhibitor of this pathway, can still be induced to undergo clonal anergy [108].

Apoptosis, or programmed cell death, on the other hand, is a process by which the cell undergoes a series of biochemical events that eventually leads to its death. The cell undergoes a number of changes including condensation of the nuclei and DNA fragmentation [110]. There are two distinct apoptotic pathways: extrinsic and intrinsic. The extrinsic pathway involves the activation of the group of receptors part of the tumour necrosis superfamily known as death receptors. Some members of this family include CD95 and TNFRI. The extrinsic pathway in T-cells is initiated when activated T-cells upregulate the death receptor CD95. Ligation of this
receptor by its ligand, CD95L, also found on activated T-cells, results in the trimerization of CD95. This trimerization leads to the recruitment of a number of adaptor molecules including FADD. This in turn leads to the recruitment and subsequent activation of caspase 8 which in turn activates caspase 3. The caspases are a class of molecules that when activated lead to the proteolytic cleavage of intracellular proteins. The intrinsic pathway also culminates in the activation of caspase 3; however, this is through the mitochondrial rather than the receptor route. When the cell encounters a stress signal such as ultra-violet light, the mitochondria release cytochrome c. This release in turn leads to the activation of caspase 3. While the two pathways have different triggers, they can work together to bring about apoptosis of the cell. For example, the Bcl-2 family member, Bid, after its cleavage by caspase 8, can lead to the release of cytochrome c from the mitochondrial membrane [111].

The Bcl-2 family of molecules plays a very important role in the mitochondrial pathway of apoptosis. Both pro- and anti-apoptotic members exist and their abundance determines whether the cell will undergo apoptosis or the third cellular fate, survival. All the members of this family are characterized by the presence of one or more homology domains known as Bcl-2 homology domains which are instrumental for the heterodimeric interactions within this protein family. Pro-apoptotic members include Bim and Bid, while anti-apoptotic molecules include Bcl-2 and Bcl-xL. The pro-survival members prevent apoptosis by preventing mitochondrial outer member permeabilization mostly by binding to and neutralizing pro-apoptotic members [110].

1.3.6 Superantigens and disease

Superantigens, as their name suggests, cause massive immune activation typified by the expansion of a large number of T-cells and a phenomenon known as a cytokine storm. During the cytokine storm, large amounts of TNF-α, IL-1, IL-6, IFN-γ and IL-2 are released leading to a strong inflammatory response [69, 112-114]. Indeed, superantigens are thought to be the inciting agent of quite a few diseases in humans including toxic shock syndrome [115]. This disease is usually initiated by the superantigens TSST-1, SEB or SEC from S. aureus and is characterized by the massive proliferation of Vβ-specific T-cells and subsequent deletion of these cells.
As mentioned earlier, superantigens have even been implicated in KD and the LCWE mouse model of disease [38-41]. T-cells activated by superantigen eventually undergo apoptosis or are rendered anergic [82, 88, 93, 116]; however, contrary to this T-cell fate after SAg activation, in humans and mice, T-cells are found to accumulate and persist at the heart, leading to coronary artery damage. A study by McCormack et. al. determined that T-cells activated by both a superantigen and a peptide-antigen for which the T-cells were specific led to reduced deletion of T-cells [117]. Indeed, for optimal presentation of a peptide antigen to a T-cell, professional antigen-presenting cells are needed. These professional APCs express high levels of costimulatory ligands, and thus costimulation could be one method by which presentation of peptide antigens to SAg-activated T-cells could lead to their survival and thus disease persistence. Several families of costimulatory receptors exist, of which two members include the B7/CD28 family of which CD28 and CTLA-4 and their ligands B7.1/B7.2 are members, and the TNF superfamily of which 4-1BB and its ligand 4-1BBL are members.

1.4 Costimulation.

1.4.1 CD28

CD28 is a 44kDa glycoprotein that exists as a homodimer on the surface of T-cells. It is expressed constitutively on 80% of human T-cells (all CD4+ and 50% of CD8+) and on all mouse T-cells. While T-cell activation leads to an increase in CD28 expression, ligation of CD28 either by an antibody or the B7 ligands leads to its downregulation [118]. Also, while a TCR signal in combination with a signal through CD28 leads to proliferation, differentiation and survival of the T-cell, ligation of just the TCR, as in the case of superantigenic stimulation, leads to apoptosis and anergy [82, 88, 93, 116, 119]. Therefore, costimulation through CD28 is instrumental for an effective and sustained immune response.

The natural ligands for CD28 are the B7 family members CD80 and CD86 (B7-1 and B7-2, respectively). Like CD28, they are cell surface membrane glycoproteins, and while both are expressed on APCs, their expression varies in terms of density and kinetics. CD86 is expressed constitutively while CD80 is upregulated upon activation of APCs. Furthermore, CD80 is
expressed at tenfold lower levels than CD86 [120]. Lastly, while both ligands bind CD28 with the same strength, CD86 dissociates at a faster rate [121].

The differential expression of CD80 and CD86 seems to suggest that these molecules might have different roles in the T-cell response. Indeed, studies in certain disease models have shown these molecules to lead to differing effects. For example, in murine polymicrobial sepsis, CD80−/− mice had better disease outcome than CD86−/− mice [122]. Similarly, in allergic rhinitis, blockade of CD80 at the induction phase of disease led to reduced immune activation, which was not seen with blockade of CD86 [123]. In contrast to these studies, blockade of CD86 but not CD80 ameliorated the inflammation accompanying hapten-induced contact sensitivity [124]. Lastly, both molecules appear to play a role in allergic pulmonary inflammation [125]. Thus, the significance of the roles played by these molecules appears to be disease process specific.

As CD28 in itself has no enzymatic activity, it appears to signal through the recruitment of other molecules. When CD28 is ligated by CD80 or CD86, it is phosphorylated on its cytoplasmic tail on its YMXM motif. This leads to the recruitment of the intracellular proteins PI 3-kinase, ITK, GRB-2/SOS, and Gads. For PI 3-kinase and GRB-2/SOS, this binding is mediated via SH2 domains [119]. Furthermore, Cai et. al showed that while mutation of the Y residue in the motif affected both PI 3-kinase and GRB-2 binding, mutation of the second M residue only affects PI 3-kinase binding. None of these mutants failed to bind ITK [126], which was shown in another study to be dispensible for CD28-mediated costimulation [127]. Furthermore, both these mutants showed a defective IL-2 response after TCR/CD28 ligation, implicating the importance of PI 3-kinase in CD28 signaling and costimulation [126]. These results are in direct contrast to the study by Crooks et.al. which showed that CD28-mediated costimulation is still possible in the absence of PI 3-kinase [128]. Grb2, on the other hand, appears to be very important in the costimulatory response [129] and was shown to activate the transcription factors NFAT and AP-1, while Gads was shown to be important in activation of the transcription factor NFκB [130].

After CD28 recruitment of the aforementioned molecules, several signaling cascades are initiated [131-133]. These signaling cascades lead to the activation and subsequent translocation into the nucleus of the transcription factors AP-1, NFAT, and NF-κB which work synergistically to upregulate IL-2 expression. CD28 also leads to increased stability of the IL-2 mRNA
transcripts [134]. Interestingly, these pathways and transcription factors are also implicated in TCR signaling. As such, a lower threshold of activation is needed for T-cell activation in the presence of costimulation [135]. Furthermore, since T-cells proliferate in the presence of IL-2, increased IL-2 production leads to increased proliferation by T-cells [136]. However, there exists the debate of whether CD28 signaling just augments TCR signaling or can initiate signaling independent of the TCR. A study by Raab et. al. determined that in the absence of a TCR signal, ligation of CD28 led to the upregulation of IL-2 and IL-4 transcription and that this effect was mediated by the transcription factor NFAT [137].

In addition to cytokine production, CD28 signaling also leads to other effects. Upon activation, the energy requirements of the T-cell increases. CD28 stimulation allows for increased glucose uptake by upregulating the expression of the glucose receptor Glut1 and promoting the movement of this receptor to the surface of the cell [138]. Furthermore, in the presence of CD28 stimulation, the expression of the survival molecule Bcl-xL is increased to greater levels than that seen with just TCR signaling alone [139]. This upregulation of Bcl-xL was shown to be dependent upon activation of NFκB [140]. As well, stimulation of the death receptor TNFRII 48 hours post-T-cell activation led to the apoptosis of these T-cells through the downregulation of both protein and mRNA expression of Bcl-xL. CD28 costimulation was able to directly counteract this apoptotic effect by increasing the levels of Bcl-xL [141]. This effect of CD28 on T-cell survival and Bcl-xL expression can nicely be demonstrated through the use of the CD28 superagonistic antibody which leads to full T-cell activation in the absence of TCR signals. While TCR activation leads to the upregulation of CD95L, T-cells activated by the CD28 superagonist do not upregulate this death ligand. Furthermore, these cells have increased levels of Bcl-xL as well as the transcription factor important in its upregulation, NFκB [142]. Lastly, the importance of CD28 signaling is evidenced through the use of mice lacking CD28. In these mice, while T-cells can get activated and initially start to proliferate, the proliferative response is weak and short-lived [143].

1.4.2 CTLA-4

CTLA-4 or CD152, like CD28, is a disulfide-linked 35-kDa homodimeric protein that also binds to the CD80/CD86 molecules. However, unlike CD28 which is expressed
constitutively on T-cells, CTLA-4 is found only on activated CD4+ and CD8+ T-cells (as well as B-cells). The expression of CTLA-4 on the surface of T-cells peaks 48 hours after activation and comes back down by 96 hours, although its mRNA can be detected within 1 hour of T-cell activation [144]. Furthermore, it is believed that this protein accumulates within intracellular vesicles and cycles between these vesicles and the surface of the cell [145].

While CD28 provides a costimulatory signal to the T-cells, the presence of CTLA-4 has inhibitory effects. This is thought to occur via two different mechanisms. First, although CTLA-4 appears to be expressed at lower levels on activated T-cells, it has a much higher affinity for the B7 ligands and a slower dissociation rate than CD28 [146]. As such, the expression of CTLA-4 can lead to the effective antagonism of the B7-CD28 interaction. It was recently shown that CTLA-4 not only interacts with the B7 ligands, but rather removes these ligands from the APCs through trans-endocytosis [147], effectively preventing the interaction between CD28 and these ligands. Second, CTLA-4, through signaling through its cytoplasmic tail, has been shown to directly affect T-cell activation and IL-2 production [148]. The effects of the presence of CTLA-4 include inhibition of T-cell activation, cell cycle progression, IL-2 production and IL-2 receptor expression [149]. Interestingly, CD4+CD25+ T-regulatory cells constitutively express CTLA-4 [150]. Indeed, the importance of T-cell activation regulation by CTLA-4 is demonstrated by the fact that mice lacking CTLA-4 display a lymphoproliferative disorder and undergo massive lethal organ failure by 3-4 weeks [151].

1.4.3 4-1BB

4-1BB (CD137), a member of the tumor necrosis factor superfamily of receptors, was first discovered in 1989 by screening cDNA libraries from activated CD4+ and CD8+ T cell clones [152]. Although it is found mainly on activated T-cells, 4-1BB has also been found to be expressed on several other immune cells including DCs, monocytes, mast cells, neutrophils, and activated NK cells [153]. On T-cells, 4-1BB expression can be detected as early as 24 hours after activation, but its pattern and duration of expression appears to be antigen and disease-model specific [153, 154]. Its ligand, 4-1BBL, is mainly found on activated APCs such as DCs, B-cells and macrophages [154].
Like CD28, 4-1BB is thought to provide costimulatory signals to T-cells. However, because the expression of 4-1BB exhibits delayed kinetics in comparison to CD28, it is thought to be more important later on in the immune response. Indeed, when mice lacking 4-1BBL are injected with the influenza virus, although the cells display a normal initial primary response, there is a decrease in the number of virus-specific CD8+ T-cells later in the primary response. This defect is then carried into the secondary response where the CD8+ T-cell expansion is of a similar level to that seen in the primary response [155]. Interestingly, the potency of 4-1BB stimulation on activated T-cells in comparison to CD28 appears to depend on the strength of the TCR signal- in the presence of a weak signal, CD28 ligation leads to greater IL-2 production by anti-CD3 stimulated T-cells, but in the presence of a strong signal, CD28 and 4-1BB ligation lead to an equal amount of IL-2 production. Furthermore, 4-1BB stimulation can lead to the production of IL-2 by T-cells even in the absence of CD28 suggesting that the presence of CD28 is not needed for 4-1BB stimulation to have an effect [156]. Lastly, 4-1BB costimulation in the presence of superantigenic stimulation was shown to lead to an increase in the recovery of live SAg-specific T-cells when compared to superantigen stimulation alone [157].

Like other members of the TNFR family, 4-1BB signal transduction is accomplished through the recruitment of TRAF adaptor molecules, specifically TRAF1 and TRAF2 (although TRAF3 has also been shown to associate with human 4-1BB) [158]. These TRAF molecules are thought to trimerize at the receptor’s cytoplasmic tails, which also form trimers. Although the ratio at which these two molecules exist at the 4-1BB cytoplasmic tail is unknown, recent studies utilizing crystal structures suggest that the preferred ratio is one TRAF1 molecule to two TRAF2 molecules [159]. Several signaling pathways are activated after formation of this trimeric complex, and the TRAF molecules have differential effects on these pathways (Figure 5).
Figure 5. Signaling downstream of 4-1BB. When 4-1BB is activated by its trimeric ligand 4-1BBL, which is found on APCs, 4-1BB itself trimerizes and recruits a trimeric complex of TRAF1 and TRAF2 molecules. While it has been well established that TRAF2 is essential for activation of the NFκB and JNK/SAPK pathways, the role of TRAF1 is more controversial [158]. It was recently shown that TRAF1 plays a role in survival of 4-1BB activated T-cells by increasing the levels of phospho-ERK which in turn causes the degradation of the pro-apoptotic molecule Bim [153, 160].

TRAF2 is the most widely expressed molecule in the TRAF family and is found downstream of many TNFR family members including TNFR1, TNFR2 and CD40 [161]. Downstream of 4-1BB, it has been found to be essential in the activation of NFκB [158], and subsequent upregulation of the survival molecules Bcl-xL and Bfl-1 [162]. Furthermore, in the absence of TRAF2, T-cells exhibit impaired proliferation, cytokine production, and MAPK activation downstream of 4-1BB ligation [153].

While the role of TRAF2 in 4-1BB signaling has been well established, that of TRAF1 is yet to be fully elucidated. Unlike TRAF2, the expression of TRAF1 is limited to the lung, spleen
and testes. Furthermore, expression of TRAF1 is low in unactivated lymphocytes, but is upregulated after NFκB activation. TRAF1 lacks the ring domain which is needed for NFκB activation, and thus unlike TRAF2 cannot activate this transcription factor [153]; however, in CD8+ T-cells TRAF1 is important in mediating the survival effects downstream of 4-1BB. This is accomplished through upregulation of phospho-ERK, which subsequently leads to the downregulation of the pro-apoptotic molecule Bim. While increased Bim levels are seen after 4-1BB activation in mice lacking TRAF1, it is unclear whether this effect is mediated directly through TRAF1 or through the ability of TRAF1 to stabilize TRAF2 at the cytoplasmic tails of 4-1BB [160].

Since 4-1BB is a costimulatory molecule, increased signaling through this receptor has been implicated in disease. In the LCWE mouse model of KD, co-injection of an agonistic antibody against 4-1BB with LCWE into mice led to increased disease incidence and severity compared to mice injected with LCWE alone (Y. Moolani, submitted manuscript). Furthermore, blocking the signaling between 4-1BB and 4-1BBL ameliorated disease in experimental autoimmune myocarditis [163]. Finally, TRAF1 is upregulated in a lot of tumours and has also been implicated in arthritis [153]. Consequently, studying this molecule and its pathway as it relates to disease susceptibility is important.

### 1.4.4 Costimulation and lineage determination

As mentioned earlier, helper T-cells can be promoted to differentiate into different subsets depending on the environment and stimulus. Interestingly, the different costimulatory and inhibitory molecules display different abilities in promoting the different helper subtypes.

CD28 stimulation has been shown to lead to an increase in the IL-12 receptor and cause the JAKs and STAT4 to become responsive to IL-12 stimulation. As such, it could be said that CD28 promotes a Th1 response [99]. The effect of CD28 on Th17 differentiation is controversial. It has been shown that when APC lacking the B7 ligands are mixed with CD4+ T-cells, less IL-17 is produced [164]. Similarly, Liu et. al. showed that treatment of primary lymphocytes with anti-CD3 upregulated production of IL-17, and that there was a slight increase with the addition of agonistic CD28 antibody [165]. In contrast to these studies, a recent study showed that when anti-CD28 was added to anti-CD3 in the presence of Th17 polarizing
cytokines (IL-1α, IL-6, IL-23, TGF-β1), T-cell differentiation into Th17 cells was inhibited. When neutralizing IFNγ antibody was added to the mix, this effect was abrogated. As well, addition of CTLA-4Ig to block interaction of CD28 with endogenous B7 ligands led to increased Th17 levels. Interestingly, when anti-CD28 was added to already differentiated Th17 cells, IL-17 production from these cells was increased [166]. It is important to note that while this study looked at Th17 frequencies, the other two studies looked at IL-17 levels. Furthermore, this study used a Th17 promoting cytokine cocktail while the first two studies used unmanipulated cells. Therefore, these conflicting results could simply be the result of differing experimental conditions.

Unlike CD28, the effect of 4-1BB stimulation on helper T-cell differentiation is clearer. Most studies report that 4-1BB ligation leads to an increase in IFN-γ and thus a Th1 phenotype [167]. Interestingly, 4-1BB activation was also shown to increase the secretion of IL-13 from CD4+ cells, perhaps as a negative feedback mechanism to prevent an excessive Th1 skew [168]. While one study reported that 4-1BB stimulation slightly but not significantly increased IL-17 production from anti-CD3 stimulated cells [165], thus far, not a lot of work has been done on the effect of 4-1BB on Th17 cells.

2  Rationale

SAgs have been implicated in KD and the LCWE mouse model of disease. Typically, SAg-activated T-cells undergo massive proliferation followed by apoptosis; however, in KD and the mouse model, these SAg-reactive T-cells persist and infiltrate the coronary arteries. Enhanced costimulation through CD28 and 4-1BB can rescue SAg-activated cells from apoptosis and can exacerbate disease in the LCWE mouse model.

3  Hypothesis

Increased costimulation can prevent deletion due to enhanced survival of SAg-activated T-cells.

4  Objectives

There were four main objectives to this study:
1. To compare and contrast the effects of CD28 and 4-1BB costimulation on the survival of SAg-activated T-cells.
2. To determine the contribution of survival versus proliferation after enhanced costimulation on T-cell persistence.
3. To characterize the helper T-cell population of the cells rescued from apoptosis.
4. To determine the role of TRAF1 in SAg-activated T-cell survival mediated by 4-1BB.

5 Materials and Methods

5.1 Experimental mice

Wildtype C57BL/6 mice were purchased from the Toronto Centre for Phenogenomics and housed under specific-pathogen-free conditions at this same centre. TRAF1−/− and 4-1BB−/− mice were generously provided by Dr. Tania Watts (University of Toronto, Toronto, Canada). CD28−/− mice were purchased from Jackson Laboratories and were housed under specific-pathogen-free conditions at the Hospital for Sick Children Research Institute. All animal procedures were in accordance with and approved by the animal care committee at the Hospital for Sick Children and Toronto Centre for Phenogenomics animal facilities.

5.2 Cell culture

To determine the effect of costimulation on SAg-activated T-cells, splenocytes from wildtype (WT), CD28−/−, 4-1BB−/− or TRAF1−/− mice (6-10 weeks old) in the C57Bl/6 background strain were stimulated with 0.3 µg/ml of the SAg Staphylococcus aureus enterotoxin B (SEB) plus either 0.5 µg/ml of the soluble agonistic CD28 mAb (clone 37.51) or 10µg/ml of plate-bound agonistic 4-1BB (clone 3H3) or appropriate isotype controls unless otherwise stated. CTLA4-Ig was used at a concentration of 50µg/ml to determine the effect of blocking CD28 costimulation at various timepoints. A standard cocktail of IL-2 (50u/ml), IL-23 (10ng/ml), and anti-CD3 (1µg/ml) was used on SEB/4-1BB-activated splenocytes to promote development of the Th17 population to determine the role of costimulation on Th17 development. Absolute cell numbers were obtained by counting cells under the microscope that were negative for the viability dye trypan blue.
5.3 Western blot analysis

Splenocytes from WT or TRAF1⁻/⁻ mice were stimulated with 0.3µg/ml SEB for 4 days, after which CD4+ T-cells were isolated using the MACS negative CD4+ T-cell isolation kit. The cells were rested for a day and then stimulated for various amounts of times with anti-4-1BB (clone 3H3) at a concentration of 10 µg/ml. Cells were then lysed, subjected to SDS PAGE, and transferred to polyvinylidene difluoride membrane. Membranes were probed with anti-phospho ERK and anti-ERK primary Abs, followed by HRP-anti-rabbit Ig Ab. The chemiluminescence signal of the protein bands was visualized by autoradiography.

5.4 Thymidine proliferation assay

WT splenocytes were cultured in 96-well plates at a concentration of 2.5 x 10⁶ cells/ml and stimulated with 0.3 µg/ml of SEB, 0.3 µg/ml SEB and 50 µg/ml CTLA4-Ig or left unstimulated. 1µCi of ³H-Thymidine was added to the wells and after a 17 hour incubation, the cells were analyzed for thymidine incorporation using a scintillation counter.

5.5 Bcl-xL expression

Splenocytes from WT mice were labeled with 5µM CFSE (Invitrogen) at a concentration of 5x10⁷ cells/ml in PBS. Cells were incubated for 10 minutes at 37°C and washed 2x with fresh media. Cells were plated as described in section 5.2. At 4 days post activation, cells were harvested, counted with trypan blue and surface stained with the fixable viability dye efluor450 (ebioscience), Vβ8-biotin (clone F23.1) and strepatividin-PE. For intracellular staining, the BD Biosciences Cytofix Cytoperm kit was utilized along with the anti-Bcl-xL antibody (clone 54H6). APC-conjugated anti-rabbit antibody was used to detect the primary Bcl-xL antibody.

5.6 Flow cytometry

Unless otherwise stated, cells were harvested 7 days post-stimulation, and the percentage of live, SEB-specific T-cells undergoing a low level of apoptosis was determined with the use of three fluorochromes: the viability dye 7-amino-actinomycin (7-AAD), AnnV-PE and Vβ8-FITC (clone F23.1). For intracellular cytokine staining, cells were fixed and permeabilized using the BD Biosciences Cytofix Cytoperm kit and stained with the fixable viability dye efluor 450 (ebioscience), CD4-FITC (clone GK1.5), Vβ8-biotin (clone F23.1), streptavidin-PE, and IFN-γ-
APC (clone XMG1.2) or IL-17-APC (clone TC11-18H10.1). To determine the expression of 4-1BB on SEB-specific T-cells, cells were stained with the viability dye propidium iodide (PI), anti-4-1BB-biotin (clone 17B5), streptavidin-APC and Vβ8-FITC (clone F23.1). To determine the expression of 4-1BBL, activated splenocytes were stained with PI and 4-1BBL-PE (clone TKS-1). For BrdU staining, BrdU at a final concentration of 10 μM was added to SEB/4-1BB activated cell cultures the day before harvesting. The cells were stained with the fixable viability dye efluor450 (ebioscience), and Vβ8-FITC (clone F23.1). For fixing, permeabilization and intracellular staining, the APC BrdU Flow kit from BD Pharamingen was utilized. The samples in all experiments were subsequently acquired and analyzed using FACSDiva and FloJo analytical software, respectively.

5.7 Statistical analysis

Where appropriate, statistics were calculated using a Student’s Paired two-tailed t-test. A P value of <0.05 was considered significant.
6 Results

6.1 Kinetics of 4-1BB and 4-1BBL upregulation

4-1BB is not constitutively expressed on T-cells, but rather upregulated upon antigenic stimulation. Upon conventional antigen stimulation, 4-1BB reaches maximal expression approximately 48 hours after stimulation and then starts to decrease thereafter [153]. To determine how long after superantigenic activation of T-cells 4-1BB could be detected at the cell surface, splenocytes from WT mice were activated with a previously optimized dose of 0.3 µg/ml of SEB, and 4-1BB expression on the Vβ8-specific subset of T-cells was determined at days 1, 2, 3, 5 and 7 using flow cytometry (Figure 6). While a third of the cells express 4-1BB as early as one day post-stimulation, almost all the cells (86.8%) express 4-1BB by day 5. By day 7, the cells begin to lose expression of this molecule as seen by a decrease in the mean fluorescence intensity of the histogram plot.

![Diagram showing 4-1BB expression levels at different time points](image)

Figure 6. 4-1BB expression of SEB-activated splenocytes. Splenocytes from WT mice were activated with SEB or left unactivated, and at days 1, 2, 3, 5 and 7 analyzed for 4-1BB expression using flow cytometry. Dead cells were excluded from analysis using PI staining. Histograms are based on live, Vβ8+ cells. Representative of 4 independent experiments.
The expression of the ligand, 4-1BBL was also assayed. 4-1BBL is expressed on activated APCs [154, 169]. Because APCs are activated by TLR ligands and agonists, the TLR4 ligand LPS and TLR2 agonist Pam3Cys were used as positive controls in inducing 4-1BBL upregulation. SEB-, LPS- or Pam3Cys-activated splenocytes were stained for 4-1BBL up to 3 days post-activation. As shown in Figure 7, while expression of 4-1BBL could be detected with LPS and Pam3Cys, no detectable expression of 4-1BBL could be seen with SEB stimulation.

**Figure 7.** 4-1BBL expression on Pam3Cys, LPS or SEB-activated splenocytes. Splenocytes from WT mice were activated with Pam3Cys, LPS or SEB and analyzed for 4-1BBL expression at days 1, 2, and 3 using flow cytometry. Histograms are gated on live cells as determined by PI exclusion. Representative of 2 independent experiments.
6.2 Costimulation through CD28 and 4-1BB can enhance survival of SAg-activated T-cells

Previous studies have shown that stimulation of superantigen-activated T-cells with a peptide lead to reduced apoptosis of the superantigen-activated cells [117]. Since APCs require costimulatory molecules to optimally activate T-cells, we hypothesized that increasing the amount of costimulation through the use of agonistic antibodies could lead to the increased survival of SAg-activated T-cells. In order to test this hypothesis, agonistic anti-4-1BB was plate-bound to wells containing SEB-activated WT splenocytes and the amount of T-cell survival was quantitated through the use of flow cytometry. To determine the extent of the enhanced survival, the percentage of 7-AAD negative, Annexin-V low, Vβ8+ cells was analyzed. Day 7 was chosen as the timepoint of analysis as previous studies had shown that this was when the superantigen cells were undergoing the greatest amount of apoptosis (Y. Moolani, master’s thesis). As shown in Figure 8A, there was a higher percentage of live, Annexin-V low, Vβ8+ cells in the condition with SEB plus agonistic anti-4-1BB antibody as compared to the condition with the isotype-matched control antibody plus SEB. This effect was not limited to the Vβ8+ population as those cells negative for Vβ8 also showed less apoptosis. The increase in the absolute number of live, Annexin-V low, Vβ8+ T-cells was also determined by multiplying the percentage of the live, Annexin-V low, Vβ8+ T-cells by the absolute number of total cells remaining in the wells in the condition on day 7. A summary of 10 experiments is depicted in Figure 8B and show that with enhanced costimulation through 4-1BB, a greater number of T-cells were induced to survive. This effect was also seen with costimulation through the CD28 receptor (Figure 8C).
Figure 8. Enhanced survival of SEB-activated splenocytes with added anti-CD28 or anti-4-1BB. A. WT splenocytes were cultured with SEB plus anti-4-1BB agonistic antibody (left panel) or SEB plus an isotype-matched control antibody (right panel). 7 days post-stimulation, cells were harvested and the amount of apoptosis of the SEB-specific population of T-cells was measured using Annexin-V-PE and a Vβ8-specific antibody. B. A summary of 10 different experiments showing the absolute number of live, Annexin-V low, Vβ8-specific cells when cells were cultured as in A. (p=0.0014) C. Same as in B., except cells were incubated with SEB plus an agonistic anti-CD28 antibody or SEB plus an isotype-matched control antibody (p=0.0064).
6.3 Enhanced survival with anti-4-1BB is dose-dependent

Previous studies in our lab have suggested that the decrease in apoptosis of SEB-activated T-cells with enhanced costimulation through CD28 is dose dependent (Y. Moolani, unpublished observation). Therefore, in order to determine whether the enhanced survival effect obtained with anti-4-1BB treatment was dependent on the dosage of the agonistic antibody, different doses at the final concentration of 2.5, 5, 10, 15 or 25 µg/ml were plate-bound to wells with SEB-activated splenocytes. The absolute number of live, Annexin-V low, Vβ8+ cells in the conditions with or without anti-4-1BB were quantified in the same manner as that in Figure 8B. The value obtained with the addition of anti-4-1BB was then divided by that obtained with the addition of isotype antibody to get the fold differences with the addition of anti-4-1BB. These results are depicted in Figure 9. Increasing doses led to increased amounts of live cells; however, this effect was diminished at higher concentrations of anti-4-1BB. A possible reason for this will be discussed in section 7.1.

Figure 9. Dose response of enhanced survival of SEB-activated splenocytes after coincubation with various amounts of agonistic anti-4-1BB antibody. Cells were harvested 7 days post-stimulation and the absolute number of live, Vβ8+, Annexin-V low cells in the cultures were quantitated using flow cytometry and trypan blue staining. Data are represented as the fold increase in absolute numbers of cells in cultures with costimulatory anti-4-1BB antibody as compared to isotype control. The black dotted line represents the point of no difference.
between cultures receiving agonistic or isotype antibodies. Data are representative of 3 independent experiments.

6.4 CD28 and 4-1BB function independently to mediate T-cell rescue

Previous studies have shown that in the absence of CD28 and in the presence of a TCR signal, 4-1BB stimulation can still lead to IL-2 production [156]. As well, 4-1BBL mice show no defect in their primary response, suggesting that CD28 signaling appears to be intact in the absence of 4-1BB signalling [155]. Therefore, in order to determine whether this was also true in the case of superantigen-stimulated T-cells from mice lacking the costimulatory receptors, splenocytes from CD28−/− or 4-1BB−/− mice were co-incubated with SEB and agonistic anti-4-1BB or anti-CD28, respectively, or the appropriate isotypes. 7 days post-stimulation, cells were harvested and the absolute numbers of live, Annexin-V low, Vβ8+ cells were quantitated using trypan blue staining and flow cytometry. As seen in Figure 10A, addition of agonistic anti-4-1BB to SEB-activated CD28−/− splenocytes led to a significant increase in the number of viable cells present at the end of the assay. Although this experiment also contained WT splenocyte controls, the effect of agonistic antibody on the cells from the knockout mice were not compared to that of the WT as SAg-activated T-cells from CD28−/− mice do not proliferate as well as those from WT mice [143]. As such, it would not have been possible to determine whether any differences between the two mice were due to differences in response to superantigen or differences in the response to the antibody. Although not significant, this pattern was also seen with the addition of agonistic CD28 antibody to splenocytes lacking the 4-1BB costimulatory receptor (Figure 10B).
Figure 10. Enhanced survival of CD28−/− or 4-1BB−/− SEB-activated T-cells after anti-4-1BB or anti-CD28 treatment, respectively. A. CD28−/− splenocytes were cultured with either SEB plus agonistic anti-4-1BB antibody or SEB plus an isotype-matched control antibody for 7 days. After the stimulation, cells were harvested and the amount of apoptosis of the SEB-specific population of T-cells was measured using Annexin-V-PE and a Vβ8-specific antibody. The absolute number of live, Annexin-V low, Vβ8-specific cells was measured in 5 independent experiments (p= 0.0121) B. Similar to A., except 4-1BB−/− splenocytes were incubated with SEB plus an agonistic anti-CD28 antibody or SEB plus an isotype-matched control antibody.

Since agonistic anti-4-1BB and anti-CD28 could lead to enhanced survival in the absence of the other molecule, next we determined whether addition of both the agonistic antibodies to splenocytes from WT mice in the presence of SEB had additive effects. Figure 11 depicts these experiments. In order to compare more effectively between conditions, the relative increase in the absolute number of live cells with costimulation as compared to isotype controls is depicted. While addition of anti-CD28 alone did not lead to a substantial increase in the number of cells in these experiments, the striking observation is that the addition of anti-CD28 to anti-4-1BB appears to have a negative effect on the survival effects of anti-4-1BB. Therefore, it can be concluded that the antibodies do not have additive abilities in these assay conditions. In fact, too much costimulation appears to have a deleterious effect. This will be further discussed in section 7.1.
Figure 11. No additive effects of agonistic anti-4-1BB and anti-CD28. WT splenocytes were stimulated with SEB and co-incubated with either anti-CD28, anti-4-1BB or anti-CD28 and anti-4-1BB or the respective isotypes. 7 days post-stimulation, cells were harvested and the absolute number of live, Annexin-V low, Vβ8+ cells were quantified using flow cytometry and trypan blue staining. The values obtained for the conditions with costimulatory antibody were then divided by that of the conditions with isotype control antibodies to get the fold difference. The fold difference of five independent experiments is plotted in this figure. The dotted line represents that point at which there is no difference between the conditions that received costimulatory antibody as compared to isotype control antibody.
6.5 Costimulatory signal is needed early to mediate a survival effect

Timing is very important in the immune response. The expression of CD80/86, 4-1BB and 4-1BBL are all tightly regulated and are upregulated after cellular activation [120, 153, 154, 169]. Previous studies have suggested that costimulatory signals need to be initiated within the first two hours of T-cell activation in order to block induction of anergy [170]. As well, previous experiments in our lab showed that for CD28, addition of agonistic antibody was effective up to 3 hours post-SEB stimulation (Y. Moolani, master’s thesis). Therefore, to determine the kinetics of the costimulatory signal needed to mediate T-cell survival downstream of 4-1BB, SEB-activated splenocytes were added to wells coated with agonistic anti-4-1BB at days 0, 1, 2, 3, or 4. Figure 12A shows the absolute number of live, Annexin-V low, Vβ8+ cells with the addition of anti-4-1BB or isotype on the various days. Figure 12B illustrates the fold difference in the absolute number of live, Annexin-V low, Vβ8+ cells with costimulation as compared to isotype. The dotted line indicates the point of no difference between the addition of costimulatory antibody or isotype. Although the induction of 4-1BB signaling up to 2 days post-SEB stimulation led to a significant increase in the number of live cells, a comparison of the degree of the increase in the number of live cells reveals that the antibody is most effective if given at the same time as the superantigen stimulation. The results suggest that costimulatory antibody needs to be given early to have an effect on superantigen-stimulated cells as the effect of anti-4-1BB treatment diminishes with each subsequent day post-SEB stimulation.
Figure 12. Anti-4-1BB needs to be added within 48 hours after SEB stimulation. A. WT splenocytes were incubated with SEB and agonistic anti-4-1BB was added at either the time of SEB stimulation or 1, 2, 3 or 4 days post-stimulation. Cell cultures were harvested 7 days later and the number of live, Annexin-V low, Vβ8+ cells in the conditions with costimulation as compared to that with isotype control antibody is depicted for each of the days. B. The relative increase in the number of live SEB-specific cells undergoing low amounts of apoptosis with the addition of anti-4-1BB as compared to isotype. The dotted line represents the point at which there is no difference between the conditions that receive costimulatory antibody and isotype control antibody. Summary of 3 independent experiments.
The next step was to determine the duration of costimulatory signal needed by SEB-activated T-cells for the enhanced survival effect. Because there is constitutive expression of CD28 and CD86, the fusion protein CTLA4-Ig was used to block endogenous costimulatory signals through CD28 at the time of SEB stimulation or 1,2 or 3 days post-SEB activation. As a control for any cell-cell interactions that may have been lost through the disturbance of the cells after CTLA4-Ig addition, control wells with cells and SEB were mixed at each experimental timepoint. As seen in Figure 13A, CTLA4-Ig was effective only when given within 24 hours of SEB activation. When the relative increase between conditions that had only SEB as compared to conditions with SEB and CTLA4-Ig was plotted (Figure 13B), the greatest differences were seen in the conditions that received CTLA4-Ig early at D0 and D1. Interestingly, there was a bigger difference when CTLA4-Ig was added after one day of SEB stimulation as opposed to at the time of SEB stimulation. By 48 hours the difference between the SEB and CTLA4-Ig conditions are diminished and there is no difference between the two conditions beyond 48 hours, similar to results obtained from experiments with 4-1BB.

In contrast to CD28, 4-1BB is only upregulated after activation and thus would only be found on activated T-cells. Furthermore, as shown in Figure 7, the ligand could not be detected after SEB activation. As well, anti-4-1BB, unlike anti- CD28 was plate-bound and not soluble. Therefore, in order to reduce the signalling through 4-1BB, SEB-stimulated cells were transferred from wells containing plate-bound anti-4-1BB to fresh uncoated wells at days 1,2,3, or 4 post-SEB stimulation. Decreasing the signal through 4-1BB by transferring the cells to uncoated wells had no effect on the survival level of cells as seen at D7 (Figure 14A). Although the differences between the anti-4-1BB condition and isotype is significant only for days 0 and 1, on average, the relative increase in the number of live cells with anti-4-1BB as compared to isotype is greater than or equal to what is seen when cells are treated with anti-4-1BB for the whole seven days (D0 timepoint) as seen in Figure 14B. This suggests that a signal through anti-4-1BB at the time of SEB stimulation is enough to enhance survival of the SAg-activated cells. As well, it is important to note that the general trend in Figure 14B seems to suggest that a shorter duration of signal in terms of 4-1BB is better, as abrogating the signal after 1 day leads to a larger increase in the number of live cells as compared to abrogating the signal at later timepoints. In transferring cells to the fresh wells, if the plate-binding protocol was not very effective, some anti-4-1BB antibody could have been transferred along with the cells and this
could have been leading to the results seen in Figure 13B. Therefore, as an experimental control to check for the efficiency of the antibody binding to the plate, cells in wells coated with anti-4-1BB or cells in wells with soluble anti-4-1BB were stained with rat-Ig-PE. As seen in Figure 14C, there is a marked reduction in the amount of antibody bound to the cells upon binding the anti-4-1BB to the plate as compared to the soluble antibody.

Figure 13. Co-stimulatory signal is needed within the first 24 hours for anti-CD28. A. WT splenocytes were incubated with SEB and CTLA4-Ig was added at either the time of SEB stimulation or 1, 2, or 3 days post-stimulation. The absolute number of live, Annexin-V low, Vβ8+ cells of the conditions with SEB alone and the conditions with SEB and CTLA4-Ig is plotted. B. The relative increase in the number of live, Annexin-V low, Vβ8+ cells with SEB stimulation as compared to SEB+CTLA4-Ig treatment is plotted for all the days. The dotted line represents the point at which there is no difference between the condition with SEB alone and SEB plus CTLA4-Ig
Figure 14. Short duration of signal needed for 4-1BB

A. WT splenocytes were incubated with SEB and anti-4-1BB. At days 1, 2, 3, and 4 post-SEB activation, cells were transferred to fresh wells. The number of live, Annexin-V low, Vβ8+ cells with anti-4-1BB and isotype control antibody is depicted.

B. The relative increase in the number of SEB-specific live cells with anti-4-1BB as compared to the isotype control. The dotted line represents the point at which there is no difference between the conditions that receive costimulatory antibody and isotype control antibody.

C. WT splenocytes were incubated with SEB and soluble anti-4-1BB, plate-bound unwashed anti-4-1BB and plate-bound washed anti-4-1BB. 7 days post-stimulation, cells were stained with anti-rat-Ig. Histograms as gated on live cells as determined by PI exclusion.
To determine whether the surviving cell population was functional, a thymidine proliferation assay was done on cells stimulated with SEB or SEB+CTLA4-Ig. In addition, CTLA4-Ig was also added before SEB incubation and at shorter intervals prior to the 24 hours timepoint. Plates were then analyzed at an early timepoint (day 3) (Figure 15A) and at a later time point (day 8) (Figure 15B) to look for any differences in proliferation during the course of the SAg response. As shown in Figure 15, while cells with CTLA4-Ig proliferate to the same extent earlier in the response, by day 8 there is a marked reduction in the proliferation of the cells that received CTLA4-Ig as compared to those that did not. Also, it is important to note that this effect was not seen if CTLA4-Ig was given post 24 hours SEB-activation, similar to what is seen in Figure 13A. Control wells containing SEB-activated cells were also mixed in order to account for any changes in proliferation that might have occurred due to the disturbance of cell-cell interactions caused by the addition and mixing of the wells at each timepoint. All SEB values obtained at each timepoint were similar in value and thus only the SEB value at 0 hours is displayed in order to keep the graph concise.

![Figure 15A](image1.png)
![Figure 15B](image2.png)

**Figure 15.** CTLA4-Ig addition affects proliferation if added within 24 hours of SEB stimulation. Cells were incubated with SEB and CTLA4-Ig was added at different timepoints. 17 hours before harvesting cells on day 3 (A) or day 8 (B), thymidine was added to the cells. Data are represented as the average disintegrations per minute (DPM) of wells set up in triplicate (n=3).
6.6 Enhanced costimulation increases proliferative capacity and Bcl-xL expression of SEB-activated T-cells

We have shown that increasing the amount of costimulation through the addition of agonistic antibodies against CD28 and 4-1BB leads to a greater number of live cells after superantigen stimulation. This effect could be due to either an increased proliferative capacity of the cells that received costimulation, or an upregulation of molecules such as Bcl-xL, indicative of enhanced survival signaling. Therefore, in order to elucidate the roles these two pathways play in increasing the number of live cells at the end of the assay, cells were stained with CFSE and incubated with SEB and agonistic anti-CD28 or anti-4-1BB or the appropriate isotype controls. Ideally, we wanted to compare, peak for peak, the difference in Bcl-xL expression obtained with costimulation as compared to the isotype control. Several different CFSE staining protocols were used including experiments where the CFSE cell concentration or the CFSE concentration itself were varied. As well, several different plating conditions were tried such as different SEB or cell concentrations. Regardless of the conditions used, only two CFSE peaks could be discerned (Figure 16A, left panel). Consequently, the CFSE low and CFSE high populations were gated, followed by the Vβ8+ population (Figure 16A, right panel) in order to determine whether or not any differences exist in Bcl-xL expression in the costimulatory and isotype conditions in the SEB-reactive population that had and had not proliferated. Interestingly, as seen in Figure 16B, there was a greater percentage of Bcl-xL negative cells in the conditions lacking costimulatory molecules. Furthermore, cells that had not proliferated had lower amounts of Bcl-xL and the percentages were not different between the two conditions. This pattern is representative of 5 independent experiments for anti-CD28 and 3 independent experiments for anti-4-1BB.
Figure 16. Increased Bcl-xL expression with anti-CD28 or anti-4-1BB. A. WT splenocytes were labelled with CFSE and incubated with SEB and anti-CD28 or anti-4-1BB. Dead cells were excluded using the efluor450 fixable viability dye. The CFSE low and high population (left panel) followed by the Vβ8+ population (right panel) were gated in order to determine the percentage of Bcl-xL+ cells B. Bcl-xL+ population of the cells gated as in A.
By day 7 all the CFSE had been diluted out. Therefore, BrdU was used instead to determine the amount of proliferation the cells were undergoing. Previous studies in the lab have shown that with increased costimulation through CD28 or 4-1BB, there is increased proliferation by SEB-activated cells as seen by increased BrdU incorporation (Y. Moolani, master’s thesis). However, there are also more cells in the condition with costimulatory antibody. Therefore, this effect could just have been due to an increased number of cells proliferating. Alternatively, this effect could also have been due to an increased capacity of each individual cell to proliferate. In order to differentiate between these two possibilities, cells in the condition with the costimulatory antibody were equated to those in the condition with the isotype-matched control antibody before the addition of BrdU. **Figure 17A** shows BrdU incorporation of cells with agonistic anti-4-1BB or isotype that were just mixed, but not equated in cell number. **Figure 17B** on the other hand, represents cell cultures where the cell number and concentration were equated between the costimulatory and isotype condition. In both situations, SEB-specific Vβ8 cells that received anti-4-1BB antibody proliferated to a greater extent than those that received only the isotype antibody, suggesting that each individual cell has a greater propensity to proliferate.
Figure 17. Increased BrdU incorporation after SEB and anti-4-1BB stimulation. A. WT splenocytes were stimulated with SEB and anti-4-1BB or a control isotype antibody. 18 hours prior to harvesting, BrdU was added to the wells. B. Cells were cultured as in A., except before adding BrdU, cells in the anti-4-1BB and isotype conditions were counted and replated at equal cell numbers and concentration. Dead cells were excluded from analysis using the efluor450 fixable viability dye.
6.7 Anti-4-1BB stimulation skews SEB-activated T-cells towards a Th1 phenotype

As mentioned earlier, both Th1 and Th17 cells have been implicated in Kawasaki disease as well as the LCWE mouse model of disease [10, 11, 19, 67, 107]. Therefore, in order to determine whether enhanced costimulation modulates differentiation into either of these subsets, WT splenocytes were stimulated with SEB and anti-4-1BB or its isotype control for seven days. After this incubation period, cells were stained intracellularly for IFNγ and surface stained for CD4 and Vβ8 to look at the effect of anti-4-1BB on Th1 cells. As seen in Figure 18A, stimulation with anti-4-1BB increases the percentage of Vβ8+ Th1 cells. This effect was reproducible over 5 different experiments. This is consistent with findings in our lab with anti-CD28 which shows that this agonistic costimulatory antibody increases the population of Vβ8+IFNγ+ cells within the CD4+ subset as well (L. Liang, unpublished observation). Median fluorescent intensities (MFI) for the five experiments are plotted in Figure 18B. There was no significant difference between the values in conditions with anti-4-1BB versus isotype suggesting that while there was an increase in the percentage of Th1 cells, each cell was not producing a greater amount of the IFNγ cytokine. Because Th17 cells were very low in number at day 7, cultures were restimulated with a cocktail of anti-CD3, IL-2 and the Th17 survival factor IL-23. Even after this enrichment, cell percentages were fairly low (Figure 18C) and no consistent difference between the condition with anti-4-1BB and isotype could be observed.

In order to determine whether increasing the doses of anti-4-1BB has an effect on the differentiation of these cells, cultures were set up as in Figure 18, except with 2.5, 5, 10 or 15 µg/ml of anti-4-1BB. As depicted in Figure 19A, there was no effect of varying the doses on the Th1 population or the Vβ8-specific Th1 population. This was also the case for the Th17 population (Figure 19B).
Figure 18. Anti-4-1BB increases percentage of SEB-specific Th1 but not Th17 cells. A. WT splenocytes were stimulated with SEB and anti-4-1BB or a control isotype antibody. 7 days post-stimulation, the cells were assayed for the presence of Th1 cells using flow cytometry. FACS plots are gated on the live, Vβ8 population. Representative of 5 independent experiments. B. MFI of the IFNγ+ cells in the Vβ8+CD4+ population. C. Cells were cultured as in A., except restimulated with anti-CD3, IL-2 and IL-23 at day 7 and with IL-2 on day 9 in order to promote the survival of the Th17 population. Cultures were harvested on day 11 post-SEB stimulation. Because populations were so small in percentage, FACS plots are gated on live cells. Representative of 4 independent experiments.
Figure 19. No dose-dependent effect of anti-4-1BB on Th1 or Th17 phenotype of SEB-activated cells. A. WT splenocytes were stimulated with SEB and different doses of anti-4-1BB. Cells were harvested on day 7 and the percentage of IFN-γ+ cells within the CD4+ and Vβ8+CD4+ subsets were determined using flow cytometry (n=4). Values are based on live cells as determined by eFluor450 fixable viability dye exclusion. B. Cells were plated and stimulated as in Figure 18 B except various concentrations of anti-4-1BB were used (n=4). Gating analysis was performed as in A except only the % of IL-17+ cells in the CD4+ population was determined as the numbers were too small to perform any further gates.
6.8 TRAF1 loss leads to diminished T-cell survival

Within the CD8+ T-cell population, the absence of TRAF1 results in decreased cell survival. Furthermore, this effect is mediated through an increase in phospho-ERK levels which in turn leads to a downregulation of the pro-apoptotic family member Bim [160]. In the LCWE mouse model, the CD4+ subset of T-cells are thought to be the inciting agent in disease formation as mice with only functional CD4+ T-cells get worse disease than those with both subsets [66]. Therefore, to determine whether TRAF1 plays the same role in survival in CD4+ T-cells as was found in the CD8+ population, WT or TRAF1−/− splenocytes were stimulated with SEB and anti-4-1BB or its isotype. Seven days post-stimulation, cell cultures were harvested and the absolute number of live, Annexin-V low, Vβ8+ T-cells were quantitated using flow cytometry. The fold difference of the condition with anti-4-1BB as compared to isotype of the WT and TRAF1−/− mice splenocytes was then plotted as shown in Figure 20A. While there were more live cells on average in the TRAF1−/− cells with anti-4-1BB as compared to isotype, the difference was not as pronounced as that found with the WT T-cells, suggesting a survival defect. Next, in order to determine whether 4-1BB stimulation leads to the phosphorylation of ERK and whether or not this is TRAF dependent, CD4+ SEB-activated T-cells from WT or TRAF1−/− mice were isolated and stimulated with anti-4-1BB or an isotype control for 2.5, 5, 10, and 15 minutes. Preliminary results suggest that 4-1BB stimulation does lead to the upregulation of phospho-ERK and this is TRAF dependent as the upregulation was not evident in the TRAF1 deficient mice (Figure 20B).
Figure 20. Effect of SEB and anti-4-1BB stimulation on TRAF1+/− T-cells. **A.** TRAF1+/− splenocytes were incubated with SEB plus agonistic anti-4-1BB or SEB plus an isotype matched control antibody. 7 days post-stimulation, cells were harvested and the absolute number of live, Annexin-V low, Vβ8 cells was determined using flow cytometry and trypan blue staining. The dotted line represents the point of no difference between conditions with anti-4-1BB or the isotype control antibody. Summary of 3 independent experiments. **B.** WT or TRAF1+/− splenocytes were incubated with SEB for 4 days. CD4+ T-cells were then isolated and cells were left to rest for 1 day. Following the rest period, cells were activated with agonistic anti-4-1BB for various amounts of time. A Western blot was then performed on the lysates of the cells to determine phospho-ERK expression.
7 Discussion

Superantigens have been implicated in Kawasaki Disease [38-41]. Consequently, in order to study this disease a mouse model wherein the bacterial extract, LCWE, known to have superantigenic activity, is used to cause disease similar to that seen in humans [58, 64, 65]. Paradoxically, when superantigens stimulate T-cells, these T-cells undergo massive proliferation but eventually apoptose leading to deletion [82, 88, 93, 116]; however, in KD and the mouse model these T-cells persist and infiltrate the heart and coronary arteries and appear to be a subset expressing SAg-reactive TCRs. Previous studies in our lab have shown that enhanced costimulation through CD28 or 4-1BB can rescue T-cells from apoptosis, and in the case of 4-1BB, can even exacerbate disease in the mouse model. Therefore, in this thesis, we have further investigated this concept of enhanced survival of SAg-stimulated T-cells after increased costimulatory signals. As well, because LCWE is a crude heterogeneous extract, the prototypical superantigen, SEB, was used in this study as a proof-of-principle concept that may be generalized to all other substances with superantigenic activity, including LCWE.

7.1 The role of costimulation on SEB-activated T-cells

In 1993, McCormack et. al. discovered the phenomenon in which SEA-reactive T-cells could be rescued from apoptosis with concurrent stimulation with cytochrome c, a peptide for which the SEA-reactive T-cells were specific [117]. Antigen presentation is accomplished through the use of antigen presenting cells which also express a number of costimulatory ligands. Consequently, this rescue from apoptosis effect could potentially also be achieved through enhanced costimulatory signals. Indeed, studies in our lab and others have shown that with increased signaling through the CD28 receptor, the proliferative response and IL-2 production of SAg-activated T-cells increase (Y. Moolani, master’s thesis, [171-175]). Furthermore, a study by Takahashi et. al. showed that after coinjection of anti-4-1BB and the SAg SEA into mice, a greater number of SEA-reactive T-cells are recovered 21 days post-injection [157]. In our study, in line with all these studies in the field of superantigens and costimulation, we also found that in our survival assay and with our method of measuring survival, increased number of SAg-reactive T-cells were present at the end of the assay when agonistic costimulatory antibody against either CD28 or 4-1BB was added to the culture along with the SAg as compared to isotype controls and SAg. As noted earlier, the percentage of live, annexin-V low cells in the non-Vβ8 population
also increased. SAgs in general activate more than one Vβ family and SEB is no exception. Therefore, the fact that these cells were not Vβ8+, does not necessarily mean they were not SEB-reactive. Although non-SEB reactive Vβ families were not stained for in these experiments, other members in our lab have shown that while anti-CD28 stimulation leads to reduced apoptosis in the Vβ8+ population after SEB stimulation, this effect is not seen in the Vβ6+ population, which is not SEB reactive (Y. Moolani, unpublished observation). In addition, memory T-cells have been shown to express 4-1BB in the presence of IL-2 and IL-15 without antigen stimulation [153]. Consequently, because whole splenocyte populations were used and not just naïve cells, the enhanced survival effect may have been due to the effect of 4-1BB on memory and not superantigen activated T-cells. While the effect of anti-4-1BB on unactivated splenocytes was not assessed, Figure 6 shows that in the absence of superantigen stimulation, 4-1BB could not be detected. As well, in the absence of 4-1BB a defect in memory of the CD8+ T-cells appears to be localized to the bone-marrow and not the spleen [153]. Consequently, the addition of anti-4-1BB to unactivated splenocytes would presumably have negligible effects, if any.

Although CD28 and 4-1BB are both costimulatory receptors, they display different expression kinetics with CD28 being constitutively expressed [118] and 4-1BB coming up later in the immune response [153, 154]. Downstream of TCR activation, 4-1BB stimulation was able to augment IL-2 production by T-cells in the absence of CD28 [156]. Similarly, we wanted to determine whether the enhanced survival effect obtained with agonistic anti-4-1BB treatment of WT SEB-activated T-cells could also be obtained with agonistic anti-4-1BB treatment of CD28−/− SEB-activated T-cells. Indeed, stimulation through anti-4-1BB led to a significant increase in the absolute number of SEB-reactive T-cells at the end of the survival assay. We also looked at the effect of treatment of SEB-activated 4-1BB−/− splenocytes with agonistic anti-CD28. Due to the small differences between experimental conditions, more replicates are needed to detect statistical significance.

Since both agonistic anti-CD28 and anti-4-BB could lead to the enhanced survival of SEB-activated T-cells, and since activation of both receptors lead to similar downstream pathways such as the NFκB pathway [158, 176], we next tried to test whether these two pathways could synergize and lead to additive effects. We could find no such effect. A study by Bukczynski et. al. had a similar objective – to determine whether CD80 and 4-1BBL could
synergize in costimulating HIV-specific CD8 T-cells. The conclusions from this study were that while CD80 and 4-1BB could individually lead to maximal expansion of antigen specific T-cells, there was no additive effect on the expansion with combination of the two molecules. Interestingly enough however, there was an additive effect when only levels of cytokines were measured. Furthermore, a synergistic effect was seen when regulatory cytokines such as TNF-α, IL-10 and particularly IFN-γ were neutralized. This suggests that dual costimulation triggers high doses of these regulatory cytokines which inhibit expansion of T-cells [177]. A similar phenomenon could have been taking place with dual costimulation of the SEB-activated splenocytes and thus no synergistic effects were seen. Consistent with too much costimulation having a negative effect on T-cell expansion, we also found that at higher concentrations of agonistic anti-4-1BB, the enhanced survival effect was diminished as seen in Figure 10.

Timing is very important in an immune response. We therefore wanted to determine the kinetics involved in the phenomenon of costimulation-mediated rescue of SAg-stimulated T-cells from apoptosis. Earlier studies in our lab with anti-CD28 had found that the antibody needed to be added within 3 hours of SEB-stimulation in order to have an effect (Y. Moolani, master’s thesis). In this study, we have shown that the greatest enhancement of survival is seen when agonistic anti-4-1BB co-stimulatory antibody is added to the cultures at the same time as superantigen. There is also enhanced survival seen when anti-4-1BB is added to cultures 24 hours post-SEB stimulation, albeit to a lesser degree. However, addition of antibody 48 hours or later after SEB-stimulation, leads to a severely diminished survival effect that is completely gone by day 4. These results were unexpected as 4-1BB was expressed maximally around day 5 after stimulation. However, around 30% of the SEB-specific T-cells expressed 4-1BB 24 hours after SEB-stimulation and thus one hypothesis is that it is these cells that express 4-1BB early that go on to survive at the end of the assay. Alternatively, in line with the finding that too much costimulation has a negative effect on the survival of the cells, another hypothesis for the diminished survival of the T-cells when anti-4-1BB is given later could be that at later time-points, there is more 4-1BB expressed. As a result, the cells get increased signals through the receptor and this is detrimental to the survival of the cells. However, considering the fact that for anti-CD28, it was shown that the signal is needed close in terms of timing to the SAg signal, the first hypothesis about anti-4-1BB appears to be more likely. One possibility for this early requirement of costimulation could be that pathways leading apoptosis and death are initiated
early after superantigen stimulation and after a certain time-frame, these pathways cannot be regulated or altered. Consequently, costimulation is needed early to counteract these pathways in order to enhance the survival of the SAg-activated T-cells.

In addition to timing, we also wanted to determine duration of signal needed. Because the B7 molecules are expressed constitutively, CTLA4-Ig was used to answer this question. CTLA4-Ig was effective only if added within 24 hours of SEB stimulation. That is, CD28 signaling was needed up to a day after superantigen stimulation. Interestingly, if given after one day of SEB stimulation, there appeared to be a greater difference between conditions with SEB and SEB plus CTLA4-Ig as compared to conditions that received CTLA4-Ig at the time of SEB stimulation. Studies in our lab have shown that the CD86 molecule is upregulated 24 hours after stimulation with SEB (A. Wong, master’s thesis). Consequently, this 24 hour timepoint could be pivotal and thus freshly added CTLA4-Ig as opposed to CTLA4-Ig that was added the day before could have been more effective in blocking the upregulated costimulatory ligand. Anti-4-1BB, on the other hand, just needed to be present at the time of SEB stimulation as abrogation of the signal by transferring the cells out the well still led to an enhanced survival effect. However, there was greater cell survival if anti-4-1BB was present in the cultures for only a day. Interestingly, if anti-4-1BB stimulation continued for a longer periods of time, less of an effect was seen. This seems counterintuitive as increased signaling through 4-1BB should lead to increased survival. However, as mentioned earlier, high doses of anti-4-1BB appeared to have a negative effect on cell survival. Similarly, the addition of anti-CD28 and anti-4-1BB together to SEB activated cells led to a reduced survival effect as compared to anti-4-1BB alone. In line with these experimental results, as the percentage of 4-1BB+ cells increase over time, leaving the cells with the anti-4-1BB could have led to increased signaling through 4-1BB and thus a dampening effect on the survival effect.

While statistical significance was achieved in the survival assay looking at the effect of anti-4-1BB or anti-CD28 on SEB-activated T-cells, one of the limitations with using this assay to measure enhanced T-cell survival was the high level of variability in the experimental results. As a result, a trend rather than statistical significance had to be used in some experiments carried out to further examine this survival effect as was done with the experiment looking at the effect of anti-CD28 on 4-1BB−/− splenocytes. While conclusions cannot be made based on these trends,
they provide rationale for further experimentation and allow for the prediction of the number of repeats needed in order to reach statistical significance. For example, using the mean and standard deviation of the samples in the experiment with the 4-1BB\textsuperscript{+}, and the accepted power value of 0.8, an estimate of the number of mice needed in the 4-1BB\textsuperscript{−} experiment to reach a statistical significance level of 0.05 would be 12. Although the sex of mice was kept constant within experiments, because there have been no reports of sexual dimorphism in our mouse model of disease, the sex of mouse was not kept constant between experiments. However, at least one study has shown that females have a more exaggerated TNF-\(\alpha\) response with SEB stimulation [178]. While the role of TNF-\(\alpha\) was not explored in this thesis, studies in our lab have shown that this cytokine plays an important role in reducing the apoptosis of SEB-activated T-cells through upregulation of costimulatory ligands, particularly CD86 (A. Wong, master’s thesis). Consequently, the sex differences of the mice could have added some variability between replicates of the assay.

7.2 The role of proliferation and survival molecule expression on SEB-activated T-cells

Previous studies in our lab looking at the role of costimulation have shown that anti-CD28 and anti-4-1BB treatment of SEB-activated cells results in both increased proliferation and expression of the survival molecule Bcl-xL (Y. Moolani, master’s thesis). However, we wanted to determine whether the effect of the enhanced stimulation is evident early, when the cells are in their proliferation phase, or later when the cells are undergoing apoptosis. In order to accomplish the above task, the dye CFSE, an antibody against Bcl-xL and an antibody against V\(\beta\)8 were utilized. The goal was to first gate on the SEB-specific V\(\beta\)8 population, and then compare, peak for peak, the percentage of cells in each peak, which would be a measurement of proliferation, and the percentage of Bcl-xL positive cells in each peak which would be a measurement of survival molecule expression. Unfortunately, regardless of the extensive experiments done to optimize the staining and increase resolution of the peaks, only two peaks could be discerned and thus only a population of divided and undivided cells could be used for analysis. One possibility for this could be that the cells all divided at similar times and thus instead of distinct peaks, there was only one merged peak of divided cells. Alternatively, different lots of CFSE appear to have different efficacies in proliferation assays.
Although distinct peaks could not be seen, the percentage of Bcl-xL+ cells was nevertheless measured in the divided and undivided cells. Interestingly, Bcl-xL showed enhanced expression in cultures with costimulatory antibodies early in the response. As well, populations that had undergone more proliferation contained a greater percentage of Bcl-xL+ cells. Furthermore, proliferation assays using CTLA4-Ig and thymidine incorporation showed that proliferation later in the SAg response is affected. In the case of anti-4-1BB and BrdU incorporation, each cell was shown to have a greater capacity for proliferation late in the SAg response, similar to the results with CTLA4-Ig. These results suggest that costimulation appears to increase the expression of the survival molecule Bcl-xL early in the response, allowing for more cells to persist and proliferate towards the end of the SAg response.

7.3 The role of anti-4-1BB on naïve T-helper cell differentiation

Both Th1 and/or Th17 cells have been implicated in a number of autoimmune diseases such as multiple sclerosis [179], diabetes [180] and even Kawasaki disease [10, 11, 19, 67, 107]. Consequently, we thought it would be important to determine whether either of these subsets is preferentially induced after SAg stimulation. Our results suggest that while an increase in the Th1 subset was seen consistently multiple times after the addition of agonistic anti-4-1BB to SEB activated WT splenocytes, there did not appear to be any effect on the Th17 population. In fact, the percentage of the Th17 population was so miniscule after SAg stimulation that no conclusion could be made as to the effect of 4-1BB treatment on this population.

SEB, as well as other superantigens, have been shown to preferentially lead to Th1 differentiation after CD4+ T-cell activation [181, 182]. Furthermore, the main cytokine secreted by cells of the Th1 subset, IFN-γ, has been shown to negatively regulate development of the Th17 population [105]. Consequently, this could have been one of the reasons for the low percentages of Th17 cells even after cytokines were added to enhance Th17 numbers. In addition, similarly to SEB, 4-1BB tends to favor a Th1 response after conventional peptide stimulation [167] and after superantigen stimulation as shown in this thesis and thus this further would have added to the IFN-γ inhibition of the Th17 population. Interestingly, IFN-γ expression in LCWE-injected mice is biphasic, with the first peak corresponding to the appearance of inflammatory cells at the coronary arteries and the second peak corresponding to the vascular
wall disruption [67]. Indeed, Th1 cells could be the subset contributing to the persistant inflammation seen in KD.

Within the mouse model, IL-6 was shown to be important for the coronary artery damage but inflammation was still evident in the absence of IL-6 [107]. IL-6 is an important cytokine in the differentiation of Th0 cells into Th17 cells [105]. Thus, while cytokines such as IFN-γ produced by Th1 cells could be contributing to the inflammation, as mentioned above, Th17 cells could play a role later on in the immune response and thus be contributing to the elastin breakdown of the coronary arteries. Unfortunately, as the experiments done were in an in vitro setting, this concept of temporal segregation could not be evaluated. Interestingly, both IFN-γ and IL-17/Th17 cells are found to be upregulated in the acute phase in humans with KD. Consequently, the complex relationship between and the role of Th1 and Th17 cells in KD would be better studied using the mouse model.

7.4 The role of TRAF1 in SEB-mediated T-cell survival

A 2006 study by Bueno et. al. determined that signaling downstream of the TCR after superantigenic stimulation follows a different pathway from that initiated by a conventional peptide [94]. Furthermore, in 2008 Sabbagh et. al. determined that in CD8+ T-cells downstream of 4-1BB, TRAF1 appears to play a critical role in survival that was mediated by phospho-ERK upregulation and Bim downregulation [160]. Consequently, because SAg-activation appears to initiate a different pathway downstream of the TCR, we wanted to determine whether this TRAF1 effect is applicable to SAg-activated T-cells since SAg-mediated T-cell activation differs from peptide-mediated activation. Indeed, our preliminary results suggest that this same pathway might play a role in CD4+ T-cells even though SAg activation leads to a different signaling pathway. While we did not look at Bim levels, the level of phospho-ERK increased in WT cells, an effect not observed in cells lacking TRAF1.

Interestingly, while the signaling downstream of 4-1BB appears to be similar after SAg-activation, anti-4-1BB was still able to enhance the survival of TRAF1−/− SEB-activated T-cells. However, when the means of the levels of enhanced survival were compared between TRAF1−/− and WT cultures, there appeared to be a survival defect with the TRAF1−/− cells. One possibility for the enhanced survival still seen in the absence of TRAF1 could be that these cells may have
compensated for the loss of TRAF1 through increased signaling via the NFκB or JNK/SAPK pathways which have also been shown to be important in survival and proliferation [153, 158].

8 Conclusion

While the exact etiology of KD remains unknown, there is uniform agreement for an infectious trigger. Furthermore, while the infections found in KD are varied, one common theme appears to connect them all – the presence of a SAg. In keeping with this view, the LCWE mouse model of disease, a disease model used to study KD, also appears to be SAg-driven. While SAgs lead to massive proliferation of T-cells, they also lead to subsequent deletion of the SAg-reactive T-cells. Signals through CD28 or 4-1BB at the time of SEB activation can prevent cell death after SEB stimulation. Furthermore, co-administration of anti-4-1BB and LCWE can exacerbate disease in the mouse model (Y. Moolani, submitted manuscript).

In this thesis, we have characterized costimulation-mediated rescue of T-cells from apoptosis after SAg activation. We have shown that costimulation through CD28 and 4-1BB leads to enhanced T-cell survival and that this effect is independent of one another which is not surprising as the two receptors exhibit some similarities in signaling such as induction of the transcription factor NFκB [162, 176]. Interestingly, the enhanced survival effect downstream of the receptors is not additive, likely due to the accumulation of regulatory cytokines. Furthermore, we determined that this costimulatory signal needs to be present early, not more than a day after the superantigenic stimulation of the T-cells. This was demonstrated by the fact that addition of anti-4-1BB only had an effect on T-cell survival if given within the first 24 hours of SEB activation, and addition of CTLA4-Ig had no effect on survival if given after 24 hours. As well, both increased proliferation and the upregulation of the survival molecule Bcl-xL appear to contribute to the presence of higher cell numbers in conditions with agonistic costimulatory antibodies, albeit at different times during the SAg response. While a difference in Bcl-xL expression was seen early, differences in cell proliferation were seen at a later time-point. Furthermore, 4-1BB stimulation in the presence of SEB leads to a predominantly Th1 response and the enhanced survival downstream of this receptor appears to depend, at least partially, on TRAF1, leading to the following disease model.
Figure 21. **Disease model.** An early optimal level of a costimulatory signal either through CD28 or 4-1BB leads to earlier expression of Bcl-xL and greater proliferation of SAg-activated T-cells as compared with cells that just receive SAg activation. Furthermore, stimulation through 4-1BB appears to induce a Th1 phenotype and the survival effect is partly dependent upon the presence of TRAF1 and its ability to upregulate phospho-ERK. These T-cells which are rescued from SAg-mediated apoptosis can then go on to accumulate at the coronary arteries and lead to persistent inflammation and coronary artery damage, similar to what is seen in patients with KD and in the LCWE mouse model of disease.
9 Future Directions

While this project looked at several aspects of the role of costimulation in SAg-mediated T-cell responses, several questions still remain to be answered. One of the most interesting results in this study was the lack of an additive response of dual costimulation by anti-CD28 and anti-4-1BB. Therefore, it would be interesting to look at whether the accumulation of regulatory cytokines play a role as was found by Bukczynski et. al.. This particular study also found that reducing the strength of the TCR signal led to synergistic effects with CD28 and 4-1BB costimulation [177]. Consequently, one possible future experiment could be to look at whether reducing the concentration of SEB or decreasing the doses of the agonistic antibodies will lead to additive effects as determined by cell numbers and accumulation of cytokines.

We also found that the costimulatory signal had to be given early, temporally close to the SAg activation. As a result, early treatment of cultures with CTLA4-Ig led to reduced T-cell survival at the end of the assay as this molecule blocked any endogenous B7-CD28 interaction. In order for these experiments to have disease relevance, the next step would be to repeat the CTLA4-Ig in the mouse model as this molecule is already being used as a therapy in autoimmune diseases and thus could serve as a potential therapy for patients with KD. It is important to note that one of the diagnostic criteria of KD is the presence of fever for at least 5 days. If the in vivo results indeed correspond to those that were found in vitro in that costimulation needs to be blocked within the first few days of SAg-activation, then blockade of costimulation may not be a feasible option for therapy.

An interesting finding of this study was that even for 4-1BB, a molecule that is expressed after T-cell activation, signaling needed to be initiated early in order to counteract pathways initiated downstream of SAg activation that promote apoptosis. We hypothesized that it could be those cells that express 4-1BB early that are the ones that go on to survive later on in the assay. One way of testing this hypothesis would be to activate splenocytes from mice expressing different cell markers such as Thy1.1 and Thy1.2 with SEB and sort the T-cells that express and do not express 4-1BB early. Then, combining cells with one marker that express 4-1BB early with cells of the other marker that do not express 4-1BB back into cultures with SEB and APCs and then examining the proportion of cells with either marker that have survived at the end of the
assay would give an indication of the role of early expression of 4-1BB. Furthermore, memory and naïve cells could also be sorted to determine the effect of superantigen and anti-4-1BB treatment on these two subsets.

Another important study would be to examine the role of Th1 and Th17 cells in vivo. As mentioned in the previous section, the relationship of Th1 and Th17 cells and their role in disease might be too complicated to fully explore in an in vitro system. As well, if the roles of these two subsets are temporally different in the model, this would be very hard to visualize in an in vitro setting. A good starting point would be to measure the levels and presence of the two cytokines associated with these cell types, IFN-γ and IL-17, at various timepoints in the course of disease formation in the periphery and at the heart. Furthermore, as the IL-6−/− mice are already available and we already know that these mice do not develop coronary artery damage, the presence of IL-17 or Th17 cells could be analyzed to determine whether this effect is due to the absence of this subset.

Lastly, further analysis of the signaling downstream of 4-1BB and the role of the TRAF proteins in the enhanced survival after 4-1BB stimulation of SAg-activated T-cells would be a good area to pursue. While phospho-ERK was shown to be upregulated, the next step would be to determine whether the pro-apoptotic molecule Bim is indeed downregulated. Furthermore, as there was still an enhanced survival effect after 4-1BB stimulation in the absence of TRAF1, it would be interesting to see whether these mice have any compensatory mechanisms that would account for this observation.
10 Literature Cited


