The Role of the Gut Microbiome in Modulating the Pathogenesis of Kawasaki Disease

By

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Graduate Department of Immunology,
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Abstract

The gut microbiome consists of trillions of microorganisms in constant interaction with the body. There is increasing evidence that these microbes have immunomodulatory effects and an important role in the pathogenesis of disease and autoimmunity. Kawasaki Disease (KD) is an inflammatory condition with a predilection for the coronary arteries. In this study, a murine model of KD using Lactobacillus casei cell wall extract (LCWE) was used to assess the effects of the microbiome in the pathogenesis of KD. We found marked changes in the microbiome between two animal facilities, the MaxBell Animal Research Facility (MBB) and the Toronto Centre for Phenogenomics (TCP), which correlated with disease susceptibility. Furthermore, depletion of the microbiome in susceptible mice at MBB was able to reduce disease incidence, while the transfer of MBB cecal samples into TCP mice was able to increase disease incidence. Segmented filamentous bacteria (SFB), a microbe associated with autoimmunity, was reliably found in susceptible mice and was absent in more resistant mice. Consistent with its effects on Th17 differentiation, we report elevated levels of IL-17 in the serum of SFB positive mice, and postulate that the changes in disease incidence are associated with this pathway. This project demonstrates an important role of the microbiome in modulating the development of coronary inflammation in a murine model of KD.
Acknowledgements

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<th>Description</th>
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<tbody>
<tr>
<td>Axillary Lymph Node</td>
<td>ALN</td>
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<tr>
<td>Antigen Presenting Cell</td>
<td>APC</td>
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<tr>
<td>Antimicrobial peptide</td>
<td>AMP</td>
</tr>
<tr>
<td>B Lymphoid Tyrosine Kinase</td>
<td>BLK</td>
</tr>
<tr>
<td>Candida Albicans water-soluble fraction</td>
<td>CAWS</td>
</tr>
<tr>
<td>Coronary Artery Lesions</td>
<td>CAL</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>CNS</td>
</tr>
<tr>
<td>Dendritic Cell</td>
<td>DC</td>
</tr>
<tr>
<td>Experimental Autoimmune Encephalitis</td>
<td>EAE</td>
</tr>
<tr>
<td>Enzyme linked immunosorbent assay</td>
<td>ELISA</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>FBS</td>
</tr>
<tr>
<td>Germ Free</td>
<td>GF</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>GAPDH</td>
</tr>
<tr>
<td>Hospital for Sick Children</td>
<td>HSC</td>
</tr>
<tr>
<td>Human Leukocyte Antigen</td>
<td>HLA</td>
</tr>
<tr>
<td>Inositol-triphosphate 3 kinase C</td>
<td>ITPKC</td>
</tr>
<tr>
<td>Interferon gamma</td>
<td>IFNγ</td>
</tr>
<tr>
<td>Intraepithelial lymphocyte</td>
<td>IEL</td>
</tr>
<tr>
<td>Intravenous Immunoglobulin</td>
<td>IVIG</td>
</tr>
<tr>
<td>Jackson Laboratories</td>
<td>JAX</td>
</tr>
<tr>
<td>Kawasaki Disease</td>
<td>KD</td>
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<tr>
<td>Lactobacillus casei Cell Wall Extract</td>
<td>LCWE</td>
</tr>
<tr>
<td>Lamina Propria Lymphocyte</td>
<td>LPL</td>
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<tr>
<td>Lipopolysaccharide</td>
<td>LPS</td>
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<tr>
<td>Matrix Metalloproteinase 9</td>
<td>MMP9</td>
</tr>
<tr>
<td>MaxBell Animal Research Facility</td>
<td>MBB</td>
</tr>
<tr>
<td>Mesenteric Lymph Node</td>
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<td>Major Histocompatibility complex</td>
<td>MHC</td>
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<td>Microfold Cells</td>
<td>M-cells</td>
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<td>Monocyte chemoattractant protein-1</td>
<td>MCP-1</td>
</tr>
<tr>
<td>Mouse Norovirus</td>
<td>MNV</td>
</tr>
<tr>
<td>Mouse Vascular Smooth Muscle Cells</td>
<td>MOVAS</td>
</tr>
<tr>
<td>Natural Killer Cell</td>
<td>NK Cell</td>
</tr>
<tr>
<td>Non-obese diabetic</td>
<td>NOD</td>
</tr>
<tr>
<td>Ontario Cancer Institute</td>
<td>OCI</td>
</tr>
<tr>
<td>Peripheral Blood Mononuclear Cells</td>
<td>PBMCs</td>
</tr>
<tr>
<td>Peptidoglycan</td>
<td>PGN</td>
</tr>
<tr>
<td>Phosphate buffered saline</td>
<td>PBS</td>
</tr>
<tr>
<td>Polysaccharide A</td>
<td>PSA</td>
</tr>
<tr>
<td>Quantitative Polymerase Chain Reaction</td>
<td>qPCR</td>
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<tr>
<td>Recombination-activating Gene</td>
<td>RAG</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-----------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>Tregs</td>
</tr>
<tr>
<td>Segmented Filamentous Bacteria</td>
<td>SFB</td>
</tr>
<tr>
<td>Serum Amyloid A</td>
<td>SAA</td>
</tr>
<tr>
<td>Specific Pathogen Free</td>
<td>SPF</td>
</tr>
<tr>
<td>T helper Cell 17</td>
<td>TH17</td>
</tr>
<tr>
<td>Taconic Farms</td>
<td>TAC</td>
</tr>
<tr>
<td>T cell Receptor</td>
<td>TCR</td>
</tr>
<tr>
<td>Toll like Receptor</td>
<td>TLR</td>
</tr>
<tr>
<td>Toronto Centre for Phenogenomics</td>
<td>TCP</td>
</tr>
<tr>
<td>Transforming Growth Factor Beta</td>
<td>TGFβ</td>
</tr>
<tr>
<td>Tumor Necrosis Factor alpha</td>
<td>TNFα</td>
</tr>
<tr>
<td>University of Toronto</td>
<td>UofT</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor</td>
<td>VEGF</td>
</tr>
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INTRODUCTION

Kawasaki Disease

History and Clinical Findings

Kawasaki Disease (KD) is the most common cause of acquired heart disease in children in the industrialized world. It was first described in 1967 by Dr. Tomisaki Kawasaki as mucocutaneous lymph node syndrome. [1] The classical symptoms of KD consist of a prolonged fever, ocular conjunctivitis, oral mucosal inflammation, changes in peripheral extremities, presence of a polymorphous rash, and swelling of the cervical lymph nodes. [2] While KD is diagnosed with the presence of a fever and four of the classical symptoms listed above, there are important clinical findings in a number of other body systems. Patients often present with gastrointestinal (71%), neurological (60%), and respiratory symptoms (50%), but the most serious and long lasting of these are in the cardiovascular system. [3, 4] Untreated, up to 30% of children can develop coronary aneurysms, and even in the current era of treatment, 5% of children with KD still develop coronary artery aneurysms. [5]

The current gold standard for the treatment of KD is high dose aspirin and intravenous immunoglobulin (IVIG), which has been shown to reduce the risk of coronary complications by 85%. Several mechanisms of action of IVIG have been postulated: neutralization of bacterial agents/by-products and pathogenic autoantibodies, down-regulation of MHC Class II on APCs and its subsequent induction of pro-inflammatory cytokines, modulation the function of endothelial cell function and their expression of adhesion molecules, and the inhibition of reactive oxygen species by neutrophils. [6, 7] IVIG has also been shown to affect IL-17 production. This mechanism is thought to be due to a direct inhibition of Th17 differentiation.
and their release of effector cytokines through the interference of RORγT and STAT3 – key transcription factors in the Th17 differentiation pathway – as opposed to a neutralizing effect on IL-17. Furthermore, IVIG was able to enhance FoxP3 expression and increase levels of Tregs, which have a reciprocal relationship with Th17 cells. [8, 9]

**Epidemiology & Etiology**

Despite its discovery almost 40 years ago, there is still much to understand about the etiology of KD. Most of what is known is derived from epidemiological evidence. For instance, there is a substantial difference in incidence of KD between different ethnic groups that persists in spite of geographical location, which points towards a genetic factor. For example, Australia has the lowest disease incidence in the world at 3.7/100,000. In contrast, Canada has a disease incidence of 26.2/100,000. It is higher in children of East Asian descent; in Taiwan, disease incidence in 69/100,000 and in Korea, it is 113.1/100,000. This pales in comparison to Japan, where disease incidence is almost 10 times as high as Canada’s. This incidence is maintained in children of the same ethnicity who are living in other countries – Japanese Americans have an incidence of 210/100,000 as compared to their Caucasian counterparts (13/100,000). [10] In addition to the epidemiological evidence for genetic factors in KD, genome-wide association studies have indicated several genes associated with an increased susceptibility to KD such as CD40, ITPKC, BLK, CASP3 and HLA haplotypes. [11]

On the other hand, epidemiological evidence also points towards an environmental trigger. A variety of non-infectious environmental factors have been associated with KD, including carpet shampoo [12], residence near bodies of water [13] and wind patterns [14]. There is also seasonal variation in the incidence of KD, albeit different between regions. In North
America, KD peaks during the winter and spring, but peak incidence in China and Korea occur during the summer months. [10] There have also been several reports of outbreaks in KD that cluster geographically. The fluctuation and localization in the occurrence of KD suggest that an environmental factor is involved in the pathogenesis of disease. [5] Furthermore, the clinical symptoms of KD – fever, rash, conjunctivitis and cervical adenopathy – are common features in childhood illnesses involving an infectious agent.

KD has been associated with multiple viral and bacterial agents (see Table 1). It is most common in young children, with a sharp increase after 6 months of age [15, 16]. The lack of cases before 6 months suggests a protective immunity to an infectious trigger due to maternal antibodies. The decrease in incidence in older children and the low recurrence rate (1-4%) indicates that a protective immunity by the self has been built up, alluding to the notion of a ubiquitous environmental agent that has been encountered without incident. [1, 5, 17] In spite of all this evidence for an infectious trigger, no one infectious agent has been proven to be the cause for KD. There are several alternative hypotheses regarding the etiologic trigger for KD. In addition to the notion of a yet-to-be-discovered conventional pathogen, it is possible that KD can occur in response to a number of microbial agents with similar properties, as supported by the multiple causative agents that have been associated with KD. Another theory is that KD occurs due to an immune response to a superantigen, supported by reports of Vβ skewing in T cells and a murine model of KD. [3] Although the debate between these hypotheses continues, there is consensus in that it eventually leads to a massive systemic inflammatory response. This eventually localizes to the vasculature, particularly that of the coronary arteries, where the production of chemokines and pro-inflammatory cytokines exacerbates inflammation and
recruits additional cells. Ultimately, this can lead to vascular injury and the formation of coronary aneurysms. [10, 18, 19]

Table 1: Infectious agents associated with KD (adapted from Burgner et al.) [5]

<table>
<thead>
<tr>
<th>Pathogens implicated in KD</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Adenovirus</td>
<td>[20]</td>
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<tr>
<td>Herpes Virus</td>
<td>[21]</td>
</tr>
<tr>
<td>Mycoplasma species</td>
<td>[22]</td>
</tr>
<tr>
<td>Streptococci</td>
<td>[23-25]</td>
</tr>
<tr>
<td>Staphylococci</td>
<td>[26, 27]</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em></td>
<td>[28, 29]</td>
</tr>
<tr>
<td><em>Ehrlichia chaffeensis</em></td>
<td>[30, 31]</td>
</tr>
<tr>
<td>Rickettsia species</td>
<td>[32]</td>
</tr>
<tr>
<td>Epstein Barr Virus</td>
<td>[33-35]</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>[36, 37]</td>
</tr>
<tr>
<td>Human coronavirus New Haven</td>
<td>[38]</td>
</tr>
<tr>
<td>Measles virus</td>
<td>[39, 40]</td>
</tr>
<tr>
<td><em>Chlamydia pneumonia</em></td>
<td>[41, 42]</td>
</tr>
<tr>
<td><em>Bartonella henselae</em></td>
<td>[31]</td>
</tr>
<tr>
<td><em>Coxiella burnetii</em></td>
<td>[43, 44]</td>
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The Gut Microbiome

The Gastrointestinal Tract as a Lymphoid Organ

Microbes occupy every site on the body that interacts with the environment, each with a distinct ‘microbiome’. For example, populations found within the skin were found to differ from that of the mouth, and both of these populations, from the gut. Variations in these commensal communities have been associated with disease. A change in the microflora on the skin has been associated with acne and dermatitis, [45, 46] while changes in the oral microflora have been linked with dental and even respiratory conditions. [47-50] The most important may be the
human gastrointestinal tract, which is the largest source and point of interaction between a host and bacterial microorganisms. There has been extensive investigation, and evidence, on its role in disease and immunity. [51, 52]

The gastrointestinal tract is colonized by approximately 100 trillion micro-organisms – such that microbial cells outnumber their host by a factor of 10 and microbial genes by a factor of 150. Not only are there a large proportion of bacteria, the human gastrointestinal tract contains the largest amount of lymphoid tissue in the body – \(10^6\) lymphocytes per gram tissue. [53] There is a T cell for every 10 epithelial cells in the small intestine, and approximately 2/3 of the cells in the intestinal lamina propria are lymphocytes. [54]

Secondary lymphoid organs known as Peyer’s patches and lymphoid follicles are scattered throughout the gastrointestinal tract. Microfold cells (M-cells) are a specialized epithelial cell found in Peyer’s patches that facilitate the entry of luminal antigens for uptake by dendritic cells in the region. They migrate to the mesenteric lymph node (MLN) and present these antigens to T cells, which can then re-enter the bloodstream through the thoracic duct.

These regions also contain germinal centres with B cell follicles and T cell areas akin to other lymphoid organs which maintain a basal amount of inflammation and can facilitate immune activation when necessary. There are a host of physical barriers that limit microbial interaction – such as the tight junctions between gut epithelial cells and the mucus layer secreted by goblet cells. [55] The cells of the intestinal epithelium also undergo rapid turnover, limiting the ability of pathogens to persist and facilitating their removal. [56] Anti-microbial peptides (AMP) from Paneth cells and IgA secretion from plasma cells are also released into the lumen of the gut to neutralize microbes before they even come into contact with the intestinal epithelial cells. [57,
In short, the gastrointestinal tract is actively monitoring the luminal milieu to maintain an inflammatory homeostasis in response to the plethora of microorganisms found within the gut.

**Composition of the microbiome**

The majority of the commensal microbiome is beneficial and exists symbiotically with the host. They compete with intestinal pathogens for resources and limit their ability to damage the host. For example, dysbiosis of the gut microflora, such as that mediated through the use of antibiotics, can lead to inflammation and a breach of the gut epithelial barrier and increase susceptibility to infection and autoimmunity. Reconstitution of the microflora through the use of probiotics [59], or even a fecal transplant has been shown to restore immune homeostasis and even treat these diseases. [60] These reports demonstrate the importance of the microbiome and its composition immunity.

The complex interaction between host and microbe has begged the question of what constitutes a healthy or unhealthy microbial composition, and its contribution to disease. With the advent of improved sequencing technology and the ability to look more in depth into the composition of the gut microbiome, it is now possible to examine specific microbes or groups of microbes and assess their physiological effects.

The problem that arises is that preliminary studies report large variations between the microbiome in different individuals. [51] While some studies have found similarities within a proportion of sample populations, there are others which show a striking lack of a commonality. These observations question whether a ‘core microbiome’ – a basic community of microbes consistently found in healthy individuals – even exists. The search is further complicated by certain limitations; the functional necessity of key components of the microbiome may vary with age, food, genetics, geographic location or a plethora of other variables. Nevertheless, there are
still trends – for example, the majority of gut bacteria fall within the phyla of Bacteroidetes, Firmicutes, Actinobacteria or Proteobacteria. [61] There is also evidence to suggest that there are a limited number of microbial compositions that constitute a ‘healthy’ microbiome, especially when assessing data further up on the taxonomical ladder. [62] The trends in phylum observed in the microbiome, in conjunction with the variations in genus or species between individuals, suggest that there may be a functional redundancy in the ability of these microbes to sustain a symbiotic relationship with their host. And while it remains to be seen whether a ‘core’ microbiome truly exists, there is strong evidence to suggest the microbiome modulates extra-intestinal functions and immunity. [63]

**Figure 1: Taxonomic representation of four major phyla in the gut**

Above is a representation of the taxonomic ladder. Each group can be further subdivided as one goes down the taxonomic ladder. Bacteria within the microbiome can be classified by their taxonomic group. This is done using the genes for the 16S rRNA, which have conserved and hypervariable regions. The gut microbiome is comprised primarily of 4 phyla: Bacteroidetes, Firmicutes, Actinobacteria, and Proteobacteria. Each phylum can be further divided into more specific subsets that can differentiate important niches of bacteria within the gut.
Microbiome in Disease and Autoimmunity

The importance of the microbiome in disease is well established. The use of probiotics has been shown to be protective against a variety of pathogens and is often used prophylactically against traveler’s diarrhea. [64-67] Fecal microbiota transplants have been demonstrated to be effective in treating C. difficile infection; a retrospective analysis of 11 clinical studies and 273 participants showed that 89% of patients experienced clinical resolution. [68] However, the microbiome has a role in the development of autoimmunity as well.

One example demonstrating the immunomodulatory role of bacterial interactions, both inside and outside of the gut, is the hygiene hypothesis. The hygiene hypothesis posits that interactions with environmental microorganisms, both symbiotic and pathogenic, can lead to a ‘protection’ against the development of autoimmunity. The increase in sanitation is thought to have modulated the natural development the Th1/Th2 immune response, priming individuals towards a dysregulated immune system. This is supported by the inverse relationship in the incidence of bacterial infections, and autoimmunity. Multiple sclerosis, Crohn’s disease, type 1 diabetes and asthma have all been on the rise as the incidence of bacterial infections have gone down. These increased incidence of autoimmunity have been associated with increased sanitation, city living and the number of siblings – all pointing towards the notion that bacterial interactions are important in the development of a balanced immune system. [64, 66, 69]

The best evidence demonstrating the importance and complexity of the microbiome in immunity has been shown using animal models of disease. One of the most studied examples illustrating the protective effects of the gut microbiome is observed in the development of Type I Diabetes. Non-obese diabetic (NOD) mice spontaneous develop diabetes, but disease incidence has been observed to vary between different regions. Incidence was found to be elevated in NOD mice housed in animal facilities with more stringent hygiene protocols, such that almost all the
mice housed in germ-free (GF) conditions would spontaneously develop diabetes. [70] In this model of diabetes, the protective effect was shown to be independent of toll-like receptor (TLR) signaling. Investigators demonstrated that mice deficient in MyD88 – a downstream adaptor of TLRs – could still be protected when colonized with microbiota. This suggests that specific microbes or microbial byproducts, as opposed to non-specific microbial signaling, may be responsible for the observed effects. King & Sarvetnick showed that mono-colonization with just \textit{Bacillus cereus} was sufficient to convey a protective effect in this model [71]. Furthermore, Kriegal et al. exploited the difference in segmented filamentous bacteria (SFB) colonization between mice, ordered from Taconic Farms (TAC) and Jackson Laboratories (JAX), to demonstrate that SFB positive NOD mice were protected from diabetes. [70] Markle et al. showed that microbial contents from male mice, when transferred to the more susceptible females, were able to reduce disease incidence. [72] These observations suggest that under certain conditions, specific components of the microbiome may be sufficient to modulate the pathogenesis of a disease.

In contrast to these protective effects, the microbiome has also been shown to exacerbate autoimmunity as well. Several studies found a correlation with increased intestinal permeability and patients with diabetes, ankylosing spondylitis, IgA nephropathy, non-alcoholic steatohepatitis and multiple sclerosis. [73-78] Thus, one possibility is that disease exacerbating microbes are originating from the gut microbiome. Another theory postulated by Eksteen et al. is that activated lymphocytes originating from the gut may be responsible for the pathogenesis of disease, such as in the case of primary sclerosing cholangitis. [79] Activated lamina propria lymphocytes (LPL) have been shown to bind to peripheral lymph nodes, [80] and endothelial cells in the liver reported expression of adhesion molecules complementary to these activated
Essentially, LPL activated by microbes within the gut are thought to migrate to extra-intestinal sites and cause unwarranted inflammation. These examples exhibit the importance of maintaining gut homeostasis and the capability of the microbiome in instigating autoimmunity.

Alternatively, the microbiome may modulate the pathogenesis of disease instead of triggering autoimmunity. Several animal models support this notion and have given insight into the mechanisms by which disease is achieved. In an induced animal model of EAE, depletion of the gut microbiome could completely ameliorate disease. GF mice had lower levels of IFN-γ, IL-17 and elevated numbers of Tregs. [82, 83] The addition of a single microbial community – SFB – was shown to remove this protective effect. Re-colonization with SFB resulted in reduced numbers of Tregs, elevated levels of IL-17, IFN-γ and a higher EAE score. The introduction of additional SPF microbes further exacerbated the symptoms, demonstrating a potential role of the microbiome as amplifiers, or perhaps, moderators of disease. [84]

Similarly, in the B10.BR model of ankylosing spondylitis, reconstitution with lactobacillus species were insufficient to restore disease, but a mixture of several microbial communities were. GF mice and Lactobacillus mono-colonized mice did not develop disease at all. SPF colonization increased disease incidence to 14% (13/90), and colonization with a conventional bacterial mixture with potentially pathogenic microbes further increases it to 16% (19/121). [85] These observations validate the notion that particular microbes may be more important, or that the interplay between several communities of bacteria are essential to mediate their effects on disease and immunity.

The K/BxN model of autoimmune arthritis was also shown to be dependent on the microbiome. In this study, GF mice did not develop disease while SPF colonized mice did. The authors also revealed that a single community of SFB was sufficient to restore disease. This
animal model of arthritis was shown to be dependent on IL-17 and the restoration of the Th17 immune response by SFB within the gut. The fact that mono-colonization with SFB was able to restore disease again demonstrates the impact a single microbial community can have on the immune response. [86]

In another model of arthritis, mice deficient in IL-1 receptor antagonist (IL-1rn−/−) spontaneously develop arthritis. Similar to the previous examples, disease is also abrogated under GF conditions. The investigators of this study demonstrated that TLR signaling through the microbiome was required for the development by using TLR deficient mice. Interestingly, the knockout of specific TLRs had different phenotypes. TLR9−/− did not differ from the control, TLR2−/− had a more severe phenotype characterized by elevated levels of IFNγ producing cells in the spleen, while TLR4−/− developed a milder form of disease with markedly lower amounts of IL-17 producing cells in the spleen. [87] As these TLRs recognize different ligands, it is likely that these observations are because different communities of bacteria within the gut mediate different effects on the immune response.

These gnotobiotic animal models of autoimmunity have given insight on the complex interactions between the microbiome and host. The microbiome can have both protective and detrimental effects on the development of autoimmunity. They can be mediated by the same community of bacteria, depending on the disease model; SFB was shown to be essential for the development of EAE, but protective against diabetes. The mechanism by which disease is modulated also appears to vary, as shown by the importance of TLR signaling in arthritis and a lack thereof in a model of diabetes. Furthermore, while specific microbes were shown to be sufficient to induce disease, the addition of other bacteria exacerbated disease, perhaps alluding to a synergistic effect of multiple communities. These observations serve to illustrate the
capability of the microbiome to modulate the peripheral immune response, the multiple pathways by which these effects can occur and the influence a single bacterial community may have on disease.

**Microbiome and Immune Development**

It is clear that the relationship between the microbiome and the host immune system is not limited to the gut – it has extra-intestinal functions as well. Several studies comparing conventionally colonized mice and GF mice revealed the importance of the microbiome in the peripheral immune response. Neutrophils were shown to have decreased function in the absence of peptidoglycan (PGN), and were not as able to kill pathogens. DCs and NK cells harvested from GF mice were also not as responsive when stimulated with poly(I:C). This evidence suggests that constant interaction with microbial products is necessary for the maintenance of innate immunity. The timing of this interaction is also crucial. GF mice that were colonized with SPF bacteria – a gnotobiotic mixture that has been tested for pathogens – at 3 weeks of age showed similar levels of cytokines and mononuclear cells to that of control germ free mice, but different levels compared to those of conventionally raised mice. This suggests that the exposure at 3 weeks of age was insufficient to restore the immunodeficiency in GF mice to those of conventionally raised mice. [88] Consistent with the hygiene hypothesis – exposure to microbes early in life may provide some protection against the development of allergies and autoimmune disease – bacterial interactions at a young age may be essential for proper immune development. [64]

In addition to its effects on the innate arm of immunity, the microbiome has been demonstrated to affect the adaptive immune response as well. GF mice were reported to have smaller and underdeveloped spleens and peripheral lymph nodes, and lower proportions of CD4+
T cells. [89, 90] There are fewer germinal centres in the spleens and proportions of B cells are significantly lower when compared to conventionally housed mice. While there is some normalization in IgM levels as they age, the deficiency in IgG and IgA persist. This data is indicative of a potential role that the microbiome has on B cell development and class-switching. Furthermore, the differentiation and development of peripheral T cells appear to be influenced by the gut microbiome; GF mice have approximately half the amount of splenic T cells as their colonized counterparts, and there is a skewing towards a Th2 profile. [89]

Interestingly, specific communities of microbes have been sufficient to correct these defects and modulate the differentiation of T cells. One example is Bacteroides fragilis, which has been shown to induce the expansion of Th1 helper T cells and restore levels of CD4+ splenic T cells to normal. [89] These mice are also protected against H. hepaticus, chemically induced colitis and EAE. These protective effects are due to the induction of IL-10, which was shown to be mediated primarily through polysaccharide A (PSA). PSA deficient B. fragilis were shown to be unable to mediate their beneficial and protective effects. [91, 92]

Another community of microbes known for their anti-inflammatory effects and their ability to induce FoxP3+ Tregs are Clostridium species. Colonization of mice with a mixture of clostridium species led to elevated levels of TGF-β, leading into Treg differentiation. [93] In this investigation, 46 species of Clostridia species were introduced into GF mice and resulted in elevated proportions of Tregs, not only in the gut, but in the lung, liver and spleen of the newly colonized mice – levels which were higher than their conventionally housed counterparts. [94, 95]

Lactobacillus and Bifidobacterium are two communities of bacteria that colonize the gut at an early age. While their population decreases with age, their beneficial effects have led to
their use as probiotics. They have important roles in digestion, nutrient absorption, and immunity. They have been demonstrated to be protective against several forms of colitis, capable of suppressing inflammatory cytokines such as TNFα, IL-1β and IL-6, and inducing Tregs. [63, 96] The gut microbiota as a whole, as well as specific communities, has exhibited the ability to modulate immune development and the peripheral immune response. These observations lend support to the notion that variations within the microbiome may predispose, or protect against the development of certain diseases and modulate their pathogenesis.

**Segmented Filamentous Bacteria**

One of the most interesting commensal microbes is the SFB – a gram positive community of spore-forming bacteria. SFB cannot be cultured *in vitro*, thus, they lack an official taxonomic designation, though they appear to be related to Clostridia species. They have been found in a broad range of animals, including humans. While morphologically similar, the SFB found in one species are unable to colonize another, suggesting a strong symbiotic, or at least dependent, relationship. Unlike the majority of other commensal microbes, SFB adhere to the epithelium of the intestine, allowing for direct interaction with the gut immune system. In mice, SFB constitute a considerable proportion of the microbiome at weaning, eventually decreasing as they age. It can be presumed that they have a relatively quick lifecycle due to their adherence to the rapidly shedding cells of the intestinal epithelium. Several analyses of the SFB genome have revealed a considerable lack of genes associated with biosynthesis, and abundance in molecular transport, germination and sporulation. These results are consistent and support the current theories regarding the symbiotic and adherent lifecycle of SFB. [97, 98]
SFB also has potent effects on the immune system. They can promote the production of IgA plasma cells [99, 100] and modulate T-cell activity. SFB colonization leads to an increase in cytotoxic activity, and a rise in numbers of natural killer and CD8+ intraepithelial lymphocytes (IEL). [101] Their presence and immunological effects are protective against certain pathogens, as shown in murine models against Salmonella and Citrobacter rodentium. [102, 103]

Most interesting is their effect on the Th17 immune response. SFB alone was shown to be sufficient for Th17 differentiation, while other bacterial communities were not. A mixture of several Bacteroides and Clostridia species were used to colonize the gut of GF mice and did not increase the proportion of IL-17, in contrast to SFB. One proposed mechanism by which SFB could induce Th17 differentiation is its adherence to the epithelial cells and the subsequent release of Serum Amyloid A (SAA). SAA is an acute phase protein that is upregulated during inflammation. [104] In the context of the Th17 immune response, Ivanov et al. reported elevated levels of SAA transcripts in the ileum of SFB colonized mice. In vitro, the addition of SAA to naïve CD4+ T cells was sufficient to induce the transcription factor RORγT and thus, Th17 differentiation. [103] The role of Th17 in the pathogenesis of many autoimmune diseases has led to further inquiry regarding the effect of modulating populations of SFB in many disease models. Currently, SFB has been demonstrated to be sufficient in the development of at least two autoimmune conditions [83]. A murine model of autoimmune arthritis was drastically reduced in GF conditions, and re-colonization of SFB restored the development of disease. This was demonstrated to be dependent on IL-17, as neutralizing antibodies was able to ameliorate this effect [86]. It is likely that the effects of SFB are mediated through the Th17 immune response. Similarly, a model of EAE protected under GF conditions would develop disease upon re-colonization with SFB. SFB was able to induce Th17 differentiation and there were elevated
levels of Th17 cells in the central nervous system (CNS). [70] In essence, the unique characteristics of this gut microbe, its effects on the local and peripheral immune response, and its role in Th17 differentiation make it a crucial player in the study of host-bacteria interactions and the pathogenesis of several autoimmune diseases.

The Th17 Immune Response

Th17 cells are one of the predominant T helper cell subset within the gastrointestinal tract and comprise of 10-20% of CD4⁺ TCRβ⁺ T cells. [103, 105] Their effector cytokines have an important role in maintaining barrier function and homeostasis, and as stated above, are affected by compositional changes in the microbiome. Furthermore, these cells have strong pro-inflammatory capabilities that are important in combating resilient pathogens. However, this can also lead to autoimmunity; Th17 cells have been implicated in arthritis, diabetes and multiple sclerosis. In essence, their relationship with the gut microbiome, role in gastrointestinal homeostasis, and involvement in autoimmunity suggest that the Th17 immune response may be one central mechanism by which the gut microbiome modulates peripheral immunity and the pathogenesis of systemic diseases.

Differentiation and function of Th17

The primary function of Th17 cells is in the clearance of particularly resistant pathogens. For example, Lin et al. showed that clearance of *S. aureus* and *C. albicans* is partly dependent on the Th17 immune response. [106] Another illustration is that IL-17 knockout mice had a delayed immune response to *K. pneumonia*. Many of these pathogens are resilient against both the Th1 and Th2 immune response, and require the induction of a massive inflammatory response by Th17. In addition to the examples listen above, the protective effect of the Th17 immune
response has been established against a large variety of fungal, viral and bacterial infections, including *P. acnes*, *C. rodentium*, *M. tuberculosis*, and *P. carinii*. [107, 108]

Another facet of the Th17 immune response is that their development often counteracts that of the Treg; in the absence of IL-6, TGF-β stimulates the differentiation of naïve T cells into Tregs. This opposition to Tregs is one of the reasons for its potent pro-inflammatory effects. TGF-β signaling increases the expression of RORγT; however it also represses its function. It does, however, also increase the expression of the IL-6 receptor. Alone, IL-6 is insufficient to induce RORγT, but it is able to mitigate the suppressive effects of TGF-β and together, these cytokines promote Th17 differentiation.

IL-17, the primary cytokine secreted by Th17 cells, mediates a host of pro-inflammatory effects. The receptor for IL-17 is found on a variety of cell types including hematopoietic cells, fibroblasts, endothelial cells and epithelial cells allowing it to alter responses throughout the body. Through these cells, IL-17 has been known recruit neutrophils, induce the production of MMPs [109] and increase the production of pro-inflammatory cytokines such as TNFα, IL1β, IL6 and GM-CSF. [110] Furthermore, the production of IL-17 is not limited to Th17 cells, but also neutrophils, macrophages, γδ T cells, and NK cells. These effects are of particular interest because many of them are involved in the pathogenesis of KD. Neutrophil infiltration has been observed during the acute stage of KD and may facilitate vascular injury. These neutrophils from children with KD, have been shown to produce more nitric oxide compared to febrile and healthy controls, and this may be one mechanism of pathology. Alternatively, they have also been shown to produce abnormal amounts of VEGF and this may lead to vascular remodeling. These observations, in conjunction with their recruitment during the acute phase of disease, suggest that they may have a role in KD. [111-113] TNFα has been shown to be crucial in the development of
coronary lesions and is a therapeutic target in the treatment of KD. TNFα is important in leukocyte recruitment, regulating cytokine production and inducing the production of MMPs. [3, 114] MMPs are a family of enzymes responsible for the breakdown of extracellular matrix. In KD, these enzymes degrade elastin in blood vessels, and promote vascular injury and the formation of coronary lesions. Inhibition of MMP-9 activity through the use of doxycycline was able to significantly reduce the incidence of elastin breakdown in a murine model. [115, 116] Furthermore, many of the pro-inflammatory cytokines induced by IL-17 (IL-1β, TNFα, IL-6) are elevated in KD patients and have been shown in murine models to be important in the pathogenesis of disease. [117-120] These observations support a potential role for the Th17 cells in the pathogenesis of KD.

**The Th17 Response in KD**

The importance of T cells in KD has been well documented in both humans and animal models. RAG-1−/− mice do not develop KD, [121] and there is a prominent lymphocyte population in human patients. [3] Kimura et al. investigated the T cell population in KD to dissect their role in the pathogenesis of disease. Interestingly, what they found was a decrease in both Th1 and Th2 transcription factors and their corresponding cytokines. [122] There are conflicting reports on the roles of the Th1/Th2 networks in KD. IFN-γ producing CD3+ T cells were reported to be lower during the acute phase in KD patients, while other studies reported elevated levels of this cytokine in the serum. [123-125] Similar trends were found in the Th2 cytokine IL-4 as well. [122, 125, 126] This has led to the investigation of another T cell subset – Th17 helper T cells. In conjunction with their well-documented roles in autoimmunity and disease, [108] it is suspected that the Th17 subset of helper T cells may be important in the pathogenesis of KD.
Preliminary studies have found that levels of IL-17 and cytokines important in the Th17 immune response, IL-6, IL-8 and IL-23, are all higher in the serum of KD patients during the acute phase of disease when compared to both healthy and disease-infected controls. Furthermore, analysis by flow cytometry revealed increased proportions of Th17 cells and decreased levels of Tregs. This study also characterized KD patients under two conditions: their response to IVIG and the presence of coronary artery lesions (CAL). Interestingly, KD patients who had CAL also had significantly higher levels of Th17 cells compared to KD patients without CAL. CAL+ patients also had elevated plasma levels of IL-17, IL-6 and IL-23. KD patients that were resistant to IVIG had markedly higher numbers of Th17 during the acute phase and have higher plasma concentrations of IL-17 and IL-6. The elevation in Th17 and its effector cytokines in patients with a more severe form of KD suggest that the Th17 immune response may have a role in exacerbating disease. [117, 127]

Genome wide association studies have given insight into the pathogenesis of KD. Hits in the genes for CD40L, CASP3 and ITPKC have led to and support the importance of costimulation, the apoptotic pathway and T cells in the pathogenesis of disease. [11, 128] One of these candidate genes has been postulated to mediate its effects by modulating the production of IL-17. B-lymphoid tyrosine kinase (BLK) was shown to be essential for the development of IL-17 producing γδ T cells. This is of particular interest as both circulating levels of IL-17 as well as γδ T cells are elevated during the acute phase of KD. (See Fig.2) [11, 129] On the other hand, Lee et al. reported that IL-17 knock-out mice were still able to develop coronary lesions. However it is important to note that IL-17 has been absent since development and it is possible that some compensatory mechanisms have emerged. There have been no studies investigating IL-17 conditional knock-outs or the effects of IL-17 depletion in KD. And while IL-17 may not
be necessary for KD, the elevated levels of Th17 cells and its effector cytokines in KD patients with more severe form of disease point towards a exacerbating role for the IL-17 immune response [118]

Figure 2: The BLK pathway in the development of IL-17 producing γδ T-cells

Global gene profiling revealed that there is an elevation in BLK in γδ T cells compared to conventional αβ T cells. Laird et al. was able to demonstrate several key roles for BLK in the differentiation towards IL-17 producing γδ T cells. 1. BLK controls the proliferative capacity of precursor thymocytes in the DN stage. BLK−/− thymocytes had reduced proliferation, particularly during the DN4 stage. Signaling strength through the αβ or γδ TCR then determines whether T cells differentiate into 2. αβ T cells or 3. γδ T cells. Differentiation at this point did not differ between naïve and BLK−/− mice. 4. The presence of strong γδ TCR signaling downregulates BLK and promotes differentiation into IFNγ producing cells. 5. The mechanism by which BLK promotes IL-17 differentiation is unclear, although BLK−/− mice had significantly lower amounts of IL-17 producing γδ T cells. One hypothesis is that BLK regulates the signaling threshold thus determining whether they differentiate into IL-17 or IFNγ producing γδ T cells. [130]
The Gut and Kawasaki Disease

Several preliminary studies have searched for phenotypic differences within the gastrointestinal tract of Kawasaki disease patients. An analysis of intestinal biopsies from 16 KD patients using immunofluorescent staining found increased numbers of activated T cells (HLA-DR^+CD3^+) and helper T cells (HLA-DR^+CD4^+) (P<0.01), and a decrease in CD8^+ T cells (P<0.05) within the lamina propria. Furthermore, KD patients have been reported to have reduced levels of lactobacillus species. They were identified by morphological, biochemical and gas-chromatographic analysis of cultures isolated from fecal samples. Of the 11 bacterial communities examined, lactobacillus was the only community in KD patients that were different from both febrile and healthy controls. They were detected in only 2 of the 20 KD patients. In a separate study, Yamashiro et al. found 7 species of streptococci and staphylococci in jejunal swabs collected from KD patients that were absent in controls. They were identified by gram staining, morphology, and catalase and oxidase tests. The supernatant from these bacterial cultures were then cultured with PBMCs collected from the subjects. Bacterial specimens harvested from KD patients induced a higher proliferative capacity in the PBMCs and the production of pro-inflammatory cytokines IFN_γ and TNFα. They also had skewing of V_β2 as determined by flow cytometry – suggestive of superantigenic activity. [131] The discovery of IgA plasma cells within the vascular tissue of KD patients also fueled the idea that the gastrointestinal tract could be one potential source/entry point of the environmental trigger in KD. [132] The high incidence of gastrointestinal symptoms and complications in KD [133] and phenotypic variations in microbial and immune composition within the gut support the hypothesis that it may be contributing to the pathogenesis of disease [134].
Animal Models of Kawasaki Disease

Animal models of KD have allowed us to understand and investigate the pathogenesis, etiology and efficacy of treatments in KD. Rabbit, swine, canine and murine models have all been developed. In this study we will use one of the two murine models. One model is the *Candida albicans* water-soluble fraction (CAWS) model of KD. *C. albicans* was found to induce coronary arteritis when injected intraperitoneally (i.p.) in mice. An alkali extract from *C. albicans* consisting primarily of polysaccharides induced arteritis 70% of the time in susceptible strains. This could be increased to 100% by a water-soluble component of the polysaccharides. However, mice had to be repeatedly injected with 4 mg/mouse i.p. for 5 days during the first week. If longer periods of assessment are required, a second dose would be required at week 5. The high dosage and repeated encounters to this agent is a situation that is unlikely in humans that develop KD. The second murine model is the Lactobacillus casei cell wall extract (LCWE) model.

LCWE Murine Model of KD

In 1985, the LCWE model of KD was discovered by Lehman et al. In contrast to the CAWS model, only a single injection of LCWE was required for the induction of coronary arteritis, at much lower concentrations (1mg/mouse). Furthermore, this model has multiple similarities with their human counterparts. First, it follows a similar time course and pathology with coronary inflammation peaking approximately 28 days after induction and elastin breakdown occurring at 42 days. Like in human patients, younger mice are more susceptible to disease and are responsive to IVIG, the standard treatment of care. [135]

In order to prepare LCWE, *L. casei* is harvested, lysed and enzymatically treated to remove adherent material to the cell wall. The cell wall is then fragmented, collected and
injected into young mice to induce disease. Injection of LCWE results in a vast proliferative response and \( \text{V}\beta \) skewing, characteristic of superantigenic activity. [121] Rosenkranz et al. also show using TLR2\(^{-/-}\) mice that LCWE-induced coronary arteritis is dependent on TLR2 signaling. TLR2\(^{-/-}\) mice had lower levels of IL-6, NF\(\kappa\)B activation and coronary inflammation. These results were also revealed to be independent of TLR4. [136] While all the components within this extract have not been elucidated, these results indicate the presence and importance of a superantigen and a TLR2 ligand in this disease model.

The LCWE murine model has revealed many key checkpoints in the pathogenesis of KD that fits with clinical findings. A single intraperitoneal injection of LCWE administered at 4-5 weeks age leads to a robust inflammatory response mediated by T cells.[19] While a subset of these cells undergoes apoptosis, a pathogenic subset persists due to survival signals mediated through co-stimulation. Infiltration of the cardiac tissue by T cells, macrophages and dendritic cells[137] is subsequently followed by the release of inflammatory cytokines: IFN\(\gamma\) [138], IL-1\(\beta\) [118], IL-6 [139], and TNF\(\alpha\). TNF\(\alpha\) induces the production of matrix metalloproteinase-9 (MMP9), which mediates elastin breakdown and the formation of coronary aneurysms. [114, 115, 140] Inflammation of the coronary arteries peaks 28 days after LCWE, with elastin breakdown occurring 42 days later. [114] This corresponds to the kinetics of disease in human patients, wherein aneurysm formation occurs approximately in 4-8 weeks, during the subacute phase. [3] And again, this model has been shown to be responsive to IVIG, the current standard of care in human KD patients, supporting its use in understanding pathogenic mechanisms in disease. [135]
**Lactobacillus species**

The use of LCWE to induce coronary arteritis is intriguing, as species of the genus lactobacillus, as well as *L. casei* are commonly used as a probiotic in humans due to their beneficial effects on health. They are a community of well-characterized rod-shaped, gram-positive bacterium. Not only do they assist in nutrient absorption and gastrointestinal functions, they are reported to have anti-inflammatory effects as well. [141] Administration of *L. casei* in the drinking water of BALB/c mice led to the downregulation of NF-κB translocation and IL-6 production in a model of colitis. The investigators postulate that this may be through TLR2 signaling as DCs and macrophages harvested from Peyer’s patches had increased TLR2 expression during the period of probiotic administration. [142] These anti-inflammatory effects were not only observed within the gut; production of IL-6 by LPS-stimulated PBMCs isolated from humans was also inhibited when cultured with extracts of the probiotic. [143] They have also been shown to promote the production of AMPs and [144] their ability to competitively exclude pathogens from damaging the gut has led to their use as a prophylactic against enteric infections. In contrast to their anti-inflammatory effects, there has also been some evidence showing that lactobacillus species can augment the immune response. This study showed that probiotic-fed mice had macrophages with increased phagocytic activity, and up to an 8-fold increase in circulating antibodies. [142, 145] Furthermore, a retrospective analysis of lactobacillus species in disease by Cannon et al. found the presence of two species commonly found in probiotics – *L. casei* and *L. rhamnosus* – in a large proportion of infections. [146] Regardless of the known beneficial effects of lactobacillus species, it is possible that they may have detrimental effects as well, particularly in certain situations or with certain strains. Most interesting, particularly in the context of KD, is that the LCWE murine model of Kawasaki disease is induced by the cell wall products of *L. casei.*
Decreasing Disease Incidence between Animal Facilities

An interesting observation in the disease incidence of the KD murine model has brought up many questions regarding the role of the environmental factors in KD. In 1985, the Lehman laboratory reported a disease incidence of 69%. A retrospective analysis of disease incidence in this model over a decade, through the progression of several animal housing facilities in Toronto revealed a noticeable decrease in disease incidence. Mice were housed at the animal facility in the University of Toronto (UofT, n=70) from 2002-2005, and disease incidence was reported to be 50%. The UofT animal facility is a conventional animal facility compared to the specific pathogen free status (SPF) of the other facilities and reported the highest disease incidence. In the Hospital for Sick Children (HSC, n=167), from 2004-2011, incidence decreased to 33.5%. Mice were eventually housed at the Toronto Centre for Phenogenomics (TCP, n=95) in 2009, where disease incidence dropped to 26.3%. Established in 2007, this was the newest of the three animal facilities and the use and implementation of new technologies and protocols has allowed for the ability to maintain cleaner and pathogen free environments. During our transfers, mice were taken from previous animal facilities to ensure there were no changes to their genetic background. Pups were C-sectioned or embryonically re-derived to ensure no transfer of bacteria. Despite the lack of changes in genetics and LCWE production, we observed a change in disease – a change associated with a reconstitution of the microbiome during its transfer to a new animal facility (Fig.3).
Figure 3: Decrease in Disease Incidence in Newer Animal Facilities

KD was induced with a single intraperitoneal injection of LCWE at 4 weeks of age. LCWE is produced in-house and each batch is tested for its ability to induce coronary arteritis. Retrospective analysis of disease incidence from 2002-2011 in three animal facilities is represented as a percentage.
RATIONALE

Epidemiological evidence suggests that infectious or environmental agent(s) are involved in the etiopathogenesis of KD. The gastrointestinal tract is a focal point of bacterial interaction in the body. Furthermore, the gut microbiome has been shown to have immunomodulatory effects in the peripheral immune system and plays a critical part in the development of several autoimmune conditions. There is also evidence suggesting a role for the gut in KD; patients often present with gastrointestinal symptoms, preliminary studies have found changes in lymphoid populations and in the microbial composition of the gut. The gut has also demonstrated an important relationship with the Th17 response. Th17 cells and its effector cytokines have been implicated in KD, and many of the downstream effects coincide with pathological features in KD. In a murine model of KD, we have also observed a decrease in disease incidence associated with the transfer to different animal facilities and the composition of the microbiome.
HYPOTHESIS

The gut microbiome modulates the pathogenesis of coronary inflammation in a murine model of Kawasaki Disease.
EXPERIMENTAL AIMS

1. Characterize the gut microbiome in the different animal facilities and determine the association with coronary disease

2. Determine the necessity of the gut microbiome in Kawasaki disease.

3. Determine the role of the candidate microbes, SFB and Lactobacillus species, from the gut microbiome in the pathogenesis of disease

4. Assess the effect of the candidate microbes on the Th17 immune response and its subsequent effects on TNFα, MMP9 and coronary inflammation
MATERIALS AND METHODS

Experimental Mice
Wild-type C57/B6 mice were obtained from The Jackson Laboratory (Bar Harbour, ME) or Taconic Farms Inc (New York, NY) and housed at the Toronto Center for Phenogenomics (TCP) or the Max Bell Animal Research Centre (MBB). Jackson mice were used for all in vivo studies and Taconic mice were used as a source of SFB for co-housing experiments. They were housed under specific-pathogen-free conditions and all procedures were approved by the animal care committee at their respective animal facilities.

LCWE Production
LCWE was prepared following established protocols developed by Lehman et al. [147] Briefly, *Lactobacillus casei* (ATCC 11578) was cultured in Lactobacillus MRS Broth for 18 hours at 37°C. Bacteria was harvested by centrifugation and then lysed in 4% SDS at room temperature for 18 hours. Pellets were thoroughly washed with PBS to remove any remaining SDS and treated sequentially with DNase I, RNAse and Trypsin for 4 hours to remove components adherent to the cell wall. The cell wall was fragmented by sonication at 4°C, centrifuged and the supernatant was harvested to obtain the cell wall fragments. The concentration was quantified by phenol/sulfuric acid colorimetric determination of rhamnose content and aliquoted at a final concentration of 1 mg/mL in PBS. LCWE is then frozen at -20°C until use.

In Vivo Studies
Female mice were injected with 1mg of LCWE or 1 mL of PBS at 4-5 weeks of age. Modulation of the microbiome through a cecal gavage or with antibiotics was conducted at 3 weeks of age, prior to LCWE injection. To assess disease incidence, hearts were harvested 28 days post injection in formalin. Sections of the coronary arteries were prepared and histological staining
with hematoxylin & eosin (H&E) was used to assess for inflammation and lymphocyte infiltration. To determine expression of IL-17, various organs were harvested 3, 7, 14, 21 and 28 days post injection and snap frozen at 80°C until use.

**Fecal Collection and DNA Extraction**

In preliminary experiments, mice were euthanized with CO2 as per protocol, colons exposed surgically and fecal contents extracted. In subsequent experiments samples were collected directly from the anus of live mice in order to assess multiple time points. Samples were snap frozen on dry ice and stored at -80°C until use. Fecal bacteria were lysed by bead-beating for 4 min at 30 Hz, following an optimized protocol developed by Smith et al. (TissueLyser II, Qiagen, Toronto, ON) [148]. DNA was extracted using the QiaGen Mini-stool extraction kit (QiaGen, Toronto, ON) according to the manufacturer’s instructions.

**qPCR Analysis of Microbiome**

qPCR was used to assess specific bacterial communities in the microbiome with immunomodulatory capabilities. (Bacteroides, Lactobacillus, Clostridia, Bifidobacteria, Enterobacteriaceae and SFB) These communities are representative of the four major phyla and provide a simplified perspective on the composition of the microbiome. qPCR was run using Power SYBR Green (Applied Biosystems) and primers (ACGT Corporation, Toronto, ON) selected utilizing previous published protocols [149, 150] in the ABI 7900HT (Applied Biosystems). *(See Table 2)*
Table 2: Bacterial Primers for qPCR

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<tr>
<th>Gene Name</th>
<th>FW Primer Sequence</th>
<th>RV Primer Sequence</th>
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<td>ATTACCGGGCTGCTGGGC</td>
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<tr>
<td>SFB 16S</td>
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</table>

**RNA Extraction**

Organs were harvested (Heart, Axillary LN, Mesenteric LN, Spleen, Colon) and snap frozen in dry ice and stored at -80°C until use. Samples were homogenized in Trizol reagent (Life Technologies) using the PT1200 Homogenizer (Kinetmatica, Bohemia, NY). The organ-Trizol suspension was centrifuged at 13,000 rpm for 10 minutes and the supernatant was collected into a new tube. Two chloroform (Sigma) extractions were performed and RNA was precipitated using isopropanol (Sigma). The supernatant was discarded following centrifugation (13,000 rpm, 10 min) and the RNA pellet was washed with 70% cold ethanol and re-suspended in 0.01% DEPC H₂O. RNA concentrations were determined using spectrophotometry.

**qPCR Analysis of mRNA Expression**

Complimentary DNA was synthesized from RNA using murine leukemia virus reverse transcriptase (Applied Biosystems) and the GeneAmp RNA PCR kit. Converted cDNA was assessed in triplicate using primers and probes for GAPDH, IL-17A, and MMP-9 (Assays-on-
demand, Applied Biosystems). A standard curve was generated using cDNA from a wild type mouse. Values were compared to a standard curve and normalized to the expression of GAPDH.

**Microbiota Transfer**
Transfer of MBB microbiota into TCP mice was performed by oral gavage with 200ul of cecal contents weekly starting at 3-4 weeks of age. Control mice were gavaged with homogenates from TCP mice. Briefly, cecal contents from 10 mice were harvested and pooled in 50mL of PBS at TCP and MBB. Following homogenization of the cecal suspension, they were snap frozen at 80°C in 1 mL aliquots before use. Two aliquots were sent to Charles River Research Animal Diagnostic Services to screen for murine pathogens using the Mouse Surveillance Plus PCR Rodent Infectious agent (PRIA) Panel.

**Antibiotic Treatment**
Ampicillin (Fisher Scientific, 1g/L) and Vancomycin (Acros Organics, 0.5 g/L) was administered through the drinking water at 3 weeks of age following established protocols [151]. The antibiotic solution was freshly prepared and replaced every week. Fecal pellets were collected and quantification of bacterial DNA in the feces was used as a surrogate for bacterial presence in the gut.

**Proliferation Assay**
Mice were euthanized by CO2 and splenocytes were harvested in sterile 1X PBS and plated at 1x10^6 cells/ml in 96 well plates. Cells were cultured in IMDM Culture media (Lonza) supplemented with 10% heat-inactivated FBS, 1mM sodium pyruvate, 2mM of L-glutamine, 0.1 mM of non-essential amino acids, 10mM HEPES and 50uM β-mercaptoethanol. Splenocytes left untreated or stimulated with LCWE (2ug/mL) together with ampicillin (Fisher Scientific),
vancomycin (Acros Organics), neomycin (Sigma) and metronidazole (MP Biomedicals) with doses ranging from 5 to 500 ug/mL. 1 μCi of Thymidine ($^3$H) was added for 18 hours and then analyzed for $^3$H incorporation 3 days post LCWE addition.

**ELISA**
Mice were euthanized by CO$_2$ and splenocytes were harvested in sterile 1X PBS and plated at 1x$10^6$ cells/ml in 24 well plates. Cells were cultured in complete IMDM (Lonza) and were left untreated or stimulated with LCWE (10ug/ml) together with ampicillin (Fisher Scientific), vancomycin (Acros Organics), neomycin (Sigma) and metronidazole (MP Biomedicals) with doses ranging from 5 to 500 ug/mL for 24 hours. Supernatant was collected and assayed for TNF$\alpha$ using an enzyme-linked immunosorbent assay (ELISA) kit as per the manufacturer’s protocol. (TNF$\alpha$ Ready-Set-Go kit, eBioscience) IL-17 ELISA was performed on serum harvested from mice 28 days after treatment with LCWE as per the manufacturer’s protocol. (IL-17A Platinum kit, eBioscience)

**Cell Culture (MOVAS)**
The MOVAS cell line is derived from murine vascular aortic smooth muscle cells [152]. MMP9 is produced by vascular smooth muscle cells in response to TNF$\alpha$. To assess the effects of antibiotics on MMP-9 production, MOVAS cells were plated at 2.5 x$10^5$ cells/ml in 12 well plates. They were seeded for 18 hours in complete DMEM (Lonza). MOVAS cells were left untreated or cultured with recombinant TNF$\alpha$ (eBioscience, 20ng/mL) plus a range of antibiotics (Ampicillin, Vancomycin, Neomycin, Metronidazole) from 5 to 100 ug/mL for 6 hours. Cells were harvested in Trizol, and RNA isolation, cDNA conversion and qPCR was performed as previously described.
Flow Cytometry
Mice were euthanized by CO₂, and splenocytes were harvested in sterile 1X PBS and cultured for 9 days in complete IMDM untreated, with LCWE (2ug/ml) SEB (0.3ug/mL) or a CD3 agonistic antibody. Media was replaced with IMDM and CD3 agonist (Clone 145-2C11, BD Pharmingen, 1ug/mL), IL-23 (R&D Systems, 10ng/mL) and IL-2 (eBioscience, 5ng/mL) on day 5 to promote the expansion and survival of the Th17 subset. Media was replaced again on day 7 with IMDM and IL-2 (5ng/mL). On day 9, cells were stimulated with PMA, Ionomycin and Golgistop for 6 hours and harvested for flow cytometry. Viable cells were quantified using standard trypan blue exclusion and 1 x 10⁶ cells/tube were used for immunostaining. Non-viable cells were stained with Fixable Viability Dye eFluor®450 (eBioscience). Cells were washed in staining buffer and stained CD4-FITC (Clone GK1.5, BD Biosciences). Cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and then stained with IL17-APC (Clone TC11-18H10.1, Biolegend). Stained cells were re-suspended in staining buffer and acquired using a flow cytometer. (LSRII, BD Biosciences)

Statistical Analysis
Statistical significance was determined by paired or unpaired student’s T test for experiments at a single time point or a two-way ANOVA for experiments conducted over several time points using GraphPad Prism 5. Disease incidence was assessed using a χ² test. Values are generally expressed at mean +/- standard error of measurement. P values less than 0.05 were considered statistically significant.
RESULTS

Marked differences in expression of bacterial communities between MBB and TCP

To determine the variations between the microbiome between animal facilities, fecal samples were collected from mice aged 7-11 weeks from four different animal facilities in Toronto – Ontario Cancer Institute (data not shown), The Hospital for Sick Children (data not shown), Toronto Centre for Phenogenomics (TCP, n=15) and MaxBell Animal Research Centre (MBB, n=14). These communities are expressed as a percentage of all Eubacteria present within the samples collected. qPCR analysis of stool samples collected from MBB showed a marked elevation in Bacteroides (P=0.0448), Bifidobacteria (P=0.0082), Lactobacillus (P=<0.0001), and SFB (P=0.0005). The population of Clostridia (P=0.0104) was significantly higher at TCP. (Fig. 4) Because bacterial expression has been demonstrated to vary with age, we collected and assessed bacterial communities in age and sex matched mice at TCP and MBB (n=5) during the period of the disease model (4-8 weeks old). While there is variation in microbial expression, SFB was the only community where proportions significantly decreased over time (P=0.001). Additionally, the same communities remained significantly different in these matched mice. It is also interesting to note that SFB was consistently found at MBB and below the detection limit at TCP (Fig. 5). Recent studies have demonstrated a potential role of this bacterial community in the pathogenesis of multiple autoimmune conditions. In conclusion, there observations indicate that the location in which mice are housed lead to significant differences in the composition of the microbiome.
Figure 4: Marked differences in expression of bacterial communities between MBB and TCP

Fecal samples were collected from mice aged 7-11 weeks from four different animal facilities in Toronto – Ontario Cancer Institute (data not shown), The Hospital for Sick Children (data not shown), Toronto Centre for Phenogenomics (TCP, n=15) and MaxBell Animal Research Centre (MBB, n=14). These communities are expressed as a percentage of all Eubacteria present within the samples collected. Statistical significance was determined by the student's unpaired T-test. A * denotes a P value <0.05.
Figure 5: Microbial communities do not vary significantly with the exception of SFB

Bacterial expression in aged-matched, untreated mice (n=5) at TCP (unbroken line) and MBB (dashed line) are expressed as a percent change from d0 to assess changes in microbial expression with the exception of SFB. SFB is expressed as a percentage of all Eubacteria as it was completely absent from TCP. Statistical significance was determined using a two-way ANOVA. A * denotes a P value <0.05.
**Disease incidence and severity is higher at MBB after injection with LCWE**

To determine the effects of the microbiome on disease, mice (n=5) from MBB and TCP received an intra-peritoneal injection with LCWE or PBS at 4 weeks of age and assessed for disease 28 days later. The presence and extent of lymphocytic infiltration in the coronary arteries were used to evaluate disease incidence and disease severity. Samples were evaluated by a blinded analysis of histological inflammation of the coronary arteries. Both disease incidence (P=0.0384) and severity (P=0.0006) were significantly higher at MBB. *(Table 3, Fig. 7)* These observations correlated with significantly higher proportions of several microbial communities within the gut at MBB. *(Bifidobacteria: P=0.0472, Lactobacillus: P=0.0067, SFB: P=0.0092)* *(Fig.6)*. Again, SFB was consistently found at MBB at all time points while remaining below detectable limits at TCP. This data suggests that mice housed at MBB are more susceptible to the development of coronary arteritis and that this susceptibility may be due to the increased proportions of Bifidobacteria, Lactobacillus and SFB.
Figure 6: SFB colonize the gut of susceptible mice housed at MBB

Mice (n=5) from MBB (dashed line) and TCP (unbroken line) were injected i.p. with LCWE or PBS at 4 weeks of age and assessed for disease 28 days later. Expression of bacterial communities was also measured through the collection of fecal samples throughout the experiment. Three communities of bacteria remained significantly different (Bifidobacteria-P=0.0472, Lactobacillus-P=<0.0067, SFB-P=0.0092). Of note are the SFB, which was consistently found at MBB at all time points while remaining below detectable limits at TCP. Levels of SFB also remained stable and did not decrease significantly over time. A * denotes a P value <0.05.
Figure 7: Disease incidence and severity is higher in MBB after LCWE induction

Mice (n=5) from MBB and TCP were injected i.p. with LCWE or PBS at 4 weeks of age and assessed for disease 28 days later. Statistical significance in disease incidence was assessed by a $\chi^2$ test. Disease severity was scored by coronary infiltration and significance was determined by a student’s unpaired t-test. A * denotes a P value <0.05.
Table 3: Disease incidence after modulating the microbiome

Mice were either injected with LCWE or PBS at 4 weeks of age. This table depicts the number of mice that developed disease over the total number treated under several conditions. Disease was compared between MBB and TCP, mice treated or untreated with antibiotics and mice that received a cecal gavage from susceptible mice or from donors at the same facility.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SFB</th>
<th>LCWE Injection</th>
<th>PBS Injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBB</td>
<td>+</td>
<td>5/5</td>
<td>1/5</td>
</tr>
<tr>
<td>TCP</td>
<td>-</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>No Antibiotics</td>
<td>+</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>-</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>MBB Cecal Gavage</td>
<td>+</td>
<td>10/11</td>
<td>0/11</td>
</tr>
<tr>
<td>TCP Cecal Gavage</td>
<td>-</td>
<td>6/10</td>
<td>0/9</td>
</tr>
</tbody>
</table>
Antibiotic treatment does not alter key immunological parameters in KD

In order to determine the necessity of the microbiome, we used antibiotics to decontaminate the gut. To ensure that broad spectrum antibiotics (Ampicillin, Vancomycin, Neomycin, and Metronidazole) were not confounding our analysis of disease pathogenesis, we evaluated their effects on key immunological parameters in vitro. The key steps in the pathogenesis of KD start with a massive immune activation in response to an infectious trigger. This is followed by an increase in levels of pro-inflammatory cytokines such as TNFα, leading to the production of MMP-9 by smooth muscle cells and resulting in elastin breakdown, vascular injury and the formation of coronary aneurysms.

To determine the effects of antibiotics on T-cell proliferation in response to LCWE, we measured thymidine incorporation in splenocytes that were cultured for 3 days in 5ug/ml of LCWE in the presence of a broad concentration of the antibiotics in question. LCWE induced a proliferative response, however we did not observe a significant difference between those cultured with, or without antibiotics in the dose range corresponding to those used in vivo. (Fig. 8A)

To determine the effects of antibiotics on TNFα production, we measured TNFα levels using an ELISA on supernatant collected from splenocytes cultured with 5ug/ml of LCWE +/- antibiotics for 24 hours. Similarly, LCWE induced the production of TNFα within 24 hours, but again, we did not observe any significant difference in cells cultured with antibiotics (Fig. 8B).

In order to determine the effects of antibiotics on MMP-9 production, we assessed its mRNA expression in Mouse Vascular Smooth Muscle Cells (MOVAS) stimulated with TNFα +/- antibiotics for 6 hours using qPCR. TNFα induced the production of MMP-9 in MOVAS. There were no significant differences (Fig. 8C).
Antibiotic administration has been associated with a bitter flavor and may lead to slower weight gain in mice. To ensure that treated mice were not in a weakened state, their weight was monitored as a sign of health. Although, mice treated with antibiotics had a reduction in weight gain (P=0.0010), they remained within the expected range for healthy mice. (Fig. 9).

Therapeutic levels of these antibiotics range from 2-40 ug/ml in the serum. These experiments serve to verify that even at doses beyond those normally used in vivo, antibiotics do not affect key steps in the pathogenesis of KD. These antibiotics have a half-life of up to 12 hours. While their effects may diminish during the course of these experiments, their administration during the initial stimulations indicate that there are no long-term effects. Additionally, vancomycin and neomycin do not cross the gastrointestinal lining and are should not have physiological effects when administered orally. Thus, the use of antibiotics in vivo should not have direct effects on the immune response in KD, and any observed changes are expected to be due to the depletion of the microbiome.
Figure 8: Antibiotics do not have an effect on LCWE induced proliferation, TNFα production and MMP-9 expression

The effects of antibiotics were tested on several key immunological parameters in LCWE-induced KD to ensure observed effects were due to gut decontamination. **A** Splenocytes were cultured for 2 days with LCWE and varying doses of antibiotics (Ampicillin, Vancomycin, Neomycin, and Metronidazole). Thymidine was added for 16 hours and proliferative capacity was assessed by $^3$H incorporation. **B** Splenocytes were cultured with LCWE and varying doses of antibiotics (AVNM) for 24 hours. Supernatant was harvested and assessed for TNF-α by ELISA. **C** MOVAS were cultured for 6 hours with recombinant TNF-α and varying doses of antibiotics. Cells were harvested and RNA was collected in Trizol. MMP9 mRNA was assessed using qPCR. Statistical significance for all experiments was determined using the paired student’s t-test.
Figure 9: Antibiotic treated mice maintain a healthy weight

The weight of mice was monitored to ensure antibiotic administration did not affect water intake or cause a significant detriment to health. While mice treated with antibiotics had a slower weight gain than untreated ones, they were within the standard range of weight for healthy mice.

Antibiotics successfully reduce bacterial diversity within the gut

To assess depletion of microbiota within the gut, the amount of DNA extracted from the feces was used as a surrogate measure of bacterial content within the gut. We administered ampicillin (1mg/ml) and vancomycin (0.5 mg/ml) in the drinking water of mice and collected fecal pellets at several time points. Antibiotic treatment was refreshed every 7 days, and administered for the entire 28-day duration of the study. Thus, samples were collected at d3, d6 and d28 to ensure continued depletion of the microbiome. (Fig. 10A) Antibiotic treated mice had a significant reduction of bacterial content in the gut. Furthermore, the efficacy did not appear to diminish, and despite the slight increase in bacterial content by d28, the levels observed were still significantly lower than untreated mice. The composition of the gut microbiome was also assessed by qPCR. There was a significant reduction in all the bacterial communities assessed.
after treatment with antibiotics. Thus, antibiotic administration results in a significant reduction in bacterial content and diversity within the gut, including the elimination of SFB (Fig. 10B).

**Antibiotic treatment reduced disease incidence and severity**

Having established that antibiotics were sufficient to deplete the microbiome, we assayed their effects on disease development. Coronary arteritis was induced with LCWE as per protocol and mice sacrificed at day 28 for histologic assessment. An asymmetric infiltration can be observed by day 3 post injection, and is primarily comprised of lymphocytes. The infiltrate starts at the adventitia and migrates inward towards the intima. Disease is characterized by coronary arteritis and reaches a maximum 28 days post injection, and has been shown to persist for up to 90 days. [115] In our preliminary results, only 2/5 mice that were treated with antibiotics developed coronary arteritis, compared to the 5/5 that were untreated. Antibiotic decontamination of the gut reduced susceptibility to LCWE-induced disease (P=0.038) (Table 3) and the severity of coronary infiltration (P=0.0203) (Fig. 11). The microbiome may not be necessary for the pathogenesis of disease, as coronary arteritis was still present following antibiotic mediated depletion of the gut microbiome. However, we still observed a significant reduction, suggesting that the microbiome has a role in increasing disease susceptibility or in exacerbating the pathology of disease.
Figure 10: Antibiotics led to significant changes in microbiome diversity and reduced microbial content

Mice were gavaged with the cecal contents of a MBB donor to colonize the mice with SFB. Antibiotics (Ampicillin, 1mg/mL, Vancomycin, 0.5 mg/mL) were administered in the drinking water 2 days after the gavage. LCWE was injected i.p. at 4-5 weeks of age and fecal pellets were collected and assessed for microbial content. Treatment was changed every 7 days. A untreated wild-type mice were treated with antibiotics and fecal samples were collected at day 3 and day 6. DNA in fecal samples is used as a surrogate for microbial content. B Mice were injected with LCWE and were treated with antibiotics. Fecal samples were collected 28 days later prior to sacrifice. Colonization was assessed at d28 to negate the possibility of gut re-colonization during antibiotic treatment. Antibiotic treated mice had significantly lower levels of fecal DNA. C Fecal samples collected 28 days post-LCWE were assessed for microbial content. There are significantly lower levels in all microbial communities in antibiotic treated mice.
Figure 11: Antibiotic decontamination of the gut microbiome diminishes incidence and severity of coronary arteritis

Mice (n=5) were gavaged with the cecal contents of a MBB donor to colonize the mice with SFB. Antibiotics (Ampicillin, 1mg/mL, Vancomycin, 0.5 mg/mL) were administered in the drinking water 2 days after the gavage. LCWE was injected i.p. at 4-5 weeks of age. Treatment was changed every 7 days. Mice treated with antibiotics had a significant reduction in disease incidence (P=0.038) and severity (P=0.0203).
Cecal gavage transfers disease susceptibility

Mice housed at MBB appeared to be more susceptible to disease compared to their TCP counterparts. In this study, it was shown that ablation of the microbiome using antibiotics could reduce disease susceptibility. To determine whether members of the microbiome in MBB mice were responsible for the increased disease susceptibility, we transferred the cecal contents of MBB mice into the mice housed at TCP.

Cecal contents from MBB and TCP were collected and pooled into 50 mL of PBS (n=10), aliquoted and frozen until use. These samples were screened by the Charles River Research Animal Diagnostic Services for murine pathogens to ensure that only commensal microorganisms would be transferred. (See Table 4) Mice at TCP were gavaged with 200ul/week of cecal contents starting at 3-4 weeks of age until sacrifice. Bacterial DNA was harvested from three aliquots of cecal content and assessed. The largest differences in the cecal contents from these two animal facilities were observed in Bacteroides, Bifidobacteria, Lactobacillus and SFB (Fig. 12). These communities were then monitored in the fecal samples of the treated mice and expressed as percentage of Eubacteria. Cecal gavage resulted in observed increases in SFB (P=0.0004), and Bifidobacteria (P=0.0168) within MBB-gavaged mice (Fig. 13). SFB was undetected at TCP before the gavage, and it was only found in mice that received a gavage from a MBB donor. These results are representative of two independent experiments.

Mice were sacrificed 28 days after LCWE disease induction and presence and severity of coronary inflammation determined by blinded histological assessment. Results compiled from two independent experiments revealed a trend for increased disease incidence (P=0.0967) and severity (P=0.0926) after a MBB gavage (10/11) compared to a TCP gavage. (6/10) (Table 3, Fig. 14) These observations show that the increased disease susceptibility found in mice housed
at MBB may be transferrable, and that it is likely due to factor(s) within the microbiome.

Furthermore, it is probable that this factor is the SFB; SFB is consistently elevated in susceptible mice, it is one of only two communities measured that increased in response to a cecal gavage, and its pro-inflammatory effects and role in autoimmunity has been well established.

<table>
<thead>
<tr>
<th>Murine Pathogens Tested - Mouse Surveillance Plus PRIA (Charles River)</th>
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<tbody>
<tr>
<td>MHV PCR</td>
</tr>
<tr>
<td>MPV/MVM PCR</td>
</tr>
<tr>
<td>MRV (EDIM) PCR</td>
</tr>
<tr>
<td>MNV PCR</td>
</tr>
<tr>
<td>TMEV/GDVII PCR</td>
</tr>
<tr>
<td>MAV 1 &amp; 2 PCR</td>
</tr>
<tr>
<td>Beta Strep Grp B PCR</td>
</tr>
<tr>
<td>Beta Strep Grp C PCR</td>
</tr>
<tr>
<td>Beta Strep Grp G PCR</td>
</tr>
<tr>
<td>B. bronchiseptica PCR</td>
</tr>
<tr>
<td>Campylobacter Genus PCR</td>
</tr>
<tr>
<td>CAR Bacillus PCR</td>
</tr>
</tbody>
</table>

Table 4: Murine Pathogens tested at Charles River Research Animal Diagnostic Services

Two aliquots of the cecal contents harvested from MBB mice were sent to Charles River for pathogen testing. Mouse Norovirus (MNV), *P. pneumotropia-Heyl*, *S. xylosus* were detected, but deemed to be acceptable for gavage into TCP.
Figure 12: Variations in the cecal contents between mice from MBB and TCP

Cecal contents from MBB and TCP were collected and pooled into 50 mL of PBS (n=10), aliquoted and frozen until use. Bacterial DNA was harvested from cecal contents and assessed. The largest differences in the cecal contents from these two animal facilities were observed in Bacteroides, Bifidobacteria, Lactobacillus and SFB species.
Figure 13: Transfer of SFB through cecal gavage

Mice at TCP (n=5-6) were gavaged with 200 ul of cecal contents starting at 3-4 weeks of age until sacrifice. Fecal samples were collected from mice that were gavaged with cecal contents from a MBB or a TCP donor and assessed for the 4 different bacterial communities that had the largest variation. Proportions of SFB and Bifidobacteria were significantly higher after gavage. Statistical significance was determined using a two-way ANOVA.
Figure 14: Cecal gavage from MBB trended towards increased disease incidence

Mice housed at TCP were gavaged at 3 weeks of age, and each subsequent week thereafter. They were injected with LCWE i.p. at 4-5 weeks of age, and sacrificed 28 days later. The difference in disease incidence (P=0.0967) and severity (P=0.0926) approached significance and appears to be elevated in MBB treated mice.
**IL-17 levels in serum are elevated in SFB colonized mice**

SFB has been shown to have a crucial role in the Th17 immune response, and there is evidence supporting a role for a Th17 immune response in KD. Furthermore, our previous data has revealed a strong correlation with coronary arteritis and SFB colonization within the gut. Knowing that SFB colonization is associated with an elevation in IL-17, we measured serum levels of IL-17 using an ELISA. IL-17 was found at significantly higher levels in mice housed at MBB over those housed at TCP (P=0.0013). IL-17 trended higher in mice gavaged with cecal contents from a MBB donor, albeit non-significant (P=0.0676) (Fig. 15). In both conditions, this observed change was independent of LCWE treatment. This suggests that changes in serum levels of IL-17 are in response to changes in the microbiome, particularly the colonization of SFB, due to its consistent elevation in these samples and its reported capability to induce Th17 differentiation. However, LCWE does not appear to trigger the production of IL-17, and that any changes in disease because of IL-17 are due to a basal increase in response to SFB.

![Figure 15: Serum levels of IL-17 are elevated in SFB colonized mice](image)

Serum was harvested from mice 28 days post-LCWE and IL-17 was assessed by ELISA. A Mice were housed at MBB or TCP B Mice were gavaged with SFB at 3-4 weeks of age. Both were injected with LCWE at 4-5 weeks of age.
**IL17 expression is elevated after injection of LCWE**

Serum levels of IL-17 do not appear to be dependent on LCWE treatment, however preliminary studies in humans show that the elevation in IL-17 is short and occurs only during the acute phase of disease. We hypothesized that a response by IL-17 to LCWE may be occurring at an earlier time point. Thus, to evaluate the role of the Th17 immune response in this model of KD, coronary arteritis was induced with LCWE as per our protocol and mice were sacrificed at various time points until d28. Mesenteric lymph nodes, axillary lymph nodes, spleen, colon, heart and serum were collected. IL17 mRNA expression was assessed by quantitative RT-PCR in the organs to determine the kinetics of the Th17 immune response in KD. IL17 was significantly elevated at d14 (P=0.0293) and d28 (P=0.0103) in the axillary lymph nodes (ALN). (Fig. 16A). In the spleen, there was a significant increase at d7. (P=0.0493) The expression of IL-17 mRNA in the spleen of PBS injected mice were below detection limits while low levels were detected in LCWE injected mice at several time points (Fig. 16C). Similar to the results found in the ALN, the levels of IL-17 mRNA in the colon are higher at d14 and d28, although not statistically significant (Fig. 16D). Levels of IL-17 mRNA in the heart were below detection limits. An enzyme linked immunosorbant assay (ELISA) was used to measure IL-17 protein levels in the serum. There were no significant differences in IL-17 in the sera of LCWE injected mice compared to PBS controls (Fig. 17).

The role of IL-17 in this model of KD is unclear. Protein levels of IL-17 do not change in response to LCWE, however, there is a significant increase in IL-17 mRNA production in the ALN and in the spleen. The fact that there is an elevation in the lymph nodes draining from the heart may be pointing to an immune response of cardiac origin. This increase appears to be brief, which may suggest that the IL-17 response to LCWE, if any, is short-acting. This can also
explain the absence of change in serum concentrations as the production of IL-17 protein may be localized. Further experiments need to be performed to characterize the kinetics and localization of IL-17 during development of coronary arteritis.

Figure 16: IL-17 mRNA expression increases in the draining axillary lymph nodes at d14 and d28

SFB-negative mice were injected with LCWE at 4 weeks of age and organs were harvested at several time points. IL-17 mRNA expression was assessed using qPCR to determine the kinetics of IL-17 in the pathogenesis of KD. IL-17 was significantly elevated in the ALN at day 14 and 28, and a similar trend appeared in the colon. IL-17 was only detected in LCWE treated mice, and only at day 3, 7 and 28 post LCWE injection.
SFB-negative mice were injected with LCWE at 4 weeks of age and serum was harvested at several time points. Serum levels of IL-17 were measured using an ELISA. There was no significant difference between untreated mice and mice treated with LCWE.

Colonization by SFB does not alter response to LCWE in vitro

SFB has been shown to have immunomodulatory abilities, and in this study, has been associated with an increased susceptibility to disease. Thus, to assess the immune response to LCWE in cells harvested from SFB-positive mice, we first colonized mice with SFB. Ivanov et al. showed that cohousing SFB negative mice with SFB positive mice resulted in the horizontal transfer and colonization of SFB [103]. As an alternative to gavage, mice were cohoused for 5 days with a SFB-colonized host, or not cohoused at all. Within 5 days, mice that were cohoused were colonized with SFB, in contrast to mice that were not cohoused (Fig 18A).
To determine if SFB colonization increased the proliferative capacity of splenocytes to LCWE, splenocytes were harvested and cultured for 3 days at $5 \times 10^5$ cells/ml with incremental doses of LCWE and proliferation. We did not observe a difference between splenocytes obtained from SFB positive and SFB negative mice. (Fig 18B) SFB has been strongly associated with the Th17 immune response. We decided to assess if SFB colonization led to an increased capacity to differentiate into Th17 cells. Splenocytes were left untreated, or cultured with LCWE, SEB, or a CD3 agonistic antibody. The Th17 cell population was selectively allowed to expand using a combination of IL-23, IL-2 and anti-CD3. Cells were gated for CD4$^+$ and IL-17$^+$ expression to assess the Th17 population. The Th17 immune response does not vary significantly due to SFB colonization; however we did observe an increase of Th17 cells in response to LCWE compared to SEB (Fig 18C). Thus, it would appear that SFB colonization does not lead to long-term effects on peripheral splenocytes, and that their effects in vivo require a constant interaction in the gut.
Figure 18: Splenocytes from SFB-colonized mice do not have an altered response to LCWE in vitro

As an alternative to cecal gavage, mice were colonized with SFB through co-housing with a SFB-colonized donor. A Within 5 days of co-housing, horizontal transfer of SFB led to colonization in previously uncolonized mice. B Splenocytes were harvested and cultured for 2 days with LCWE. \(^3\)H was then added for 16 additional hours and proliferation was assessed with \(^3\)H incorporation. C Splenocytes were harvested from SFB–colonized or non-colonized mice. They were cultured for 5 days with LCWE, SEB, agonistic CD3 antibody or media. The Th17 population was selectively expanded for 4 more days. Cells were then stimulated with PMA and ionomycin for 6 hours and stained for CD4 and IL-17 expression for flow cytometry.
DISCUSSION

The gut microbiome modulates the pathogenesis of Kawasaki Disease

Epidemiological evidence in humans supports the notion of an environmental factor in the pathogenesis of disease. Furthermore, observations in the LCWE murine model of KD may also allude to an immunomodulatory role for environmental agents. For example, several groups using the LCWE murine model of KD have reported differences in disease incidence. [114, 118, 147] Although these observations are not controlled, our laboratory has also reported a change in disease incidence corresponding to different animal facilities. (Fig. 3) Of the many possibilities to interact with environmental antigens, the gastrointestinal tract is the largest interface between microbial factors and their host. Furthermore, it has been demonstrated to have important roles in immune development and autoimmunity. The substantial and growing evidence of the capability of gut microbes to modulate the peripheral immune response has led to this investigation of the role of the gut microbiome in modulating the pathogenesis of KD.

While the notion that the gastrointestinal tract is important in the pathogenesis of KD is not new, its role remains unclear. Previous theories have postulated that the gastrointestinal tract is the source for the missing etiologic trigger in KD – either as a microbe or microbial toxin, but again, no one trigger has been consistently present. [131, 153, 154] Elevation of IgA in the vasculature of KD patients garnered some support for the notion of gastrointestinal origins for an etiologic cause, although further evidence pointed towards a respiratory portal of entry instead. [132, 155] Nevertheless, the hypothesis that the gastrointestinal tract is the etiologic cause and source for KD remains uncertain.

In this study we propose an alternative theory: the microbiome has a modulating or contributory role in the pathogenesis of disease. Our initial findings between two animal
facilities reveal significant differences in disease incidence and severity correlating with marked variations in the composition of the microbiome, consistent with this hypothesis. Animal models of EAE and arthritis were demonstrated to be protected under GF conditions, while diabetes was exacerbated. Furthermore, specific communities of microbes have been shown to alter immune development and the peripheral immune response. We tested the effects of depleting the microbiome through the use of broad spectrum antibiotics prior to disease induction and observed a marked decrease in disease incidence and severity. This suggests that the microbiome is necessary to exacerbate disease and increase disease susceptibility. To further validate this hypothesis, we conducted a cecal gavage to ascertain whether the microbiome at MBB was responsible for the observed differences in disease incidence and severity. We introduced the cecal contents of susceptible mice (MBB) into the gastrointestinal tracts of mice that were not as susceptible (TCP). While the difference was not significant, there was an observable trend towards increased disease incidence. This data provides convincing evidence that the microbiome is modulating the pathogenesis of Kawasaki Disease (Table 3).

Members of the microbiome have demonstrated both protective and detrimental effects in the context of disease. [63, 84, 156] It is unclear whether an exacerbating or moderating agent is acting upon the pathogenesis of KD. However, our use of antibiotics to mediate widespread depletion of the gut and its subsequent reduction in disease incidence makes the notion of a protective microbe within the gut less likely. We observed massive reduction of microbial diversity and content, suggesting that any protective microbes would have been removed. Thus, in the scenario a protective agent was eliminated, we should have observed an increase in disease incidence, as opposed to decrease that did occur. This suggests that the increase in disease
incidence conveyed by the cecal gavage, and the decrease conveyed by antibiotic treatment, was most likely due to an exacerbating agent.

**Bacterial communities in the pathogenesis of KD**

Specific microbial communities have been sufficient to modulate the peripheral response and may even be necessary for the development of autoimmunity. [63, 84] In order to assess the effects of specific microbial communities, we started with a broad assessment of the microbiome by evaluating the proportions of the four major phyla found within the gut – Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria. However we did not observe significant variations, thus we decided to narrow our search by evaluating 7 bacterial communities further down the taxonomic tree – Bacteroides, Bifidobacteria, Lactobacillus, Clostridia, Enterobacteriaceae, and SFB. These communities were selected for their reported roles in immune development, autoimmunity, and their ability to modulate the peripheral immune response. Additionally, this group contains at least one representative from each of the major phyla found within the gut. By focusing our search, we observed several trends. In untreated mice at TCP, there was an elevation in the proportion of Clostridia, (Fig. 4) however, it fell to levels comparable to those at MBB after LCWE injection. Because Clostridia species are generally known to be anti-inflammatory, it is possible that the inflammatory milieu induced by LCWE is altering the gut composition. Consistent with this theory, the proportion of SFB, which was shown to decline with age in naïve mice, stayed at relatively high and consistent levels after injection with LCWE. (Fig.5, Fig.6) Not only have changes in the microbiome been shown to mediate changes in the periphery, the reverse has been shown as well. Animal models of acquired immunodeficiency syndrome (AIDS) and liver disease, where pathology is induced, revealed distinct changes in the microbiome and increased intestinal permeability. [157, 158] In
addition to the initial effects mediated by the microbiome, it is possible that inflammation perpetuates a dysbiosis within the gut that feeds back into disease exacerbation. The hypothesis where the cycle between gut dysbiosis and aberrant immune activation sustains disease is one theory for the continuous inflammation in IBD, and it may a potential mechanism by which it exacerbates disease in this model. [156]

Interestingly, of all the bacterial communities assessed in this study, SFB and Lactobacillus were consistently elevated at MBB. In fact, SFB was only found at MBB – it was undetectable at TCP. These observations are of particular relevance as lactobacillus is the genus wherein the disease inducing agent of this animal model, LCWE, is derived. Perhaps characteristics of this genus within the intestinal tract facilitate the progression of disease. On the other hand, SFB is known for its pro-inflammatory effects, its role in the Th17 response, and has been reported to be essential in several models of autoimmunity. The Th17 has also been implicated in KD. Thus, we decided to focus on these particular communities and investigate their role and the potential mechanisms by which they could be modulating disease.

**SFB in the pathogenesis of KD**

Germ-free mice have been shown to be protected against several models of autoimmunity. The re-introduction of SFB was demonstrated to be able to exacerbate or restore disease. [83, 84, 87] In the K/BxN model of arthritis, mono-colonization of SFB was sufficient to restore the development of disease that was ameliorated in GF mice. [86] Similarly, SFB re-colonization was found to be able to overcome the protection conveyed by GF status in EAE. [83] Here, we show that mice colonized with SFB at MBB are more susceptible to disease than the SFB negative population at TCP. Furthermore, reconstitution of a SFB negative population with the microbiome of SFB-positive mice increased disease incidence from 60% to 91%, albeit
not statistically significant (P=0.0967, **Fig. 14**). This suggests a potential role for SFB in the pathogenesis of KD.

While the increase in disease incidence of mice gavaged with SFB containing cecal contents was not statistically significant, there are several important caveats to consider. In contrast to the reconstitution of SFB into GF mice, the recipients in this experiment already had a homeostatic community of gut microbes. Studies have shown that indigenous flora can inhibit colonization of new bacteria, even using established techniques designed for modulating the microbiota. [159, 160] This may have limited that ability for the SFB to adhere, colonize the gut and mediate its downstream effects. However, this scenario is unlikely in this study, as we have demonstrated that SFB was able to successfully colonize the gut. (**Fig. 13**)

The timing in which these interactions occur may also be crucial. The epithelial barrier in the neonatal gut has been shown to have increased permeability, and homeostatic mechanisms such as the production of anti-microbial peptides are still developing. [161, 162] These properties can facilitate microbial interactions and subsequently, the development of the immune system. Hansen et al. showed that colonized GF mice had levels of IL-10, TGF-β and splenocyte populations similar to untreated GF controls, both of which differ from SPF conventionally colonized mice. The proportion of splenic Tregs in GF mice colonized at 1 week of age were more similar to that of SPF conventionally colonized mice than mice colonized at 3 weeks of age. This suggests that a delay in microbial colonization may have long term effects on the immune system. [88] Additionally, many important facets of mucosal immunity also develop during the neonatal stages which are absent in GF mice. [161-163] In this study, SFB was administered starting at 3 weeks of age where key stages in immune development may have already occurred. This delay may be responsible for the diminished change in disease incidence.
There are also limitations using an oral gavage to manipulate the microbiome. The gavage is essentially a large and sudden influx of microbes, an event not often encountered in nature. The gut is usually colonized after exposure to microbial factors during birth, and any further exposure is primarily due to ingestion. Mice participate in sacrophagy, and are constantly exposed to microbes within the feces of their littermates. Foster-nursing of newborn pups may be one method of addressing these issues. It would allow for microbial interactions starting at neonatal ages and promote a more natural transfer of the microbiome [159]. Our study also uses a mixture of flora found within the cecum of MBB donors, and thus it is possible that the observed effects are due to any number of factor(s) within the sample. However, SFB cannot be cultured in vitro, although Petrof et al. have recently demonstrated a method to generate synthetic stool samples by generating an in vitro environment similar to the gut. With improving microbiologic techniques, it may be possible isolate SFB and evaluate its effects in the absence of confounding factors. Until then, investigators have used SFB mono-colonized mice in order to specifically test this community, but again, there may be issues with purity[164].

Despite these caveats, this study establishes a potential role for SFB in the pathogenesis of KD. The role of SFB on disease incidence was evaluated and tested under several conditions: 1. The presence of SFB and its association with KD (Fig. 7), 2. The effects of adding SFB to the microbiome in KD (Fig. 14), and 3. The effects of eliminating SFB from the microbiome in KD (Fig. 11). We observed significant differences in disease incidence in the first and third condition, which correlated with the presence of SFB, and a trend towards increasing disease incidence in gavaged mice. Essentially, mice colonized with SFB were more likely to develop KD, depletion of SFB from the gut decreased disease incidence, and the addition of SFB in previously un-colonized mice was able to confer some increase in disease susceptibility.
Furthermore, we observed elevated levels of IL-17 in the serum of mice colonized with SFB. Previous reports in the literature have established a clear link between SFB and the Th17 immune response [103], and a potential role for Th17 in the pathogenesis of KD [127]. Together, this suggests that the colonization of SFB may be modulating the peripheral immune response and potentially, exacerbate the pathogenesis of Kawasaki disease.

**Potential mechanisms of disease modulation**

Based on published results on the effects of SFB in the Th17 immune response, an observed elevation in the guts of susceptible mice, and the discernible elevation in disease after reconstitution of the gut with SFB, we decided to evaluate the Th17 response in our murine model. The role of Th17 and IL-17 in KD has been controversial. To address their role in KD, we evaluated IL-17 mRNA levels in a various organs: the heart, axillary lymph node (ALN), spleen, mesenteric lymph node (MLN), and colon. While we did observe significant elevations in IL-17 mRNA in the spleen and ALN at several time points, the data is preliminary (Fig. 16). Furthermore, the differences in mRNA expression did not translate to an increase in serum levels of IL-17, nor was it found in the heart – our primary organ of focus. Splenocytes harvested from SFB-colonized mice did not appear to have variations in their response to LCWE in vitro, suggesting that SFB colonization does not lead to long term changes in T cell reactivity – at least to LCWE. Lee et al. reported coronary arteritis in IL-17 KO mice [118] and in conjunction with this data, does imply that IL-17 may not be necessary for the development of KD.

However, as opposed to animal models, the development of KD in humans is much more variable. Additional factors, genetic and environmental, can impact the development and pathogenesis of KD. One such variable may be the level of circulating IL-17. Jia et al. reported that patients who develop CAL and patients which are resistant to IVIG treatment have higher
levels of Th17 cells and their effector cytokines. [127] Consistent with these reports, this study found elevated serum levels of IL-17 in mice that were colonized with SFB. The addition of SFB through an oral gavage also resulted in some increase in IL-17. (Fig.15) Our data indicates that changes in IL17 are independent of LCWE and thus, that the pathogenesis of KD may not require the Th17 immune response. However, we do observe increased disease susceptibility in mice that have higher levels of circulating IL-17. These results, taken with the observations in human patients, suggest that IL-17 may increase disease susceptibility or exacerbate disease.

Th17 helper T cells and its prototypic cytokine, IL-17, mediate a host of pro-inflammatory effects. IL-17 has been known to induce the production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α by macrophages and T cells. IL-17 was also demonstrated to induce stromal cells to produce IL-6, IL-8 and G-CSF.[165] Furthermore, IL-17 was shown to induce the production of MMP-9, a fundamental component in the formation of coronary lesions. Neutrophils, which are recruited early to sites of inflammation in KD and are thought to prime the ensuing damage in the vasculature, are recruited and activated by IL-17. Additionally, our laboratory has shown a clear and essential role for the TNF-α mediated production of MMP-9 in the pathogenesis of the murine model of KD. Here, we show that SFB colonization is associated with elevated levels of IL-17, and with an increase in disease susceptibility. Reports in the literature have established a role for IL-17 in mediating the production of TNF-α, and MMP-9, among other pro-inflammatory functions. We hypothesize that the increase in circulating levels of IL-17 leads to a pro-inflammatory environment that is able to perpetuate T cell activation and exacerbate coronary infiltration and elastin breakdown, potentially through the induction of TNF-α and MMP-9. (Fig. 19)
Through the use of various methods of modulating gut composition and assessing disease, we have demonstrated a strong correlation between the microbiome and the pathogenesis of KD. The use of antibiotics to abolish the natural flora was able to reduce disease incidence and severity. The use of cecal gavage for microbiota transplantation alluded to the sufficiency of specific member(s) – SFB – of the microbiome to aggravate disease. These observations also correlated with increased levels of circulating IL-17. Based upon this evidence, we propose that in this model of KD, the microbiome exacerbates the pathogenesis of disease and one potential mechanism through the Th17 pathway.
Figure 19: SFB induce the differentiation of Th17 and exacerbate the pathogenesis of KD

This diagram represents a hypothesized mechanism by which SFB can modulate the immune response in a murine model of KD. SFB induces the differentiation of Th17 and a general elevation in IL-17 within the peripheral immune system. A superantigenic trigger (LCWE) activates a portion of T cells, wherein a pathogenic subset persists, resulting in the initiation of the pathogenesis of KD. The elevated levels of IL-17 in the presence of SFB exacerbate disease through a variety of pro-inflammatory functions. This can facilitate the release of TNF-a, which has been shown to induce the production of MMP-9. IL-17 can also induce the production of MMP9 in macrophages. This results in coronary inflammation and eventually, elastin breakdown and the formation of coronary lesions.
FUTURE DIRECTIONS

While our data suggests a role for the microbiome in KD, more investigation is required on the extent and mechanism by which these effects are mediated. It would be interesting to investigate the pathogenesis of KD in GF mice. Several animal models where the microbiome has an exacerbating role, such as arthritis and ankylosing spondylitis, are completely protected from disease under GF conditions. [84] In contrast, we were still able to observe the presence of disease in antibiotic-treated mice, indicating that the microbiome is not necessary for disease to develop, per se. One of the caveats in using antibiotics to deplete the gut is that resilient microbes and microbial products may still be present, factors that have demonstrated a capability to modulate the peripheral immune response. [166-168] Furthermore, unlike the antibiotic-treated mice our study, GF mice have no bacterial interactions starting from birth. Many of the techniques we used to modulate the microbiome took place at 3 weeks of age, which occurs after important periods of immune development in neonatal mice. [88, 169] This limitation is also present when conducting a cecal gavage. SFB is more predominant in the gut at early ages, and its effects on the Th17 response has been shown to vary depending on the age of colonization. [105] It would be interesting to determine if the effects of SFB colonization, or gut microbial modulation, from birth would have a greater exacerbating effect on the pathogenesis of KD. This could be achieved with foster nursing neonatal mice to facilitate a natural horizontal transfer of microbiota. This would provide insight on the importance of timing for the microbiome to mediate its immunomodulatory effects. In order to further dissect the roles of specific microbes, selective use of antibiotics could be used to deplete groups of bacteria within in the gut. SFB is gram-positive, and the use of vancomycin alone could remove this community (and other gram-positive microbes) without harming other groups of bacteria. Thus we would be able to gain a better understanding of their particular role in disease. Alternatively, there are also methods to
generate mice that are mono-colonized with SFB and again, their use would be important to
dissect and validate its role in the pathogenesis of KD. [164]

Another potential candidate within the microbiome is the Lactobacillus species. The
majority of lactobacillus species tend to be anti-inflammatory and generally seem to be
beneficial for health, particularly during the early stages of life. However, in this model of KD,
lactobacillus casei cell wall extract (LCWE) is used to induce disease, and proportions of
lactobacillus species are consistently elevated in the susceptible mice at MBB. Probiotic
administration of L. casei has also been shown to increase expression of TLR2. [142] TLR2
ligands present in LCWE exacerbate inflammation and facilitate localization to the coronary
arteries. Lactobacillus is commonly used as a probiotic, and has already been used in several
studies to evaluate their effects on the immune system and disease[170]. Continued investigation
of additional members of the microbiome that have immunomodulatory effects may reveal
additional mechanisms by which the microbiome can exacerbate, or moderate the pathogenesis
of KD.

CONCLUSIONS

There is growing evidence for the importance of the microbiome in disease and
autoimmunity, and the concept of modulating the microbiome as a therapeutic intervention has
been done for several diseases. This study demonstrates a role for the microbiome in the LCWE-
induced coronary arteritis model of KD, and perhaps suggests a role for the microbiome in the
pathogenesis of KD in humans as well. While previous studies have investigated small subsets of
bacteria in the gut of human KD patients, newer studies are in progress that will refine our
understanding of the microbiota and their role in human KD. [120] This study provides evidence
for the role of the microbiome in the pathogenesis of KD, provides one potential mechanism by which this can be done, and brings us closer to understanding the role environmental agents can have on disease.
REFERENCES


