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The Impact of Microbial Experience on the Murine Innate Immune Response

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Abstract

The hygiene hypothesis predicts that certain environmental factors shape overall immune system function in animals and humans. While current specific pathogen free (SPF) mouse models are invaluable for studying the immune system, they have limitations for comparison with humans who have microbial exposures throughout their lifetimes. Several studies have shown that the composition of the immune system of SPF mice more closely resembles that of newborns, whereas the immune system from mice exposed to microbial pathogens more closely reflect adult immunity. In this study we have established a model using traditional SPF mice (“clean mice”) and SPF mice that were cohoused with pet store mice (CoH mice). The goal of establishing this model was to observe if the cells of the innate immune system are more numerous and activated in mice who have been exposed to more microbial challenges during their development. Additionally, the goal of the model is to establish a framework for studying macrophage activity in CoH mice, including the potential for macrophages to develop a “learned response” as is commonly seen in adaptive immunity.

The initial data demonstrated that CoH mice could be successfully infected with a variety of microbes, while the control SPF mice remained “clean” without the use of a BSL-3 facility. With regard to innate immunity, F4/80+ CD11b+ peritoneal macrophages were more abundant in the CoH mice than in the SPF mice. In addition, CoH mouse white blood cells and peritoneal macrophages had increased MHC-II+ expression when compared to SPF mice. Using our model, we propose ways to characterize innate immune cell activation profiles in clean and CoH mice in order to better understand how microbial experiences impact innate immune profiles and activation.

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Abbreviations

SPF= specific pathogen free; standard protocol for keeping laboratory mice

CoH= co-housed mice; SPF mice co-housed with dirty mice for 30+ days

I.P. wash= peritoneal lavage

TLR= Toll-like receptor

ELISA= enzyme-linked immunosorbent assay

PAMP= pathogen associated molecular pattern

PRR= pathogen recognition receptors

DC= dendritic cell

NK= natural killer cell

Chapter 1: Introduction

The hygiene hypothesis predicts that environmental factors contribute to overall immune system function in animals and humans. The innate immune system is the first line of defense against pathogens and recognizes microbial components of pathogens and/or injured host cells to initiate a response to potential infection. This activates a series of signaling cascades that ultimately activate the transcription factors necessary for an inflammatory response. While current mouse models are invaluable for studying the immune system, they have limitations when correlating mouse and human immunity. Since mouse models are often used to inform prophylactic and therapeutic treatment for humans, improved mouse models for studying human immunity need to be established.

Our model successfully co-housed SPF C57BL/6 mice with dirty pet store mice for 30 days or more (hereafter called co-housed or CoH mice), allowing for CoH mice to become exposed to various microbes while keeping a control group of mice under specific pathogen free (SPF) conditions. Our mouse studies were modeled after experiments performed by a group of researchers at the University of Minnesota, who have implemented models that intentionally expose their mice to various microbial pathogens (Beura et al., 2016). By exposing SPF mice to microbes and pathogenic challenges, researchers believe they have recreated an immune system model in CoH mice that is a more accurate representation of that seen in human adults. It was concluded that the immune system of a clean, SPF lab mouse more closely reflects the immune system of a neonatal human than that of an adult human (Beura et al., 2016).

Other researchers have also attempted to create a more accurate representation of murine immunity using their own models of SPF vs CoH mice. A study by Abolins et al., (2017) emphasizes the importance laboratory mice have to researchers of the immune system and how it might be more beneficial to start using wild mouse models. It is not known whether the immune response of laboratory mice can properly represent that of free-living, outbred mouse populations (Abolins et al., 2017). Laboratory mice are typically housed in highly controlled and optimal environments optimized to support their survival and well-being. They have unlimited access to food and water and are kept free of pathogens (Abolins et al., 2017). Wild mice, however, are continually being exposed to new and old environmental antigens, and are more likely to be infected by bacteria, viruses, and parasites (Abolins et al., 2017). Taking into consideration the substantial differences between laboratory mice and wild mice, it is no surprise that differences may exist in their baseline immune parameters, immune responses to model antigens, and functional immune competence (Abolins et al., 2017).

Our lab is also interested in examining the effects of microbial experience on these mice. I was particularly interested in trying to get a better idea of what happens in the innate immune system of SPF mice after they are co-housed with dirty mice and acquire various pathogenic microbes. More specifically, I wanted to determine whether the number of macrophages would increase with pathogen exposure, and whether CoH macrophages would be more activated than macrophages in the control group of SPF mice. While this project could have been approached in a variety of different ways, I was most interested in trying to re-create the environment established by researchers at the University of Minnesota. Even though their research was performed in a BSL-3

facility, we felt confident that we could successfully reproduce the environmental set-up using the BSL-2 facility available at Grand Valley State University.

It is not well understood how inflammatory cells of the innate immune system differ between microbially experienced mice (“dirty” mice) and “clean” C57BL/6 (SPF) lab mice. To explore these differences, we have developed an effective mouse model to examine how environmental factors impact macrophage activation and function. Microbially experienced mice were compared to conventional “clean” mice with regard to their macrophage responses. We hypothesized that the microbially experienced mice would have a higher percentage of myeloid cells and peritoneal macrophages as well as a more activated population of macrophages than the clean lab mice. Using this mouse model to study myeloid cells and peritoneal macrophage presence will help us to better understand to what extent environmental factors influence innate immunity.

Peritoneal lavage was performed on SPF and CoH mice and the cells were analyzed for macrophage-specific markers using flow cytometry to compare the relative number of macrophages in the 2 groups at various time points. Next, we evaluated the activation state of the macrophages isolated from “dirty” and “clean” mice. Specifically, we analyzed the indirect activation status of TLRs by measuring cytokines that are produced after TLR stimulation and observed differences in cell-surface macrophage activation markers using flow cytometry. This project extends the current work in immunology where conventional murine models are used and will reveal how environmental factors can influence activity and quantity of macrophages in the innate immune system. Our proposed mouse model will be valuable for future studies comparing murine and human immune mechanisms.

My hypothesis was that co-housing microbially-experienced (dirty) mice with clean C57BL/6 specific-pathogen-free (SPF) mice would cause changes in the innate immune response of the co-housed (CoH) mice. I hypothesized that cohousing clean mice with dirty mice would cause CoH mice to be more immunologically active or "prepared" with regard to their macrophage quantity and activation when compared with the control SPF mice. The proposed co-housing model was meant to simulate an environment where microbial exposure is increased, mimicking the environmental factors that are described in the hygiene hypothesis.

Purpose

Once we have demonstrated successful implementation of the mouse model of microbial experience after co-housing SPF mice with microbially experienced (ME) mice, our aim is to further investigate the cellular innate immune response in CoH versus SPF mice. In this study, we examined how microbial experience affects the number of macrophages and their state of activation. To do this, macrophages from blood and from peritoneal lavage were identified by flow cytometry and also stimulated *in* vitro with TLR ligands to measure for the production of TNF-α or other macrophage-secreted cytokines.

Scope

This project is limited to the evaluation of macrophage function as an indicator of the innate immune response. While there are many types of innate immune cells, we have focused on the activity of myeloid cells and peritoneal macrophages. In addition, we

limited our study to the stimulation of TLR 4 and TLR 5 with appropriate agonists even though there are a number of other Toll-like receptors on macrophages that could be used.

Research Questions

Hypothesis 1: There is an increase in the number of macrophages that are F4/80+ CD11b+ in CoH mice and these cells have higher expression of MHC-II than SPF mice, reflecting a quicker and more functionally activated state.

Hypothesis 2: Macrophages from CoH mice exhibit more robust activation in terms of inflammatory cytokine production *in vitro* upon Toll-like receptor (TLR) signaling when compared to macrophages from SPF mice.

Significance

CoH mice have the potential to provide a more refined and viable representation of the human innate immune system than current SPF models. If macrophages of microbially experienced mice are greater in number, are more quickly and functionally activated, and are more efficient in phagocytosis and inflammatory cytokine production, than those in SPF mice, it suggests that environmental exposure may contribute to the innate immunity response. A mouse model with more microbial exposure and antigenic experience better simulates the various environmental stimuli that adult humans have faced during development and could provide valuable answers as to how our environment shapes innate immunity.

Chapter 2: Literature Review

The Hygiene Hypothesis

The hygiene hypothesis is centered on the idea that the sooner an individual is exposed to “unclean conditions”, the more efficiently their immune system can distinguish between true pathogens and innocuous antigens that cause asthma, allergies, and autoimmune disorders. The immune response relies on an intricate and malleable system where many environmental and genetic factors contribute to the functionality and efficiency of newly developing host immunity, as is illustrated in the figure below (Figure 1).

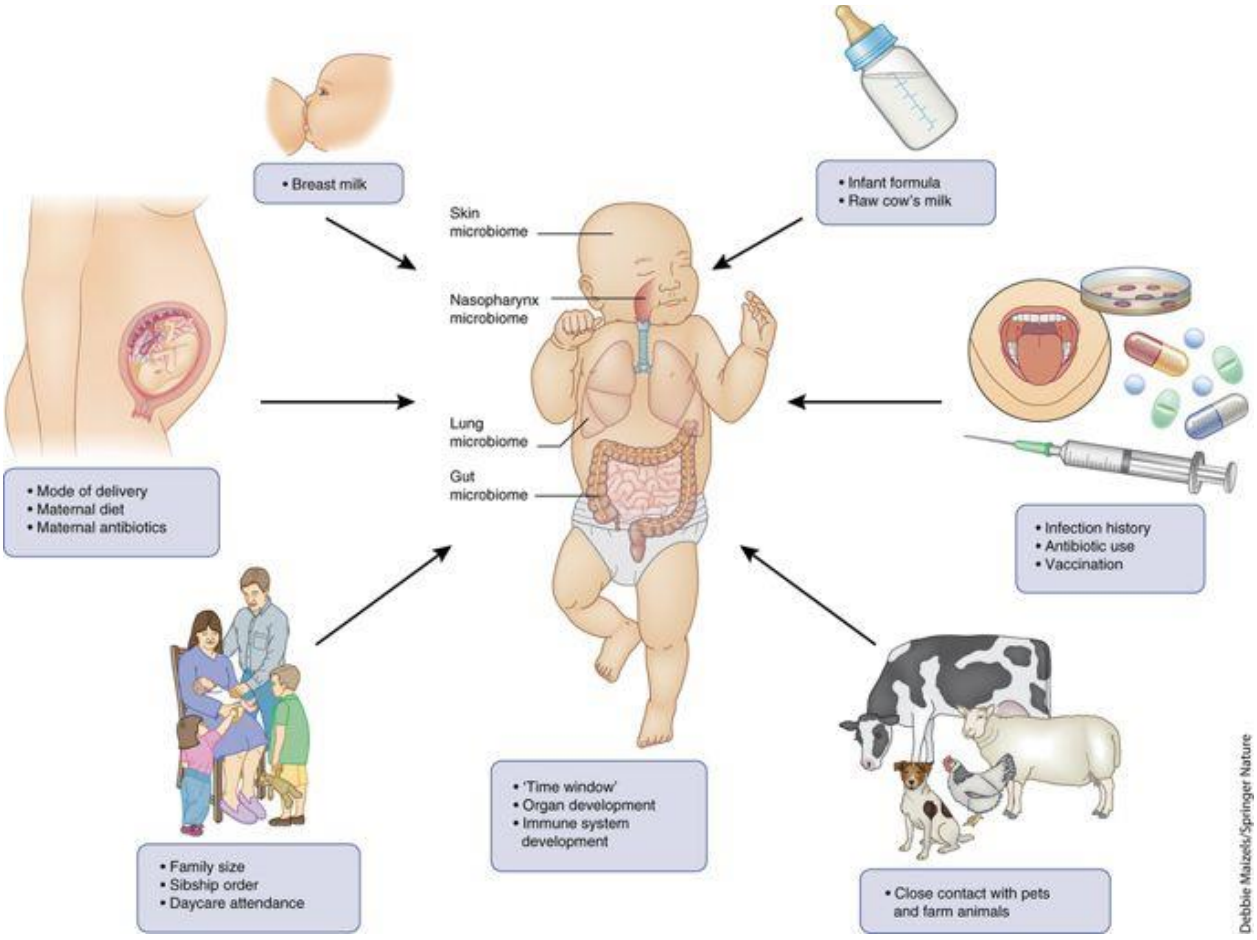

Figure 1. **The Hygiene Hypothesis and factors influencing immunity (Lambrecht et al., 2017).**

The History and Epidemiology of the Hygiene Hypothesis

One of the first scientists to publish research pertaining to the hygiene hypothesis was epidemiologist David Strachan. In Strachan's original publication offering support for the hygiene hypothesis, he observed whether there was any correlation between the incidence of hay fever and the size of an individual's household in British children (Strachan, 1989). Through his work, Strachan found that the likelihood of contracting hay fever was dependent on a child's order of birth in the family as well as how large the family was in size (Strachan, 1989). These results remained consistent after Strachan observed and questioned more than 17,000 children born during the same week in 1958 and following them up until they were all 23 years old. This was more commonly known as the National Child Development Study. Those who were the only child in their household had a higher rate of disease incidence when compared to children who had multiple siblings in their household (Strachan, 1989). Furthermore, it was noted that disease incidence got increasingly smaller as the number of siblings in the household continued to rise. Strachan hypothesized that there was a protective effect with regard to hay fever that was derived from having more siblings in a household (Strachan,1989; Strachan, 2000).

Using the hygiene hypothesis, Strachan was also able to offer an explanation based on something called the socioeconomic gradient (Figure 2). It has been shown that there is a higher prevalence of hay fever and eczema in individuals that come from more "well-off" families (Broder et al., 1974; Butland et al., 1997; Williams et al., 1994; Williams et al., 2006). Multiple studies (Kilpelainen et al., 2000; Braun-Fahrlander et al., 1999; von Ehrenstein et al., 2000) support the idea that there is a reduced occurrence

of allergic rhinitis and atopy among children who come from farm families. Strachan believed this pattern was due to the fact that these children received a greater exposure to infection and would therefore be better off later in life immunologically (Strachan, 2000).

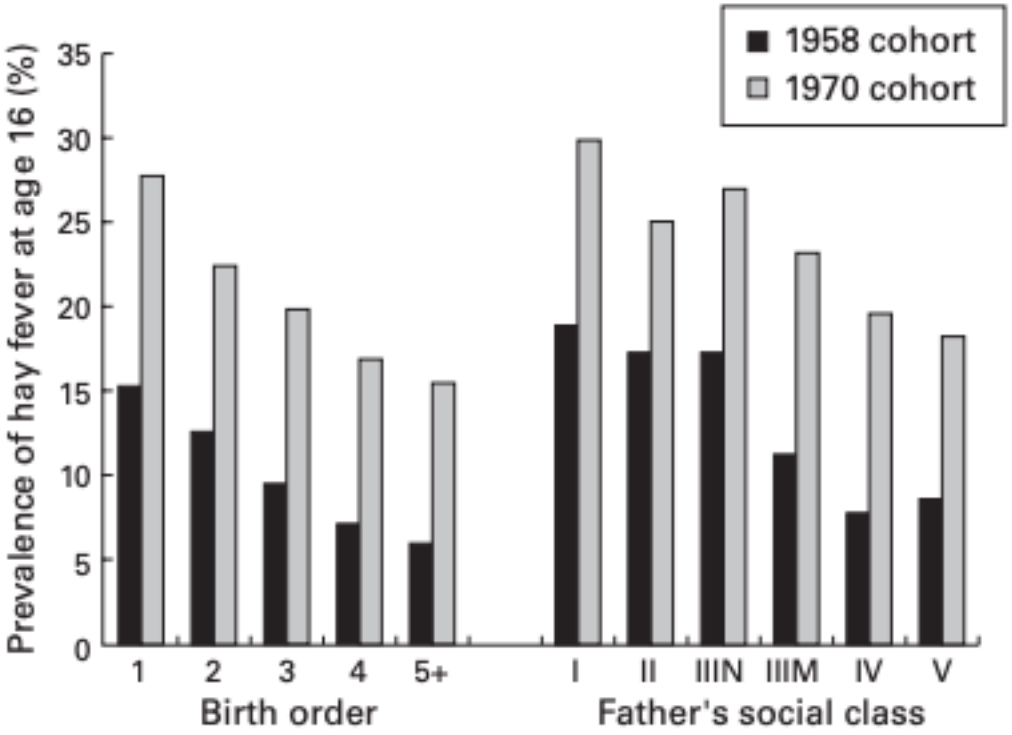

Figure 2. **Hay fever prevalence by birth order and father's social class. Cohort 1 and 2 consist of British children born in 1958 and 1970 (Strachan, 2000).** The graph on the left shows the prevalence of hay fever in relation to the order of birth a child is born into a family. The right graph shows the prevalence of hay fever in relation to the social class of the child's father. I indicates professional occupations. II indicates managerial and technical occupations. IIIN indicates skilled non-manual occupations. IIIM indicates skilled manual occupations. IV indicates partly skilled occupations. V indicates unskilled occupations.

In the 18th century prior to the start of the industrial revolution, the importance of having sanitary living conditions was not as well understood as it is today. Before industrialization took place, populations of people around the world were suffering from things such as inadequate sewage systems, contaminated water supplies, and rapid disease transmission due to overcrowding (Wilde, 2019). After the industrial revolution started, public health measures were implemented to limit the spread of infectious diseases. This included pasteurization and decontamination of dairy and food products, vaccinations against diseases, and the development of antibiotics (Okada et al., 2010). In countries where the focus on public health is still lacking, allergy prevalence remains low (Okada et al., 2010). The question remains that, in countries with reduced allergy prevalence, what is different about the overall immunity of a population? And what factors play a role in altering immunity from one group of people to the next?

Asthma Risk in Amish vs. Hutterite Farm Children

Over the last 50 years there has been an exponential increase in the number of asthma cases in industrialized countries, suggesting that environmental conditions must influence the likelihood of developing asthma (Bach, 2002). Strachan's original findings of increased disease prevalence in those with higher socioeconomic status supports this (Strachan, 2000). While the association between childhood microbial exposure and the presence of farm animals suggests a pattern of reduced disease risk, little is known about the impact of living on a farm on the immune response (Stein et al., 2016).

A study by Stein et al. was designed to address what is happening at the immunological level that would cause a reduction in disease prevalence. The study's goal was to look at two different groups of children living in a farm environment. The first group was the Amish of Indiana, and the second group was the Hutterites of South Dakota (Stein et al., 2016). The two groups had many lifestyle characteristics in common, including a large number of siblings, high rates of childhood vaccination, diet, and low rates of childhood obesity. The big difference between the two populations, however, was in their farming practices. The Amish tended to farm using more traditional methods and the Amish children began working on the farm at a very young age. Conversely, the Hutterites had large, highly industrialized farms where new technology was often used and children didn't work on the farm with their parents until they were much older (Stein et al., 2016). Interestingly, Stein and her colleagues noted that the prevalence of allergies among Amish children was much lower (7.2%) than that of the Hutterite children (33.3%). Asthma prevalence was only 5.2% in the Amish population while it was 21.3% in the Hutterite population (Stein et al., 2016).

Table 1. Demographic and Clinical Characteristics of the Study Populations.*

Characteristic	Amish (N=30)	Hutterite (N=30)
Age (yr)		
Median	11	12
Range	8–14	7–14
Girls (no.)	10	10
Sibships (no.)	15	14
Children with asthma (no.)	0	6
Positivity for allergen-specific IgE (no.)		
>0.7 kUA/liter	5	9
>3.5 kUA/liter	2	9
Serum IgE (kU/liter)		
Median	21	64
Interquartile range	10–57	15–288

* UA denotes allergen-specific unit.

Figure 3. **Amish vs. Hutterite population characteristics (Stein et al., 2016).** Two notable features of the data are the prevalence of asthma in the Hutterite population and the large increase in serum IgE (kUA/liter) in the Hutterite population.

Immune profiles of both populations were characterized, and a mouse model was used to study the effect of the environment on the prevalence of asthma. Data was collected from 30 children for each population (Figure 3). Blood was collected from individuals and the serum was frozen and stored to perform tests such as an ELISA assay (Stein et al., 2016). Blood was also used to analyze peripheral-blood leukocytes and additional serum was collected for immunoglobulin E (IgE) studies. Dust was collected from the bedrooms of those in each population, which was analyzed for endotoxin and allergen levels. The dust samples were also used to inject mice intranasally for the environmental study (Stein et al., 2016).

The study from Stein et al. found that there were common allergens in the airborne dust from 4 of the 10 Amish homes and from only 1 of the 10 Hutterite homes (Stein et al., 2016). The increased endotoxin levels in the Amish home dust are shown in Figure 4.

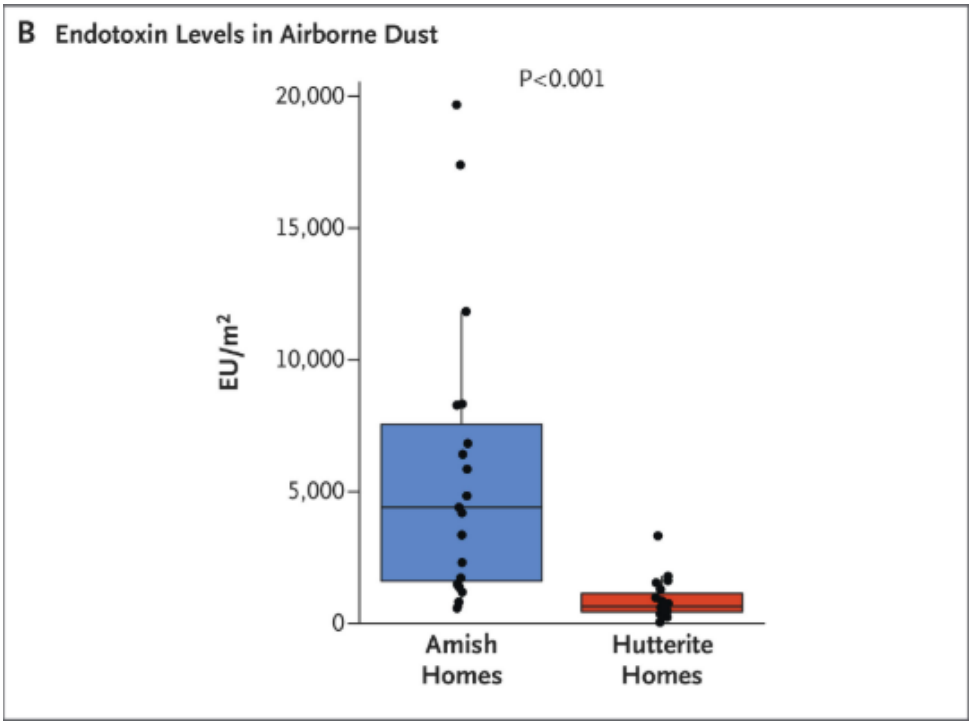

Figure 4. **Endotoxin levels in airborne dust in Amish vs. Hutterite homes (Stein et al., 2016).**

Flow cytometry data was also collected from both populations. The Amish subjects had higher numbers of neutrophils, monocytes, and immunoglobulin-like transcripts (ILTs), specifically, ILT3 – a cell-surface marker on monocytes (Stein et al., 2016). The Amish children also had less eosinophils than Hutterite children, which could potentially be an indicator of a less atopic immune system (Stein et al., 2016). Stein and her colleagues also noted that although the number of monocytes in the Amish children was higher, the

Amish children monocytes expressed a reduced amount of human leukocyte antigen DR (HLA-DR), which is one of the MHC-II isoforms found in humans (Stein et al., 2016). This reduced HLA-DR expression can be caused by repeated microbial stimulation (Meisel et al., 2009; Gomez et al., 2014) and is suggestive of anti-inflammatory function (Stein et al., 2016). Finally, the results of the mouse studies looking to find a relationship between dust particles and asthma favored the hypothesis that the Hutterite children would have increased asthma prevalence. Stein and her colleagues found that the airways of the mice inoculated with Hutterite dust were more likely to have airway resistance suggestive of asthma (Stein et al., 2016). The study concluded that the Amish environment activated the innate immune system to provide a protective effect. This directly relates to the belief that the hygiene hypothesis is describing. Because the children in the Amish farming population had less industrialized equipment and were being required to work on the farms at an earlier age, they were found to have a more protective innate immune system.

Hygiene Hypothesis Skepticism

Many scientists have been skeptical of Strachan's original findings in support of the hygiene hypothesis. Because Strachan's cohort of subjects consisted of parental or self-reported incidences of hay fever and eczema, one could argue the potential for bias in the study (Strachan, 2000). However, subsequent research was published by others that supported Strachan's observations about environmental conditions and immunity (Jarvis et al., 1997; Forastiere et al., 1997; Svanes et al., 1999). For example, asthma, hay fever, and atopic dermatitis doubled in children living in Sweden over a 12-year

period (Aberg et al., 1995). Additionally, cases of multiple sclerosis, which is considered an autoimmune disease, also doubled over a 17-year period in Germany (Poser et al., 1989). Additionally, a paper featured in The New England Journal of Medicine (Figure 5) shows how there has been a steady decrease in the incidence of infectious diseases, while the incidence of immune disorders has increased (Bach, 2002).

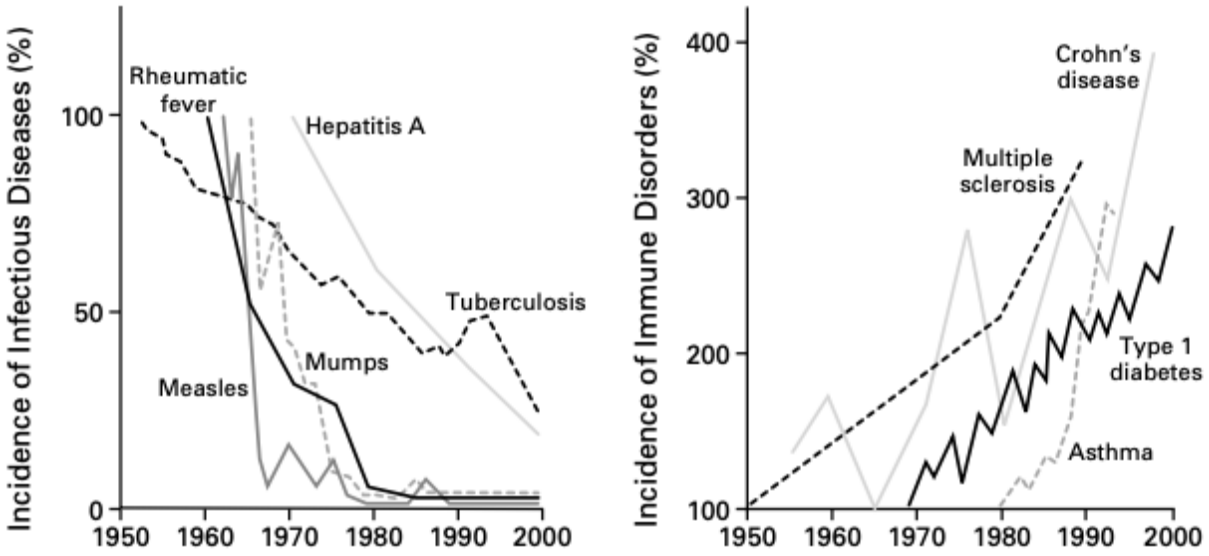

Figure 5. **Inverse relationship between infectious disease prevalence and immune disorder prevalence in the United States.** The graph on the left shows the incidence of infectious disease in The United States from 1950-2000. The graph on the right shows the incidence of immune disorders in the United States from 1950-2000 (Bach, 2002).

Studies have shown that the global incidence of allergies, autoimmune disorders, and chronic inflammation is higher in industrialized nations (Okada et al., 2010). Due to lifestyle changes being made by those living in industrialized countries, the rate of infectious disease burden has decreased while the rate of allergies and autoimmune disorders in individuals has steadily increased over time (Okada et al., 2010). Diseases such as asthma and atopic dermatitis have both increased over time in areas where

more industrialization has occurred; asthma is an epidemic in some parts of the world (Beasley, 1998; Masoli et al., 2004). In a study by Beasley, over 400,000 children aged 13 to 14 years old were given a questionnaire asking them questions about their experiences with asthma, allergic rhinoconjuctivitis, and atopic eczema (Beasley, 1998). This study found that the occurrence of asthma-like symptoms was highest in the United Kingdom, Australia, New Zealand, and Ireland, while the prevalence of asthma-like symptoms was lowest in less industrialized countries such as Indonesia, Greece, Taiwan, India, and Ethiopia (Beasley, 1998).

Others have also shown that the prevalence of atopic dermatitis, a skin disease closely associated with asthma and allergic rhinitis, has tripled over the past 20-30 years, largely impacting children more than adults (Bieber, 2008; Williams, 1994). The etiology of atopic dermatitis is believed to be a disturbance in the immune system that results in immunoglobulin E (IgE)-mediated sensitization to host epithelial barriers -- this being the result of local inflammation in the body (Bieber, 2008; Illi, 2004). Because atopic dermatitis is less prevalent in less industrialized areas of the world, one could hypothesize there is a connection between atopic dermatitis and the hygiene hypothesis (Bieber, 2008; Strachan, 1989).

Innate Immunity

Atopy, as defined by The American Academy of Allergy, Asthma, & Immunology (AAAAI), is one's genetic predisposition for developing an allergic disease. Atopy is also often associated with a heightened immune response to environmental objects that would normally not trigger an immune response in a healthy individual (AAAAI, 2019).

To fully understand how a normal-functioning immune system is cognizant but tolerant of particular allergens and how immune function can be altered, it is important to understand the mechanisms that control the activity of the innate immune system. The innate immune system is the first line of defense for the body against infections and injuries. The adaptive immune system offers a powerful line of defense; however, it has a slower response time and requires the activation and clonal expansion of specialized immune cells in order to operate at full capacity. The innate immune system includes physical barriers such as the skin and mucosal epithelium. Leukocytes of the innate immune system include natural killer (NK) cells, mast cells, eosinophils, basophils, macrophages, neutrophils, and dendritic (DC) cells. Cells such as macrophages within the innate immune system can be activated by the presence of pathogen-associated molecular patterns (PAMPs) binding to specific innate cell receptors, or by the production of cytokines – chemical messengers responsible for various biological effects in the immune system. Regardless of what kind of activation occurs, the innate immune system is crucial for a host's survival because it is the fastest acting line of defense and is required for activation of adaptive immunity.

Molecular Signaling in Innate Immunity

The innate immune system protects against invading pathogens through the inflammatory response via various intracellular signaling pathways. Innate immune cells, such as macrophages and neutrophils, reside in or migrate to the tissues and are able to recognize invading pathogens or self-damage with pattern recognition receptors (PRRs) (Newton et al., 2012). Both intracellular as well as extracellular PRRs are

present to detect pathogen-associated molecular patterns (PAMPs). PAMPs include characteristics unique to pathogens, while also being distinguishable from self-antigens (Mogensen 2009; Janeway, 1989). Some of the most common PAMPs include microbial nucleic acids, lipoproteins, and carbohydrates, or cytokines released by damaged host cells called damage-associated molecular patterns (DAMPs) (Newton et al., 2012).

Inflammatory Signaling Through Toll-Like Receptors (TLRs)

Perhaps one of the most well-characterized type of PRR is the Toll-like Receptor (TLR). There are at least ten different families of TLRs in humans, and their function and gene expression are based on which cell is activating them and which cell they are bound to (Newton et al., 2012). The overall goal of TLR-mediated cell activation is to trigger a host cell gene expression program in response to a pathogen (Alberts et al., 2002). One of the most-studied TLRs is TLR4, a receptor on the surface of a macrophage that binds to lipopolysaccharide (LPS), a PAMP of Gram-negative bacteria (Alberts et al., 2002). In response to TLR4 engagement, adaptor proteins bind to the cytoplasmic tail of TLR4 (Figure 6). The engagement of these adaptor molecules stimulates the recruitment and activation of IL-1R associated kinases (IRAKs), TNF-receptor-associated factor 6 (TRAF6), and β-activated kinase 1 (TAK1) (Molteni et al., 2016). TAK1 activation eventually leads to the phosphorylation and activation of the IκB kinase (IKK) (Alberts et al., 2002). IKK will phosphorylate the NF-κB inhibitor, IκB, causing it to be released and degraded. This allows NF-κB to translocate to the nucleus where it acts as a master transcription factor, upregulating expression of genes involved in immune and inflammatory responses (Alberts et al., 2002).

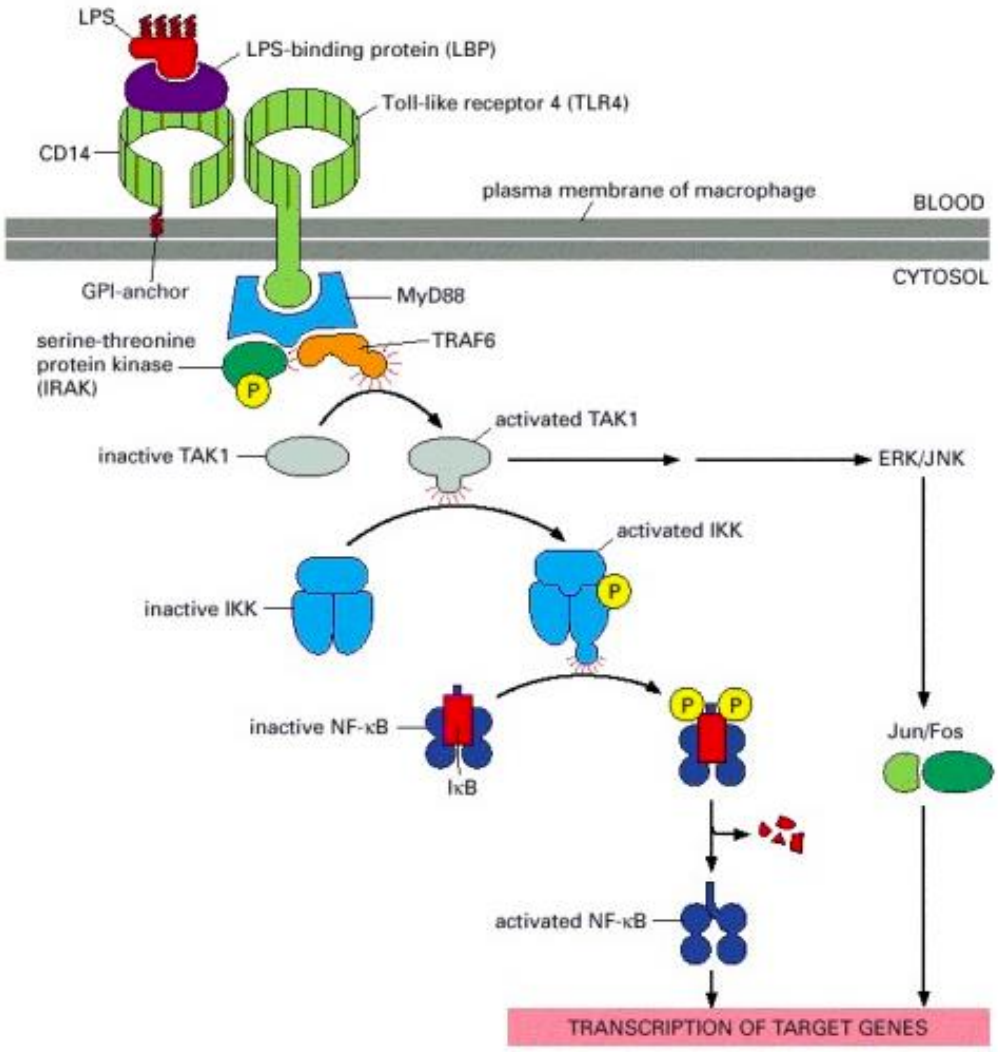

Figure 6. **Activation of NF-κB via TLR4 engagement with LPS (Alberts et al., 2002).**

The Immune System of SPF Lab Mice and Dirty Mice

Immunologists have historically conducted their studies using laboratory strains of mice, and from these studies, scientists have been able to provide information on the etiology and pathophysiology of immune-related abnormalities such as autoimmune disorders or allergies (Russell, 1985). Laboratory mice have been long-standing experimental models of immune disorders for quite some time, as working with lab mice is relatively

easy and cheap. However, though lab mice are relatively cheap and easy to use, there are considerable differences in the immune systems of lab mice compared to humans (Abolins et al., 2017). In a research laboratory, outside variables such pathogenic exposure, temperature, diet, and lighting are kept regulated and controlled. While controlling a lab mouse's environment undoubtedly has benefits, scientists are discovering that this may not be the best way to study the intricacies of the innate and adaptive immune systems in humans (Masoput et al., 2017; Abolins et al., 2017). In adults, our innate and adaptive immune systems become differentiated through exposure to various microbial antigens, chemicals, and other environmental factors. During the course of a laboratory mouse's life, there is little to no contact with microbial exposure. Lab mice are kept in specific pathogen free (SPF) environments to limit environmental variables in studies being performed. Because of this, researchers suggest that using SPF mice to study the immune system is an insufficient model when trying to compare mouse immunity to adult human immunity (Abolins et al., 2017; Beura et al., 2016; Reese et al., 2016; Rosshart et al., 2017).

In a study by Abolins et al., it was shown that immune responses of wild mice varied drastically from that seen in SPF lab mice. This was indicated by data showing that wild mice had increased production of cytokines, higher numbers of CD4+ T cells, along with more activated macrophages, natural killer cells, and dendritic cells (Abolins et al., 2017). Similar findings were also observed in commercially available pet store mice (Beura et al., 2016). The biggest limitation of these comparisons is the differing genetic backgrounds of SPF and wild/pet store mice.

Studies such as these suggest the potential importance environmental and pathogen exposure may play on developing the immune system of mice. Researchers also discovered that the immune system of lab mice might more closely resemble that of a human newborn, whereas the adaptive immune system of wild and pet store mice is more closely related to that of an adult human (Abolins et al., 2017; Beura et al., 2016).

Co-housing Mice is and Effective Model for Studying Adaptive Immunity

The original idea and coining of the phrase “co-housed” (CoH) mice for immunology research was developed by the University of Minnesota (Beura et al., 2016). Co-housing is a form of mouse housing where SPF mice are placed in the same cage with a commercially available pet store mouse. This co-housing of mice leads to the eventual infection of the SPF mice with various pathogens and zoonotic infections (Beura et al., 2016). The unique feature with the Beura et al. study was that they used genetically identical mice for their SPF and CoH populations, and the only variable in their study was microbial exposure. This caveat eliminated issues pertaining to not being fully aware of the genetic composition of a wild mouse. The research group at Minnesota kept their CoH mice and SPF control mice in strict BSL-3 laboratories to reduce the spread of contamination to other lab mice. Table 1 shows that after a period of co-housing, CoH mice were infected with nearly half of the pathogens found in the pet store mice. Additionally, the SPF control group (“laboratory”) had no microbial exposure to any of the pathogens (Beura et al., 2016).

	Pet store	Laboratory	Co-housed
Viruses			
Rotavirus (EDIM)	0	0	0
Mouse Hepatitis Virus	93.3	0	61.5
Murine norovirus	60	0	38.5
Mouse parvovirus NS1	53.3	0	0
Mouse parvovirus type 1	40	0	7.7
Mouse parvovirus type 2	46.7	0	0
Minute virus of mice	46.7	0	0
Theiler's murine encephalomyelitis virus	60	0	38.5
Sendai virus	66.7	0	23.1
Ectromelia virus	0	0	0
Lymphocytic Choriomeningitis virus	6.7	0	7.7
Mouse adenovirus 1 and 2	0	0	0
Mouse cytomegalo virus	0	0	0
Polyoma virus	6.7	0	0
Pneumonia virus of mouse	53.3	0	0
Reovirus	0	0	0
Bacteria			
Cillia-Associated Respiratory Bacillus	0	0	0
Mycoplasma pulmonis	73.3	0	30.8
Clostridium piliforme	26.7	0	0
Parasites/Protozoa/Fungi			
Enchephalitozoon cuniculi	40	0	0
Pinworm	100	0	100
Mites	100	0	100

Table 1. **Co-housed mice are infected by nearly half of the pathogens carried by pet store mice.** Co-housed mice (SPF mice co-housed with pet store mice for 30+ days) successfully acquire pathogens from pet store mice. Additionally, laboratory (SPF) mice stayed clean. Blood and fecal samples were tested via PCR and serology in all three groups before and after co-housing. Table is compiled results (Beura et al., 2016).

Beura et al., discovered that infections from pet store mice alter the immune system of CoH mice significantly. Other published research has focused on observing CD8+ T cell activity, which act as important players of the adaptive immune system (Gerritsen & Pandit, 2016; Gulzar & Copeland, 2004; Ioannides & Whiteside, 1993). However, there

were very few labs before the Beura et al. group that were quantifying the effect of microbial exposure on CD8+ T cell activity. After examining CD8+ T cells in wild, pet store, and SPF mice, researchers were able to observe notable differences. They discovered that, when it comes to antigen experienced CD8+ T cells, SPF mice had the least experienced and pet store mice had the most experienced (Beura et al., 2016). Using cell specific markers to indicate antigen experience (CD44, KLRG1, CXCR3, and Granzyme B), it was found that co-housing SPF mice was effective at increasing the antigen experienced CD8+ T cells, as CoH mice had a much higher percentage of CD44+ T cells, along with the other markers of T cell differentiation and experience (Beura et al., 2016). Microbial exposure studies have focused primarily on the adaptive, T cell response so far, and very little is known about how microbial exposure influences the innate immune system.

Chapter 3: Materials and Methods

Co-Housing

Mouse cohorts consisted of 25-35 female C57BL/6 clean (SPF) mice that were four weeks old. The mice were ordered from Charles River Laboratories (Mattawan, MI) and were dispersed amongst six cages with 4-5 mice in each cage. Two cages housed the control group with five female SPF mice in each cage. In order for our SPF control group to remain a legitimate and reliable source of data, all handling, feeding, and supply changes were performed using extreme caution to keep the clean and dirty mice separate. SPF mice from the same original SPF cohort (N=15-20) were co-housed with female pet store mice (purchased from a PetCo store in Walker, MI) for 30 days or more to generate “dirty” co-housed (CoH) mice. Mice were fed as needed and were monitored daily. Weekly cage changes were done according to a strict protocol to keep SPF and CoH mice separate from one another. For example, the SPF cages were always washed and changed before the CoH mice were taken care of to avoid cross contamination. Cages, lids, and water bottles were sprayed and soaked weekly in a solution of concentrated Clidox (1:3:1 dilution) and all of the housing supplies for the mice were washed with Rescue disinfectant as another safeguard against contamination. The mice were kept in a vivarium completely separate from other research animals on Grand Valley State University’s campus. All animals used in the study were used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Grand Valley State University (Protocol Number: 19-08-A).

Pathogen Testing

Fecal and blood samples were collected on day 0 and day 30 from SPF, CoH, and pet store mice and were sent to Charles River Research Animal Diagnostic Services (CR RADS) for testing against specific pathogens. Fecal samples were pooled from mice in the cage in order to get a more representative sample from the mouse population as a whole.

Blood Collection

During the course of co-habitation, mice were anesthetized using isoflurane and bled retro-orbitally once a week into heparinized or non-heparinized tubes. Heparinized blood was used for RNA isolation and flow cytometry preparation and non-heparinized blood was used for serum collection.

Flow cytometry preparation**.** Red blood cells were lysed with 300 µL of ACK lysis buffer (Ammonium Chloride Potassium, ACK; KD Medical), vortexed, and incubated at room temperature for three minutes. 500 µL of PBS was then added to dilute the samples and stop the ACK lysis buffer. The tubes were then centrifuged for 4 minutes at 4,000 RPM and the cell pellet was then incubated with the appropriate flow cytometry antibodies as described in more detail below.

RNA isolation**.** RNA was isolated from 30 µL of the heparinized blood (100 U/mL in DMEM) collected by retro-orbital bleed. Red blood cells were lysed as described above. The tubes were then centrifuged for 4 minutes at 4,000 RPM and the cell pellet was retained for RNA isolation according to the manufacturer's protocol (Quiagen RNeasy mini kit). 350 µL of RLT buffer was used for each tube and we included the

optional drying step in the procedure. Isolated RNA was then stored at -80 C for further analysis.

Serum collection. Blood samples were allowed to clot and were centrifuged at 8,000 RPM for 8 minutes. After centrifugation was complete, a minimum amount of 30 µL of serum was pipetted and collected into Eppendorf tubes. The samples were frozen at -80 C and stored for further analysis.

Tissue Collection and Processing

After 30+ days of co-housing, the collection and processing of various tissue samples was performed. Mice were euthanized with a lethal dose of isoflurane and secondary cervical dislocation. Tissues were then harvested, including blood (via cardiac puncture or retro-orbital bleed), spleen, cervical and inguinal lymph nodes and peritoneal cells (see below). Spleens and lymph nodes were collected in 3 mL of RPMI or PBS with 2% FBS. The blood was aliquoted into heparinized tubes for flow cytometry analysis and non-heparinized tubes for serum collection, as described above. The spleens and lymph nodes were processed using a syringe and a 70 µM filter to produce a single-cell suspension. Spleen samples were lysed with ACK buffer in a similar manner to heparinized blood, but the exposure to the ACK lysis was much shorter (~one minute). Samples went through multiple washing steps with FACS buffer, and samples were finally resuspended in FACS buffer (1x PBS with 1% FBS) and incubated with the appropriate antibodies for at least 20 minutes.

Peritoneal Lavage

Peritoneal cells were harvested according to the method of Herzenberg et al. (1988) for subsequent flow cytometry or cell culture experimentation. Based on cell staining with F4/80 and CD11b, macrophages made up 30-50% of the peritoneal cell population in SPF mice.

In vitro Macrophage Stimulation

To look at macrophage TLR activation *in vitro*, we used the mouse TLR agonists, LPS-EK (TLR 4) and FLA-ST (TLR 5) at 1 and 10 ng/mL respectively (TLR1-9 Agonist Kit, InvivoGen). Peritoneal lavage cells were cultured in 24-well plates with RPMI-complete medium. The effect of TLR 4 and TLR 5 stimulation was examined one day and three days after culture initiation by flow cytometry, using the same F4/80+ CD11b+ MHC-II+ cell markers. Cells were stained with a live/dead fluorescent dye to determine macrophage viability. Macrophages were identified by staining with F4/80 and CD11b, and their activation states were assessed by the increased expression of CD80+, CD86+, and MHC-II+.

Flow Cytometry and Data Collection

Flow cytometry samples were analyzed using a Beckman & Coulter CytoFlex four channel flow cytometer. Spleen, lymph node, blood, and peritoneal cells were resuspended in 200 µL of FACS buffer (1x PBS with 2% fetal-bovine serum) before being run at a speed of approximately 1,000-5,000 events/sec for 2 minutes. Samples

were fixed with a Fix/Perm FoxP3 kit (Tonbo Biosciences) to eliminate RBCs and allow for intracellular antibody staining. Fixed samples were incubated for an additional 30 minutes in 100 µL of PermFix per sample and washed twice with 300 µL PermWash. Samples were run using the specific antibody panels indicated in table 2.

Blood and I.P. Macrophage Wash Antibodies	Fluorescent Tag	Company	Cat No.	Dilution
CD11b	PerCp-Cy5.5	Tonbo	65-0112-U025	1:400
F4/80	PE-Cy7	Tonbo	50-4801-U025	1:400
MHC-II	PE	Tonbo	50-5321-U025	1:400
CD80*	FITC	Tonbo	35-0801-U025	1:400
Cell Culture Days 1 and 3 Antibodies				
CD11b	PerCp-Cy5.5	Tonbo	65-0112-U025	1:400
F4/80	PE-Cy7	Tonbo	50-4801-U025	1:400
Live/Dead Red	PE	Thermo Fisher	L34971	1 uL per mL
CD86**	FITC	Biolegend	105005	1:400
Other Antibodies Used				
OR MHC-II**	FITC	Tonbo	35-5321	1:400
OR CD86*	FITC	Biolegend	105005	1:400

Table 2. **Antibody panels used to stain blood myeloid cells and peritoneal macrophages, and cell cultured cells (data points for cohorts 1-4).** Antibody was switched from CD80 antibody to CD86 for cohort 4.

Statistical Analysis

Flow cytometry data was acquired on the pre-installed software CytExpert (Beckman Coulter). Graphs and statistical testing were both done using Prism 8 (GraphPad). Significance was determined by p-values < 0.05 using an unpaired student's T-test or multiple T-tests.

Chapter 4: Results

CoH Mice Acquire Infections Over 30+ Days of Co-Housing While SPF Mice Remain Pathogen Free

Although the concept of co-housing SPF lab mice with “dirty” mice from either wild populations or pet stores is not an overly complicated idea, implementing a mouse model of this nature in an animal care setting came with its own list of difficulties. In order for a dirty or CoH mouse model to be effective, precautions must be put in place to avoid the transfer of pathogens or zoonotic infections from dirty mice to SPF control mice. In most settings where CoH mice are used, the labs operate under strict Biosafety-level 3 (BSL-3) guidelines in vivariums. Although the potential for pathogenic and zoonotic infection incidence is reduced in these BSL-3 facilities, the use of non-BSL-3 labs is a more expensive and difficult lab space to establish (Beura et al., 2016). In this study, co-housing was set up using a satellite vivarium facility. We successfully co-housed SPF mice with pet store mice that were purchased from a local pet store, all while keeping a control group of SPF mice completely free of any pathogenic contamination or zoonotic infection. Monitoring for pathogens or zoonotic infections was performed by the Charles River Research Animal Diagnostic Services (CR-RADS) team. Fecal and blood samples were collected from SPF and pet store mice at the initiation of co-housing, and then from SPF and CoH mice ~30 days after co-housing began. RT-PCR and immunoserology tests indicated there were no pathogens or zoonotic infections present in our SPF control group, while the pet store mice contained various types of bacterial, viral, and parasitic infections. Table 3 shows that after the co-housing period, nearly half of the pathogens seen in pet store mice had infected the CoH mice. Acquisition of pathogens could also be observed through visible signs of

illness in the CoH mice. In the second half of the co-housing period, CoH mice became lethargic, ate poorly, and developed a “scruffy” appearance. While a small number of the mice did in fact die due to their acquisition of pathogens (data not shown), the CR-RADS data confirmed that we developed an effective co-housing protocol despite not having the resources available for a BSL-3 lab environment.

Charles River Pathogen Screening	SPF Day 0 (%)	SPF Day 30 (%)	Total PS Day 0 (%)	Total CoH Day 30(%)
Lymphocytic choriomeningitis virus	0.0	0.0	0.0	0.0
Mouse adenovirus 1 & 2	0.0	0.0	100.0	50.0
Mouse hepatitis virus	0.0	0.0	0.0	0.0
Mouse norovirus	0.0	0.0	66.6	50.0
Mousepox (Extromelia virus)	0.0	0.0	0.0	0.0
Mouse parvovirus	0.0	0.0	66.6	50.0
MPV 1	0.0	0.0	50.0	0.0
MPV 2	0.0	0.0	100.0	0.0
MPV 3	0.0	0.0	100.0	50.0
MPV 4	0.0	0.0	0.0	0.0
Mouse rotavirus	0.0	0.0	0.0	0.0
Pneumonia virus of mice	0.0	0.0	0.0	0.0
Reovirus	0.0	0.0	0.0	0.0
Sendaivirus	0.0	0.0	0.0	0.0
Theiloviruses	0.0	0.0	100.0	100.0
Corynebacter bovis	0.0	0.0	0.0	0.0
Cilia-associated respiratory bacillus	0.0	0.0	0.0	0.0
Clostridium piliforme	0.0	0.0	0.0	0.0
Mycoplasma pulmonis	0.0	0.0	0.0	25.0
Mites	0.0	0.0	33.3	0.0
Pinworms	0.0	0.0	33.3	50.0
Aspiculuris tetraptera (pinworm)	0.0	0.0	0.0	50.0
Syphacia muris (pinworm)	0.0	0.0	0.0	0.0
Syphacia obevelata (pinworm)	0.0	0.0	100.0	100.0

Table 3. **CoH mice get infected by various pathogen types during 30+ days of co-housing while SPF mice stay clean.** Data was compiled for cohorts 2 and 4 and served as a representative sample for other cohorts. Fecal and blood samples were collected from 2 separate cages for each group of mice for each sample.

CoH Mice Developed More Numerous and More Activated Myeloid Cells in Blood Samples than SPF Mice

I was interested in measuring macrophage activity due to their important role in directing the innate immune response upon pathogen challenge. Specifically, I wanted to observe whether the activity and expression of myeloid cells in the blood of SPF and CoH mice differed, and how that might lead to more activated macrophages once they migrate to their respective tissues. Although we don't expect activated myeloid-lineage cells in the blood of SPF mice, it is unclear whether activated myeloid cells are present in CoH mice. In order to compare the myeloid cells in SPF versus CoH mice, groups of 5 mice were bled weekly via retro-orbital bleed.

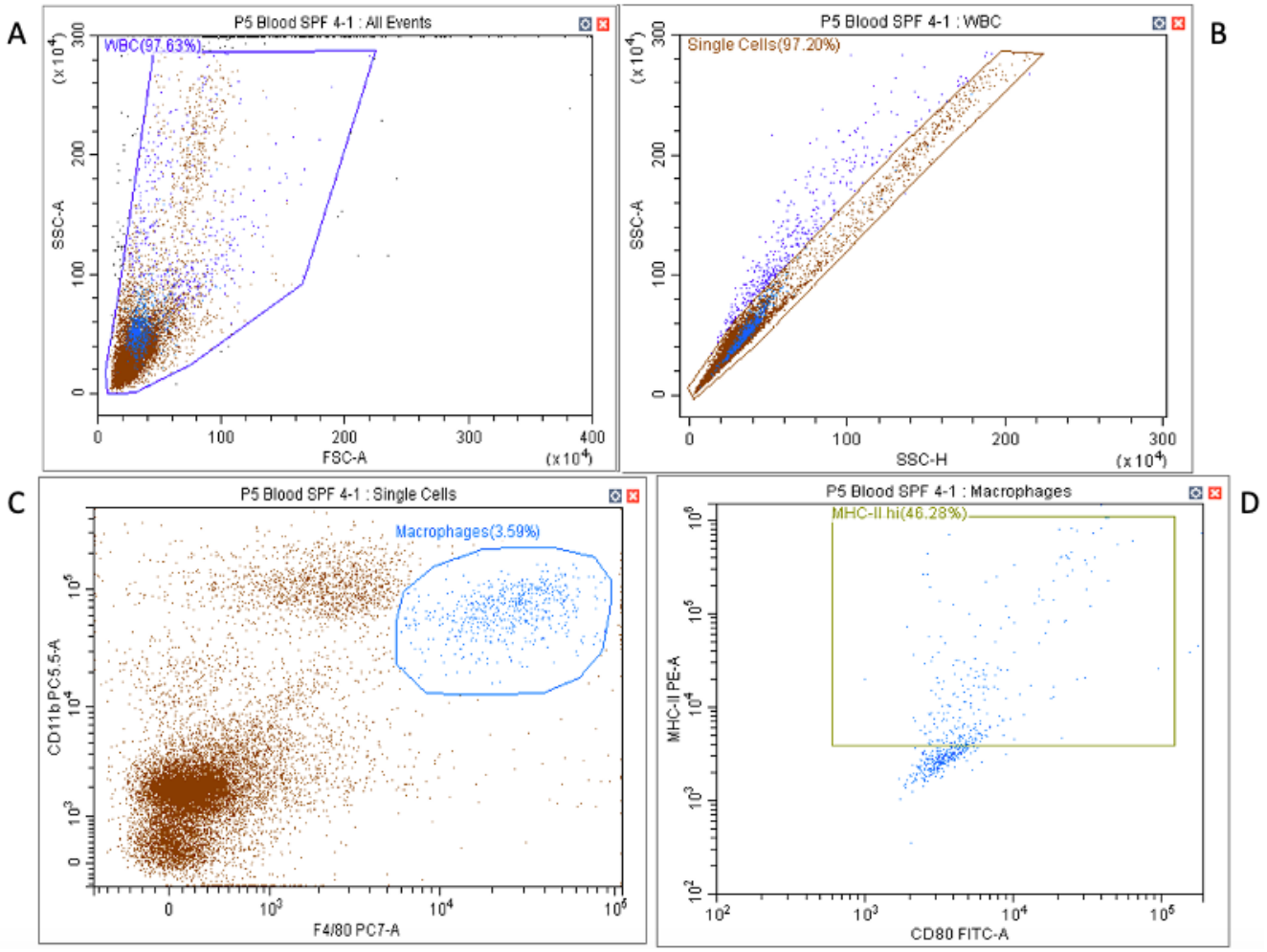

Figure 7. **Representative gating strategies used for weekly retro-orbital bleeds and harvests.** Gating strategies used in the CytoFLEX software to compare the frequency of F4/80+, CD11b+, and MHC-II+ cells in SPF and CoH mice. White blood cells were gated on forward-and side-scatter axes (A), which were then more finely gated into single cells by using side-scatter height and side-scatter area (B). Out of the single white blood cells gated, F4/80+ and CD11b+ were identified and gated as a population of macrophages, which is the typical surface stain for tissue macrophages (C). From this macrophage population, MHC-II+ cells were isolated and quantified (D). Identical gating was used for all blood samples per time point during co-housing, and analysis was done similarly across bleeds for all cohorts. The above sample gating technique example is from an SPF mouse in cohort 4 on a day 7 bleed.

After collection of SPF and CoH blood, samples were stained with specific antibodies to quantify the number (F4/80+ and CD11b+ cells) and activation state (MHC-II) of the myeloid cells. Over the course of our weekly bleeding during co-housing, CoH mice, but not SPF control mice, showed an increase in a number of cells expressing F4/80 and CD11b cell surface markers. CoH mice also demonstrated an increased population of MHC-II+ cells, which are found on activated antigen-presenting cells when activated, while SPF mice did not. Figure 7 shows the gating strategy used in the CytoFLEX software to isolate these cell populations. SPF mice did not gain microbial exposure through the process of co-housing (Table 3), and therefore predictably did not show an increase in CD11b+ F4/80+ macrophages over 30 days. Figure 8 shows that at day 14 of co-housing, 5-7% of CoH cells were F4/80+ CD11b+.

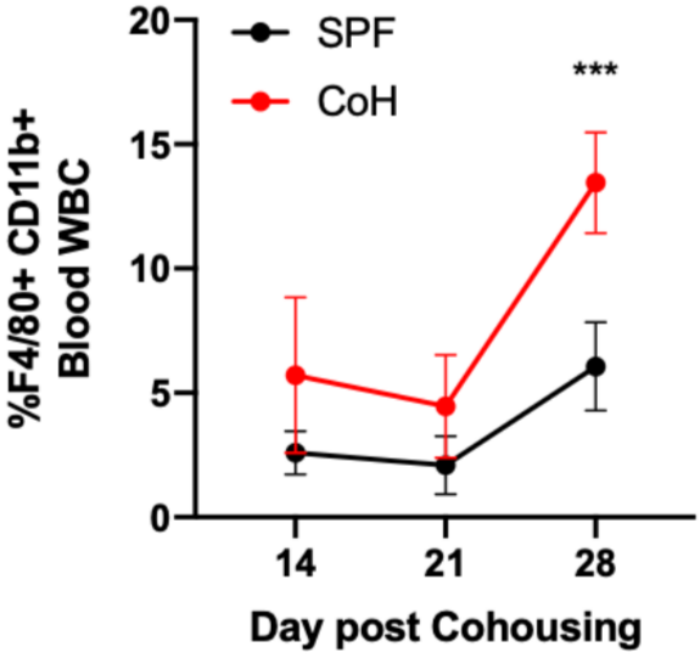

Figure 8. **CoH mice developed more numerous macrophage cells than SPF mice.** CoH mice developed a higher number of more experienced F4/80+ CD11b+ macrophages than SPF mice over a co-housing period of 30 days. Each occurrence of weekly bleeding consisted of 4-5 mice. Data is from cohort 2 and has been repeated for 4 cohorts of mice. T-tests were used to compare groups for each time point with day 28 having a p-value < 0.01.

MHC-II is one of two cell surface proteins that plays an important role in adaptive immunity (Lipski et al., 2017). MHC-II is expressed by activated antigen presenting cells (APCs) like macrophages and dendritic cells when they are exposed to pathogen-associated molecular patterns (PAMPs) and is used to process and present external antigens to naïve T helper cells (Lipski et al., 2017). With this information in mind, we used MHC-II as a marker of macrophage activation in our SPF and CoH mice (R&D Systems). As previously stated, Figure 9 shows the process of gating for MHC-II+ cells, and this technique was used for both SPF and CoH mice across all cohorts. MHC-II was found to be significantly higher in macrophages from CoH mice compared to SPF mice.

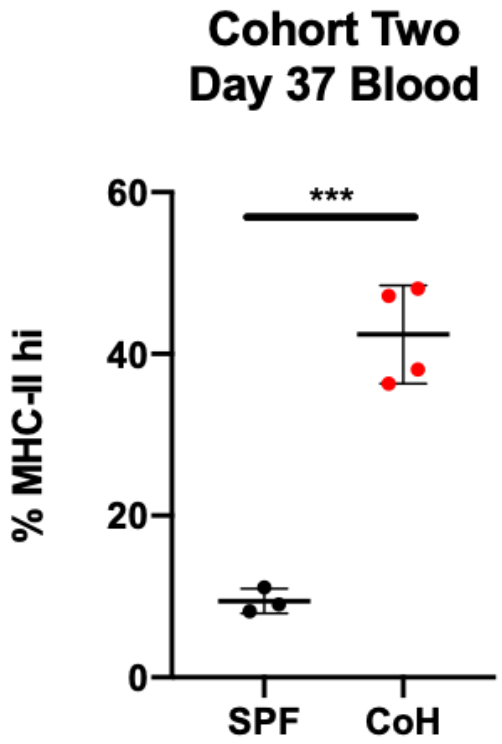

Figure 9. **CoH mice developed more functionally activated macrophage cells than SPF mice.** The data shows that F4/80+ CD11b+ cells also express increased MHC-II+. 4-5 mice were bled for each group and cohort. Data are shown for cohort 2 and are representative of all cohorts.

At day 37, there were 20% more MHC-II+ cells in CoH mice than in SPF mice. As expected in SPF mice, the number of MHC-II cells remained relatively low due to the lack of pathogenic exposure.

Overall, CoH mice developed a significantly higher frequency of F4/80+ and CD11b+ macrophage cells compared to SPF mice during their 30-day co-housing period. In addition, the frequency of MHC-II+ macrophages were higher in the F4/80+ and CD11b+ subset of macrophages in CoH mice than in SPF mice at nearly every bleed day post-cohousing.

CD80 was another cell surface marker used to differentiate between active and inactive macrophages. Both CD80 and CD86 are cell surface markers that can be found on antigen-presenting cells (e.g. macrophages) when activated. These cell surface markers bind to specific T-cell ligands, which will activate or inhibit the function of the T

cell it is acting on. Like MHC-II, CD80 and CD86 are hypothesized to be more frequently expressed by activated macrophage cells. However, we found no significant difference in expression of CD80 between SPF and CoH macrophage populations. It was suggested that we switch to using the CD86 marker instead (Jesse Williams PhD, personal communication). Figure 11 shows the gating strategy implemented with the CD86+ cell marker.

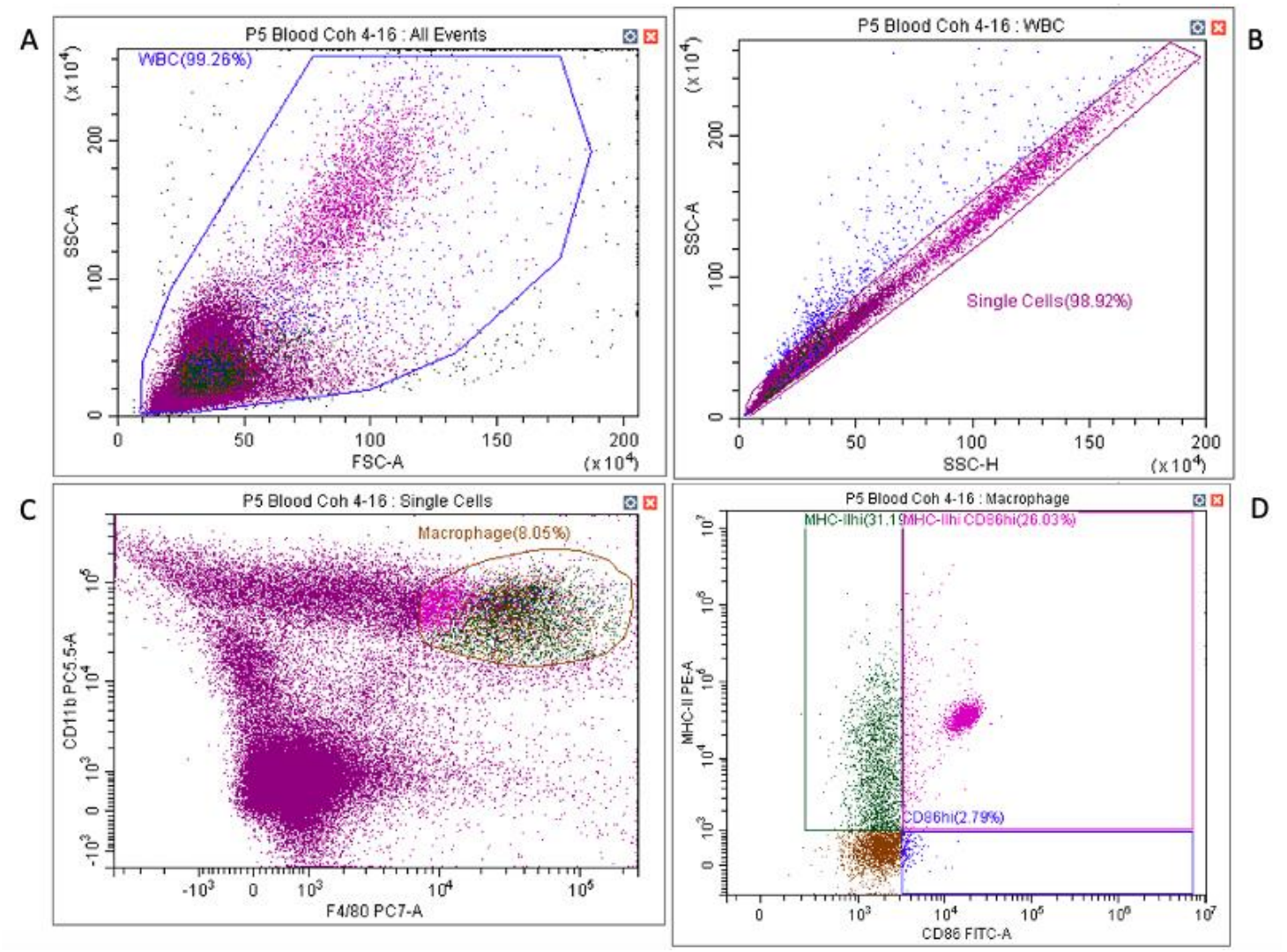

Figure 10. **Gating strategy used for CD86+ cells.** Previous gating strategies were used in the CytoFLEX software to compare the frequency of F4/80+, CD11b+, and MHC-II+ cells in SPF and CoH mice. The new gating was used for CD86. White blood cells were gated on forward-and side-scatter axes (A), which were then more finely gated into single cells by using side-scatter height and side-scatter area (B). Out of the single, white blood cells gated, F4/80+ and CD11b+ were identified and separated into a gated population of macrophages (C). From this macrophage population, MHC-II+ cells were isolated and quantified into their own separate gate, as were new cell populations consisting of CD86+, and MHC-II+ CD86+ (D). Gating was identical for all blood samples per time point during co-housing, and analysis was done similarly across bleed days for cohort 4 specifically. The above gating example is from a CoH mouse in cohort 4.

Though we were only able to use this new macrophage marker in one cohort of mice, the data was significant and gave us more information on the activation state of CoH macrophages versus SPF macrophages. Interestingly, figure 11 shows us that the number of CD86+ cells was significantly increased in SPF mice than in CoH mice. This is not what we hypothesized, as CD86 expression is an important ligand associated with the co-stimulation of T lymphocytes, and influences the activation of T cells, and therefore we would expect it to be increased in activated macrophages (Axelsson et al., 2020).

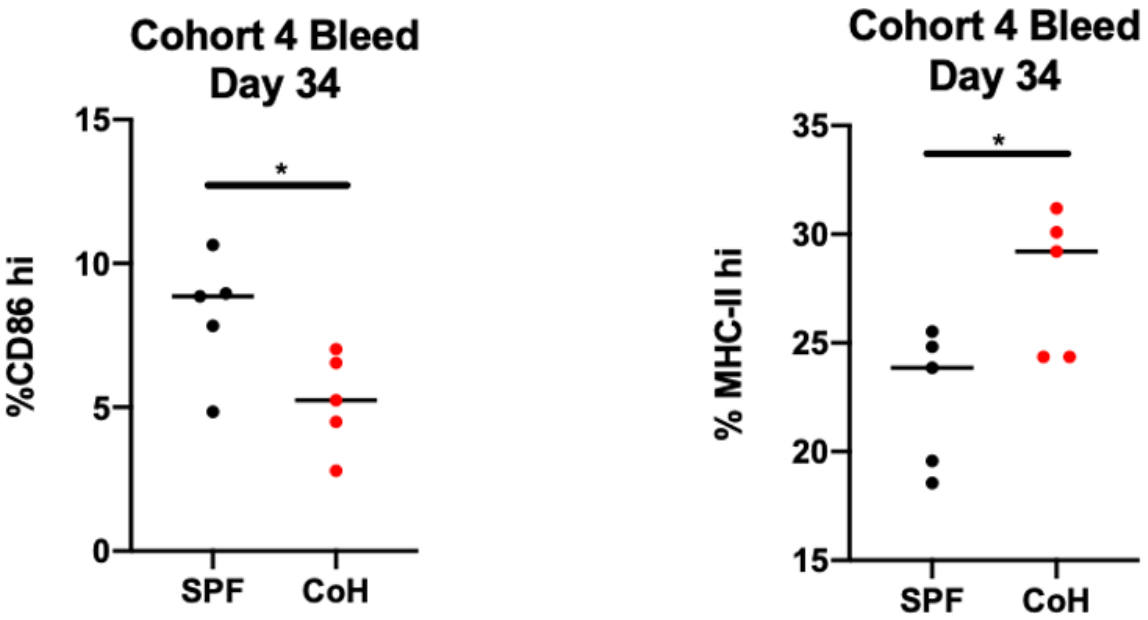

Figure 11. **CoH mice have a decreased number of CD86+ cells and elevated MHC-II+ cells after 30+ days of co-housing.** As previous data has shown, CoH mice had significantly higher MHC-II+ cells in blood samples than SPF mice. Interestingly, SPF mice had significantly higher occurrences of CD86+ cells on day 34 of co-housing than CoH mice.

CoH Mice Developed More Numerous Activated Macrophages than SPF Mice in Peritoneal Macrophage Wash Samples

After successful co-housing experiments were done across multiple cohorts of mice, we used our initial data to improve our mouse model. We noted that macrophage quantity and activation in CoH mice were difficult to measure in CoH organs such as lymph node and spleen samples. While these samples could be stained with macrophage-specific cell surface antibodies, no useful data could be ascertained due to low cell numbers. We turned to peritoneal lavage to obtain an enriched population of macrophages in the SPF and CoH mice. Gating for the peritoneal macrophages was done similarly to the gating strategies used in the blood samples. Figure 12 shows the specific gating strategies that were used.

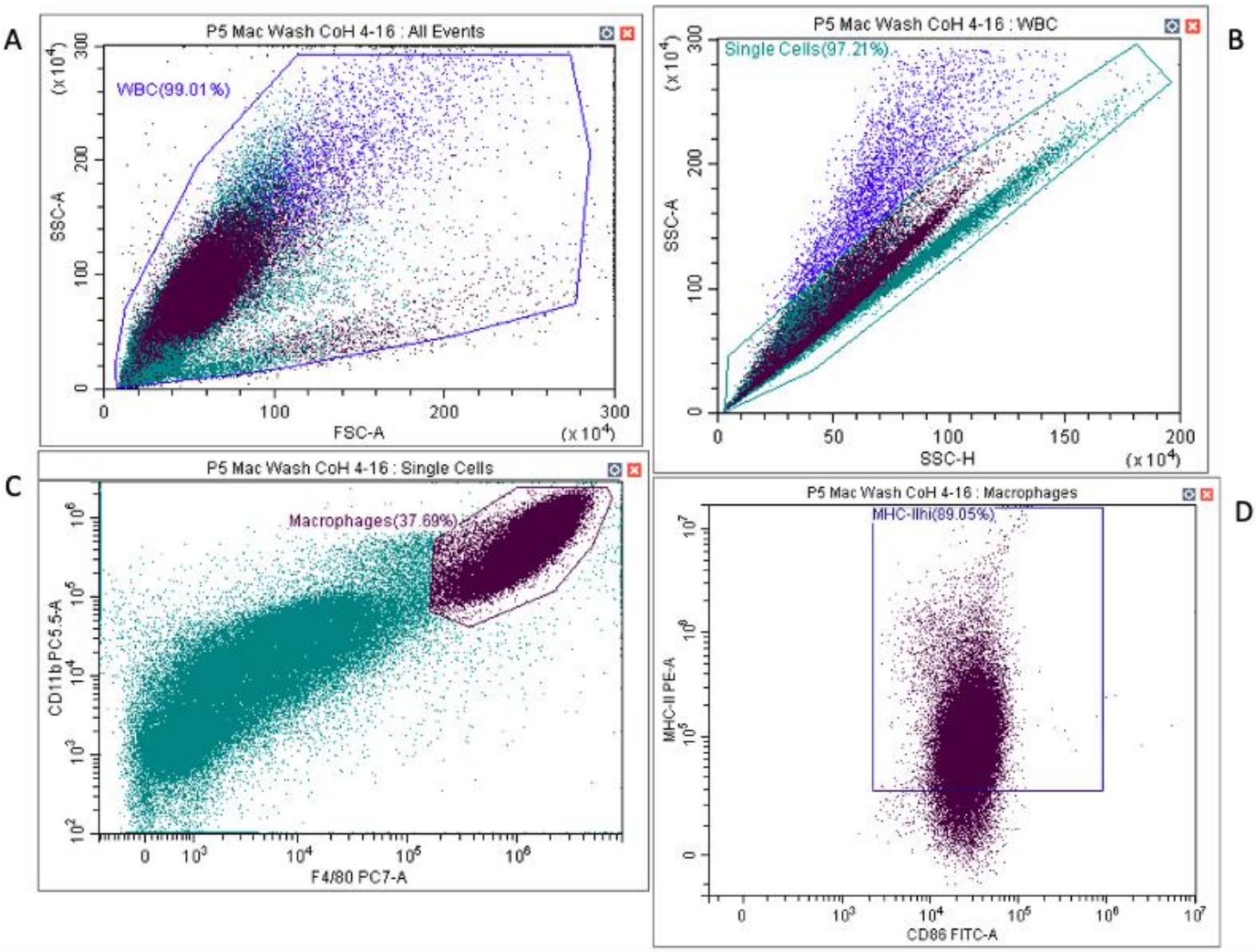

Figure 12. **Representative gating strategy used for peritoneal macrophages.** Gating strategies used in the CytoFLEX software to compare the frequency of F4/80+, CD11b+, and MHC-II+ cells in SPF and CoH mouse peritoneal lavage samples. White blood cells were gated on forward-and side-scatter axes (A), which were then more finely gated into single cells by using side-scatter height and side-scatter area (B). Out of the single, white blood cells gated, F4/80+ and CD11b+ were identified and separated into a gated population of macrophages (C). From this macrophage population, MHC-II+ or MHC-II^{hi} cells were isolated and quantified into their own separate gate (D). Gating was identical for all peritoneal lavage samples per time point during co-housing, and analysis was done similarly across all cohorts. The above gating example is from an CoH mouse in cohort 4.

Similar to the myeloid cells where the CoH mice showed increased activated macrophages, Figure 13 shows that CoH mice had significantly higher numbers of F480+ CD11b+ MHC-II+ cells than SPF mice did. This data was a nice source of validation for our hypothesis that macrophages in CoH mice would be more activated, and the peritoneal lavage gave us an enriched population of macrophages present. CoH mice had over 60% more F480+ CD11b+ MHC-II+ cells at day 30 than SPF mice.

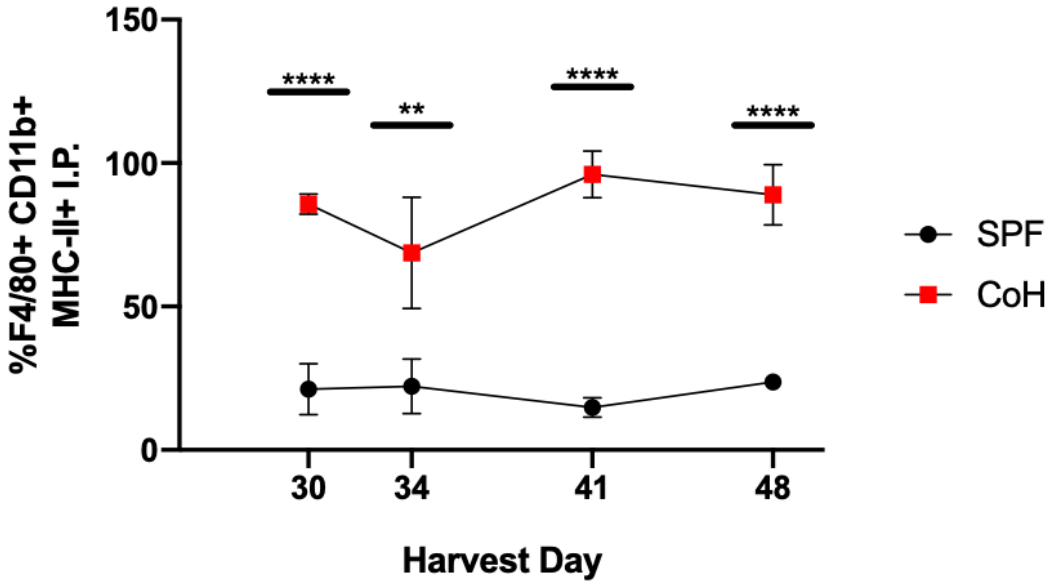

Figure 13. **CoH mice have more activated and functionally effective macrophages than SPF mice.** CoH have significantly more activated and effective F480+ CD11b+ MHC-II+ macrophages on days 30, 34, 41, and 48 than SPF mice. Coh F480+ CD11b+ MHC-II+ cells decreased from 80-85% to 75-80% over a four-day period, but the data was still significant and the F480+ CD11b+ MHC-II+ cells increased once again on day 41. F480+ CD11b+ MHC-II+ macrophages in SPF mice stayed relatively consistent at about 20%. Data are shown for 4-5 mice per group per peritoneal lavage sample for days 30-48 in cohort 4. This has been repeated and has been consistent with other cohorts where the peritoneal lavage protocol was utilized.

Because the specific antigen-presenting cell antibodies showed increased expression in CoH mice, we hypothesized that these peritoneal macrophages were more activated than their SPF counterparts. To test this hypothesis, we did *in vitro* TLR stimulation of peritoneal macrophages to observe if there were increased activation levels of MHC-II, CD86, or CD80 antibodies. We also performed preliminary ELISA testing to observe whether or not CoH macrophages produced larger quantities of inflammatory cytokines.

Peritoneal Macrophages are not more Activated After *in vitro* Stimulation

Studies have shown that in MHC-II knock-out mice, there is a decreased responsiveness to TLR stimulation (Frei et al., 2010), supporting that MHC-II might enhance TLR-mediated innate immune responses. We hypothesized that the production of inflammatory cytokines in or macrophage populations might be directly correlated with the increased expression of MHC-II as shown by some of the macrophages. Our goal with *in vitro* stimulation of TLR 4 and TLR 5 was to examine the macrophage cell response in SPF versus CoH mice. Peritoneal lavage cells were cultured *in vitro* for 1 or 3 days with TLR agonists LPS-EK (TLR 4) or FLA-ST (TLR 5). At the start of each day of assay, 1 mL of supernatant was pipetted collected and stored at -80 C for future ELISA analysis. Our preliminary data suggests that macrophages were producing TNF-α upon stimulation (data not shown). The remaining amounts of cells were analyzed via flow cytometry.

Since our data showed us that CoH mice have increased frequencies of activated phenotype macrophages, we measured MHC-II expression in the cultured peritoneal macrophages as well. As previously mentioned, MHC-II aids in the antigen presentation

of macrophages to CD4+ T helper cells. Figure 14 shows the gating strategy used for analyzing MHC-II expression in the cultured cells, using the CytoFLEX software. As in blood or peritoneal macrophage samples, we compared the percentage of F4/80+ CD11b+ MHC-II+ cells between SPF and CoH mice 1 day (approximately 24 hours) and 3 days (approximately 72 hours) after stimulation with TLR agonists. Additionally, we only looked at cells that were alive, so that any of the cells that died during incubation would not alter results.

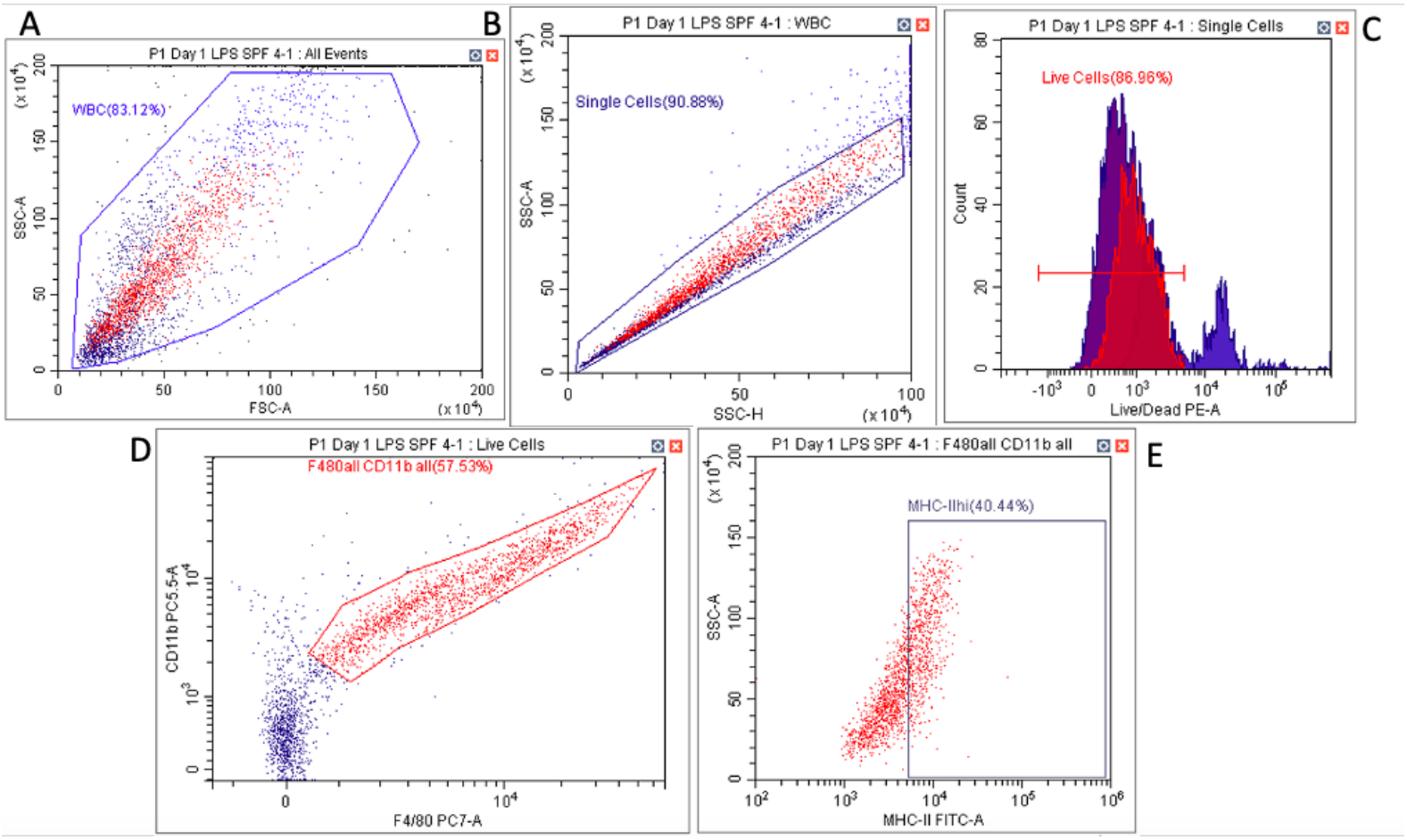

Figure 14. **Representative gating strategy used for cell culture data between LPS and FLA TLR agonists.** Gating strategies used in the CytoFLEX software to compare the frequency of F4/80+, CD11b+, MHC-II+, and live/dead cells in SPF and CoH mice cell culture samples. White blood cells were gated on forward-and side-scatter axes (A), which were then more finely gated into single cells by using side-scatter height and side-scatter area (B). Out of the single, white blood cells gated, F4/80+ and CD11b+ were identified and separated into a gated population of macrophages(C). Our gating for F4/80 and CD11b for cell cultures was done in a slightly different way and was done with the intentions of gating for F4/80 CD11b low, middle, and high cells. From this macrophage population, MHC-II+ or MHC-II^{hi} cells were isolated and quantified into their own separate gate (D). Gating for CD86+ was done in an identical manner to MHC-II+ (not pictured). Gating was identical for all cell culture samples per time point during co-housing, and analysis was only done for cohort 4. The above gating technique example is from a SPF mouse in cohort 4.

We were unable to observe any significant, repeatable differences between SPF and CoH F4/80+, CD11b+, MHC-II+, and live/dead macrophage cells on either day 1 or day 3 of *in vitro* TLR stimulation (Figure 15). This was consistent for both LPS-EK and FLA-ST TLR agonists. There was, however, significant data for some of the LPS stimulation data. On day 3 of LPS stimulation, the frequency of live F4/80+ CD11b+ macrophages were significantly higher in CoH macrophage cells, and on day 1 of LPS stimulation, SPF cells had significantly higher expression of F4/80+ CD11b+ MHC-II+. Data such as this was only seen once and in all other cell culture data showed no significant difference for these two variables. In addition, we did not control for the starting number of macrophages in each sample, and therefore initial *ex vivo* differences in macrophage numbers and activation status could impact our analysis after *in vitro* stimulation.

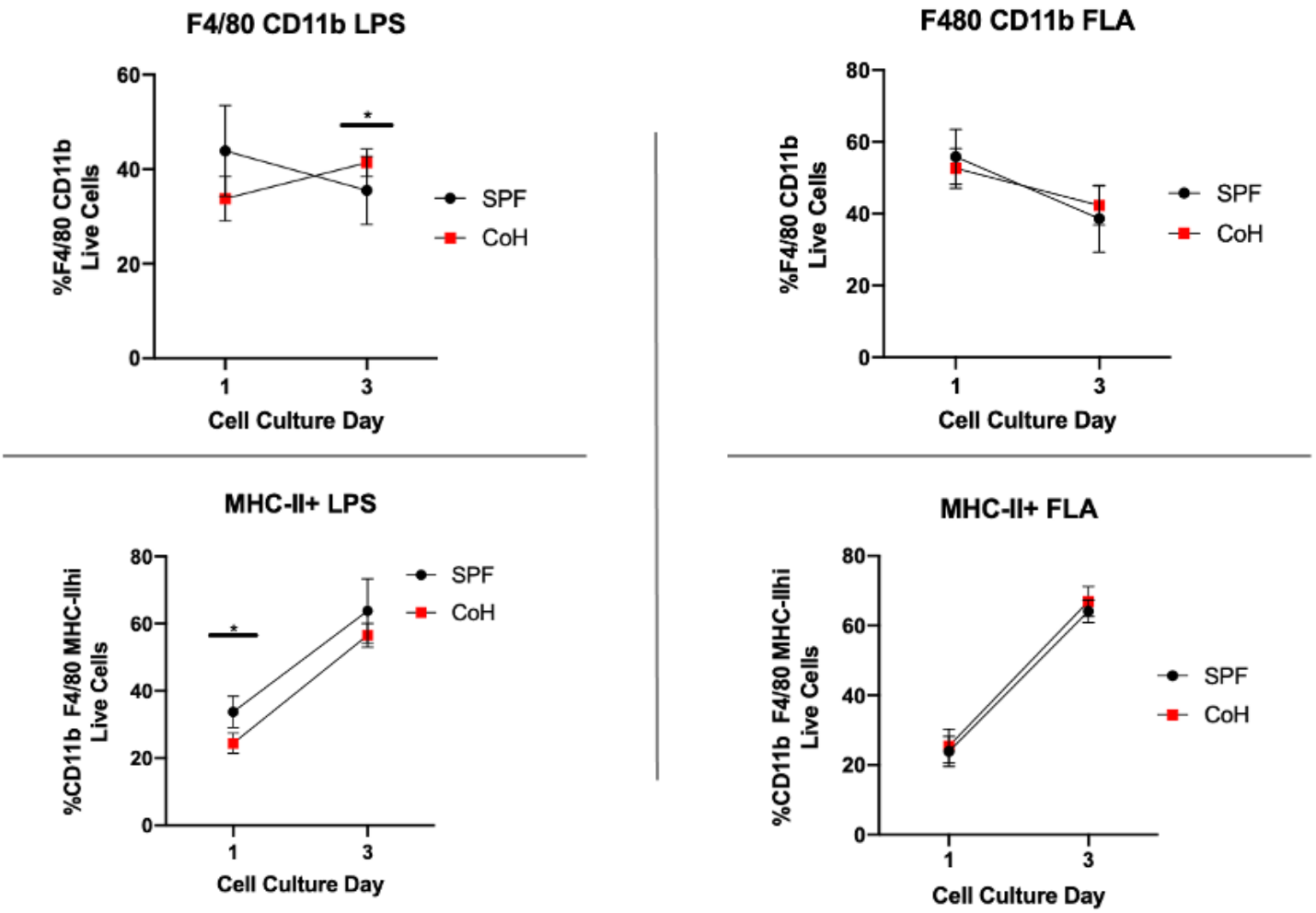

Figure 15. **The in vitro stimulation of macrophage Toll-like receptors.** CoH macrophages had increased expression of F4/80+ CD11b+ LPS activity on day 3, and SPF macrophages had increased expression of F4/80+ CD11b+ MHC-II+ on day 1. Though these data points were significant, we were unable to reproduce this data in any other SPF versus CoH mouse groups. Data comes from a single cohort of SPF and CoH mice.

During our *in vitro* TLR stimulation, we also wanted to see if there were any differences in expression of CD86 between SPF and CoH mice. Representative gating for cell culture data was used for all CD86-associated findings. Figure 16 shows that there were no significant differences on day 1 when stimulated with LPS-EK and FLA-

ST. Day 3 values for CD86 expression could not be shown, because for our day 3 stimulation we were looking for MHC-II levels, and not CD86 expression.

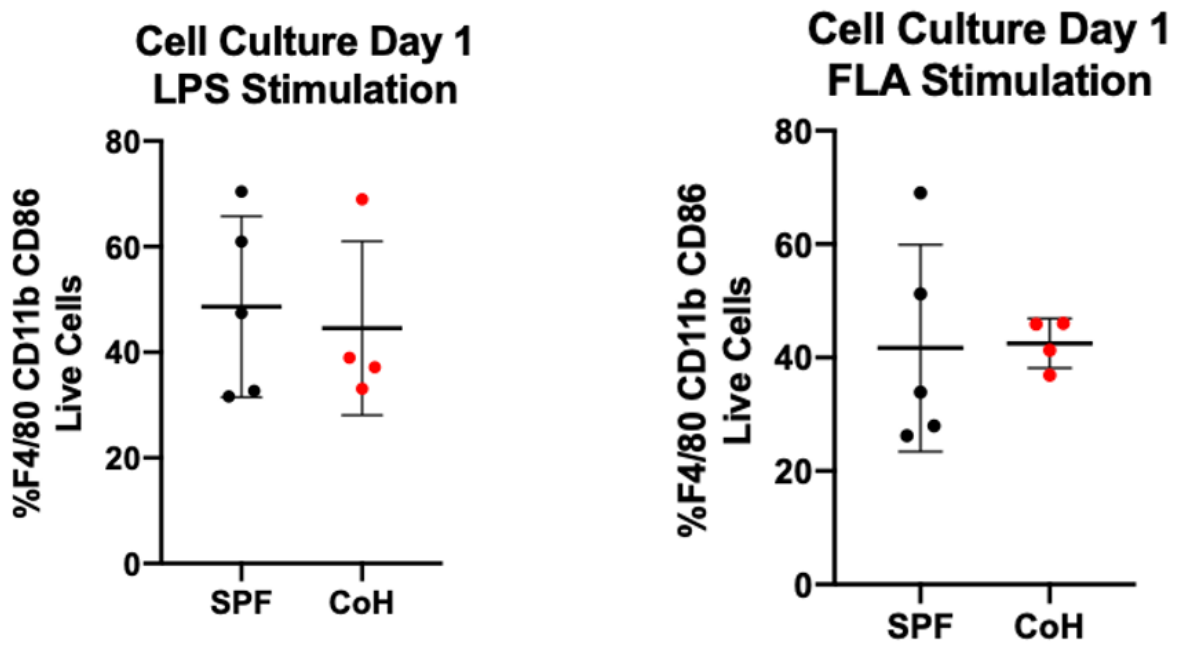

Figure 16. **In vitro Toll-like receptor stimulation data day one.** Both LPS-EK and FLA-ST stimulation data sets showed no significant differences between SPF and CoH mice. Data was performed for one group of mice in cohort 4, and day 3 cell culture data could not be included.

In summary, we could observe some minor trends in TLR stimulation between SPF and CoH mice. The significant observations we were able to make, however, were not consistently obtained across all cell culture data or individual mouse samples. Most of the data from our cell culture experiments can be deemed insignificant. Additionally, there were significant differences in the increased expression of F4/80+ CD11b+ MHC-II+ in CoH myeloid cells, and increased expression in peritoneal lavage samples.

Chapter 5: Discussion

Even though most immunology research utilizes traditional SPF lab mice, researchers are using microbially experienced mice more frequently. Because of the pathogenic nature of microbially experienced mice, it is no surprise that the process of having a more widely used “dirty” mouse model is an arduous task to establish in scientific communities. Because of the safety precautions necessary to work with dirty mouse models, existing data is preliminary and unexplored at best, and there are very few labs that study the effects that environmental exposure could have on innate immune function. To our knowledge, we are currently the only lab doing research on macrophage activity in dirty mice.

We were able to observe significant differences in active F4/80+ CD11b+ MHC-II+ myeloid cells in CoH blood when compared to SPF blood. This data was reproduced amongst 4 different cohorts. In cohort 2, when we began to analyze peritoneal macrophages, the data also showed significantly more active F4/80+ CD11b+ MHC-II+ macrophages in CoH peritoneal samples than SPF samples. This was consistent for both cohort 3 and cohort 4. In addition, peritoneal lavage gave us a more concentrated sample of macrophages to work with, whereas it was sometimes difficult to find macrophages in blood samples due to the fact that macrophages in the blood are considered monocytes and don’t actually become macrophages until they migrate into tissues. We also measured the quantity and activation of macrophage cells in lymph node and spleen samples, but this data was inconclusive and insignificant, as it was often rather difficult to isolate enough macrophages to work with. For future studies, it could be beneficial to establish or research various skin digestion protocols to

potentially further isolate macrophages in order to see how macrophages in other locations differ from those we have examined.

Unexpectedly, when we started to test for CD86+ expression in the blood and peritoneal samples, SPF mice showed a significant increase in CD86+ expression when compared to CoH mice. As previously stated, CD86 is a cell surface marker specific to activated antigen-presenting cells – a population of cells we wouldn't expect to see in SPF mice. If the data could be reproduced in the future, perhaps SPF mice have the ability to bind to particular T cell ligands that were not observed in CoH mice. Alternatively, perhaps SPF upregulate the expression of CD86 and downregulate the expression of CD80 in response to no pathogenic challenges, Future research is needed to obtain a more definitive answer. We were only able to test for the expression of CD86 once in our SPF and CoH samples (in cohort 4). We hope to validate these results with future mouse cohorts where CD86 will be used rather than CD80, which proved unreliable. Since we now know that CD80 was not the best cell surface marker to be using when looking for macrophage activation, future studies can start off in the right direction using the preliminary data from this study.

A more detailed analysis of *in vitro* TLR stimulation should be done with peritoneal macrophages, as we noted there were few statistically significant findings in these cell cultures. Since we cannot control which specific pathogens infect our CoH mice, it is difficult to tell exactly what TLR agonists are most beneficial for studying differences in the functionality and efficacy of macrophages. We used TLR 4 and TLR 5 as baseline, preliminary agonists since TLR 4 and 5 are widely known and studied. They are also able to bind to very common bacterial structures, making them a good fit for initial

testing. The potential for other TLR agonists to show significant data is high and should be explored as a potential future experiment.

In future mouse cohorts, we would also like to include a more in-depth analysis of TNF-α concentrations in the supernatant of macrophage cell samples. Because of limited research time, we were only able to perform preliminary ELISA data on one of our cohorts of mice, but the testing was not reproduced. Analyzing TNF-α secretion confirmed the TLR agonists actually stimulated the macrophages in our cell culture experiments, leading to potentially more telling data to come if the testing were to be performed again.

Herzenberg et al. (2009) have described the peritoneal macrophages as two distinct subsets referred to as large peritoneal macrophages (LPM), and the small peritoneal macrophages (SPM). Herzenberg discovered that the LPM subset is the most abundant (90% of all peritoneal macrophages) macrophage subset that resides in the peritoneal cavity of adult mice, and that their size, surface marker phenotype, and functions vary greatly from the SPM subset (Herzenberg et al., 2009). The LPM expressed three times higher CD11b and approximately 80 times higher F4/80 as the SPM subset of cells found in the peritoneum (Herzenberg et al., 2009). It was also noted that the SPM subset expressed MHC-II whereas LPM did not. Other surface markers specific to macrophages like CD80 and CD86 were also found to be expressed at higher levels in LPMs.

Herzenberg noted that there was a distinct population of monocytes in the blood of adult mice, whereas the subset groups of LPM and SPM subsets are completely absent in blood samples, even though it was observed that SPM are derived from blood

monocytes (Herzenberg et al., 2009). Monocytes are cells present in the blood that become resident macrophages when they migrate into tissues. The ability of LPM and SPM subsets to respond to pathogenic challenges was also assessed and it was found that TLR 4 was readily detectable on LPMs but was less detectable in the SPM population of macrophages (Herzenberg et al., 2009). This suggests that, depending on the type of macrophage cells present in the peritoneum that are responding to an infection, the response to the pathogen exposure can be very different.

Lastly, when the peritoneal cavity is stimulated with LPS or thioglycolate, it was found that SPM's were the predominant subset of macrophages found in the peritoneal cavity (Herzenberg et al., 2009). These SPM subset cells are derived from blood monocytes that rapidly enter the peritoneal cavity after stimulation occurs. It was also discovered that the phagocytic activity of these two subsets can vary depending on whether you stimulate LPM's or SPM's, and whether the stimulation takes place *in vitro* or *in vivo*. *In vitro*, LPS stimulated the LPM subset of cells that Herzenberg was working with, but when the same stimulus was used *in vivo* both subsets of macrophages produced phagocytic products such as nitric oxide, but responded in different ways (Herzenberg et al., 2009).

With regard to the work of Herzenberg et al., there are important questions to be asked about what subsets of macrophages were isolated from the peritoneal lavage. In the future, it would be beneficial to first ascertain which subset of macrophages we were using, so that future stimulation and observation of these cells could be steered in the proper direction. Herzenberg mentioned that the primary subset of macrophages in the peritoneal cavity are the LPM's. However, these were the dominant cell type in

unstimulated adult mice, and the LPM population rapidly decreases once LPS or thioglycolate stimulation occurs, in which case SPM's become the dominant macrophage subset. Given the insignificant amounts of expression seen in our TLR stimulation studies, it is my guess that we were not stimulating the appropriate macrophages, because LPM's do not show much expression of MHC-II+ while SPM's do when there is exposure to LPS or thioglycollate. Given this information, future experiments would benefit from somehow isolating these SPM subsets and performing stimulatory experiments on them instead of how we performed our *in vitro* TLR stimulation. A hallmark of the SPM's is that they are blood monocytes that become resident macrophages in the peritoneal cavity. Perhaps if we were to intentionally expose our mice to LPS or thioglycolate this would act as a catalyst to cause a migration of monocytes into the peritoneal cavity. TLR stimulation could then be done on these macrophages that would hopefully more closely resemble the SPMs in the Herzenberg et al. study.

Another interesting study that could take place in the future is one that could look at the potential for peritoneal macrophages and myeloid cells to become primed from previous encounters with pathogenic agents. Feurstein et al. (2020) found that dermal macrophages in mice that were previously infected with *Staphylococcus aureus* showed faster monocyte recruitment, increased bacterial killing, and improved healing upon the presence of a secondary infection of *S.* aureus. Although the idea of innate immunity largely involves the fact that the innate immune system is a non-specific, rapid response to pathogens, maybe macrophages have the ability to become specialized and are able to be more efficient in their phagocytic functions if they are exposed to the same

stimulus multiple times. This could easily be tested if a lab was able to isolate resident macrophage cells from either tissues or from the LPM subset of macrophages in the peritoneal cavity, as these were found to be more efficient phagocytic cells (Herzenberg et al., 2009). After the cells are obtained, they could be stimulated with various bacterial stimuli, and one could record the response the macrophages showed by tracking expression levels of surface cell markers. Shortly after the first stimulation occurred, one could repeat the process and see if the macrophages had any increased or more functionally efficient responses.

In conclusion, we have successfully shown that our lab kept SPF mice clean and that CoH mice were able to contract various pathogens in a co-housing model over the course of 4 cohorts. CoH mice were also shown to have more numerous and more activated myeloid cells in blood and macrophages in peritoneal lavage samples across multiple cohorts. This leads us to accept our first hypothesis, which stated that macrophages have larger population sizes as indicated by elevated cell markers of F4/80+ CD11b+ cell population and are more activated (higher levels of MHC-II) in CoH mice than in SPF mice *in vitro*. The data we collected led us to reject our second hypothesis, which stated that macrophages from CoH mice exhibit more robust activation in terms of inflammatory cytokine production *in vitro* upon Toll-like receptor (TLR) signaling when compared to macrophages from SPF mice. Further experimental procedures and analysis need to be done to determine whether macrophage TLR stimulation is more active in CoH mice versus SPF mice.

Adding CoH mice as an additional model in immunology research has the potential for improving our understanding of adult human immune systems and how the hygiene

hypothesis (or exposure to microbial antigens) can impact the innate immune system. Because SPF mouse models fail to adequately simulate an adult human immune system, we have helped show that dirty mouse models are valuable tools for immunology research. Though SPF mice have historically been incredibly important for immunology research, dirty mice may be the key to answering questions regarding how persistent environmental exposures may play a role in shaping the overall efficiency of the immune system. Dirty mice could be used to test for autoimmune disorders and allergy susceptibilities, which in turn could potentially offer relief and answers to those struggling with immune-related disorders. In summary, co-housing lab mice could serve as a valuable model to increase the understanding of how environmental exposure can impact adult human immune-related disorders and imbalances.

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