Dissecting The Role Of TNFα In Kawasaki Disease: Alteration Of Cell Fate By TNFα After Superantigen Activation

by

Aaron Wong

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Department of Immunology
University of Toronto

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Abstract

Kawasaki disease (KD) is an acute inflammatory disease characterized by persistent inflammation of the coronary arteries. KD is characterized by the release of cytokines such as tumor necrosis factor alpha (TNFα) and is thought to be initiated by a superantigen (SAg). The *Lactobacillus casei* cell wall extract model of KD demonstrates a critical requirement for TNFα and its receptor during pathogenesis, although the precise effect of TNFα is unknown. A persistent T cell infiltrate in the coronary artery disagrees with established fates of SAg activated cells, which undergo apoptosis. In this work, TNFα was found to promote the survival of SEB-reactive T cells. The results demonstrate that TNFα regulates B7.2 molecule expression on antigen presenting cells, and that TNFα indirectly promotes the survival of SEB-stimulated T cells by driving costimulation. These observations demonstrate how TNFα prevents T cell apoptosis and lend support to KD therapies which target TNFα and B7.
Acknowledgments

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<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
<tr>
<td>AP-1</td>
<td>Activator Protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>Bcl</td>
<td>B cell lymphoma</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>Ca</td>
<td>Calcium</td>
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<tr>
<td>CAD</td>
<td>Caspase activated DNase</td>
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<td>CAWS</td>
<td>Candida Albicans Water Soluble</td>
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<tr>
<td>Cbl-b</td>
<td>Casitas B-lineage Lymphoma</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentation</td>
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<tr>
<td>cDNA</td>
<td>Complmentary Deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>c-IAP</td>
<td>Cellular Inhibitor of Apoptosis</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte-associated molecule-4</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per minute</td>
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<tr>
<td>ERK</td>
<td>Extracellular regulated MAP-Kinase</td>
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<tr>
<td>EVG</td>
<td>Elastic Van Gieson</td>
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<tr>
<td>FADD</td>
<td>Fas-Associated protein with Death Domain</td>
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<tr>
<td>FoxP3</td>
<td>Forkhead box P3</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GITR</td>
<td>Glucocorticoid-induced TNFR-related protein</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IKK</td>
<td>IκB Kinase</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol trisphosphate</td>
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<tr>
<td>ITAM</td>
<td>Immunoreceptor tyrosine-based activation motif</td>
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<tr>
<td>ITPKC</td>
<td>Inositol 1,4,5-trisphosphate 3-kinase</td>
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<tr>
<td>IVIG</td>
<td>Intervenous immunoglobulin</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>KD</td>
<td>Kawasaki Disease</td>
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<tr>
<td>LCWE</td>
<td>Lactobacillus casei cell wall extract</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>Major histocompatibility complex II</td>
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MMP  Matrix metalloproteinase
MMTV  Mouse mammary tumor virus
NFAT  Nuclear factor of activated T-cells
NFKB  Nuclear factor kappa B
NOD  Non obese diabetic mice
PCC  Pigeon cytochrome C
PDK1  Pyruvate dehydrogenase
PIP2  Phosphatidylinositol 4,5 bisphosphate
PLAD  Pre-ligand-binding assembly domain
PLCγ  Phospholipase C gamma
RA  Rheumatoid arthritis
RIP  Receptor-interacting protein
SAg  Superantigen
SEA  Staphylococcal enterotoxin A
SEB  Staphylococcal enterotoxin B
SEM  Standard error of measurement
SLP-76  Lymphocyte cytosolic protein 2
SPE A  Streptococcal pyrogenic exotoxin A
T1DM  Type 1 Diabetes Mellitus
TCR  T cell receptor
Th1  T helper cell 1
Th17  T helper cell 17
Th2  T helper cell 2
TNFα  Tumor necrosis factor alpha
TNFR  TNFα receptor
TRADD  TNFα-associated DEATH domain protein
TRAF  TNFα receptor associated factor
Treg  T regulatory cell
TSST-1  Toxic shock syndrome toxin -1
Vb  Variable beta
VEGF  Vascular endothelial growth factor
ZAP-70  Zeta-chain-associated protein kinase 70
1 Introduction

1.1 Kawasaki Disease

1.1.1 Overview of Kawasaki Disease

Kawasaki Disease (KD) was first characterized by Dr. Tomasaku Kawasaki in 1967, as a puzzling inflammatory condition in children, which had lymphoid involvement and specific desquamation of the fingers and toes [1]. Also known as “mucocutaneous lymph node syndrome”, it has since been characterized as an acute, pediatric self-limiting, multi-systemic inflammatory condition, where children have the potential to develop life-threatening cardiac abnormalities, such as coronary artery aneurysms or ectasia, as well as ischemic heart disease and sudden death [2]. The persistent inflammation of the coronary arteries has been shown to lead to vessel wall breakdown and cardiac complications are seen in 20-25% of untreated children [3]. As a result, KD is the most common cause of multisystem vasculitis and acquired heart disease in the US and Japan [4]. Currently, the etiology of KD is unknown, although many epidemiological studies suggest that an infectious trigger may be involved [2]. Treatment of KD begins with careful diagnosis of KD by clinical criteria which include prolonged fever of ≥ 5 days, and 4 of 5 other principal features: cervical adenopathy, polymorphous skin rash, extremity changes, nonpurulent conjunctivitis, and oral mucosal changes [5]. After KD is diagnosed, children are treated within 10 days of the fever onset with high dose aspirin and intravenous immunoglobulin therapy [2]. Despite ongoing research in KD, many aspects of the pathogenesis remain unknown. The etiology of the disease is still unclear and mechanisms governing how autoimmunity arises in this condition remain elusive.

1.1.2 Clinical Kawasaki Disease

Kawasaki Disease occurs mostly in children from 6 months to 5 years of age, although it may occur in both younger and older individuals too [6]. The disease is broken down into three phases: the acute, sub-acute, and convalescent phases. The acute phase lasts 7 to 14 days and is characterized by fever, conjunctival injection, mouth and lip changes, swelling and erythema of the hands and feet, rash and cervical lymphadenopathy [7]. The subacute phase
starts after the fever resolves and lasts until the resolution of inflammation. During this phase, patients may have desquamation of the fingers and toes. The convalescent phase normally occurs between 6-8 weeks after the onset and during this phase clinical signs are absent and the erythrocyte sedimentation rate (ESR) returns to normal.

Diagnosis of KD remains difficult and based entirely on clinical features because there is no specific test or biomarker available for the disease. Aside from the clinical signs and symptoms of KD, there are also significant immunological laboratory findings. Leukocytosis is common during the acute phase, with approximately 50% of patients having white blood cell counts above 15,000/mm³ [2]. Classic markers of inflammation are also elevated, including acute phase reactants like ESR and C-reactive protein (CRP). During the subacute phase, thrombocytosis occurs with elevated platelet counts often reaching greater than 1,000,000/mm³. Although these laboratory findings are non-specific, they add additional information to help diagnose patients who are suspected, but do not fulfill, the complete criteria for KD [2].

After a diagnosis of KD is made, therapy begins with the aim to prevent cardiac disease by decreasing inflammation. In North America, the American Heart Association’s recommendations include a single dose of intravenous immunoglobulin (IVIG) given during the acute phase of KD. This has been found to significantly reduce the prevalence of coronary artery abnormalities if given during the acute phase, typically before the 10th day of fever [8-11]. The mechanism of action of IVIG in KD is unknown, but it appears to have a generalized anti-inflammatory effect. Potential mechanisms of IVIG action include: modulation of cytokine production, neutralization of bacterial or viral proteins, augmentation of suppressor T cell activity, or inhibition of antibody synthesis [2]. In addition to IVIG, high-dose aspirin is also used as an important anti-inflammatory. Although treatment with aspirin alone does not seem to lower the frequency of coronary abnormalities [8], when used in combination with IVIG, there seems to be a synergistic effect [2].
Treatment with aspirin and IVIG results in subsidence of inflammatory symptoms within 48 hours after IVIG infusion [12]. However, approximately 5-15% of patients will fail to respond to this treatment, and have persistent fever and elevated CRP levels. In these cases, a second course of IVIG therapy is often given and 70-80% of patients will typically respond to treatment. In cases of IVIG-refractory KD, corticosteroids and other therapies that target tumor necrosis factor alpha (TNFα) have also been used. Corticosteroids have been shown to reduce fever [13, 14] but the effects on coronary artery abnormalities are still unclear. TNFα-based therapies have also been effective in salvage therapy and include pentoxifylline, which specially inhibits TNFα messenger TNF transcription [15], and Infliximab, a humanized monoclonal antibody against TNFα which neutralizes it [16, 17].

1.1.3 Etiology

The etiology of KD is unknown although most investigators agree that it involves an infectious trigger. Epidemiological studies have shown that KD is endemic, with seasonal fluctuations [18, 19]. In North America, KD is more common during the winter and early spring months [20], and appears to occur in geographical outbreaks. Furthermore, many different viruses and bacteria have been isolated from KD patients and include: coronavirus [21], cytomegalovirus [22-25], Epstein-Barr virus [24, 26-28], parvovirus [29], and members of the streptococci [30, 31], staphylococci [30], and mycoplasma [32, 33] families of bacteria. Despite these findings, no single bacterium or virus has been consistently isolated from KD patients.

It has also become clear that genetics play a strong role in the susceptibility of children to KD. Asian children of Japanese, Korean or Chinese decent have the highest incidence, with the disease being most prevalent in Japan. A recent epidemiological study has reported an average annual incidence rate of 184.6 per 100000 Japanese children aged 0-4 years [34]. Other ethnic groups are affected as well, but Japanese children have incidence rates approximately tenfold higher than those of Caucasians [20, 34]. This increased incidence seems to be independent of geography as Japanese children living in a Western environment are still more prone to develop KD [20]. These data are further supported by studies which have looked
at the incidences of KD within families. The risk of occurrence in twins is 13% [35, 36], and within 1 year after onset of the first case in a family, the rate in a sibling is 2.1% (which is a relative risk of approximately ten-fold) [37, 38].

Although the precise etiology of KD remains unknown, clinical and epidemiological studies strongly suggest an infectious agent which causes disease in young susceptible children, who likely share a genetic predisposition to an inflammatory response to infection. The clinical signs of a self-limited, inflammatory disease, with fever, rash, enanthenm, conjunctival injection, and cervical adenopathy correspond nicely with an infectious etiology. The epidemiological features such as the seasonality, punctuated outbreaks, and geographical spread also align closely with this hypothesis.

1.1.4 Immunological Findings

Research on the immune system in KD patients has not been completely straightforward. There are numerous ethical and practical considerations that need to be made when studying a pediatric population. Over the last 40 years, studies on the immune system have come from the analysis of patient plasma, immunohistochemical analysis of damaged vessels on autopsy and, more recently, the use of molecular genetics. The findings can be categorized in terms of: cells types, cytokines, growth factors, and enzymes implicated in KD.

Although neutrophils make up the dominant inflammatory cell type in the peripheral blood of KD patients, at the coronary artery, they are not the predominant cell type [39]. Instead, histological studies have shown that the inflammatory infiltrate in KD tissues contains large mononuclear cells, T cells, macrophages, dendritic cells, plasma cells, and a limited number of neutrophils [39-42]. In the early stages of KD, an influx of neutrophils is seen, transitioning after 7-9 days to large mononuclear cells with a high proportion of T cells [40]. Although other cell types have been studied in KD including B cells [41, 43] and macrophages [44, 45], T cells appear to play a key role in disease as they are seen to co-localize and
accumulate with myeloid dendritic cells in coronary artery lesions [46] and also appear at the other sites of KD inflammation [47]. The functional state of T cells in KD has also been investigated. Studies demonstrated that mRNA expression levels of T-bet and GATA-3 (which play critical roles in Th1 and Th2 polarization, respectively), are significantly decreased in the blood mononuclear cells from KD patients [48]. Proportions of regulatory CD4+ CD25+ T cells were significantly lower in patients with acute-phase KD and mRNA expression levels of Foxp3, CTLA4, and GITH were also found to be decreased [49]. Supporting these results, current investigations have focused on the more recently discovered Th17 subset of helper cells [50] which are posited to be the reciprocal arm to regulatory T cells [51]. It has been shown that Th17 cell proportions and frequencies of CD4+ IL-17+ T cells are increased in patients with KD, and had a tendency to decrease after treatment of IVIG [52]. This group also confirmed that regulatory T cell frequencies were lower among KD patients. Taken together, these findings suggest that T cells play a role in disease pathogenesis. These observations are also in keeping with the view that KD is an inflammatory disease which results from a dyregulated immune response to an infectious agent.

Results from a genome wide association study (GWAS) have implicated particular genes involved in T cell activation in the pathogenesis of KD [53]. Different signaling molecules have since been reported to play important roles in predisposing individuals to KD [54]. For example, a single nucleotide polymorphism in inositol 1,4,5-triphosphate 3-kinase (ITPKC) was found to associate with disease susceptibility and coronary artery lesion formation [55]. ITPKC is an important kinase that regulates TCR signaling and T-cell activation by phosphorylating inositol 1,4,5-triphosphate (IP3) [56, 57]. During T cell activation, stimulation of the TCR leads to activation of phospholipase C-gamma (PLC-γ), which then acts to produce IP3 by cleavage of phosphatidylinositol bisphosphate (PIP2). IP3 binds to IP3 receptors present on the endoplasmic reticulum and calcium is released, leading to the activation of the phosphatase, calcineurin. Calcineurin activates the transcription factor nuclear factor of activated T cells (NFAT), which then drives specific gene transcription leading to cell proliferation and differentiation. ITPKC is thus a negative regulator of T cell activation because it downregulates the calcium/NFAT signaling [55]. Impairment in the activity of ITPKC, as in the KD associated polymorphism identified, would contribute to a hyperactive T cell phenotype.
Other T cell associated genes encoding costimulatory receptors were identified in studies of children with KD. In particular, members of the TNF-superfamily of costimulatory receptors such as CD40 ligand (CD40L) and programmed death-1 (PD-1) were associated with susceptibility and predisposition to poor coronary outcomes [58-60]. CD40L and PD-1 have been shown to be expressed on the surface of activated T cells stimulated with anti-CD3 antibodies [61, 62] and have both been demonstrated to regulate the activation of T cells [63, 64]. During the acute phase of KD, it has also been reported that CD40L expression is higher on CD4+ T cells and that expression correlated to coronary artery lesions and disease progression [65]. Platelet expressed CD40L has been found to trigger thrombotic events and inflammatory reactions on endothelial cells [66, 67]. These observations fit nicely with clinical observations as thrombocytosis is one key pathognomic feature of KD.

The characteristic cytokines which accompany KD symptoms match those of an overwhelming infection. Proinflammatory cytokines such as TNFα [68, 69], TNFβ [68], IFNγ [70, 71], IL-6 [68, 72], IL-8 [72], IL-1[73], and IL-17 [52, 74] have all been reported to increase during the acute phase of disease. This cytokine profile may be reflective of Th1 and Th17 responses, as TNFα, IFNγ, and TNFβ are produced by Th1 cells [75] and IL-1 and IL-6 promote the differentiation of IL-17-producing Th17 cells [76]. In particular, increases in TNFα [68] and also its shed receptor (p60) [77] were found in the sera of KD patients, and the extent of ligand and receptor levels positively correlated with the severity of coronary artery lesions. These findings are important because they support the use of biologics that interfere with TNFα as a therapy for the treatment of KD [2]. Interestingly, plasma levels of IL-4 and IL-10 were also found to be significantly higher in the acute phase, but their levels correlated inversely with each other [70]. Since this study, genetic approaches have shown that IL-10 polymorphism are associated with coronary artery lesions in acute KD [78], however, conflicting studies have also reported both positive and negative roles of IL-4 [79, 80].

Other enzymes and soluble factors such as MMP9 and MMP2 have also been reported to have roles in KD [81] and have previously been reported to be produced by T cells and macrophages [82, 83]. Coronary artery aneurysms had high levels of MMP-9 expression,
while MMP-2 expression was most prominent in the thickened neointima and in endothelial cells [84]. Although circulating levels of MMP-9 were reported to be increased in sera from patients with acute KD [85], systemic MMP-9 concentrations and enzymatic activity did not correlate with coronary outcomes [81]. Instead, it is thought that MMP-9 plays a localized role in aneurysm formation, by degrading elastin in an animal model of KD [86, 87].

1.2 Superantigens

1.2.1 Superantigens and SEB

Superantigens (SAg) are bacterial and viral proteins that are potent activators of T cells. In contrast to conventional antigens, which are first processed within antigen presenting cells, loaded into the peptide binding groove of MHC, and presented to T cells bearing an antigen specific TCR, SAg are able to circumvent these processes. Superantigens bind directly to MHC, outside the peptide groove and away from the TCR, enabling them to bypass MHC restriction and TCR specificity (Figure 1). This SAg binding mechanism allows for activation of a large proportion of T cells (up to 25% of all T cells) in comparison to conventional antigens (0.0001% of all T cells) [88].

There are two classifications of SAg: endogenous and exogenous. Endogenous SAg include the minor lymphocyte stimulating (mls) determinants, which are the result of chromosome integrated mouse mammary tumor virus (MMTV) [89, 90]. The existence of these determinants within the mouse genome results in their expression in the thymus and subsequent negative selection of lymphocytes which react with MMTV SAg. This results in resistance to exogenous infection by MMTV [88].
Figure 1: Superantigens bind to the variable beta chain of the T cell receptor

Activation of naive T cells with conventional antigens proceeds when peptide is presented on MHC class II molecules to T cells bearing a specific TCR. This process activates ~0.0001% of all T cells and requires antigen processing. In contrast to conventional antigens, superantigens bind to the TCR outside of the peptide groove; bypassing antigen processing and peptide specificity. This binding mechanism results in activation of large proportion of T cells (up to ~25% of all T cells).
Exogenous SAg are produced by many types of bacteria and viruses. The most well studied groups of SAg are those produced by *Staphylococcus* and *Streptococcus* bacteria. Crystallization studies of bacterial SAg have determined that SAg bind to specific variable beta (Vβ) chains of the TCR [91-93]. Importantly, they can also bind to multiple Vβ chains, inducing specific activation and proliferation of T cells which bear the target Vβ TCRs. This binding leads to a characteristic Vβ “signature”, where specific Vβ families are expanded. For example, Staphylococcal Enterotoxin B (SEB) is known to expand Vβ 3, 7, 8.1, 8.2, 8.3, and 17 in mice [94], while staphylococcal Enterotoxin A (SEA) expands Vβ 1, 5.3, 6.3, 6.4, 6.9, 7.4, 9.1, and 23 [95].

Teleologically, SAg are thought to function as virulence factor bacteria allowing them to evade the immune system. Expression of SAg has been shown to prevent the development of a normal immune response [96]. Antigen-specific T cells are noted to fail to proliferate in response to antigens that are presented normally by MHC class II because they are rendered “anergic” (see 1.2.2 Cell fates). In addition, SAg stimulation of T cells has also been shown to induce IL-2 production initially, but result in inhibition of IL-2 release thereafter [97]. Thus, SAg might act to assist microbes in evading the immune system by depleting IL-2, interfering with T cell help, and by rendering T cells anergic [88].

### 1.2.2 Cell Fate after SEB Stimulation

Similar to *in vitro* studies [94, 98-100], after mice are injected with SEB, Vβ8 reactive CD4 and CD8+ T cells undergo multiple rounds of proliferation [101]. Reactive Vβ8+ T cells are seen to undergo increased cell cycling and expand for about 4 days following injection. However, after this phase of rapid cell expansion, the reactive cells undergo apoptosis or are rendered “anergic” [102], and the number of SEB-reactive cells eventually reach lower levels than those found in untreated mice. The decrease is mainly in CD4+ Vβ8+ T cells and represents apoptotic processes [103], although the specific mechanism for inducing death is still unclear [101]. Many investigators have suggested that survival of SEB-stimulated cells is heavily dependent on the growth factor IL-2, but initial reports were controversial [104, 105].
addition, it has become clear that other molecules, such as Fas [106], TNF and its receptors [107], and the lymphoid environment (antigen presenting cells) [108] might also have potent roles in SEB-induced apoptosis.

Although large proportions of SEB-stimulated cells undergo apoptosis, another population of “anergic” Vβ8+ cells remain afterwards. They have been called anergic because they fail to proliferate after subsequent exposure to SEB in vitro, while their response to other SAg remains intact [103, 109, 110]. This lack of proliferation corresponds to a defect of the Vβ8+ T cells to secrete IL-2 in vitro upon restimulation with SEB. The cells have lower transcription of IL-2 and decreased amounts of steady state IL-2 mRNA [111]. Furthermore, SAg-induced anergy has been shown to be reversed if IL-2 is given in vivo [112]. This led to the idea that anergy results from a simple withdrawal of costimulatory cytokines from an IL-2 poor environment (due to high catabolism of IL-2 by T cells) [113]. However, later reports have demonstrated that SAg-induced anergy results mainly from cell autonomous defects [114], which likely include: impaired TCR signaling [115] and inhibited expression of transcription factors [116]. Some investigators were reluctant to acknowledge that anergic cells could truly exist, because of the response/anergy paradox: that multiple cell fates (death and anergy) can result from the same initial stimulation [101]. Instead, they proposed that unresponsiveness was due to the failure of some SAg-reactive T cells to be sufficiently activated in the first place [117], or that anergy was simply ‘slow deletion’ since Vβ8+ cells would eventually disappear upon repeated stimulation of SAg [118].

More recently, a few elegant studies have demonstrated that T cell anergy is regulated and maintained by persistent exposure to antigen. In the first study, Rocha and colleagues adoptively transferred transgenic CD8+ T cells specific for the H-Y antigen into nude male mice which were thymectomized. Much like exposure to SAg, the CD8+ T cells rapidly expanded then declined in numbers and did not proliferate upon restimulation in vitro with male spleen cells or anti-TCR antibodies. When these anergic cells were harvested and re-transferred into a second female nude recipient, the anergized cells regained their ability to proliferate. The authors concluded that antigen persistence was required to maintain the unresponsive state [119].
In addition, in another study, CD4+ TCR transgenic T cells were adoptively transferred into irradiated syngenic mice, then the mice were challenged with i.v. injections of the soluble peptide antigen. In this study, the T cells similarly expanded and a residual subset of cells were found to be unresponsive to antigen restimulation \textit{in vivo} and \textit{in vitro}. Although this anergy did reverse over the course of 7 weeks, if peptide was reinjected every week the anergy was maintained [120]. In support of both of these studies, a third report by Tanchot et al. used a double transgenic model to investigate anergy. The authors transferred CD4+ TCR transgenic T cells on a RAG2 \textsuperscript{−/−} background into a CD3ε \textsuperscript{−/−} mouse expressing the antigen pigeon cytochrome c (PCC), under an MHC class I promoter and an Ig enhancer. Since the CD4+ T cells underwent an enormous expansion (50-100 fold), a large residual population of anergic cells was observed (10-40 million) which could be followed for as long as 5 months. In keeping with previous reports, when these cells were adoptively transferred into CD3ε \textsuperscript{−/−} mice without PCC, the cells reversed their tolerance to PCC [121]. This confirmed that the anergic state is regulated by persistent exposure to antigen and can be maintained.

1.2.3 Superantigens in Kawasaki Disease

There is currently a debate over the nature of the immune response in KD, and whether it is driven by a SAg or conventional antigen. Multiple lines of evidence support a role for SAg in KD [30]. Early clinical symptoms of KD appear similar to other SAg-mediated diseases such as toxic shock syndrome (TSS), scarlet fever, and streptococcal toxic shock syndrome (STTS). These diseases are all characterized by massive T cell activation and also share the same profile of proinflammatory cytokines: IL-2, TNFα, IL-1, and IL-6 [122]. Investigators have also isolated SAg producing bacteria from children with acute KD [123-125]. In one study, 11 of 13 SAg positive cultures from KD patients contained TSST-1 secreting \textit{S. aureus} [39], which has led to many investigators focusing their efforts on TSST-1. Although a variety of bacterial and viral species have been isolated from KD patients, most share the capacity to produce SAg (for example: Coronavirus [21], Cytomegalovirus [22-25], Epstein-Barr virus [24, 26-28], Parvovirus [29], and Streptococci [30, 31], Staphylococci [30], and Mycoplasma [32, 33] family of bacteria). Furthermore, the populations of T cells of children with KD are also characteristic of SAg responses (see 1.2.1). Characteristic ‘V beta skewing’ of
Vβ2+ and Vβ8.1+ T cells was first reported in KD patients by Leung and colleagues [126]. Subsequent studies have confirmed the role of a SAg in skewing the T cell repertoire [126-132] and demonstrated infiltration of affected cardiac tissues by the polyclonal SAg-reactive TCR Vβ families [133]. Serological evidence also points to the presence of SAg in KD infections. Significant elevation of IgM antibodies against staphylococcal Enterotoxin A and B, TSST-1, and streptococcal exotoxin A (SPEA) were found in a group of 65 KD patients and also 120 control subjects [134]. Thus, investigators believe that KD may not result from a single pathogen, but rather a group of microbes which produce SAg, strongly activate the immune system, and cause disease in susceptible hosts [135].

1.3 Animal Models of Kawasaki Disease

Investigators have taken advantage of animal models to investigate the pathogenesis of KD. Numerous animal models currently exist, which include a rabbit [136, 137], canine [138], and swine model [139], as well as three different mouse models in which yeast extract from *candida albicans* [140, 141], peptidoglycan from group A *streptococcus* [142], or *lactobacillus casei* cell wall extract (LCWE) [143, 144] is injected to induce disease. All the animal models aim to model the characteristic arteritis of KD, although the swine and rabbit models fail to display specific inflammation of the coronary arteries.

In the *Candida*–induced model of KD, young mice are injected i.p. with the water soluble fraction of *Candida albicans* extract (CAWS) [140]. Inflammation can be seen within hours after the first injection and, after approximately 8 weeks, the mice develop proliferative and granulomatous inflammation of the heart with macrophages, lymphocytes, plasma cells and neutrophil infiltration that closely mimics KD [145]. Characteristic vessel damage also occurs with intimal thickening and destruction of the internal elastic lamina and media. Although this model is useful for studying KD, repeated daily injections of CAWS are required to sustain the inflammation, as well the arteritis is granulomatous and more widespread affecting the abdominal aorta as well.
Few studies have been published on the streptococcal-induced model of KD. In this model, sonicated peptidoglycan fragments from *Streptococcus pyogenes* are injected intravenously every week for 4 weeks [142]. Mice develop diffuse cellular infiltration in the vascular wall and display marked hyperplasia of the endothelial cells, necrosis of the coronary artery medial smooth muscle, and degradation of elastic fibers in the artery.

### 1.3.1 The *Lactobacillus casei* cell wall extract (LCWE) model of Kawasaki Disease

The first paper published on the LCWE-induced model was in 1985 by Lehman *et al.* [143]. After a single i.p injection of the extract into mice, the group reported a specific inflammation localized specifically to the coronary arteries. The arteritis displayed disruption of the arterial intima and media. Various inbred mice strains such as C57BL/6, A/J, Balb/c and C3H/FeJ were tested and found to be susceptible to the disease.

Further characterization of the LCWE-model demonstrated that it closely mimicked KD with respect to susceptibility of young mice, time course, histopathology, and response to IVIG therapy [146] (Figure 2). The disease can only be induced in young mice from 4 to 5 weeks of age. Proinflammatory cytokines induced with LCWE match those implicated in human KD: TNFα [147], IFNγ [148], IL-1 and IL-6 [149]. Localized inflammation of the coronary arteries can be seen at day 3 and persists until day 60 [146]. Maximum inflammation occurs at day 28 and elastin breakdown and aneurysm formation (detected by eosin von Gieson (EVG) staining) occurs at day 42. These disease kinetics approximate to those of human KD as aneurysms typically occur at weeks 4 to 8. Histopathologically, the inflammatory cell types match those found in human tissues; with specific staining of F4/80+ macrophages, activated MIDC-8+ myeloid DCs, plasmacytoid DCs, and specific colocalization of CD3+ T cells with myeloid DCs detected within coronary artery lesions [150]. Interestingly, however, the group did not detect B cells in the lesions and B cell null mice did not develop disease. These results
contrast from other histopathological findings that have demonstrated that B cells infiltrate the coronary artery of children with KD [41]. Lastly, LCWE-treated mice respond to IVIG treatment [146], which lends additional support to the use of IVIG as first line treatment in children with KD [2].

Figure 2: Overview of the LCWE model of Kawasaki Disease

*Lactobacillus casei* cell wall extract (LCWE) induces coronary arteritis in mice and mimics human KD with respect to its histopathology, time course, susceptibility in young-aged mice, and response to IVIG therapy. Disease is induced in young inbred mice (4 to 5 weeks of age) with 1mg of LCWE injected i.p. at day 0. Systemic immune activation results with the production of TNFα, IFNγ, IL-6, as well as activation of TCR Vβ 2, 4, and 6 family T cells.
1.3.2 Superantigens in the LCWE model

Our laboratory has discovered a novel SAg in LCWE [151], and has demonstrated that SAg activity correlates well to its ability to induce disease \textit{in vivo}. Stimulation with LCWE replicates many of the hallmarks of a SAg response: marked proliferation of naive T cells, non-classical MHC restriction with a hierarchy in the efficiency of different class II molecules to present SAg, a requirement for antigen presentation but not processing, and polyclonal expansion of T cells in a TCR Vβ chain-dependent fashion. It was found that TCR Vβ 2, 4, 6 and 14 specific T cells were activated.

1.3.3 Tumor Necrosis Factor alpha in the LCWE model

Stimulation with SAg is known to elicit the release of TNFα with biphasic kinetics. Activated T lymphocytes produce the ‘first wave’ of TNFα, which then drives the ‘second wave’ of TNFα production from monocytes and macrophages within 24 hours [152]. Since TNFα plays a large role in patients during the acute phase of KD [153], our lab has also characterized its role in the LCWE model [147]. TNFα was rapidly produced in the spleen 6 hours post-LCWE injection. Peak levels of TNFα mRNA were detected in murine hearts at days 3 and 28, correlating with the immune cell infiltrate on day 3 and maximal inflammation (day 28) of the heart observed by histological examination. Most importantly, TNFα activity was required for disease induction. Mice given etanercept (soluble TNF receptor fusion protein) to neutralize TNFα did not develop inflammation at any point. Additionally, TNF receptor I (TNFRI) KO mice were also completely resistant to disease. These findings are significant because they support the idea of using TNF-based pharmaceuticals as a therapy for the treatment of KD. However, further work should be done to investigate the precise mechanisms by which TNFα drives disease in this model.

Given the multitude of effects TNFα regulates, there were many potential mechanisms by which TNFα could mediate disease in the LCWE model. The absence of infiltrate in the coronary vessels of TNFRI KO mice, suggested that one function of TNFα might
be to affect local migratory signals. In agreement with this hypothesis, mRNA expression of MIP-1α, RANTES, ICAM-1, VCAM-1, and E-selectin was significantly lower in mice lacking TNFRI.

1.4 Tumor Necrosis Factor

1.4.1 Brief Overview

The history of TNFα begins from the observations of a German physician, P. Bruns, who noted the regression of tumours in humans after bacterial infection [154]. It was hypothesized then that bacterial extracts could thus be used to treat human cancers. Later work demonstrated that LPS purified from bacterial extracts was responsible for tumour regression [155], and O’Malley et al. eventually demonstrated that LPS did not act directly on tumours, but through the production of a serum factor; which they called tumour-necrotizing factor [156]. Later in 1984, the cDNA of TNFα was cloned and sequenced [157], which led to intense research of this pleiotropic cytokine over the next couple decades.

TNFα is a homotrimeric protein of 157 amino acids which is primarily produced by macrophages, but is also produced by natural killer cells, T cells, B cells, monocytes, and dendritic cells [158]. TNFα signals through two distinct cell surface receptors, TNFR1 (55kDa) and TNFR2 (75kDa) which are expressed on a variety of different tissues. TNFR1 has been shown to be widely expressed by all tissues tested so far [158] including: the brain [159, 160], heart [161], lungs [162], kidney [163], pancreas [164], immune cells [165], reproductive organs [166], and the gut [167]. This is in contrast to TNFR2, which is mainly expressed by immune cells and endothelial cells [158]. Ubiquitous expression of TNFRs has been suggested as the reason for the diversity of functions attributed to TNFα. Studies have suggested that TNFR1 mediates the majority of TNF’s inflammatory functions such as proliferation, differentiation, and regulation of apoptosis [168], although this has been debated as other groups suggest that the both receptors cooperate to transduce their signals [169].
The functions of TNFα include the regulation of apoptosis, survival, differentiation, and proliferation [158] (Figure 3). The diversity of roles stem from the different cell signaling pathway initiated. Binding of TNFα to its receptors leads to trimerization via their intracellular pre-ligand-binding assembly domain (PLAD) [170]. TNFR1 contains an intracellular death domain (DD) region which can associate with TNR receptor-associated death domain (TRADD) [171], a cytosolic adaptor protein that acts to recruit the downstream signaling adaptor molecule Fas-associated death domain (FADD) [172]. FADD is able to bind and activate caspase-8, which can then initiate apoptosis.

Both TNFR1 and TNFR2 recruit TNF receptor-associating factors (TRAFs) which serve as adaptors to drive dramatic changes in cell function [168]. One TRAF family member, TRAF2, which can bind both TNF receptors, leads to activation of JUN N-terminal kinase (JNK)/activator protein 1 (AP1), p38 mitogen-activated protein kinase (p38MAPK), and nuclear factor kappa B (NF-κB) signaling pathways [158] (Figure 3). Activation of these cellular pathways contribute to TNFα’s function as a stimulator of survival, differentiation, and proliferation.

TNFα represents a double edged sword; having both positive and negative effects. Stemming from its history, one positive effect was its ability to destroy tumor cells. Recent studies demonstrated successful treatment for locally advanced limb soft tissue sarcomas and other large tumours [173]. However, even with success, TNFα’s anticancer utility is limited by its vasculotoxic effects which include constitutional symptoms such as fever, chills, headache, and fatigue [174]. TNFα also has regulatory functions in the immune system. It has been shown to provide key signals for the morphogenesis and microarchitecture of secondary lymphoid organs. For example, mice deficient in TNFα fail to develop peyers patches [175] and TNFα/Lymphotoxin double knockouts lacked follicular dendritic cell networks within mucosal lymph nodes [176]. In addition, TNFα also contributes to the function of cytotoxic effector cells in the detection and destruction of virally infected cells [177, 178]. Owing to its production by almost all haematopoietic cells, TNF has also been implicated in the proliferation of lymphocytes [179, 180]. In addition, genetic deletion studies have also shown its importance in protection...
against microbial infections from *Listeria monocytogenes* and resistance to LPS-mediated septic shock [181]. However, despite these positive aspects of TNFα function, dysregulation of TNFα can lead to harmful effects such as autoimmunity (see 1.4.2), and other disease such as chronic heart failure [182, 183] and bone destruction [184].
Figure 3: TNFR1 and TNFR2 drive apoptosis, survival, differentiation, and proliferation through different cell signaling pathways. Adapted from [158].

TNFα activates its receptors, TNFR1 and TNFR2, leading to the recruitment of cytosolic adaptor proteins TNF receptor-associated death domain (TRADD) and TNF receptor-associating factor 2 (TRAF2). These adaptors link to multiple signaling pathways controlling different cell functions. Signaling from TRADD leads to the recruitment and activation of Fas-associated death domain (FADD), which can lead to the initiation of apoptosis via caspases 8 and 3. TNF signaling via TRAF 2 results in the activation of activator protein 1 (AP1), p38 MAP kinase, and NF-κB signaling pathways.
1.4.2 TNFα and cell survival

TNFα has a prominent role in the regulation of apoptosis (reviewed in [168]). Reports have shown both pro-apoptotic and anti-apoptotic functions for TNFα. Signaling by TNFR1 is thought to occur by the formation of two complexes [185]. When TNF binds TNFR1, the adaptor protein TNF receptor-associated death domain (TRADD), receptor interacting protein 1 (RIP1), and TNF receptor associated factor 2 (TRAF2) are recruited to form complex I. Complex I binds the I kappa B kinase (IKK) complex, and activates NFκB, resulting in the production of anti-apoptotic factors such as FLIP<sub>L</sub>. The second complex (complex II) is found in the cytoplasm and forms when TRADD and RIP1 associate with FADD and caspase-8 to drive apoptosis. In cases where complex I activates NFκB, FLIP<sub>L</sub> is able to bind complex II and inhibit caspase-8 to promote cell survival. In instances where the initial signal from complex I fails, cell death via complex II is thought to occur. In addition to this pathway, signals mediated through TRAF2 can also promote programmed cell death. TRAF2 can stimulate the MEKK pathway, resulting in the activation of JNK signaling. Induction of JNK is strongly linked with the induction of apoptosis, although the precise mechanism of action is unclear [186, 187].

TNFR1 also activates additional survival pathways through the activation of other NF-κB induced factors. For example, cellular inhibitor of apoptosis protein 2 (c-IAP2) is under the transcriptional control of NF-κB and can bind to TRAF2 to block caspase-8 activation and apoptosis [168]. A20 is another NF-κB-induced protein which can bind TRAF2 to block apoptosis [188]. Finally, another induced protein named gadd45b strongly inhibits apoptosis via downregulating the JNK cascade [189]. It was shown to bind directly to MKK7 to block its activation of JNK [190]. Thus, there are multiple pathways of survival and apoptosis which stem from TNFα signaling. However, how one pathway is chosen over another is not currently clear.
Figure 4: TNFα drives apoptotic and survival signaling pathways. Adapted from [158, 168, 185, 189].

Binding of TNF to TNFR1 is thought to result in two sequential signaling complexes which mediate apoptosis and survival. After TNF binds TNFR1, the adaptor protein TNF receptor-associated death domain (TRADD), receptor interacting protein 1 (RIP1), and TNF receptor associated factor 2 (TRAF2) are recruited to form complex I. Complex I activates the NF-κB signaling pathway via recruitment of the I kappa B kinase (IKK) complex which results in the production of NF-κB–induced factors such as FLIPL. The second signaling complex mediates apoptosis and is formed when TRADD and RIP1 associate with FADD and caspase-8 in the cytoplasm (complex II). When complex I activates NF-κB, FLIPL associates with complex II and inhibits caspase-8 to promote cell survival. In cases where complex I fails to activate NF-κB, cell death occurs. TNFα signaling can also result in survival with other NF-κB–induced factors such as A20 and cellular inhibitor of apoptosis protein 2 (c-IAP2). Both proteins can bind to TRAF2 to block caspase 8 activation. Another induced protein named gadd45b is also induced by NF-κB and inhibits apoptosis by downregulating the JNK signaling cascade.
1.4.3 TNFα during superantigen responses and autoimmunity

In KD, the immune response is thought to be initiated by a SAg-producing infectious agent, which activates T cells and drives their expansion and subsequent infiltration into the heart. However, the persistence of these T cells in the heart does not agree with the established fate of SAg-activated T cells, as the SAg-reactive cells typically undergo apoptosis or are rendered anergic (see 1.2.2). Thus, a paradox arises as it becomes unclear how autoimmunity can result from SAg responses.

A few studies have shown that the fate of T cells after SAg activation can be altered with concurrent stimulation. For example, it has been shown that dually reactive T cells from mice immunized with both SAg and conventional peptide-antigen are able to escape apoptosis [191]. Our lab has shown that this ‘rescue’ from apoptosis is mediated by enhanced costimulation [192] as murine splenocytes cultured with SEB and agonistic anti-CD28 antibodies displayed a reduction in annexin V+ staining cells within SEB-reactive Vβ8+ T cell population. In addition to this, interesting work by Vella et al [193, 194], demonstrated that LPS could also interfere with the induction of T cell death in response to the SAg SEA. In particular, it was found that TNFα was critical for this ‘rescue,’ as neutralizing TNFα antibodies prevented the survival of SEA-reactive T cells with LPS. In view of these results, it is demonstrated that T cell fate following SAg stimulation is mutable, and that the apoptosis of peripheral T cells is not yet completely understood.

Because TNFα is implicated as a pathogenic cytokine in KD (see 1.3.3), we sought to understand how TNFα might modulate T cell fate after SAg stimulation. In this work, we aim to demonstrate that TNFα is important in the regulation of costimulatory molecule expression during SEB responses, effectively controlling the apoptosis of SEB-responsive T cells.
1.5 T cell Activation and Costimulation

1.5.1 T cell receptor signaling with conventional antigens

During T cell activation with conventional peptides, naive T cells are stimulated by their T cell receptor (TCR), which interacts with peptide presented on MHC molecules by antigen presenting cells (APC). The T cell receptor is composed of the functional receptor complex (made up of α and β chains) and the additional CD3 signaling complex composed of four signaling chains (two ε, one δ, and one γ) (Figure 5). The ζ chains of CD3 are particularly important because they contain immunoreceptor tyrosine-based activation motifs (ITAM[s]) which are phosphorylated to recruit proteins containing src homology (SH) domains.

Figure 5: Adapted from Immunobiology 7th Ed. by Janeway C. (Figure 6.10) The T cell receptor complex is made up of antigen-recognition proteins and invariant signaling proteins.

The T cell receptor is made of α and β chains with variable regions (v) which allow for antigen recognition, and constant (c) regions. The CD3 complex (ε, δ, and ζ chains) also associates with the TCR and helps mediate cell signaling via their ITAMs.
Interaction between the MHC:peptide complex and the TCR specific for the peptide activates the T cell and induces multiple cell signaling cascades. After ligation of MHC and TCR, the CD4 co-receptor present on the surface of T cells binds to MHC class II. The intracellular domain of CD4 associates with a src-family kinase called Lck, which, now localized to the TCR, can phosphorylate the ITAMs present on CD3’s ζ chains (Figure 6). Phosphorylated ITAMs are able to recruit the syk-family kinase ZAP-70 via its SH2 domain, which then recruits and phosphorylates linker of activation in T cells (LAT) and another protein named SLP-76 (Figure 6). LAT serves as an adaptor protein to recruit Tec kinases, such as Itk, which is then activated by SLP-76, and initiates the phospholipase Cγ (PLC-γ) and guanine exchange factor (GEF) pathways.
Interaction between the MHC:peptide complex and TCR results in phosphorylation of the ζ-chain ITAMs by CD4-associated Lck. ZAP70 then binds these ITAM and is activated by Lck. ZAP70 phosphorylates LAT and SLP-76, which recruit and stimulate Tec kinases, which activate PLC-γ and SOS pathways. PLC-γ converts PIP2 to IP3 and DAG, which leads to NFAT, NF-κB, and RasGRP signaling. SOS activates a MAPK signaling cascade involving Ras, Raf, Mek, Erk, and Elk. NFAT, NF-κB, and AP-1 transcription factors transcribe specific genes involved in cell proliferation and differentiation.
The PLC-\(\gamma\) pathway involves the cleavage of phosphatidylinositol bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol trisphosphate (IP3) (Figure 6). Production of these second messengers results in the release of Ca\(^{2+}\) from the endoplasmic reticulum (via IP3 receptors) and activation of protein kinase C (PKC) and calcineurin which activate transcription factors NF-\(\kappa\)B and NFAT respectively. Translocation of NF-\(\kappa\)B and NFAT into the nucleus drives specific transcription of genes involved in T cell activation.

The tec kinases associated with LAT also activate mitogen-activated protein kinase (MAPK) cascades by activating the GEFs called SOS and Vav (Figure 6). Activation of SOS leads to the activation of Ras, a small G-protein, which activates the MAPK cascade [Raf (MAPKKK), MEK 1/2 (MAPKK), ERK 1/2 (MAPK)] which results in the phosphorylation and activation of transcription factor Elk, which translocates to the nucleus to transcribe genes involved in T cell activation. A similar pathway is initiated by Vav and will be expanded upon in 1.5.2 because it is associated with costimulatory CD28 signaling.

1.5.2 Costimulation with CD28, B7 ligands, CTLA4, and Cbl-b

During productive T cell responses, naive T cells must also receive a “second signal” in addition to peptide stimulation of the TCR. Ligation of the TCR alone does not induce proliferation and differentiation into effector T cells; instead, the T cells are rendered unresponsive or anergic. Thus, for proper T cell expansion and function, a second costimulatory signal, must be provided by the APC.

The most well studied costimulatory molecules are the glycoproteins B7.1 and B7.2. They are homodimeric members of the immunoglobulin superfamily that are found exclusively on the surfaces of cells that stimulate T cell proliferation. The differences between B7 ligands appear to be slight. Although B7.2 is constitutively expressed at lower levels on APC than B7.1, it is induced quickly after activation and is thought to mediate earlier events in T cell
activation. After exposure to an inflammatory stimulus (such as a foreign antigen), APC undergo a maturation process whereby they upregulate B7.1 and B7.2 on their cell surface. In this way, matured APC presenting a specific peptide also deliver a second costimulatory signal to antigen specific T cells. B7 molecules on the APC interact with the CD28 receptor found on T cells. Interaction between B7 and CD28 is required for T cell clonal expansion and has been shown to dramatically lower the threshold of T cell activation.

CD28 is a glycoprotein with a single ligand-binding domain expressed on almost all murine T cells, with CD4 T cells expressing higher levels [195]. Numerous studies have shown that CD28 promotes T cell proliferation, cytokine production (via gene transcription and mRNA stability), and cell survival [196]. Binding of B7 to CD28 results in the recruitment and activation of phosphoinositide 3-kinase (PI3K) [197], which catalyzes the conversion of PIP2 to phosphatidylinositol trisphosphate (PIP3) (Figure 7). Localized to the cell membrane, PIP3 serves as a docking site for 3-phosphoinositide-dependent protein kinase 1 (PDK1), which can then activate its target kinase called Akt. Multiple signaling events are initiated downstream of Akt’s kinase activity. Activation of Akt enhances nuclear translocation of NF-κB, which increases the expression of prosurvival genes such as Bcl-xL. In addition, Akt inhibits transcription factors that promote cell cycle arrest [198]. Akt accomplishes this by inactivating glycogen-synthase kinase 3 (GSK3), which promotes nuclear export of NFAT [199], allowing for optimal transcription of NFAT-genes such as IL-2.
Elements from TCR signaling are labelled in grey, unless they are in common with CD28 signaling. Binding of B7 ligands to CD28 results in the recruitment of PI3K which activates PDK-1, then Akt1. Akt1 is a kinase which phosphorylates many cytosolic proteins resulting in the increased NF-κB activity. One such protein is GSK3, which acts to increase NFAT activity by preventing its export. The activated CD28 receptor can also bind adaptor protein Grb2, which recruits SLP-76. Similar to TCR signaling, SLP-76 can also bind another GEF called Vav, which initiates another distinct MAPK cascade (Rac, Mekk, Jnkk, Jnk), resulting in enhanced AP-1 signaling. Costimulation is regulated by CTLA-4 and Cbl-b. CTLA-4 competes with CD28 for binding to B7 ligands. Cbl-b is an E3 ubiquitin ligase which negatively regulates PI3K and Vav.
In addition to recruiting PI3K, activated CD28 can also bind the adaptor protein growth factor receptor-bound protein 2 (Grb2), which is able to recruit SLP-76 to activate another GEF called Vav. Activation of Vav leads to the activation of Rac, a small g-protein, which activates the another MAPK cascade [Mekk (MAPKKK), Jnkk (MAPKK), Jnk (MAPK)] and results in the phosphorylation and activation of transcription factor Jun. Thus CD28 signaling can been seen to synergize with common elements from the TCR signaling pathway (NFAT, NF-κB, AP-1) (Figure 7).

Other costimulatory proteins act to taper T cell activation. Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) is another costimulatory receptor which opposes the activities of CD28 on T cells. CTLA-4 shares sequence homology with CD28 and is able to bind B7 ligands. CTLA-4 antagonizes CD28 signaling because, once induced on activated T cells, it binds B7 ligands with 20-fold greater avidity; effectively preventing CD28 signaling by competitive binding (Figure 7). In addition to competing with CD28 for B7, other studies have suggested that CTLA-4 might interact with Lck, Fyn and ZAP-70 through SHP1 phosphatase to prevent constitutive expression of downstream signaling pathways [200]. Mice lacking CTLA4 develop a strong lymphoproliferative phenotype and die prematurely [201]. Inhibition of T cell activation by CLTA-4 has been suggested as a viable therapy to treat hyperactive immune disorders [202, 203]. After its cloning in 1987 [204], a recombinant protein of CTLA-4 fused to a human heavy Ig chain was developed [203], and was found to greatly suppress T cell responses. In 2005, Abatacept (humanized CTLA4-Ig fusion protein) was approved for the treatment for severe rheumatoid arthritis that cannot be controlled by traditional anti-rheumatic drugs such as methotrexate.

Another mechanism by which T cell activation can be suppressed is by the activity of the regulatory protein Cbl-b (Figure 7). Named after Casitas B-lineage Lymphoma, Cbl-b is an E3 ubiquitin-protein ligase which acts seems to regulate costimulatory signaling in T cells [205]. By ubiquitinating target proteins such as PI3K and Vav, Cbl-b negatively regulates T cell activation. Notably, Cbl-b deficiency results in spontaneous autoimmunity as mice age [206].
1.5.3 T cell activation and costimulation with superantigens

Activation with SAg has been assumed to be similar to TCR signaling with conventional antigens [207]. Many studies have shown that particular features of conventional T cell activation remain intact after stimulation with SAg. These features include similarities in NFAT activity and calcium mobilization [208], formation of mature immunological synapses [209], as well as expression of typical activation markers CD25 and CD69 [208]. Despite these similarities, SAg stimulation seems to override the requirement for CD4 and Lck signaling. It is known that CD4 binds MHC class II to stabilize interactions with the TCR and to bring its associated Lck in proximity to the ζ chains. However, it has been shown that responses to some SAg (SEB and SEC) are not affected by blockade of CD4 with monoclonal antibodies [210], and T cell lines deficient in CD4 respond similarly to SAg [211]. Interestingly, Lck seems to be dispensable for T cell activation with SAg [208], indicating the possibility of additional signaling pathways emanating from the TCR used by bacterial toxins.

A recent study has elucidated an Lck-independent pathway of T cell activation with SAg that converges with conventional signaling at the level of ERK1/2 activation [212]. In the canonical pathway, PLC-γ is responsible for initiating the MAPK cascade leading to ERK1/2 activation. Yet, after stimulation with SAg, PLC-γ was not activated in the absence of Lck. Thus, the investigators hypothesized that another PLC was involved to allow for ERK signaling. Their study suggests that SAg bypass Lck-dependent TCR signaling by activating PLC-β via TCR-mediated activation of a Ga11 (a G protein). The activity of PLC-β leads to IP3 and DAG production, which result in activation of the MAPK cascade involving Ras, Raf, Mekk, and Erk. Signaling differences between conventional antigens and SAg are interesting because they may begin to explain how SAg-stimulation leads to apoptosis and anergy of T cells.
1.5.4 Costimulation and anti-apoptotic proteins

Signaling from CD28 results in enhanced expression of prosurvival genes such as Bcl-XL [213]. Bcl-XL is a member of the Bcl-2 family of proteins which include both pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl-XL and Bcl-2) members [214]. These proteins are integral membrane proteins located in the outer membrane of the mitochondrion [215], which regulate apoptosis by controlling the integrity of the membrane. Anti-apoptotic molecules prevent the influx of ions through the mitochondrial membrane, thereby inhibiting the release of cytochrome c, which triggers apoptosis if released into the cytosol. In contrast, pro-apoptotic molecules such as Bax have opposite functions. Elevated levels of Bax promote the influx of ions into the mitochondria and the release of cytochrome c while Bad initiates apoptosis by inhibiting the activity of Bcl-2/Bcl-XL in the membrane by binding them. Costimulation provided by CD28 also results in the activation of Akt kinase, which can also prevent the activities of pro-apoptotic molecules. Akt phosphorylates Bad, which in phosphorylated form, cannot bind to Bcl-2/Bcl-XL in the membrane and promote apoptosis. Thus, costimulation promotes the survival of T cells by regulating the expression of Bcl-2 family proteins.

In addition to regulation of apoptotic molecules, CD28 signaling has also been shown to induce high levels of IL-2 transcription [216] and stability [217]. Apoptosis is known to be prevented by the addition of IL-2 [218, 219] and thought to play a major role in the survival of activated T cells [220]. Therefore, costimulation promotes the viability of activated T cells through multiple pathways, including increased production of IL-2 and enhancement of Bcl-XL expression. Interestingly, these two mechanisms operate independently of each other [221], as it was shown that CD28 mediates intrinsic survival via Bcl-XL and that IL-2 provides an extrinsic survival signal to activated T cells [213].
2 RATIONALE

Kawasaki disease (KD) is an acute, multi-systemic inflammatory disease characterized by persistent inflammation in the coronary arteries. Acute KD is characterized by the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and is thought to be initiated by a superantigen (SAg). *Lactobacillus casei* cell wall extract (LCWE) induces coronary arteritis in mice and closely models human KD. Studies using this model have shown that TNFα and its receptor are critically required for pathogenesis- although the precise action of TNFα is/are unknown. A persistent T cell infiltrate in the coronary artery is not in keeping with established cell fates for SAg stimulated cells, which typically undergo apoptosis or are rendered anergic. TNF receptors are ubiquitously expressed in mouse tissues and are known to regulate the apoptosis of T cells. Costimulation with CD28 and B7 ligands induce signaling pathways which also prevent the apoptosis of activated T cells.

3 HYPOTHESIS

TNFα plays a key role in the survival of SAg-activated T cells.
4 EXPERIMENTAL AIMS

To evaluate the role of TNFα in KD, we aimed to investigate the direct and indirect effects of TNFα on the apoptosis of SAg-activated T cells in vitro. Previous work had shown that enhanced costimulation could rescue SAg-reactive T cells from apoptosis [192]; thus we developed 4 specific aims:

1. Determine whether TNFα contributes to the survival of SAg-reactive T cells indirectly by regulating costimulation
2. Determine whether TNFα regulates activated T cell survival directly via its own TNF receptors
3. Identify the precise mechanism(s) and cellular players that regulate TNFα-driven survival of SAg-reactive T cells
4. Characterize TNFα’s effects on T cell activity and the expression of survival molecules.
5 MATERIALS AND METHODS

5.1 Experimental Mice

Wild-type C57BL/6, TNFR1−/−R2−/−, and CD86−/− were purchased from The Jackson Laboratory (Bay Harbor, ME) and housed under specific pathogen-free conditions at the Center for Phenogenomics and the Hospital for Sick Children in Toronto under institutional guidelines. Cbl-b−/− spleens were a generous gift from Dr. Pamela Ohashi (University of Toronto). All animal procedures were approved by the animal care committee at The Hospital for Sick Children Research Institute.

5.2 Cell Culture

Splenocytes from mice between 6-8 weeks of age were homogenized and treated with Gey’s solution to lyse red blood cells. Cells were cultured in 24-, 12-, and 6-well plates at 2×10⁶ cells/ml in Iscoves minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, non-essential amino acids, 50μM 2-ME, 2mM L-glutamine, and 10mM HEPES. Cells were cultured with various combinations of stimulatory and inhibitory agents including: the prototypical superantigen Staphylococcal Enterotoxin B (SEB) (Toxin Technology (Specific doses are labelled figures), CD28 stimulatory antibody (37.51, Biolegend) (0.5 μg/ml), CTLA4-Ig (Bristol-Myers Squibb) (50 μg/ml), TNFα neutralizing antibody (MP6-XT22, eBioscience and BD Biosciences) (0.1 μg/ml), and recombinant mouse TNFα (eBioscience) (see doses in figure). During incubations, cells were placed in a humidified chamber containing 5% CO₂ at 37°C.

5.3 Purification and Culture of T cells and Antigen Presenting Cells

T cells and antigen presenting cells (APC) were purified by magnetic bead separation using the pan T isolation kit from Miltenyi Biotec as per the manufacturer’s instructions. Specifically, the kit contains biotin-conjugated antibodies against CD11b, CD11c,
CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC-class II, and Ter-119 and the isolation of T cells is achieved by depletion of magnetically labeled cells. Isolation with this kit does not disturb or activate T cells. Purity of the T cells and APC was assessed by flow cytometry using fluorescent anti-CD3 antibodies or isotype control staining of isolated cells. In all experiments, the purity of T cells was >90%. In mixing experiments involving co-culture of APC and T cells, the APC fraction was further enriched using a second immunomagnetic column. The purity of APC after this enrichment was >90%. For these mixing experiments, purified T cells were added to APC at a ratio of 1:4 and were cultured at a total concentration of 2x10^6 cells/ml in 24-well plates.

5.4 Antibodies and Flow Cytometry

Cultured single cell suspensions were stained with fluorochrome-conjugated antibodies and secondary reagents, and the immunofluorescence analyzed on a BD Bioscience flow cytometers (initial experiments on BD FACScan and BD FACSCalibur, later experiments on BD LSR II and BD Canto II) using standard labelling techniques. Briefly, 1x10^6 cells were aliquoted into 4 ml round bottom tubes (Falcon 2052) and were washed with 1ml of staining media (SM) (2% FBS, 25mM EDTA in PBS, pH 7.2). Cells were stained in 100ul of SM containing optimal concentrations of fluorescent antibodies for 20-30 minutes at 4°C. Flow cytometry analysis was performed on live cells by staining with 7AAD, propidium iodide, or fixable viability dye eFluor® 450 (eBioscience). Gating of populations were determined using isotype controls and fluorescence minus one (FMO) controls. Cells were stained extracellularly with CD19-PE (1D3), CD11c-PE (HL3), F4/80-APC (BM8), CD80-FITC (16-10A1), CD86-FITC and –PECy7 (GL-1), CD3-FITC (145-2C11), and V-beta 8 TCR-FITC (F23.1) antibodies. Cells were stained intracellularly with TNF-APC (MP6-XT22), Bcl-2-PE (3F11), Bcl-XL (54H6) antibodies. Biotinylated goat anti-rabbit antibodies were used as secondary antibodies for Bcl-XL staining and were detected by strepavidin conjugates. Purified antibodies were purchased from BD Biosciences (San Diego, CA), eBiosciences (San Diego, CA), Cedarlane (Hornby, ON, Canada), and Cell Signaling Technology (Danvers, MA). Apoptosis was measured with the Annexin V-PE Apoptosis Detection kit (BD biosciences) following the manufacturer’s protocol. Flow cytometry data was analyzed using FlowJo (Tree Star, Inc., Ashland, OR).
5.5 Measurement of Proliferation

5.5.1 $^3$H-Thymidine Incorporation

Murine splenocytes were cultured in 96-well plates at $5 \times 10^5$ cells/200ul of complete media containing different stimuli. A minimum of 3 replicate wells were plated and cultured for various durations (see figure legends). Before scintillation counting, cells were and pulsed with 1µCi/well of $[^3]$H-thymidine (GE Healthcare Bio-Sciences Inc, Baie d’Urfe, QC) for the last 18-24 hours of incubation. Quantification was performed on a Beckman Coulter LS6500 scintillation counter. Disintegrations per minute (dpm) measurements from quadruplicate wells were averaged and a stimulation index was calculated by dividing dpm counts from stimulated wells by unstimulated controls.

5.5.2 BrdU Incorporation

For measurement of specific SAg-reactive T cell proliferation a BrdU incorporation assay (BrdU-FITC Kit, BD Biosciences) was performed. Splenocytes were cultured in 12-well plates at $2 \times 10^6$ cells/ml with complete media containing various stimuli. BrdU was added during the last 18-24 hours of incubation, and the cells were stained for T cell markers and BrdU as per the manufacturer’s instructions.

5.6 Statistical Analysis and Graphics

Statistical significance was determined by either unpaired or paired two-tailed student’s t-tests where appropriate using GraphPad Prism 4 (GraphPad Software Inc, La Jolla, CA). A p-value of $P<0.05$ was considered significant. In certain experiments, values were reported as the mean ± standard error of measurement.
6 RESULTS

6.1 SEB upregulates B7.2 ligand on B cells but not B7.1

Superantigenic stimulation of T cells results in their activation and expansion, followed by their deletion. Since SAgs can bind to MHC class II directly and do not require the help of professional antigen presenting cells (APC), SAgs are thought to activate T cells without costimulation [193]. Previous studies have investigated the role of costimulatory molecules CD28 and B7 during SAg responses, but they have mainly focused on the initial activation and proliferation of T cells [222, 223]. Furthermore, the role of endogenous B7 ligands affecting survival in vitro and in vivo is still controversial [193, 194, 224-226].

To demonstrate whether B7 molecules have an endogenous role, we first characterized the costimulatory ligands B7.1 and B7.2 in response to the SAg SEB. Splenocytes from wildtype mice were cultured with SEB for 2-4 days and the expression of B7.1 and B7.2 was determined by flow cytometry. The expression of B7.2 was markedly upregulated after stimulation with SEB at days 2 and 3, then returned back to control levels at day 4 (Figure 8A). No upregulation of B7.1 was seen between days 2 – 4. On day 2, the mean increase in MFI after SEB stimulation was plotted for B7.1 (0.366 ± 2.118, p=0.8785) and B7.2 (286.5 ± 39.03, p=0.0101) (Figure 8C, left and middle). These findings support other data which demonstrate a larger role for B7.2 ligand than B7.1 in response to SEB [226]. Further characterization of the cells expressing B7.2 was performed. After 48hr culture with SEB, B cells expressed high levels of B7.2 (Figure 8B), while T cells also upregulated B7.2 but at lower levels. The mean increase in MFI after SEB stimulation was plotted for B cells (3489.0 ± 915.5, p=0.0189) (Figure 8C, right).
Figure 8: Stimulation with SEB increases B7.2 expression on B cells

(A) Splenocytes were cultured in media in the presence or absence of 0.3μg/ml SEB for 2-4 days. Staining was performed with antibodies specific for B7.1 and B7.2. Samples were analyzed by flow cytometry using a gate for viable cells. (B) Identical experiment as (A), except cells were analyzed on day 2 only and stained with antibodies to CD19 (B cells) and CD3 (T cells). Isotype control staining was performed on SEB stimulated cells and is shown in grey. Each histogram presented in this figure are representative of a minimum of n=3 independent experiments. (C) Changes in MFI on day 2 were plotted for the data shown in (A) (C left and middle) and (B) (C right). Statistical significance was determined by the student’s paired t-test.
6.2 TNFα and its receptors regulate B7.2 expression on B cells

Since superantigens can stimulate the production of TNFα [107], we sought to determine whether TNFα might contribute to the expression of costimulatory molecules directly. Splenocytes were cultured in the presence or absence of recombinant TNFα and the expression of B7.1 and B7.2 was determined by flow cytometry. Analysis was performed at day 2, because previous experiments with SEB demonstrated sustained upregulation of B7.2 on days 2 and 3 (Figure 8). Stimulation with recombinant TNFα resulted in increased levels of B7.2 (Figure 9A) but not B7.1 (data not shown). Characterization of the immune populations with specific staining for CD19 and CD3, revealed that both B cells and T cells were able to upregulate B7.2 expression after TNFα stimulation (Figure 9A). Changes in MFI after stimulation with TNFα were plotted for B cells (5055 ± 450.3, p<0.0001) and T cells (2318 ± 129.7, p<0.0001) (Figure 9B). This upregulation was also found to be dose dependent, as increasing concentrations of recombinant TNFα (2ng/ml, 12ng/ml, 62ng/ml) induced higher expression of B7.2 (Figure 9C). This effect was also mostly absent in TNFR1−/−R2−/− cultures although slight upregulation could be seen.

Although TNFα was found to directly upregulate B7.2 expression on B cells, it remained unclear whether SEB directly upregulated B7.2, or whether SEB indirectly upregulated B7.2 through production of TNFα. To address this question, splenocytes from wildtype or TNFR1−/−R2−/− mice were stimulated with SEB and the expression of B7.2 was determined. Wildtype B cells expressed high levels of B7.2, while TNFR1−/−R2−/− cells did not display any upregulation (Figure 10A). In addition, TNFR1−/−R2−/− cells had lower expression of B7.2 than wildtype cells (Figure 10B) after stimulation with SEB. These findings support the idea that endogenously produced TNFα regulates costimulatory molecules on B cells.
Figure 9: TNFα upregulates B7.2 expression on B cells in a dose dependent manner

(A) Splenocytes from wildtype mice were stimulated in the presence or absence of recombinant TNFα (62ng/ml). Specific staining for B7.1 and B7.2 was performed on day 2 and analyzed by flow cytometry with a gate on viable cells. (B) Changes in MFI were plotted for the data shown in (A). Statistical significance was determined by the student’s paired t-test. (C) Different doses of recombinant TNFα were added to wildtype and TNFR1⁻/⁻R2⁻/⁻ splenocyte cultures, and the expression of B7.2 on B cells was determined on day 2. Fluorescence minus one control staining was performed on SEB stimulated cells and is shown in grey. Each presented histogram in this figure is representative of a minimum of n=3 independent experiments.
Figure 10: TNF receptors on B cells regulate the expression of B7.2 during SEB stimulation

(A) Wildtype and TNFR1−/−R2−/− splenocytes were cultured in the presence or absence of SEB (0.03μg/ml) for 2 days. Staining was performed with antibodies specific to CD19 and B7.2 and the samples were analyzed by flow cytometry using a gate for viable cells. (B) The data in (A) were replotted to show the relative expression of B7.2 between wildtype and TNFR1−/−R2−/− in media or SEB-stimulated cultures. Fluorescence minus one control staining was performed on SEB stimulated cells and is shown in grey. Each histogram is representative of n=2 independent experiments.
6.3 Enhanced costimulation rescues SEB-reactive cells from apoptosis

Initial reports demonstrated that B7 ligands are critical for the activation and proliferation of responsive T cells, however, whether costimulation plays a role in cell survival has been somewhat controversial. Some reports have shown that disrupting B7 ligands with CTLA4-Ig antibodies prevents optimal activation and expansion of cells [194, 226] while other studies using CTLA4-Ig transgenic mouse failed to find such differences [225]. Furthermore, because it has been reported that only the proliferating SEB-reactive cells undergo death [102], it remains unclear whether survival, or the lack thereof, was due to a lack of costimulation. It may be the case that SEB-reactive cells were not effectively expanded in the first place.

To determine whether costimulation affects the survival of SEB-reactive T cells, SEB-stimulated splenocytes were cultured with or without agonistic anti-CD28 antibody. After the initial proliferative phase (days 1-3), the apoptosis of Vβ8+ T cells was detected with Annexin V and 7AAD staining at day 5. The addition of anti-CD28 decreased the total frequency of apoptotic Vβ8+ T cells from 27.1% to 19.7% in the experiment shown (Figure 11A). In addition, enhanced costimulation also decreased the percentage of apoptotic Vβ8+ T cells relative to the total Vβ8+ T cells from 72.6% to 65.6% (Figure 11B). These findings were significant (p = 0.03) and reproducible (data are shown from 4 independent experiments).
Figure 11: Enhanced costimulation rescues SEB-stimulated T cells from apoptosis

(A) Wildtype splenocytes were cultured with SEB (0.3μg/ml) in the presence or absence of costimulatory anti-CD28 antibody (0.5μg/ml) for 5 days. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain. (B) The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=4 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
To further test whether costimulation regulates the survival of T cells after SAg stimulation, additional costimulatory blockade experiments were performed using CTLA4-Ig. When CTLA4-Ig was added to SEB-stimulated splenocytes, increased apoptosis was observed in Vβ8+ T cell population (Figure 12A). In the particular experiment shown, the addition of CTLA4-Ig increased the total frequency of apoptotic Vβ8+ T cells from 17.3% to 23.7%. In addition, costimulatory blockade also increased the percentage of apoptotic Vβ8+ T cells relative to the total Vβ8+ T cells (Figure 12B). These findings were significant (p = 0.03) and reproducible (data are shown from 4 independent experiments).

Finally, experiments were also performed using Cbl-b −/− mice. These mice lack the E3 ubiquitin ligase Cbl-b, which is a negative regulator of Vav, a critical guanine-nucleotide exchange factor involved in CD28 signaling. Thus, T cells deficient in Cbl-b have enhanced costimulatory signaling [227]. Less apoptosis was seen in Cbl-b −/− Vβ8+ T cells than wildtype (Figure 13A) and adding anti-CD28 to cells lacking Cbl-b −/− could not further increase the survival of Vβ8+ T cells (data not shown). Although Vβ8+ T cells from Cbl-b −/− mice displayed a trend towards enhanced survival during 5 independent experiments (Figure 13B), statistical significance was not achieved.
Figure 12: Blocking costimulation with CTLA4-Ig increases apoptosis of Vβ8+ T cells

(A) Wildtype splenocytes were cultured with SEB (0.3μg/ml) in the presence or absence of CTLA4-Ig (50μg/ml) for 5 days. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain. (B) The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=3 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
Figure 13: Deletion of Cbl-b enhances cell survival

(A) Wildtype and Cbl-b^{-/-} splenocytes were cultured with SEB (0.3μg/ml) for 5 days. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain.

(B) The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=5 independent experiments are shown. Statistical significance was determined by the student’s unpaired t-test.
6.4 TNFα enhances cell survival in a dose dependent manner

Although TNFα has been reported to prevent the death of SAg-reactive cells in vivo [193] it still remains unclear how this occurs. Moreover, since TNFα signaling has a range of functions which include cytotoxicity, proliferation, and activation of nuclear factor kB, and act through two different TNF receptors (TNFR) expressed on the majority of cell types [228], it is unclear if TNFα acts on T cells directly, or indirectly via other cell types expressing TNFR.

To determine if TNFα plays a role in the survival of T cells in general, we performed an experiment to see whether adding TNFα could enhance the viability of T cells in the absence of SEB. Splenocytes were cultured in media in the presence or absence of recombinant mouse TNFα. The survival of T cells was measured by staining with a viability dye (sytox blue) and specific antibodies to CD3. The addition of TNFα increased the frequency of viable cells (Sytox blue negative) from 11.8% to 27.2% as well as increased the frequency of live T cells from 41.6% to 49.6% in the experiment shown (Figure 14A). Changes in the percentage of total viable T cells were also plotted for n=3 independent experiments and was found to be statistically significant (p = 0.0002) (Figure 14B). This effect was also found to be dose dependent as increasing concentrations of TNFα led to increased T cell viability (Figure 14C). Since SEB can also directly induce the production of TNFα by itself [107], we performed another experiment to determine whether neutralization of endogenously produced TNFα would affect Vβ8+ T cell death. The addition of neutralizing anti-TNFα antibody to cultures stimulated with SEB, increased the frequency of apoptotic Vβ8+ T cells from 58.9% to 67.5% in the experiment shown (Figure 15A). Changes in the percentage of apoptotic Vβ8+ T cells among total Vβ8+ cells was plotted for n=4 independent experiments and was found to be statistically significant (p = 0.0006) (Figure 15B).
Figure 14: TNFα enhances survival of T cells in a dose dependent manner

(A) Wildtype and Cbl-b -/- splenocytes were cultured with SEB (0.3μg/ml) for 5 days. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain.

(B) The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=5 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
Figure 15: Neutralization of endogenous TNFα decreases survival of SEB-reactive T cells

(A) Wildtype splenocytes were cultured with SEB (0.3μg/ml) in the presence or absence of neutralizing anti-TNFα antibody for 5 days. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain. (B) The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=4 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
6.5 TNFα receptors on APC regulate superantigen-induced death of T cells

The previous results (see 6.4) support the idea that TNFα may have a pro-survival role in SAg responses, but did not clearly demonstrate the mechanism of TNFα action. TNFRs are expressed by both APC and T cells, thus it was unclear whether TNFα acts on T cells directly, or indirectly via TNFRs on APC. To dissect the specific contributions of TNFRs, we purified T cells and APC from wildtype and TNFR1^−/−R2^−/− mice, then mixed them together in different combinations and stimulated them with SEB. In these mixing experiments, when TNFR-deficient T cells were paired with wildtype APC, we observed no difference in the levels of apoptosis of Vβ8+ T cells (mean Δ = -2.22 ± 1.172%, p=0.1539) (Figure 16, right). However, when TNFR-deficient APC were paired with wildtype T cells, we observed significant increases in the apoptosis of SAg-reactive T cells compared to wildtype APC (mean Δ% = 9.77 ± 1.234%, p=0.0002) (Figure 16, left). These findings suggest that during SAg responses, TNFα acts indirectly through the APC to affect cell survival.
Figure 16: TNFR on APC regulate survival of SEB-reactive T cells

T cells and APC from wildtype and TNFR1-/-R2-/- mice were purified then recombined and cultured with SEB (0.3μg/ml) at a ratio of 1:4. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain. The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from a minimum of n=4 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
6.6 TNFα promotes survival of T cells via TNFR on APC

It remained unclear whether TNFα induced the survival of T cells directly via TNFRs on T cells or indirectly via TNFRs present on APC. To dissect TNFα’s specific contributions to T cell survival, splenic T cells and APC from wildtype and TNFR1−/−R2−/− mice were purified, then recombined and stimulated with TNFα. After gating on CD3+ cells (Figure 17A), the percentage of dead T cells was quantified by staining with sytox blue (Figure 17B). In these mixing experiments, when TNFR-deficient T cells were paired with wildtype APC, the addition of TNFα decreased T cell death (mean $\Delta = 6.438 \pm 1.797\%$, p=0.009) (Figure 17B, top row). In contrast, when TNFR-deficient APC were paired with wildtype T cells, the addition of TNFα did not impact T cell death (mean $\Delta = -1.313 \pm 0.9255\%$, p=0.1991) (Figure 17B, bottom row). Although an increase in T cell death displayed in the experiment shown (62.6% to 67%), this difference was not statistically significant when the changes in the percentage of dead T cells were plotted for n=8 independent experiments (Figure 17C). These findings suggest that TNFα can act indirectly through the TNFR on APC to affect promote T cell survival.
Figure 17: TNFR on APC direct T cell survival

(A) T cells and APC from wildtype and TNFR1-/-R2-/- mice were purified, recombined, then cultured in the presence or absence of recombinant TNFα (12 ng/ml) for 2 days. T cell death was quantified by CD3 and sytox blue staining. T cells were gated on using light scattering properties and CD3 staining. (B) The percentage of sytox blue positive T cells (see gated) relative to all T cells was plotted. (C) Data from of n=8 independent experiments are shown here. Significance was determined using an unpaired student’s t-test.
6.7 TNFR does not affect SEB-induced proliferation

TNFα’s promotion of cell survival of SEB-reactive Vβ8+ T cells could have resulted from early differences in proliferation. For example, an increase in the initial proliferation phase might contribute to differences in cell numbers observed after the expansion. To investigate this, the proliferative potential of wildtype and TNFR1−/−R2−/− mice was assessed by [3H] thymidine incorporation. Splenocytes were stimulated with different concentrations of SEB and their proliferation measured at the peak of expansion (day 3). No significant difference in proliferation was found between wildtype and TNFR1−/−R2−/− splenocytes at three different doses of SEB (Figure 18).

Figure 18: Lack of TNFRs does not affect SEB-induced proliferation

Splenocytes from wildtype and TNFR1−/−R2−/− mice were stimulated with different doses of SEB for 3 days. [3H] thymidine was added to wells 24 hrs before scintillation counts. The stimulation index was calculated by dividing the mean disintegrations per minute (DPM) of stimulated cells by unstimulated controls. The average DPM was calculated from quadruplicate wells and data from n=3 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
6.8 Contribution of B7.2 on APC and T cells to survival of SEB-stimulated T cells

B7.2 expression occurs on B cells and T cells after SEB stimulation with SEB or TNFα (Figure 8 and 9). Thus, it was possible that B7.2 expressing T cells could promote their own survival by virtue of expression both B7.2 ligand and the CD28 receptor. To dissect the contribution of B7.2 on APC vs T cells, additional mixing experiments were performed with cells from B7.2 −/− mice. T cells and APC from wildtype and B7.2 −/− mice were purified, then mixed them together in different combinations and stimulated with SEB. When B7.2-deficient APC were paired with wildtype T cells, we observed no difference in the proportion of Vβ8+ T cells undergoing apoptosis (Figure 19A, left). Similarly, no differences were observed when B7.2-deficient T cells were paired with wildtype APCs (Figure 19A, right). Although the proportion of Vβ8+ T cells undergoing apoptosis did not differ, a trend towards lower absolute Vβ8+ T cell numbers was seen when B7.2-deficient APC were paired with wildtype T cells (Figure 19B).
Figure 19: Contribution of B7.2 on APC and T cells to survival of SEB-reactive T cells

(A) T cells and APC from wildtype and B7.2-/-- mice were purified then recombined and cultured with SEB (0.3μg/ml) at a ratio of 1:4. Apoptosis of SEB-reactive T cells was determined by annexin V and Vβ8-TCR staining. Dead cells (7AAD+) were excluded from analysis and gating was determined by an isotype control stain. The percentage of apoptotic Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=8 independent experiments are shown. (B) The absolute viable Vβ8+ T cell counts were plotted. Statistical significance was determined by the student’s paired t-test.
6.9 Enhanced costimulation increases Bcl-\textsubscript{XL} and Bcl-2 expression

We have shown that costimulation can increase the survival of SEB-stimulated T cells. Because studies had demonstrated that SAg-activated T cells downregulate pro-survival proteins Bcl-\textsubscript{XL} and Bcl-2 [229], we also tested whether costimulation would alter the levels of these anti-apoptotic proteins. Wildtype splenocyte were cultured with SEB in the presence or absence of costimulatory anti-CD28. When costimulation was enhanced, Bcl-\textsubscript{XL} and Bcl-2 expression levels increased compared to SEB and Media controls (Figure 20A). Increased Bcl-\textsubscript{XL} expression supports earlier data from Moolani et Al [192, 230] and is reproduced in Figure 20B-left. Although increased Bcl-2 expression was also found in n=2 independent experiments, repetitions will need to be performed to determine statistical significance (Figure 20B, right).
Figure 20: Enhanced costimulation increases expression of anti-apoptotic proteins

(A) Splenocytes harvested from wildtype mice were stimulated with SEB (0.3μg/ml) in the presence or absence of costimulatory anti-CD28 antibody (0.5μg/ml) for 2 (Bcl-XL staining) or 5 (Bcl-2 staining) days. Staining with antibodies specific to Vβ8-TCR, Bcl-XL, and Bcl-2 was performed. Gating was set on Vβ8+ T cells. Fluorescence minus one (FMO) control staining was performed on SEB stimulated cells and is shown in grey. Histograms represent a minimum of n=2 independent experiments. (B) Changes in MFI were plotted for the experiments shown in (A) (Bcl-XL left, Bcl-2 right). The plot of Bcl-XL MFI values (left) was taken from earlier work by Moolani et Al [230]. Statistical significance was determined by the student’s paired t-test where applicable (in this figure * denotes a significance of p<0.05).
6.10 Loss of TNFR decreases Bcl-2 and Bcl-XL in SEB-reactive T cells

It was shown that costimulation can increase the survival of SEB-stimulated T cells with specific increases in the expression of pro-survival molecules Bcl-XL and Bcl-2. Given that TNFR promotes the expression of costimulatory molecules (see 6.2), additional experiments were performed to see whether the loss of TNFR would affect the expression of Bcl-2 and Bcl-XL in Vβ8+ T cells during responses to SEB. It was shown that TNFR1−/−R2−/− T cells stimulated with SEB had decreased expression of Bcl-2 (Figure 21A) and Bcl-XL (Figure 21B) in comparison to wildtype controls after the initial expansion phase. These findings demonstrate that, after exposure to SEB, TNFR signaling contributes to the expression of pro-survival molecules.

6.11 TNFR contributes to the activity of surviving SEB-reactive T cells

In these studies, TNFα was seen to drive the survival of Vβ8+ T cells after SEB stimulation. Since TNFR signaling was found to contribute to the survival of T cells (see 6.4-6.6) and expression of pro-survival molecules (see 6.10), additional experiments were performed to determine whether TNFR would also affect the activity of surviving Vβ8+ T cells. To measure the differences in T cell activity within the SEB-reactive population, flow cytometry was used to measure proliferation by incorporation of BrdU. In comparison to wildtype, Vβ8+ T cells from TNFR1−/−R2−/− displayed decreased amounts of proliferation after stimulation with SEB for 5 days (Figure 22). This finding suggests that TNFα not only preserves the survival of SEB-reactive T cells, but also contributes to their proliferative capacity after expansion.
Figure 21: TNFR promotes the expression of survival molecules in SEB-reactive T cells

Wildtype or TNFR-deficient splenocytes were stimulated with SEB (0.3μg/ml) for 5 days. Staining with antibodies specific to Vβ8-TCR, Bcl-XL, and Bcl-2 was performed. Histograms depict the expression of (A) Bcl-2 and (B) Bcl-XL of Vβ8+ T cells. Light scatter properties and isotype controls were used to set the gates on histograms. The histograms represent a minimum of n=2 independent experiments.
Figure 22: TNFR contributes to the activity of surviving Vβ8+ T cells

(A) Wildtype or TNFR-deficient splenocytes were stimulated with SEB (0.3μg/ml) for 5 days. BrdU was added to cultures 24hrs before staining with antibodies specific to Vβ8-TCR and BrdU. Analysis was performed on SEB-reactive T cells after gating on Vβ8+ T cells with isotype controls. (B) The percentage of proliferating Vβ8+ T cells within the total Vβ8+ population was plotted. Data from n=3 independent experiments are shown. Statistical significance was determined by the student’s paired t-test.
7 DISCUSSION

7.1 TNFα drives survival of SAg-reactive T cells by costimulation

The presence of a persistent T cell infiltrate in the coronary artery of KD patients is not in keeping with the established fates of SAg-activated T cells, which undergo death and anergy. Previous studies demonstrated that TNFα has important roles in regulating apoptosis and that TNFα may also be able to prevent the death of SAg-stimulated T cells (see below and 1.4.3). In addition, work in the LCWE model has demonstrated that TNFα and its receptors are critically required for pathogenesis. In these studies, we investigated the effects of TNFα on the fate of SAg-activated T cells. Understanding how TNFα alters the fate of SAg-activated T cells may help explain how T cells persist in KD, and further legitimizes the use of therapies targeting TNFα for the treatment of KD.

The functions of TNFα and its receptors TNFR1(p55) and TNFR2(p75) have been well studied. They include necrotic and apoptotic cell death, proliferation, differentiation, and inflammation. The breadth of functions is thought to result from the diversity of proteins and cell signaling pathways TNFα activates. Although the number of activated cell signaling pathways is large, it has become clear that TNFRs have key roles in regulating apoptosis. Reports have shown both pro-apoptotic and anti-apoptotic roles for TNFα. For example, TNFR1 is understood to associate with TNF receptor associated death domain protein (TRADD), which can recruit Fas-associated protein with death domain (FADD), to activate the initiator caspase 8. While, in contrast, TNFR2 through its association with the TNF receptor associated factor 2 (TRAF2) can activate the NF-κB pathway, which transcriptionally regulates cellular inhibitor of apoptosis protein 2 (c-IAP2), and can block caspase 8 activation and apoptosis.

It has been reported that SEB-stimulated T cells undergo similar levels of expansion and deletion in triple knockout mice lacking Fas, TNFR1, and TNFR2 [229], and thus there may be alternative pathways to death in SAg-activated T cells. The authors of this paper demonstrated the existence of an intrinsic cell death pathway triggered by SAg. It was found that
SEB-activation caused Bcl-2 downregulation in T cells, and that overexpression of Bcl-2 in transgenic mice permitted the T cells to survive. Their results agree with other investigators who have suggested alternative pathways to cell death in activated T cells that involve cytokine withdrawal [231], as it has also been shown that administration of IL-2 [231] or TNFα [194] can promote the survival of SEA-activated T cells. One interesting study by Vella et al. found that coadministration of LPS could preserve survival of SAg reactive T cells in vivo [193]. Although the precise mechanism was unclear, the increased survival mediated by LPS was found to depend on the production of TNFα.

In this study, TNFα was found to alter the fate of SAg-stimulated T cells, promoting their survival though regulating costimulation. It was shown that stimulation with SEB or TNFα, which is produced during SAg-responses, can upregulate B7.2 expression on splenocytes, and more specifically, on B cells (Figure 8 and 9). Although B cells have been known to upregulate B7.2 when activated with other stimuli such as alloantigen, LPS, or concanavalin A [232], it has not been demonstrated that TNFα and its receptors could directly drive B7.2 expression. Interestingly, upregulation of B7.1 was not detected on splenocytes in response to SEB or TNFα. This might have been due to the time frame of analysis, as it has been suggested that B7.1 and B7.2 are expressed with different kinetics [233]. However, given that a kinetic analysis of B7.1 was explored in this study, and that B7.1 and B7.2 have been found to be expressed on B cells after 18-42 hrs of LPS stimulation [234], it is unlikely that B7.1 was undetected because of the time frame chosen. Instead, it could be that different stimuli, such as SEB or TNFα, drive differential expression of B7.1 and B7.2. This idea is supported by published data showing a decreased role for B7.1 than B7.2 during SAg stimulation [226].

While it was demonstrated that stimulation with recombinant TNFα could upregulate B7.2 on wildtype splenic B cells in a dose dependent manner (Figure 9B), this effect was also partially seen in TNFR1−/−R2−/− B cells. This finding could be due to the preparation of TNFα used, as commercial preparations may contain traces of endotoxin, which are known to upregulate B7 expression [234]. It is also possible that the differences are a result of inherent
experimental variability. However, a direct relationship between higher doses of TNFα and larger differences in MFI was found in TNFR1-/-R2-/- cultures (data not shown).

During SEB immune mediated responses, TNF receptors were shown to be required for B7.2 expression as upregulation of B7.2 was only seen in wildtype and not TNFR1-/-R2-/- cultures (Figure 10). This result indicated that TNF receptors are responsible for the regulation of B7.2 expression during SAg immune responses, and suggested that TNFα might alter the fate of SAg-stimulated T cells via the costimulatory pathway. It should be noted that during these experiments, multiple doses of SEB (0.03, 0.3, and 3 μg/ml) were also compared. Abrogation of B7.2 expression in TNFR1-/-R2-/- cultures was always seen at 0.03 μg/ml; however, at higher doses, B7.2 expression was consistently diminished although not completely disrupted (data not shown).

Although increases in B7.2 expression were measured when SEB or TNFα was added to cultures (Figures 8, 9 and 10), it should be acknowledged that these differences could be, in part, attributed to potential increases in the auto-fluorescence of stimulated cells. In retrospect, even though control stains (isotype and fluorescence minus one) were performed on activated cells, additional control stains on unactivated cells could have been included to determine if changes in background auto-fluorescence occurred in the experiments.

Our lab had previously demonstrated that SEB-stimulated T cells could be rescued from apoptosis by enhanced costimulation [192]. Since TNFα signaling was found to be critical for costimulatory ligand expression during SEB responses (Figure 10), further experiments were performed to explore the specific links between costimulation and the survival of SEB-reactive T cells. In addition to recapitulating initial results which demonstrated that adding anti-CD28 to SEB stimulated cultures could rescue Vβ8+ T cells from apoptosis (Figure 11), it was further demonstrated that blockade of B7 ligands with CTLA4-Ig increased the apoptosis of SEB-reactive Vβ8+ T cells (Figure 12) and deletion of Cbl-b, which negatively
regulates vav during CD28 signaling (see 1.5.2), decreased apoptosis of SEB-reactive Vβ8+ T cells (Figure 13). The results further suggest that costimulation provided by B7 plays an important role in preventing the death of SEB-activated T cells. Although statistical significance (p = 0.08) was not achieved with the number of Cbl-b −/− experiments performed (n = 6), a trend towards increased survival of T cells was observed (Figure 13B). More experiments with Cbl-b −/− mice would be expected to clearly resolve this difference in apoptosis.

Other studies which have looked at costimulatory molecules CD28 and B7 during superantigen responses have mainly focused on the initial activation and proliferation of T cells [222, 223]. Conflicting data show that the precise role of B7 ligands in regulating cell survival is unclear. For example, a study using transgenic mice expressing soluble CTLA4-Ig found no differences in the numbers of viable Vβ8+ cells after treatment with SEB [225], while another group showed that injection of CTLA4-Ig decreased the numbers of SEA-reactive cells [194]. Complicating this discrepancy further is the acknowledgement that blocking costimulation would also inhibit the initial activation of T cells, and thus might prevent a clear measurement of cell survival later on. To avoid this problem, experiments were performed where costimulation was not blocked, but enhanced with anti-CD28 antibody or with Cbl-b −/− mice (Figure 11 and 13). In this way, cells from both conditions would not have defects in the initial proliferation and the differences in the survival of cells would not be attributed to defects in expansion. However, this approach has limitations as well. In optimization experiments, increased levels of proliferation were noted in SEB stimulated cultures when anti-CD28 antibody was added (data not shown), thus it remains possible that the differences in apoptosis measured may also reflect different expansion kinetics of Vβ8+ T cells. This consideration would also affect experiments performed with Cbl-b −/− mice, as they are also noted to have increased capacity to proliferate [205]. Determining whether this difference in cell survival is due to increased costimulatory signaling or due to increased expansion remains an interesting question that could be addressed in the future. To resolve the expansion and survival components in the presence or absence of costimulation, CFSE dye could be used to track successive generations of Vβ8+ T cells. With this approach, the levels of apoptosis between conditions could be compared while normalizing between generations.
Additional experiments revealed that TNFα itself also had a pro-survival effect on resting T cells and on SEB-activated T cells. In the absence of SAg, the addition of recombinant TNFα enhanced T cell viability in a dose dependent manner (Figure 14), while in the presence of SEB-stimulation, neutralization of TNFα increased apoptosis (Figure 15). These findings suggest that TNFα contributes to T cell survival during SAg-responses and are in agreement with published data which demonstrated that the addition of recombinant TNFα promotes the survival of SEA-stimulated T cells [194]. The authors of this paper did not clarify the specific mechanism(s) by which TNFα interferes with the apoptosis of SEA-activated cells.

In this thesis, experiments also aimed to dissect the specific contributions of TNFR from various different cell types to the survival of SAg-reactive T cells. Since TNFRs are expressed on many different cells types, including B cells and T cells (see 1.4.1), TNFα-driven survival might have resulted from stimulation of T cells, by upregulation of costimulatory molecules APC, or by both. Mixing experiments revealed that TNFR present on APC drive the survival of SEB-reactive T cells while the absence of TNFR signaling on T cells did not affect survival (Figure 16A). Additional mixing experiments also demonstrated that stimulation with recombinant TNFα promotes the survival of T cells via TNFRs on the APC, and not by TNFRs present on the T cells themselves (Figure 16). Thus, these results, viewed together with previous observations, suggest that TNFα acts to promote the survival of SAg-stimulated cells indirectly, through modulating costimulatory molecule expression on APC.

Similar to previous costimulation experiments, it was also possible that differences in cell survival could be attributed to differences in proliferation between wildtype and TNFR-deficient cells as TNFR have been reported to promote proliferation [158]. However, thymidine incorporation experiments revealed no significant statistical difference in proliferation between wildtype and TNFR −/− cultures in response to three doses of SEB (Figure 18). Taken together, the results suggest that the differences in apoptosis were not due to a significant failure
of TNFR \(^-\) cells to expand, but could result from defects in survival pathways which are driven by TNFR such as B7.2.

Additional mixing experiments using B7.2 \(^-\) \(^-\) mice revealed that B7.2 present on the APC may be important for cell survival after exposure to SEB (Figure 19). Although this result did not reach statistical significance, a trend towards increased survival was seen when absolute cell counts were graphed. The inability to reach statistical significance could be due to the inherent variability between experiments and the small magnitudes of change being measured. In particular, compensatory mechanisms may diminish the effect of a single deficiency in costimulation as preliminary experiments from our lab have shown that 4-1BBL (another costimulatory ligand and receptor of the TNF superfamily [235]) is also upregulated after exposure to SAg (unpublished observation) and may thus be able to compensate for a loss in B7.2 function.

Apoptosis is triggered by the extrinsic or intrinsic pathways. In the extrinsic pathway, receptors containing death domains, such as Fas and TNFR1, induce apoptosis by recruiting the adaptor FADD which leads to activation of caspase 8 and initiation of apoptosis. In the intrinsic pathway, external stresses, growth factor withdrawal, or cytotoxic drugs are shown to initiate apoptosis in a Fas/TNFR1-independent pathway which strongly regulated by Bcl proteins Bim and Bcl-2 [236]. Bcl proteins regulate the permeability of the mitochondria and release of cytochrome c, which interacts with APAF1 and caspase 9 to initiate apoptosis. The cell death induced by exposure to SAg may involve both extrinsic and intrinsic pathways. For example, some report that SEB-activated CD4\(^+\) T cells express Fas and commit fratricide [237], while others report have shown that SEB-stimulated results in an intrinsic death pathway, independent of death receptor signaling. For example, triple knockout mice lacking Fas, TNFR1, and TNFR2 are seen to undergo similar amounts of apoptosis in response to SAg [229]. An intrinsic death pathway is thought to be activated and regulated by Bcl proteins such as Bid and Bcl-2. For example, V\(\beta\)8\(^+\) T cells from Bid \(^-\) \(^-\) mice or from Bcl-2 overexpression transgenic mice are shown to survive after stimulation with SEB. Apoptosis by the extrinsic or intrinsic pathways is thought to be due to differences in the initial stimulation has been suggested to be
due to differences in experimental design. In experiments using recurrent SAg stimulation, roles for Fas have been observed, while experiments using a single dose of SAg are found to be Fas-independent [229]. It is argued that restimulation studies are not realistic because clearance of SAg occurs quickly \textit{in vivo}, which precludes restimulation [238]. In this thesis, a single exposure to SEB was always used.

In this study, TNF\(\alpha\) was seen to drive the expression of costimulatory ligands and interfere with the apoptosis of SEB-activated T cells. This TNF\(\alpha\)-dependent survival may be driven by costimulatory signals which could alter the intrinsic pathway of cell death triggered by SEB exposure. Given that Bcl-2 is strongly downregulated after SEB stimulation and that decreased levels correlate with the death of T cells [229], experiments were performed to determine whether enhanced costimulation could alter the expression of Bcl-2. It was shown here that after SEB-activation, enhanced costimulation with anti-CD28 increased the expression of Bcl-2 and Bcl\textsubscript{XL} in V\(\beta\)8+ T cells (Figure 20). These findings suggest that costimulation can rescue V\(\beta\)8+ T cells by increasing anti-apoptotic Bcl family members, Bcl-2 and Bcl\textsubscript{XL}. This result is also in agreement with another report which demonstrated that CD28 signaling promotes T cell survival by enhancing Bcl\textsubscript{XL} expression [213]. Interestingly, the study also reported that CD28 costimulation does not affect Bcl-2 expression, while an increase in Bcl-2 was found in this work. This discrepancy could be due different initial stimulatory agents, as their study used anti-CD3 antibodies to activate T cells, and in this report, SEB was used. Thus, it could be that the increased expression of Bcl-2 seen with enhanced costimulation does not reflect a direct upregulation of Bcl-2 expression per se, but may interfere with the downregulation of Bcl-2 during SAg activation. In this study, it was also demonstrated that mice deficient in TNFRs have decreased expression of Bcl-2 and Bcl\textsubscript{XL} in response to SEB (Figure 21). This finding agrees with the idea that costimulation driven by TNFR promotes an interference of the intrinsic Bcl-2 death pathway triggered by SEB stimulation. The defect in the survival of these T cells observed after expansion also correlated with less proliferation of V\(\beta\)8+ TNFR-deficient T cells (Figure 22). These findings also help explain why growth factors, such as TNF\(\alpha\), can promote the survival of SEB-activated T cells, yet may not exert their effects directly through TNFRs expressed on T cells.
7.2 TNFα and Autoimmunity

Proinflammatory cytokines coordinate and cause most of the generalized events observed during inflammation. Since inflammation seems to accompany most forms of autoimmune disease, it seemed intuitive that key cytokines, such as TNFα, may also be involved in autoimmunity [239]. Over the years, many studies have reported key roles for TNFα in different disease models such as type 1 diabetes mellitus (T1DM) and rheumatoid arthritis (RA). Interestingly, TNFα is seen to have complex functions as it can be both harmful and helpful in the development of autoimmunity [240]. Of particular relevance to our laboratory, TNFα does seem to have a pathogenic role (see 1.3.3 TNFα in the LCWE model), although the precise mechanism(s) by which TNFα mediates disease is not well understood. By understanding how TNFα acts in different autoimmune modalities, we can begin to infer how TNFα may be acting in LCWE-initiated KD.

In the NOD mouse model of type I diabetes mellitus, TNFα is thought to have an overall pathogenic role [240]. TNFα is expressed in the intra-islet infiltrate, predominantly by CD4+ T cells, and contributes to the development of spontaneous diabetes [241]. It has also been shown that when NOD-scid mice are injected with lymphocytes from NOD donors, they develop insulitis and diabetes within 120 days; however, with early exposure to a chimeric TNFα inhibitor [242], diabetes is prevented entirely [243]. Yet, in contrast to these results, it was shown that systemic administration of TNFα has a protective effect in NOD mice [244]. In view of these results it becomes clear that TNFα’s role is complex; likely having multiple non-mutually exclusive effects. For example, it has been suggested that the harmful effects of TNFα are driven locally in the islet, while systemic TNFα has immunoregulatory functions that dampen autoimmunity [240].

TNFα has also been demonstrated to have multiple roles in RA. Clinical studies have shown that TNFα is highly expressed in the synovial fluid of RA patients [245, 246]. Numerous studies using experimental models of RA have demonstrated pathogenic roles for TNFα, in the initiation, maintenance, and progression of the local inflammatory response and
tissue injury in arthritis (reviewed in [247]). Similar to experimental models of diabetes, use of neutralizing TNFα antibodies was seen to ameliorate arthritic lesions [248, 249], yet in contrast to diabetes, systemic administration of TNFα exacerbated the disease [250]. Thus TNFα likely has disease-specific roles, despite sharing many similarities in driving and perpetuating autoimmunity.

In view of the functions of TNFα in RA and T1DM, KD shares many similarities. The LCWE model (see 1.3.1) displays characteristics of dysregulated TNFα production as well as localized TNFα expression within the target organ of autoimmunity. In fact, it has been shown by our laboratory that local TNFα expression drives the production and enzymatic activity of matrix metalloproteinase 9 (MMP9), which degrades the elastin layer of vessels resulting in aneurysm formation [86, 87]. This localized production of TNFα and subsequent destruction of target tissue in KD is quite similar to RA and T1DM, where TNFα is seen to stimulate MMP9 production from synoviocytes [251], as well as, potentiate the toxic effects of IL-1 on pancreatic islets cells [252]. Additionally, interfering with TNFα with neutralizing antibodies or by genetic deletion of the receptor is also seen to ameliorate disease [147]. Thus, with knowledge that TNFα acts in multiple capacities in different autoimmune disorders, it may be the case that TNFα plays a larger systemic role in dysregulating the immune system in KD. Aside from inducing MMP9 production locally, we suggest in this study that TNFα contributes to the survival of a subpopulation of pathogenic cells T cells which remain after SAg-induced expansion.

7.3 Future Directions

Since this work has demonstrated that TNFα alters the fate of SAg-stimulated T cells via costimulation on APC, it would be interesting to investigate the role of TNFα-driven costimulation more closely in vivo. One future experiment could be performed using mice which lack TNFR1 and TNFR2 on their APC only. The receptors could be knocked out using a Cre/Lox system with Cre recombinase expressed under an MHC class II promoter and loxP sites added to both TNFR1 and TNFR2. Injection of LCWE into these mice would determine the contribution of TNFR on APC in our disease model. This question is particularly interesting
because APC have been found to localize with T cells and accumulate in coronary artery lesions [46]. In addition, because costimulation was found to be important for the survival of SAg-reactive T cells, future experiments could also investigate this effect in our disease model by injecting LCWE into CD28<sup>−/−</sup> mice or transgenic mice which overexpress Bcl-2, as it has been shown that overexpression of Bcl-2 prevents the death of SAg-reactive T cells [229]. The severity of disease would be expected to be decreased in CD28<sup>−/−</sup> mice and increased when Bcl-2 is overexpressed. In support of these experiments, our lab has recently demonstrated that coinjection of LCWE and a 4-1BB agonist can aggravate coronary disease [230].

7.4 Conclusions

It is known that TNF<sub>α</sub> is important in human KD and the LCWE mouse model. Expression of TNF<sub>α</sub> is upregulated during disease induction and the lack of TNF<sub>α</sub> signaling in the LCWE model was seen to completely prevent disease [147]. The findings in this work suggest that TNF<sub>α</sub> promotes the survival of SEB-stimulated T cells by regulating costimulatory molecule expression on APC. These observations are important because they shed light on the mechanisms which govern T cell death after SAg exposure and can be represented in a disease model (Figure 23). Perhaps most interestingly, these findings provide a way through a paradox: that SAg-mediated autoimmunity can occur, despite the majority of activated cells being deleted. Studies in mouse models have already shown that TNF<sub>α</sub> may contribute to different autoimmune conditions such as diabetes [243, 253], KD [147], and arthritis [248, 249]. In addition, this study also lends support to the autoimmune therapies which target TNF<sub>α</sub> and B7.
Figure 23: Model for TNFα-driven survival of SAg activated T cells

A single exposure to SAg results in initiation of the intrinsic cell death pathway which is regulated by Bcl proteins [229] and not the extrinsic pathway [229, 236]. Production of TNFα from APC and T cells drives the expression of B7.2 on APC, which provides costimulation to T cells and rescues them from cell death and modulates Bcl-2 and Bcl-XL expression. Given the importance of TNFα in autoimmune diseases (see 1.4.2), autoreactive T cells stimulated by SAg might escape cell death and persist with TNFα stimulation.
8 REFERENCES


