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Microbial experience influences tumor- infiltrating T lymphocytes

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ABSTRACT

Immune cells, including T cells, have been used for anti-cancer therapy with varying degrees of success. One potential reason for immunotherapy failures in clinical trials may be that typical specific pathogen free (SPF) mice do not accurately replicate human microbial experience, which has important influence on shaping the adaptive immune response. Recently, several previous studies have shown that the immune system of SPF mice more closely resembles newborn human immunity, whereas immune systems from mice exposed to diverse pathogens more closely reflect adult human immunity.

To study the impact of microbial experience on the immune response, we have adopted a mouse model of microbial experience by co-housing SPF mice with mice purchased from a local pet store, therefore exposing the SPF mice to various viral, bacterial, fungal, and parasitic pathogens. Pathogen testing confirmed that the co-housed (CoH) mice are exposed to pathogens and that the SPF controls kept in the same vivarium remain pathogen-free. We used flow cytometry to show that CoH mice, but not SPF controls, also gain significantly more antigen- experienced and differentiated $KLRG1^{hi}CD44^{hi}$ CD8+ T cells in blood, lymph nodes and spleen.

We injected B16 melanoma cells subcutaneously into SPF and CoH mice and monitored tumor development and T cell activation ex vivo and in vitro. CoH tumors had increased frequencies of $KLRG1^{hi}CD44^{hi}$ CD8+ T cells compared to SPF tumors, and CoH tumor- infiltrating CD8+ T lymphocytes exhibited increased activation upon in vitro stimulation. Lastly, CD8+ T cells from tumors, but not spleens, of CoH mice had a higher percentage of divided cells, indicating a potentially more effective T cell phenotype.

Ultimately these findings will contribute to our understanding of how microbial experiences shape anti-tumor immunity and could have significant implications for future immunotherapy research. We hope that CoH mice, which capture more of the individual microbial variability seen in human patients, can be utilized as an additional model to improve translation from immunology and oncology research in the lab to improving success rate of human clinical trials.

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ABBREVIATIONS

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

PS= pet store mice

CoH= cohoused mice; SPF mice cohoused in the same cage with PS mice for 30+ days

KLRG1= killer-cell lectin like receptor G1; marker of antigen-experience and differentiation

GzB= Granzyme B; effector molecule of CD8+ T cells

Bd= blood samples

LN= lymph node samples

Spl= spleen samples

SLECs= short-lived effector cells

MPECs= memory-precursor effector cells

LLECs= long-lived effector cells

IFNγ= Interferon γ; cytokine released by CD8+ T cells upon activation

ELISA= enzyme-linked immunosorbent assay; used to measure concentrations of IFNγ in serum and supernatant samples

CHAPTER 1: INTRODUCTION

INTRODUCTION

Mice commercially available from different vendors have been a staple model organism in biomedical research for over 100 years (Russell, 1985). They have provided valuable insight in understanding basic physiologic and pathologic processes in humans, including understanding the functions of the human immune system (Hood et al., 1983; Russell, 1985). Evidence from various studies in recent years suggests that while specific pathogen free (SPF) mice generally used in research have many advantages; they are not the most suitable model for immunological and oncologic research (Abolins et al., 2017; Beura et al., 2016a; Reese et al., 2016; Rosshart et al., 2017). Due to limited exposure to pathogens, the adaptive immune system of SPF mice has little antigen experience and its immune state is reflective of a human newborn infant (Beura et al., 2016a; Masopust et al., 2017; Seok et al., 2013).

The immune system of mammals can be categorized into a more general, rapidly responding innate- and a more specific adaptive immune branch. Humans are continuously exposed to various microbes and infected with pathogens throughout their lifespan, which significantly shape the adaptive response, including T lymphocytes (Beura et al., 2016a; Gerritsen & Pandit, 2016). There are various subsets of T cells based on function and these can be further characterized by specific markers and CD8+ or “cytotoxic” T cells are specialized in recognizing intracellular pathogens of infected cells as well as cancerous cells (Curtsinger et al., 2003a; Gerritsen & Pandit, 2016; Gulzar & Copeland, 2004; Ioannides & Whiteside, 1993). CD8+ T cells effectively kill these cells by secreting Perforin and Granzyme B upon activation to initiate apoptosis of infected cells (Curtsinger et al., 2003a, 2005).

Microbial experience influences development of antigen experienced and differentiated CD8+ T cells. Differences in cytokine concentrations and concentrations of Granzyme B also indicate potentially more effective CD8+ T cells in microbially experienced mice (Beura et al., 2016a). Recent insight from complex mouse models, including wild and pet store mice, has demonstrated that these models more closely reflect the adult human immune experience (Abolins et al., 2017, 2018; Beura et al., 2016a; Reese et al., 2016). Additionally, various studies in the last decade have suggested a previously underestimated importance of the commensal microbiota in organisms on cancer development and therapy in both mice and humans (Iida et al., 2013; Matson et al., 2018; Rosshart et al., 2017; Vétizou et al., 2015).

These findings imply a need for a more refined mouse model to study the complex interactions of an experienced immune system with immune related diseases, such as cancer and autoimmune disorders. Cohousing standard "clean" SPF mice with pet store mice (PS) has been shown to successfully introduce microbial experience to the SPF mice generally used in research, while preserving their genetic standardization (Beura et al., 2016a). We propose that these cohoused mice (CoH) are thus an ideal complementary model to study immune- related diseases and cancer.

PURPOSE

We previously established a mouse model with microbial experience by cohousing SPF mice with PS mice and aim to further investigate the CD8+ T cell response in CoH versus SPF mice. We examine how microbial experience effects the anti-tumor CD8+ T cell response by injecting a cohort of SPF and CoH mice with B16 melanoma subcutaneously, and investigating the activation, differentiation and effectiveness for tumor- infiltrating CD8+ T cells.

SCOPE

This study utilizes flow cytometry to analyze specific markers of antigen-experience, differentiation, and effectiveness of CD8+ T cells in blood, lymph nodes, spleens and tumors of mice. Additionally, we activate T cells *in vitro* to look at differences in activation markers and Granzyme B (GzB) concentrations in different tissues and tumors of SPF versus CoH mice. We use Enzyme-linked immunosorbent assays (ELISA) for supernatant of T cell cultures and serum sample to quantify concentrations of Interferon γ (IFNγ), an important T cell cytokine.

ASSUMPTIONS

We have demonstrated previously that we can successfully cohouse SPF mice with PS mice in our lab set-up (Groeber, 2019). CoH mice acquire some pathogens over the cohousing period of 30+ days and develop distinct subsets of CD8+ T cells; SPF mice remain pathogen-free and lack antigen-experienced, differentiated CD8+ T cells. B16 melanoma cells can be cultured and injected subcutaneously at a predetermined concentration and mice will grow tumors in 5-14 days.

RESEARCH QUESTIONS

Hypothesis 1: CD8+ T cells are more antigen experienced, differentiated as indicated by elevated markers of CD44 and KLRG1 and effective (more Granzyme B and IFNγ) in CoH mice compared to SPF mice and respond more rapidly upon stimulation in vitro.

Hypothesis 2: Microbial experience in CoH mice positively influences the anti- melanoma CD8+ T- cell response.

Hypothesis 2A: There are more activated, antigen experienced ($CD44^{hi}CD69^{hi}$) and effective (more GzB and IFNγ) CD8+ T cells present in tumors of CoH versus SPF mice.

Hypothesis 2B: CoH mice have smaller tumors compared to SPF mice.

SIGNIFICANCE

CoH mice could complement and refine immune and cancer research currently done only in SPF mice. If CD8+ T-cells of microbially experienced mice target cancerous cells more effectively, this suggests that individual anti-cancer responses in different patients might in part be due to differences in "education" of T cells. A mouse model with more antigen- experience better mimics the immune state of adult humans and could improve translation from bench to bedside, especially in understanding complex immune-related diseases such as cancer.

CHAPTER 2: REVIEW OF LITERATURE

THE IMMUNE SYSTEM OF STANDARD LAB MICE

Studies done using laboratory strains derived from the common house mouse *(Mus musculus domesticus*) have provided great insight on the physiology and genetics of diseases such as cancer and characterizing the host immune response (Russell, 1985). Because mice are easy to breed, have a relatively short lifespan and high reproduction rate, they are beneficial for understanding basic physiologic and genetic principles. To examine the tumor growth, Harvard-researcher Clarence Cook Little established the first inbred mouse strains, and amongst those also created the standard lab strain C57Bl/6 in the early 20th century (Russell, 1985). In subsequent years, the addition of various other inbred mouse strains with distinct susceptibilities have provided great insight of cancer genetics (Russell, 1985). In addition to genetic and oncologic studies, mice have enabled understanding of basic components of the immune system such as classification of MHC genes (Hood et al., 1983).

While the benefit of laboratory research done in mice is undeniable, there are also considerable differences in the immune state of lab mice compared to humans (Beura et al., 2016b; Mestas & Hughes, 2004; Seok et al., 2013). To reduce influence of outside variables such as infections, temperature changes and diet, genetically identical laboratory mice are generally kept in a very clean and controlled environment. They are commonly housed in specific pathogen-free conditions (SPF), kept on a strict temperature and light cycle, and fed with a standardized diet. While this is a great way to decrease influence of environmental factors on genetic research questions, it might provide an insufficient model organism to study the adaptive immune system (Masopust et al., 2017). The human adaptive immune system is not

fully developed at birth, but instead is drastically shaped by exposure to microbes, chemical and environmental agents with aging in both humans and mice.

MICE WITH MICROBIAL EXPERIENCE REPLICATE THE ADULT HUMAN IMMUNE SYSTEM

Microbial exposure over the time span of a normal laboratory mouse kept under SPF-conditions stays consistently minimal, because novel pathogens are strictly prevented from introduction into mouse cages. Recent studies have suggested that development and differentiation of the immune system in SPF mice might thus be insufficient to model the immune system of an adult human (Abolins et al., 2017; Beura et al., 2016b; Reese et al., 2016; Rosshart et al., 2017).

Studies on wild relatives of lab mouse strains in the United Kingdom have demonstrated that the immune responses in wild mice greatly differs from that seen in laboratory animals, which was indicated by increased production of cytokines and higher antibody titers in wild compared to SPF animals (Abolins et al., 2017, 2018). Additionally, more CD4+ T cells, as well as highly activated macrophages, natural killer cells and dendritic cells could be observed in wild mice. More effector or effector- memory type T cells were present in wild and pet store mice, compared to a higher percentage of naïve T cells in SPF mice (Abolins et al., 2017, 2018).

Similarly, these findings could also be observed in commercially available pet store mice (PS) (Beura et al., 2016a; Ericsson et al., 2017). Characterization of the microbial diversity in two strains of laboratory SPF mice compared to both wild and PS mice suggested a significantly increased heterogenicity of commensal microbes living in the gut of PS mice compared to both

wild and SPF mice purchased from Jackson laboratories. Interestingly, there were also significant differences seen between the two vendors of lab mice (Ericsson et al., 2017).

These studies suggest a previously underestimated importance of the environment and pathogen exposure on shaping in the immune system of mice as a common model organism in the laboratory. This has been further researched at the University of Minnesota by specifically investigating CD8+ T cells, which are a major part of the adaptive immune response in mammals and target specifically intracellular pathogens and cancerous cells. Laboratory, pet store and wild mouse blood was compared with human blood specimens by RNA sequencing. Further analysis confirmed significant differences in cytokine levels and phenotype of CD8+ T cells in lab mice compared to adult humans (Beura et al., 2016b). Pet store mice as well as adult human patients, but not SPF mice, had more experienced CD8+ T cells due to previous antigen stimulation, as indicated by increased frequencies in CD44+ T cells observed (Beura et al., 2016b). Upregulation of CD44 has been commonly used as a marker for experience of antigens in T cells (Schumann et al., 2015). Immune signatures of laboratory mice demonstrated striking similarities in those observed in human neonate cord blood (Beura et al., 2016b). Taken together, these findings implicate that the immune state of laboratory mice compares closer to human newborns, whereas the adaptive immune system of wild and pet store mice is more reflective of the adult human state (Beura et al., 2016b).

Therefore, further studies on the immune system aimed at translating the research into adult human patients might highly benefit from a more refined complementary mouse model with more microbial exposure. While it seems like a simple concept, increasing exposure to pathogens in clean laboratory mice brings its own challenges. As detailed previously, increased

immune exposure is desirable to model a human-like immune state, but should not come at the cost of genetic standardization, which would introduce other unwanted variables to research questions, as would be the case when studying wild mice. Additionally, SPF conditions have been developed to eliminate the risk of zoonotic infections, keep maintenance, cost and sample sizes of laboratory mice as low as possible, and thus bring many benefits as well (Masopust et al., 2017). Figure 1 demonstrates some of the advantages and disadvantages of introducing microbial experience to create a "dirtier" mouse model. The next paragraph will examine different ways in how this can be achieved.

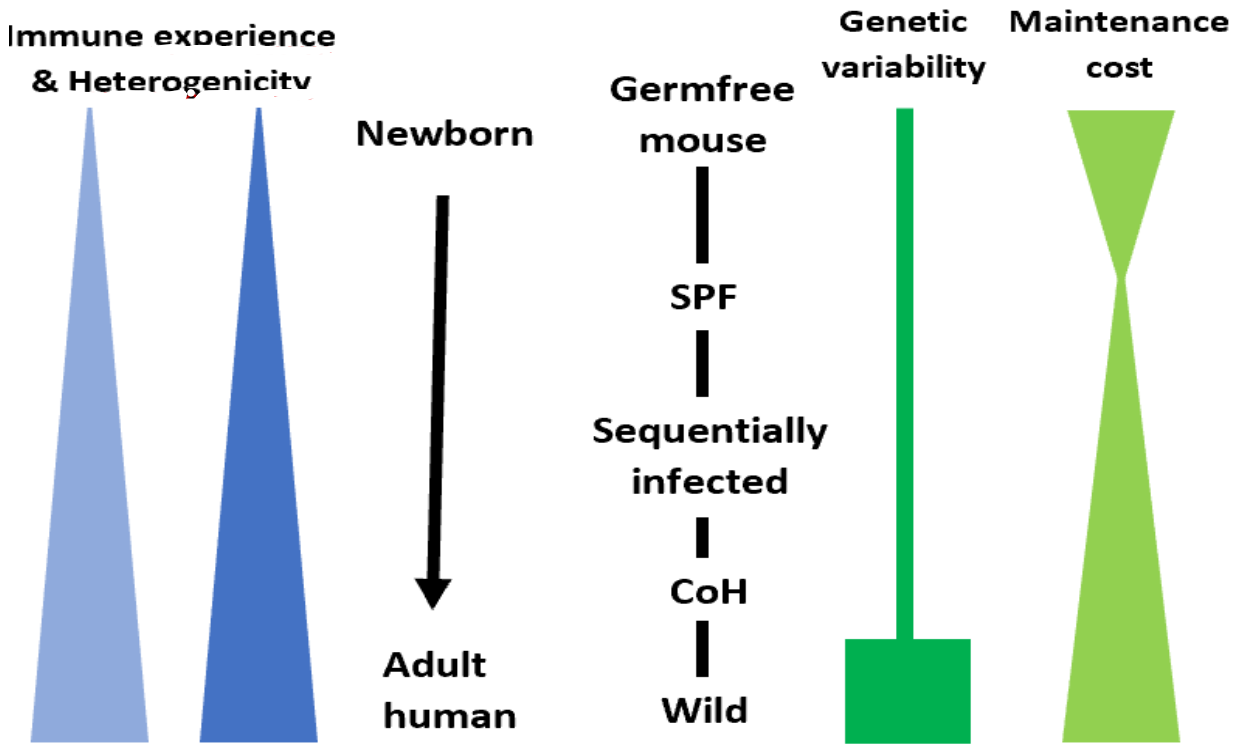

Figure 1: Cohoused mice are cost- efficient yet have a comparable immune experience to adult humans.

Cohousing SPF mice with pet store mice generates a cost-efficient mouse model that is also more representative of the human adult immune state. CoH mice have limited genetic variability but increased immune experience compared to standard SPF- mice (graphic adapted from Masopust, Sivula & Jameson, 2017).

DIFFERENT APPROACHES TO GENERATING IMMUNOLOGICALLY EXPERIENCED MICE

To fully understand the human immune system, accurate lab model organisms are necessary to understand the immune response, especially regarding the individual response to infections, vaccination and immunotherapy due to commensal microbes and microbial exposure (Beura et al., 2016b; Gopalakrishnan et al., 2018; Iida et al., 2013; Leung et al., 2018; Reese et al., 2016; Rosshart et al., 2017; Shen et al., 2014). Purposely increasing microbial exposure of SPF mice might help bridge the gap in translating research in immunology, microbiology and oncology in mice to adult humans. Several different approaches on achieving microbial experience while minimizing introduction of too many external variables are reviewed here.

Rewilding SPF mice in a seminatural environment can alter susceptibility to a parasitic infection as well as greatly influencing the immune response compared to SPF mice kept in a lab setup, as demonstrated by Leung et al., 2018. Exposing a common strain of C57Bl/6 lab mice to the external environment by housing them in an outdoor cage successfully preserves genetic standardization, but also creates a simple model with increased pathogen exposure and enhanced gut microbial diversity (Leung et al., 2018). This concept has the major disadvantage of being relatively expensive due to the necessities of large outdoor cages.

A more simplistic concept by Reese et al., 2016, focused on sequentially infecting laboratory mice with four known pathogens mimicking chronic infections commonly observed in humans. Mice were infected with two Herpesviridae, the Influenza strain WSN and the intestinal helminth *Heligomosmoides polygyrus* and later vaccinated against Yellow- Fever Virus. Immune signatures of infected and control mice were subsequently compared to human blood samples, demonstrating that infection with only four pathogens greatly alters the immune state of lab

mice and generates gene signatures comparable to adult human blood samples in contrast to cord blood (Reese et al., 2016). This could help understand individuality in anti-vaccine responses observed in different human populations, but also raises the question whether artificial introduction of certain pathogen species fully captures the microbial diversity that human individuals are exposed to over their lifespan.

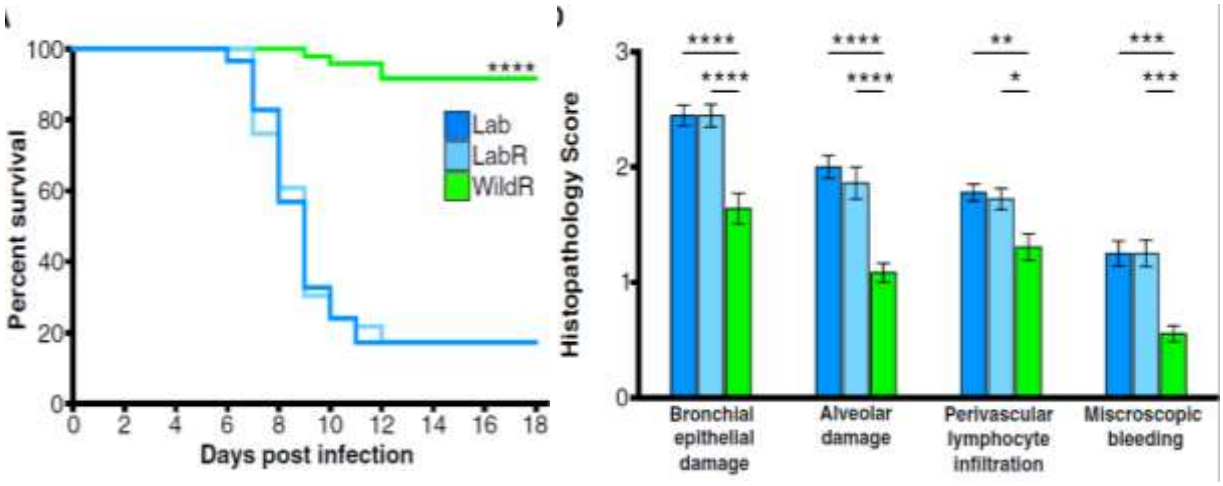

Figure 2: Diverse microbiota improve the host immune response against infection

Wild mouse gut microbiota transplanted into GF- lab mice (WildR) significantly improved the immune response against a normally lethal infection. WildR mice had better survival (left) and measures of lung damage due to influenza infection (right) compared to two control groups of GF mice with an SPF microbiota (LabR) and normal GF mice (Lab) (Rosshart et al., 2017).

To investigate the effects of gut microbiota diversity on the immune system of the host, Rosshart et al., 2017, caught wild mice and biobanked the microbiota of the genetically most similar feral population. Using fecal- oral gavage, these microbiota were then transferred to pregnant germ-free (GF) lab mice kept in sterile conditions, which successfully passed the experienced microbiota on to their pups (Rosshart et al., 2017). Their offspring (WildR mice) was less likely to die when infected with the mouse version of influenza, as demonstrated in Figure

2. To investigate the effects of gut microbial diversity on cancer progression, colorectal neoplastic development was induced in a subgroup of mice. In WildR mice, tumor burden, volume, invasiveness and inflammatory scores were greatly reduced compared to standard GF mice with an SPF microbiota (LabR) as well as normal GF mice (Lab) (Rosshart et al., 2017). While this approach might aid in understanding the influence of gut microbial diversity on the immune state of lab mice, it fails to capture external infections of pathogens. Additionally, trapping and analyzing the wild mice to identify the best-matching microbiota for transfer requires a lot of time and resources not available in many research facilities.

These previous studies demonstrate various methods to create microbial experienced mouse models that can be tailored towards specific research questions involving the microbiota-host interactions. They provide further insight on how the immune state is impacted by microbes in adult humans, but also are not always ideal in keeping cost and resources low, as seen in the models of Leung et al., 2018, and Rosshart et al., 2017. On the other hand, sequentially infected mice provide a simple model, yet might fail to capture the full extent of immune exposure. Therefore, we propose that "cohoused" mice are the ideal complementary mouse model to establish standardized, yet microbially experienced mice, which builds on a concept successfully established in a BSL-3 facility by Beura et al., 2016.

COHOUSED MICE ARE EFFECTIVE IN STUDYING THE ADAPTIVE IMMUNE SYSTEM

The idea of using "cohoused" mice (CoH) for immunology research was developed by researchers at the University of Minnesota (Beura et al., 2016b). SPF mice are therefore cohoused in the same cage together with commercially purchased pet store mice. The

	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: Cohoused mice get infected by some of the pathogens from pet store mice. Cohoused mice, which are laboratory mice cohoused with pet store mice for 30+ days successfully acquire some pathogens, that are not normally observed in SPF mice. Blood and fecal samples were tested via PCR and serology in all three groups before and after cohousing and results are compiled (Beura et al., 2016).

previously “clean” SPF mice are a genetically standardized lab strain and become infected with some of the pathogens common in “dirty” pet store (PS) mice (Beura et al., 2016b). To decrease the risk of contamination of other research animals, the CoH mice in their study were kept in a BSL-3 (Biosafety level-3) facility with normal SPF mice serving as a control group (Beura et al., 2016b).

Table 1 demonstrates that after a cohousing period, CoH mice were infected by about half of the pathogens found in the PS mice. The control group of SPF mice had no microbial exposure of the pathogens tested for by serological analysis and fecal PCR. A period of at least 30 days of cohousing has been shown to be sufficient to induce immune changes. As indicated in Figure 3 a), markers of immune experience increase only slightly beyond 4 weeks and start to decrease after about 60 days again (Beura et al., 2016b).

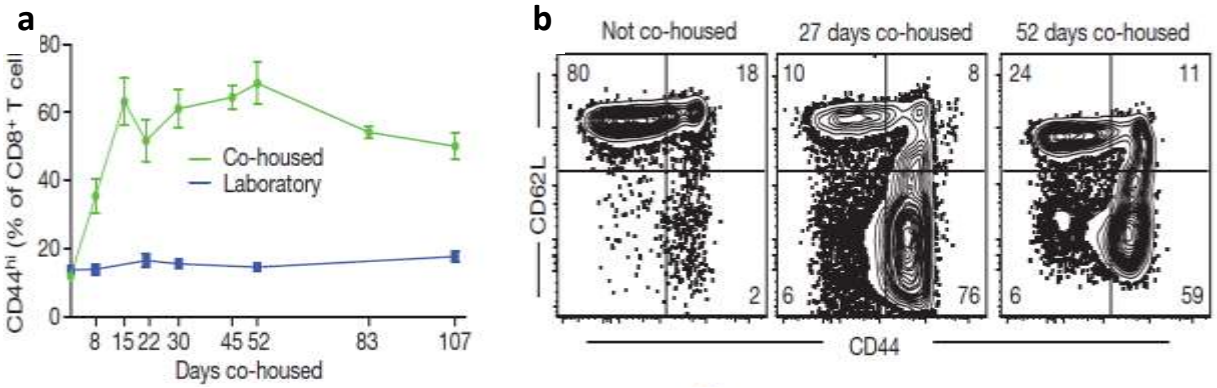

Figure 3: Cohousing SPF mice with pet store mice greatly increases the number of antigen-experienced CD8+ T cells.
As seen in a), a maximum percentage of $CD44^{hi}$ CD8+ T cells is reached after a cohousing period of approximately 30 days and levels of thereafter. B) Flow cytometry plots demonstrates the shift in $CD44^{hi}CD62L^{lo}$ T cells observed during cohousing (Beura et al., 2016).

Infections change the immune system of previously "clean" lab mice significantly. Research published so far has mainly focused on examining the CD8+ T cell response, which is a potent weapon of the adaptive immune system in organisms to combat intracellular infections and cancer cells (Gerritsen & Pandit, 2016; Gulzar & Copeland, 2004; Ioannides & Whiteside, 1993). CD8+ T cells are commonly known as "cytolytic" T cells in contrast to CD4+ T- helper cells.

Three signals are necessary for complete activation and proliferation of CD8+ T cells (Curtsinger et al., 2007). CD8+ T cells generally have a T cell receptor (TCR) composed of an external αβ domain that is assembled randomly, and an internal signaling domain (CD3). TCRs only recognize their specific antigen presented by antigen- presenting cells (APCs) such as dendritic cells. The fragment of an intracellular antigen is presented on an MHC I molecule (Mammalian Histocompatibility Complex Class I) by the APC, although cross- presentation of

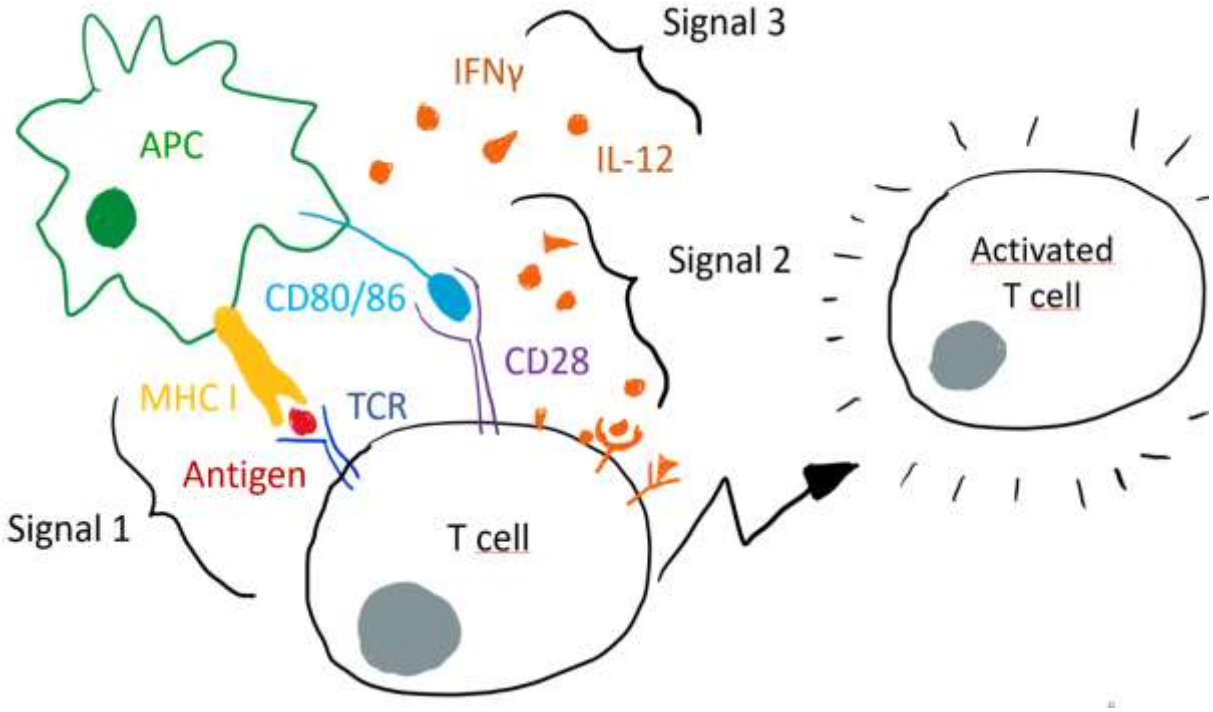

Figure 4: Three signals are necessary for full activation of T cells.
Signal 1 of T cell activation is provided by the initial interaction of MHC I, antigen fragment and T-cell receptor (TCR), as shown on the left. Co- stimulation is essential via additional receptor interaction between APC and T cell, for example through CD80/86 and CD38 (Signal 2). Full effector function is only established once the T cell gets additional stimulation through cytokines such as IFNγ or IL-12 (Signal 3).

exogenous antigens has also been observed (Curtsinger et al., 2003b). The Interaction of TCR, antigen and MHC Class I is known as the first signal. To strengthen the interaction of MHC I and TCR, co- stimulation provided by CD8 is necessary for full activation of the CD8+ T cells, which is

the second signal necessary. While this can lead to proliferation of the CD8+ T cells, development of the cytolytic effector function characteristic of CD8+ T cells is only achieved through additional stimulation by cytokines or adjuvants such as Interleukin 12 (IL-12) and Type 1 Interferons. This is referred to as the third signal of T cell activation (Curtsinger et al., 2003b). This 3-step activation process is displayed in Figure 4.

Characterization of antigen experienced CD8+ T cells in wild, pet store and SPF mice revealed significant differences: SPF mice have the least and pet store mice have the most experienced CD8+ T cells (Beura et al., 2016b). CD44 is used as a marker to indicate previous antigen- experience, as it remains upregulated on memory- type CD8+ T cells (Baaten et al., 2010). Cohousing SPF mice effectively increased the antigen experience of CD8+ T cells towards a much higher percentage of CD44+ T cells (Figure 3 b). Additionally, other markers of T cell differentiation and experience such as KLRG1, CXCR3 and Granzyme B differed visibly between CoH and SPF mice and CoH mice had significantly more CD8+ T cells in non-lymphoid tissues, as seen in Figure 5 (Beura et al., 2016b).

CoH, pet store and SPF mice were also infected with the commonly used intracellular bacterial pathogen *Listeria monocytogenes* to assess differences in immune function (Beura et al., 2016b). Bacterial load in the spleens of PS and CoH mice was significantly lower than in lab mice and comparable to the bacterial load observed in vaccinated mice. These results suggest that changes in T cell subsets of CoH mice also provide greater resistance against infection (Beura et al., 2016b). Thus, it seems desirable to investigate the effects of microbial exposure on the adaptive immune system in mice by further characterizing functionality and differentiation of CD8+ T cells in addition to established differences in antigen experience.

DIFFERENT CD8+ T CELL SUBSETS CAN BE STUDIED IN COHOUSED MICE

So far, research has focused on characterizing the adaptive immune system in CoH mice, targeting mainly CD8+ T cells. CD8+ T cells are a crucial part of the adaptive immune system in mammals and primarily target intracellular pathogens such as viruses (Gerritsen & Pandit, 2016). Additionally, tumor-infiltrating lymphocytes effectively aim to kill transformed, and thus potentially cancerous, cells (Reiser & Banerjee, 2016). Naïve T cells circulate in the blood and lymphoid tissues of organisms and will differentiate and proliferate into potent CD8+ effector cells upon antigen stimulation, as previously detailed. After successful clearance of the infected or cancerous cells, the population of effector CD8+ T cells contracts and only a small group of memory-type CD8+ T cells remains (Gerritsen & Pandit, 2016). In cases of further tumor growth, this mechanism of T cell differentiation is disrupted through inhibitory signals from the tumor microenvironment and/ or host immune system (Reiser & Banerjee, 2016).

Different subsets of antigen-experienced CD8+ T cells with several specific properties have been identified to date (Chen et al., 2018). During the primary response to antigen, effector CD8+ effector-type T cells can be further differentiated into short-lived effector cells (SLECs) or memory precursor cells (MPECs). SLECs are $KLRG1^{+}CD127^{-}$; the majority of SLECs die during contraction. In contrast, MPECs are generally $CD127^{+}KLRG1^{-}$, and these cells are much more likely to form long-lived populations (Henson & Akbar, 2009). Long-lived antigen experienced CD8+ T cells also exist such as long- lived effector cells (LLECs).

Effector cells generally die after clearance of a primary infection, but a small population of memory- type CD8+ T cells remains to protect against subsequent infections such, central memory, effector memory and resident memory cells (Joshi et al., 2007). It has already been

shown that long-lived KLRG1^{+} CD8+ effector cells (LLECs) are very effective in fighting viral infection (J. A. Olson et al., 2013). LLECs are characterized by a continued upregulation of the KLRG1-receptor long after infection. This population of T cells has already been shown to have a significant protective function against reinfection with pathogens and might thus also be beneficial in fighting tumor cells (Renkema et al., 2019). These LLECs can be found in CoH mice as well (Beura et al., 2016b). LLECs are an important part of the memory function of the immune system and are not frequent in normal SPF mice, suggesting that studying the CD8+ T cell response only in clean SPF mice misses important pieces of the adaptive immune response.

Further characterization of CD8+ T cells is necessary to fully understand differences in functionality introduced by cohousing. We aim to compare subsets of CD44^{hi}KLRG1^{hi} T cells in both SPF and CoH mice. Since we cannot usually establish a distinct positive or negative population, we term our T cell subsets as “hi” (instead of positive) and “lo” (instead of negative). Additionally, we will measure CD69, a marker that is upregulated on T cells and NK- cells in early activation (Cibrián & Sánchez-Madrid, 2017). It is used to differentiate between CD8+ T cells that are activated rapidly in response to an acute infection and those that differentiate due to previous antigen experience. (Simms & Ellis, 1996; Wang et al., 2011). CD69 will be downregulated once an infection has been cleared and similar levels of CD69^{hi} CD8+ T cells exist in both SPF and CoH mice (Masopust et al., 2017).

The functional properties of CD8+ T cells are characterized through intracellular staining for Granzyme B, which is an important effector molecule in CD8+ T cells. After complete activation, CD8+ T cells develop cytolytic functions through release of Granzyme B and Perforin upon stimulation with their target antigen. Perforin penetrates the cell membrane of infected

cells and allows Granzyme B to initiate apoptosis via activating caspases. CD8+ T cells will only remain Granzyme B+ for a few weeks without further antigen stimulation and thus, memory-type CD8+ T cells lose their cytolytic properties (Nowacki et al., 2007). Figure 5 summarizes the activation state of the CD8+ T cells and the respective presence of specific markers, such as CD69, CD44, KLRG1 and Granzyme B.

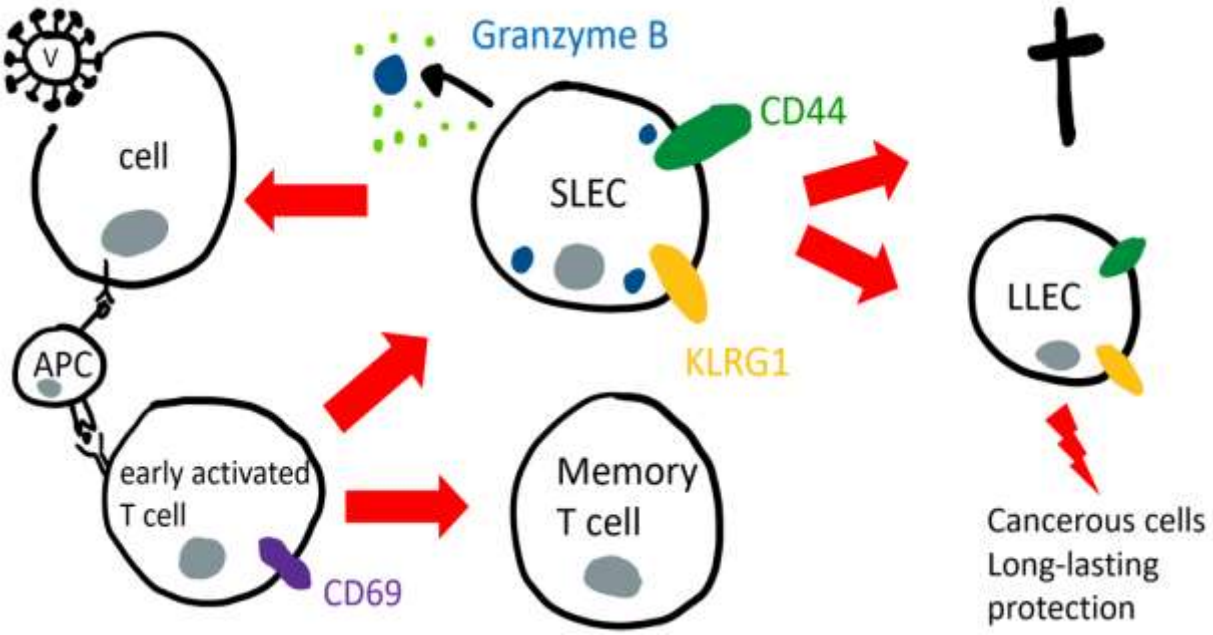

Figure 5: The activation state of CD8+ T cells can be characterized via different markers

When a CD8+ T cell is successfully activated by the APC (antigen-presenting cell) through the previously introduced 3 necessary signals, it rapidly upregulates CD69. This receptor is used as a marker for early activated T cells. These cells either develop into a small pool of memory-type CD8+ T cells that can effectively respond to a subsequent infection of the same pathogen, or into an effector-type CD8+ T cell termed SLEC (short-lived effector cell). SLECs are characterized by downregulating CD69, but instead express CD44 as a marker for antigen-experience as well as KLRG1 as an indicator of further differentiation. SLECs effectively initiate

apoptosis of the virus-infected or mutagenic cell by releasing Perforin and Granzyme B, the latter of which can be used to estimate its effector function. After successfully eliminating the target cells, most SLECs themselves die, but some also develop into so-called LLECs (long-lived effector cells), that are crucial for long-lasting protection, also against cancer (J. A. Olson et al., 2013; Reiser & Banerjee, 2016).

To assess whether T cells in CoH mice react more rapidly upon antigen stimulation, spleen cells are cultured in vitro and examined using the same markers. Thus, we hope to compare T cell characteristics across blood, spleen, lymph node and culture samples as well as investigating subsets present in mice injected with B16 melanoma cells.

IFNY STIMULATES THE CD8+ T CELL RESPONSE

Interferon γ is an important proinflammatory cytokine stimulating a variety of immune cells. In contrast to Type 1 Interferons, IFNγ is classified as a structurally different Type 2 Interferon and binds to its own specific receptor (Schroder et al., 2004). It is secreted by activated macrophages, natural killer cells, Th1 CD4+ T cells and activated CD8+ T cells (Bhat et al., 2017). CD8+ T cells receive IFNγ amongst other cytokines as a third signal of activation, which ensures full effector function of these T cells (Curtsinger et al., 2003b). It is then also secreted in an autocrine fashion by activated CD8+ T cells to further increase the local immune response (Bhat et al., 2017). It has been demonstrated to be a potent necessary mediator in the anti-tumor response of CD8+ T cells as well as their migration to antigen-presenting cells (Bhat et al., 2017).

There are differences in quantities of IFNγ secreted by T cells of different inbred mouse strains. It has been demonstrated that T cells of C57/BL6 mice release higher amounts of this cytokine than Balb/c mice, indicating better effector function of their T cells (Schroder et al., 2004). Studies have found elevated signaling through Type I Interferon- related pathways in both microbially experienced pet store mice as well as human adults versus unexperienced neonates but have focused on Type I Interferons so far. IFNγ signaling has not been specifically investigated in cohoused mice before (Beura et al., 2016b; Huggins et al., 2019). Interestingly, Reese et al., 2016 have demonstrated higher concentrations of IFNγ as well as induction of Interferon- related genes in their model of sequentially infected mice, but this has not been studied in CoH mice to date (Reese et al., 2016).

Studies have also focused on characterizing the role of IFNγ in the tumor microenvironment and specifically its contribution to anti-cancer T cell activation (Lugade et al., 2008; Ni & Lu, 2018). IFNγ and its associated signaling pathways are crucial to stimulate the immune response in tumors due to its effects as a proinflammatory mediator (Blankenstein & Qin, 2003; Dobrzanski et al., 2001) . Its tumor- decreasing influence has been shown to be due to caspase activation, induction of MHC class I and increasing chemokine expression in tumors to promote migration and recognition of tumor cells by tumor-infiltrating T lymphocytes (Lugade et al., 2008). Absence of IFNγ on the other hand, contributes to increased tumor growth, possible via impaired STAT1 phosphorylation (Lugade et al., 2008). Overall, we aim to investigate Interferon γ concentrations in CoH versus SPF mice as it seems to be one crucial mediator in promoting improved tumor cell recognition and local migration of tumor-infiltrating CD8+ T cells.

MICROBIOTA INFLUENCE CANCER DEVELOPMENT AND THERAPY

Many promising improvements in cancer treatment over the last decade focus on an immunotherapeutic approach. Monoclonal antibodies against immune-regulators such as PD1, PDL1 and CTLA4 are the most common immune drugs used in human cancer patients so far, but adoptive immunotherapy is another important field of research based on the observation that tumor- specific CD8+ T cells may directly kill tumor cells (Reiser & Banerjee, 2016). Adoptive immunotherapy has been implemented successfully on some B-cell lymphomas but has been of very limited use in solid tumors (Martinez & Moon, 2019). Some studies indicate promising results in patients with metastatic melanoma, using autologous T cell therapy (Rosenberg et al., 2011; Rosenberg & Dudley, 2009). Still, much remains unknown about the specific T cell interactions within the tumor microenvironment, and how microbial experience may influence these interactions.

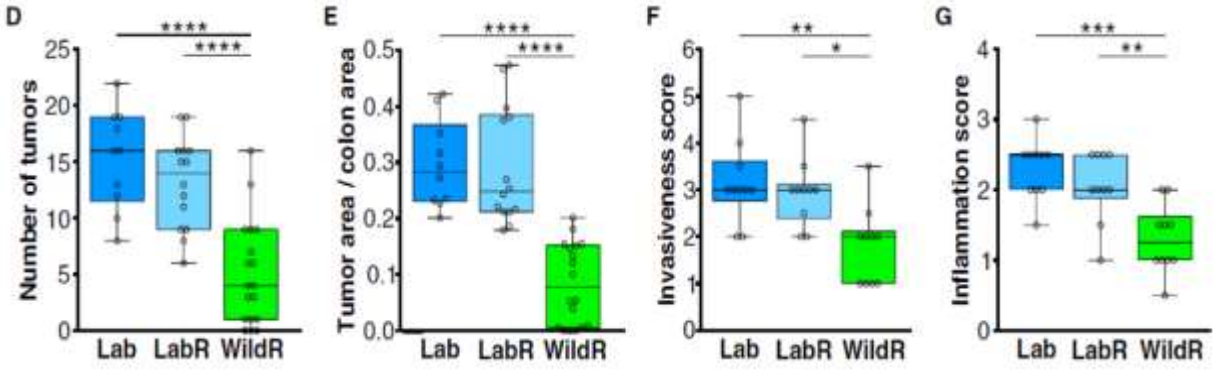

Figure 6: A diverse microbiome improves cancer development and outcome in mice with colon cancer.
Germ-free mice receiving wild mouse gut microbiota (WildR) showed reduced markers of colon cancer**.** Tumor burden (D), tumor size (E), tumor invasiveness (F) and inflammation markers (G) were significantly reduced in WildR compared to both SPF- mice (Lab) and GF- mice that had received SPF-mouse microbiota by a similar procedure (LabR) (Rosshart et al., 2017).

Highly individual responses to immunotherapy due to variable microbiota-immune interactions in humans have been investigated by various recent studies (Dzutsev et al., 2015; Gopalakrishnan et al., 2018; Vétizou et al., 2015; Zitvogel et al., 2018). Several studies have demonstrated a significant and previously underestimated influence of the gut microbiota on cancer development and therapy both in mice and humans.

Rosshart et al., 2017, examined the effects of transfer of wild mouse gut microbiota into laboratory strains. They have demonstrated that the diverse wild mouse microbiota of feral mice could be successfully transferred and maintained in Germ-free laboratory mice (GF mice). GF mice that have received the wild mouse microbiota (WildR) showed greatly improved anti-tumor responses compared to both normal lab mice (Lab) as well as GF-mice receiving SPF mouse microbiota (LabR) (Rosshart et al., 2017). As seen in Figure 6, several markers to quantify

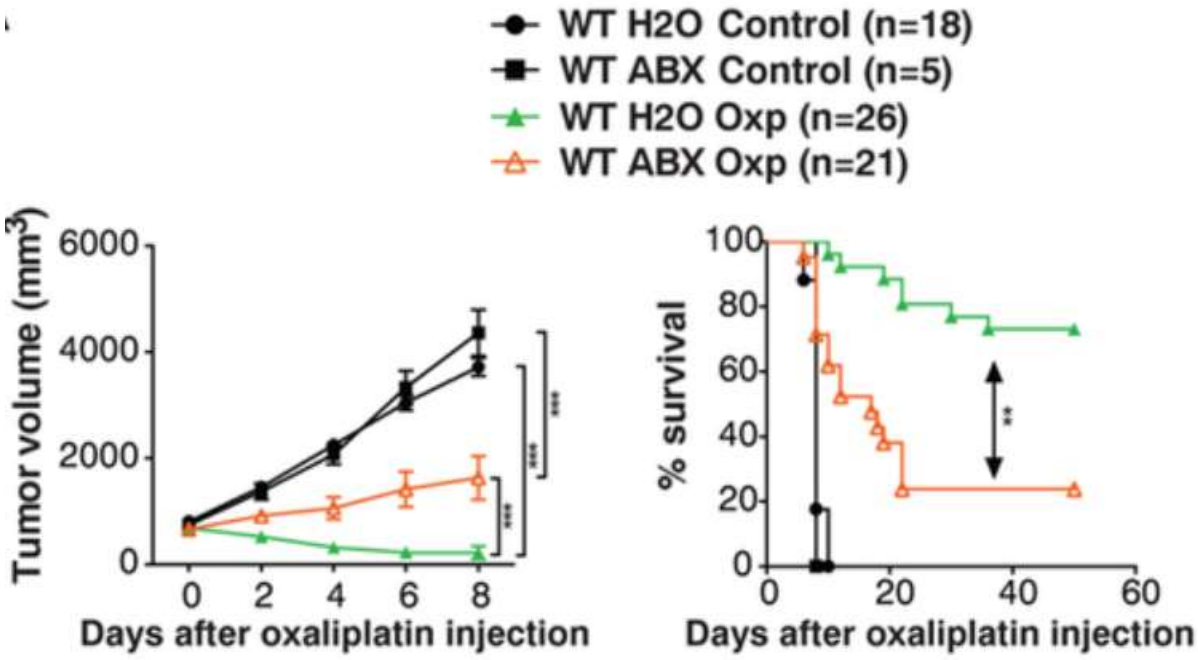

Figure 7 Microbiota impact cancer treatment in mice.

Treatment of lab mice with an artificially depleted gut microbiome through treatment with an antibiotic (ABX) showed significantly decreased cancer survival and increased tumor volume after chemotherapeutic treatment with Oxaliplatin (Oxp) (Iida et al., 2013).

severity of mutagen-induced colon cancer were significantly reduced in WildR-mice compared to both control groups with normal SPF- microbiota (Rosshart et al., 2017).

Beyond tumor development, cancer treatments such as chemotherapy might also be influenced by commensal bacteria. This interaction was presented in a mouse study by Iida et al., 2013, where SPF mice were treated Oxaliplatin, a potent chemotherapeutic that also induces the immune response. As illustrated in Figure 7, artificial depletion of the mouse microbiota by antibiotic treatment before chemotherapy significantly reduced effectiveness of anti-cancer therapy and post-treatment survival (Iida et al., 2013). This suggests that microbial influence is not limited to immunotherapy, but also extends to other cancer treatments.

In addition to gut bacteria, recent evidence also points towards commensal skin microbes influencing T cell priming and development, as demonstrated in laboratory mice (Shen et al., 2014). Further investigation on different treatments and of other cancer types is necessary. A more accurate mouse model that captures microbial experience, such as CoH mice, could support future studies.

CoH MICE WITH B16 MELANOMA TO STUDY TUMOR- INFILTRATING CD8+ T LYMPHOCYTES

The effects of cancer have not been published a model of cohoused mice so far. As an initial cancer model, we suggest using B16 melanoma. Cancer might be fought more effectively by a microbially experienced mouse model compared to the standard SPF-mice. B16 melanoma is a common, well- characterized and effective tumor model in C57BL/6 mice and has poor immunogenicity (Turk et al., 2004). B16 melanoma in mice, just like human melanoma, is notoriously difficult to treat as these tumors escape immune the immune system (Lechner et al.,

2013; Overwijk & Restifo, 2001). B16 melanoma is considered a T-cell inflamed tumor, which is a type commonly seen in human patients as well and the function and cytokine concentrations of T cells are substantially decreased in the tumor environment (Gajewski et al., 2013).

Since recent studies have demonstrated such a significant impact of microbiota and cancer, we aim to characterize the immune-tumor interactions in a model with microbial experience and compare it to standard SPF- mice. It has been hypothesized that one of the reasons for failed translation of mouse studies are because of increased immunosuppressive cytokines and specific chemokines in humans that cannot be observed in mice (Farrar et al., 2000; T. S. Olson & Ley, 2002). The differences in mouse vs. human immune system are not limited to signaling molecules (Mestas & Hughes, 2004). For example, SPF mice lack certain subsets of CD8+ T cells that can be observed in humans and recapitulated in CoH mice by introducing microbial experience (Beura et al., 2016b; Ericsson et al., 2017; Masopust et al., 2017).

In conclusion, mice with microbial experience might provide a better living tumor model that can replicate the human immune state and microbial influence on shaping the immune system more closely (Dzutsev et al., 2015; Gopalakrishnan et al., 2018; Yaddanapudi et al., 2013). Differences in antigen- experienced and differentiated tumor-infiltrating CD8+ T cells in mice with microbial experience could indicate a previously underestimated importance of antigen experience in humans on the anti-cancer response. Studying the immune-cancer interactions using cohoused mice as a simple and cost-effective model could be of major significance in providing a better additional model in translating immunological research from bench to bedside.

CHAPTER 3: METHODS

MOUSE COHOUSING

Per cohort, 25- 35 young female C57/BL6 mice were ordered from Charles River, spread equally to cages with 4-5 mice each and allowed a transition period. 2-3 cages with 5 mice each served as the control group and were designated clean (SPF). Their handling and supplies were strictly separated from the remaining dirty mice. 15-20 dirty mice (CoH) were cohoused with 3-5 female pet store mice for 30+ days purchased from a conventional pet store (PETCO) as feeding mice to allow for infection with pathogens (Appendix, Figure 15). Mice were fed ad libitum and monitored daily. Cages were changed weekly according to a strict protocol separating SPF and CoH mice and respective supplies. Therefore, cages, lids, wires, and bottles were sprayed and soaked in concentrated Clidox solution (1:3:1 dilution). Additionally, they were washed with Rescue to eliminate contamination. The cohoused mice and the corresponding SPF-mice were kept in a satellite vivarium separated from other research animals on campus. The animals were used in accordance to the guidelines of the Institutional Animal Care and Use Committee (IACUC) at Grand Valley State University.

PATHOGEN TESTING

Fecal and blood samples were collected on day 0 and day 30 from pet store, CoH and SPF mice and sent to Charles River Research Animal Diagnostic Services (CR RADS) for testing against specific pathogens. Fecal samples were pooled from designated cages of either SPF or CoH mice.

BLEEDS AND RNA ISOLATION

Mice were anesthetized using isoflurane and bled retro-orbitally weekly during the cohousing span. Serum was collected from some of the blood, and the remainder of the blood was collected into heparinized tubes for flow cytometry analysis and potential RNA isolation (for future studies). Red blood cells were lysed with ACK lysis buffer and prepared for either RNA isolation or flow cytometry. RNA isolation was performed according to the manufacture's protocol (Quiagen RNeasy mini kit). Eluted RNA was stored at -80 C for further analysis.

B16F10- OVA CELL CULTURE AND SUBCUTANEOUS B16 MELANOMA INJECTION

B16 cells were purchased and cultured in RMPI 10% complete medium (RPMI + 10% FBS + streptomycin/ penicillin at 5000 µg/ml and 5000 u/ml respectively + 50 µM 2-Mercaptoethanol). We injected 2.5 x 10^5 cells per flank in 200 µl PBS subcutaneously. Each mouse was anesthetized with isoflurane to decrease stress, facilitate handling, and ensure proper injection in each flank. Mice were checked daily for tumors and were harvested before day 14 or development of necrosis. Tumor growth and mouse weight were recorded daily.

HARVESTING MICE

The mouse cohorts were harvested on 3 time points after the 30-day cohousing mark to collect blood via cardiac puncture, spleens and cervical, as well as inguinal lymph nodes (illustrated in Figure 15, appendix). Therefore, mice were euthanized with a heavy dose of isoflurane and cervically dislocated to ensure death. The blood was divided into heparinized tubes for further flow cytometry analysis as well as serum collection. Spleen and lymph nodes

were processed immediately, with an additional ACK lysis performed on spleen samples. Tumors of mice injected with B16 melanoma were collected in separate tubes and processed similarly to the lymph nodes. Samples were finally resuspended in FACS buffer (1x PBS with 1% FBS) and stained with antibodies for 20+ minutes.

T-CELL IN-VITRO STIMULATION

To look at T-cell activation ex-vivo, splenocytes and tumor cells were cultured in 24-well plates coated with an antibody cocktail (10 µg/ml anti-CD28, 5 µg/ml of anti-CD3). T cells were cultured in RPMI- complete medium and addition of Il-2 (10 U/ml) to keep cells alive. T-cell stimulation was examined on day 1 and day 3/ 4 of culturing by flow cytometry. Cells were stained with markers for antigen experience (CD44), differentiation (KLRG1), effector function (Granzyme B) and proliferation (CFSE). The level of IFNγ was measured in the supernatant of cultures by ELISA.

FLOW CYTOMETRY AND DATA ACQUISITION

A Beckman & Coulter CytoFlex 4 channel flow cytometer was used for analysis. Samples were prepared according to a standard protocol, resuspended in 200µl FACS buffer and were generally run at medium speed. Blood and spleen samples as well as samples stained with Granzyme B were fixed before analysis with a Fix/Perm FoxP3 kit, to eliminate possibly cross-reacting RBCs and release intracellular Granzyme B. Samples were generally run with the same specific antibody combinations on the harvests and bleeds, as well as for analysis of the cell cultures. T-cells were surface- stained with antibodies purchased from Tonbo and Biolegend against CD4, CD8, CD44, CD69 and KLRG1. Samples were fixed first and then stained with

antibody against intracellular Granzyme B. Cell cultures were additionally stained for live/dead cells (Tonbo) and with CFSE on day 3/4 analysis.

IFNγ- ELISA

Concentrations of the cytokine Interferon γ were measured in supernatant and serum samples via an enzyme-linked immunosorbent assay (ELISA). A complete ELISA- Kit was purchased from Tonbo, with a standard of IFNγ provided. ELISAs were performed according to the manufacturer's protocol. Serum samples were diluted before analysis, spleen supernatant samples were used undiluted for our pilot ELISA, but we suggest diluting them 1:2 prior to analysis for future tests.

STATISTICAL ANALYSIS

Initial flow cytometry data were acquired on the preinstalled software CytExpert and imported for detailed analysis into FlowJo LLC 10.6. Design and statistical testing were done using Graphpad Prism 8. Significance was determined by p- Values < 0.05 using an unpaired Student's t- Test or multiple t-tests.

CHAPTER 4: RESULTS

COH MICE GET INFECTED BY VARIOUS PATHOGENS OVER 30+ DAYS OF COHOUSING WITH PS MICE

Cohousing standard lab mice kept under SPF conditions with "dirtier" bunk mates purchased from a commercial pet store is not a novel concept. CoH mice are used for research at other universities, but not without major safety precautions employed by the University's administration to prevent any potential contamination or zoonotic infections. CoH mice are kept in Biosafety- level 3 (BSL-3) facilities in most places and thus, experiments with CoH mice have several limitations due to the strict safety regulations (Beura et al., 2016b).

Here, we report a successful and simple cohousing setup employed in a satellite vivarium on Grand Valley State University's campus. We cohoused SPF mice with pet store mice purchased from a commercial vendor in the area (PETCO) and kept a control group of standard SPF mice completely free of any pathogen contamination over a period of at least 65 days. To test for pathogen transfer, fecal and blood samples were compiled from 2 cages of SPF and CoH as well as 1-2 PS mice and send to Charles River Research Animal Diagnostic Services (CR-RADS) for testing. RT-PCR on fecal samples and immunoserology performed on blood samples revealed no novel pathogens in the SPF control group and several common bacteria, viruses and parasites in PS mice.

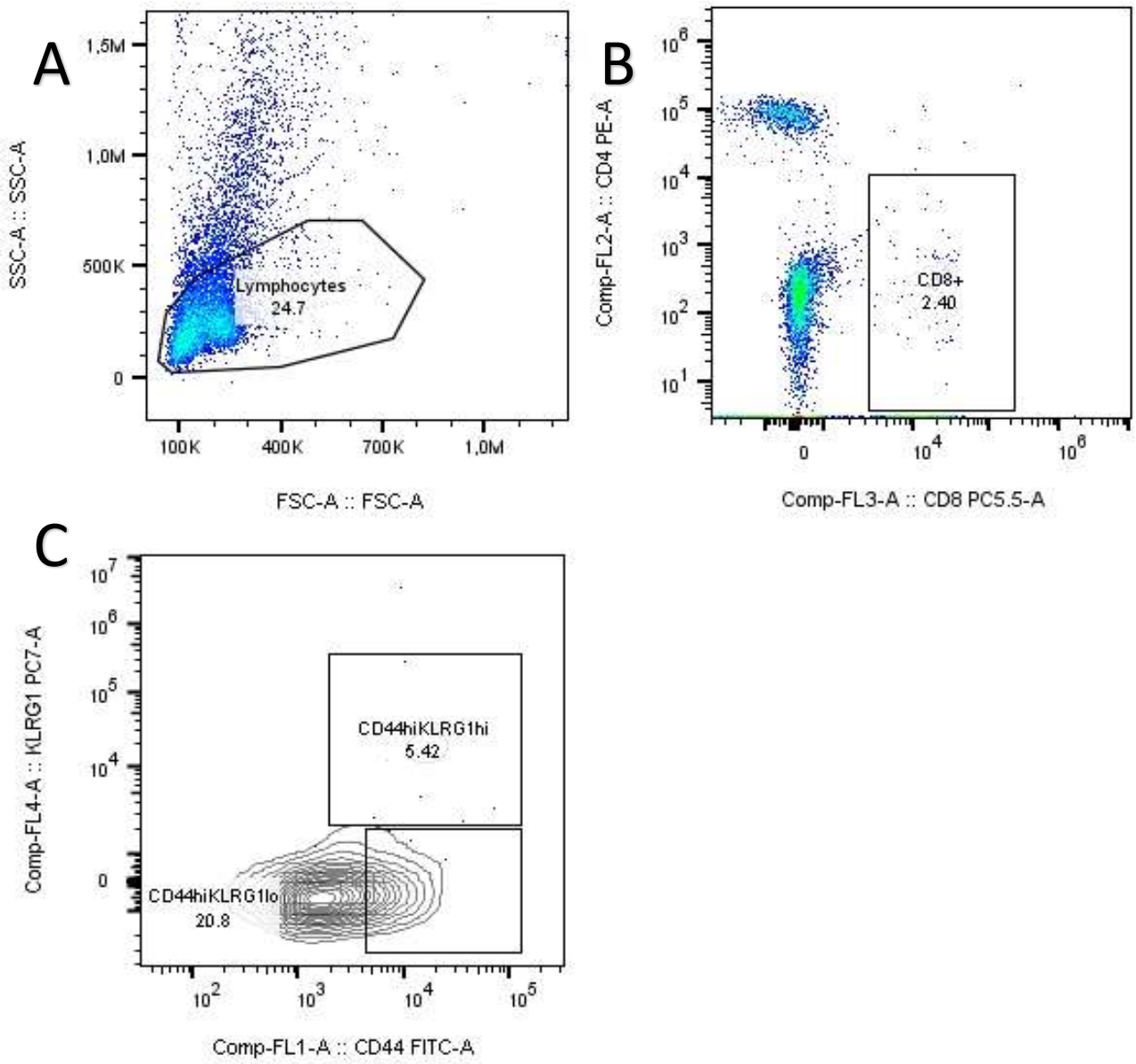

Figure 8: Gating strategy on weekly bleeds and harvests

Gating strategy employed in FlowJo to compare frequencies of $CD44^{hi}KLRG1^{hi}$ and $KLRG1^{lo}$ cells on weekly bleeds in both CoH and SPF mice. Lymphocytes were roughly gated off forward- and side scatter (A), and CD8+ cells were obtained using CD8 and excluding CD4+ cells (B). Out of the CD8+ T cells, $CD44^{hi}$ cells were identified and separated into a $CD44^{hi}KLRG1^{hi}$ and a $CD44^{hi}KLRG1^{lo}$ population, as seen in C. Gates were identical for all blood samples per time point, and analysis was done similarly across all bleed days. Day 21 bleed of CoH 3-1 is shown here as an example.

After a cohousing period of 30 days, about half of the pathogens from PS mice had infected most of the CoH mice. Among the most prevalent microbes in CoH mice were Mouse parvoviruses, Mouse adenoviruses and Theiloviruses (table 2, appendix). This caused visible sickness in the CoH mice, usually around Day 14-23 of cohousing as indicated by apathy, runny crusted eyes, scruffy appearance, and no appetite. Between 5-15% of CoH mice per cohort succumbed to the infections, less than previously reported (Beura et al., 2016b).

Overall, CR-RADS testing confirmed our cohousing protocol and lab setup as well as standard cleaning procedures as successful, as seen in 3 independent cohorts of 25-35 mice. CoH mice were infected with several pathogens from the PS mice, while our SPF mice control group remained completely pathogen-free.

CoH MICE DEVELOP MORE ANTIGEN EXPERIENCED, DIFFERENTIATED AND EFFECTIVE CD8+ T CELLS

To examine antigen experience and differentiation of CD8+ T cells in CoH versus SPF mice, 5 mice per group were bled weekly. Blood samples were stained with specific antibodies to quantify upregulation of certain markers on and in the CD8+ T cells of the mice. After 10-17 days of cohousing, CoH mice, but not control SPF mice, develop a distinct population of $CD44^{hi}KLRG1^{hi}$ CD8+ T cells, as illustrated in the gating strategy employed in FlowJo (Figure 8). SPF mice do not gain any additional antigen exposure through infection and thus do not upregulate KLRG1 as a receptor on their CD8+ T cells to a significant degree. Therefore, in contrast to the “dirtier” CoH mice, SPF mice do not develop this population of antigen experienced, differentiated T cells. Initially, about 5-7% of CoH CD8+ T cells are $CD44^{hi}KLRG1^{hi}$ and this percentage steadily increases over the cohousing period (Figure 9). After about 24 days,

the percentage of CD44^{hi}KLRG1^{hi} CD8+ T cells starts to level off at about 10-20% depending on the cohort of mice and eventually decreases after about 70+ days of cohousing (Beura et al., 2016b).

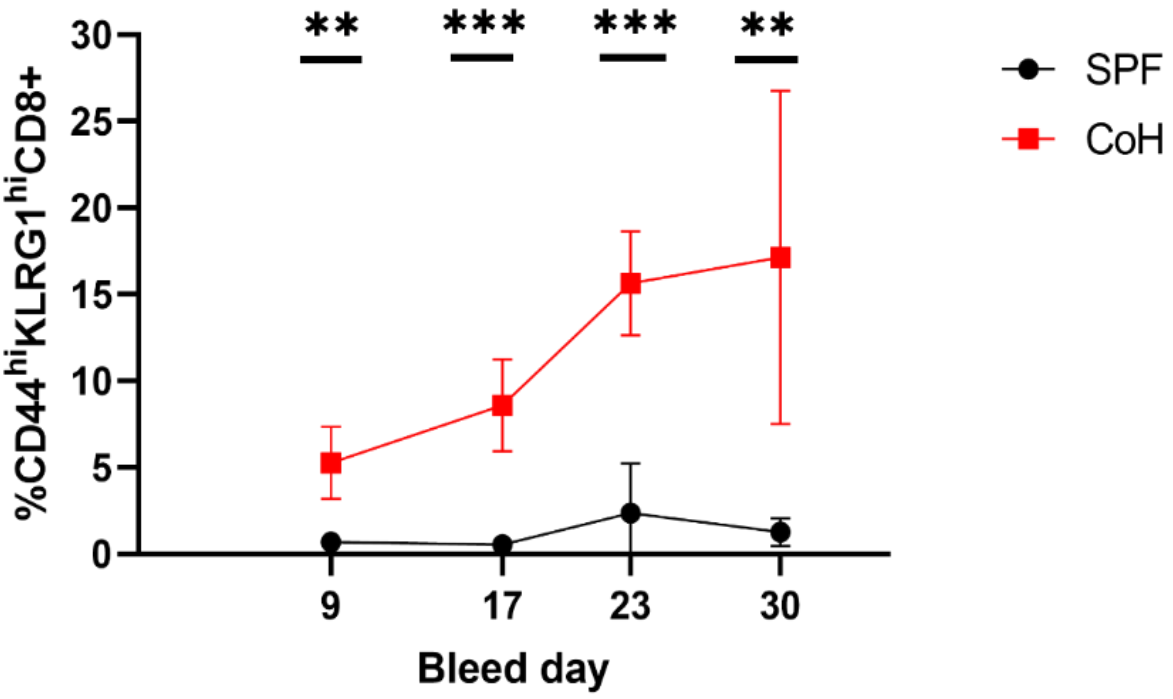

Figure 9: CoH mice develop more antigen experienced and differentiated CD8+ T cells. Cohoused mice develop more antigen experienced, differentiated CD44^{hi}KLRG1^{hi} CD8+ T cells over a cohousing period of 30 days, as seen in roughly weekly bleeds. 4-5 mice were bled for each group and each time point. Data are shown for Cohort #2 and have been repeated n=3 times. T-tests were used to compare groups for each time point with p-Values consistently <0.01 (Groeber, 2019).

To examine whether the CD8+ T cells of CoH mice are not just more antigen experienced and differentiated, but also more effective, we stained for the intracellular marker molecule Granzyme B (GzB). GzB gets secreted only by fully activated CD8+ T cells and initiates apoptosis in its infected target cells (Curtsinger et al., 2005). We used a similar gating strategy to obtain a Granzyme B- positive population as seen in the first gating schematic in Figure 8 and illustrated

in Figure 16 in the appendix section. Thus, we compared $CD44^{hi}GzB^{hi}$ CD8+ T cells in CoH versus SPF mice during 3 different bleed days. Consistent with the previous observations, CoH mice also have significantly more $CD44^{hi}GzB^{hi}$ CD8+ T cells at all bleed dates investigated. Granzyme B was quantified in antigen experienced $CD44^{hi}$ CD8+ T cells.

Interestingly, as seen in Figure 10, in contrast to a steady increase in $CD44^{hi}KLRG1^{hi}$ CD8+ T cells observed in CoH mice over 30 days of pathogen exposure, the percentage of $CD44^{hi}GzB^{hi}$ CD8+ T cells in CoH mice decreased over time. There is a rapid development of effective CD8+ T

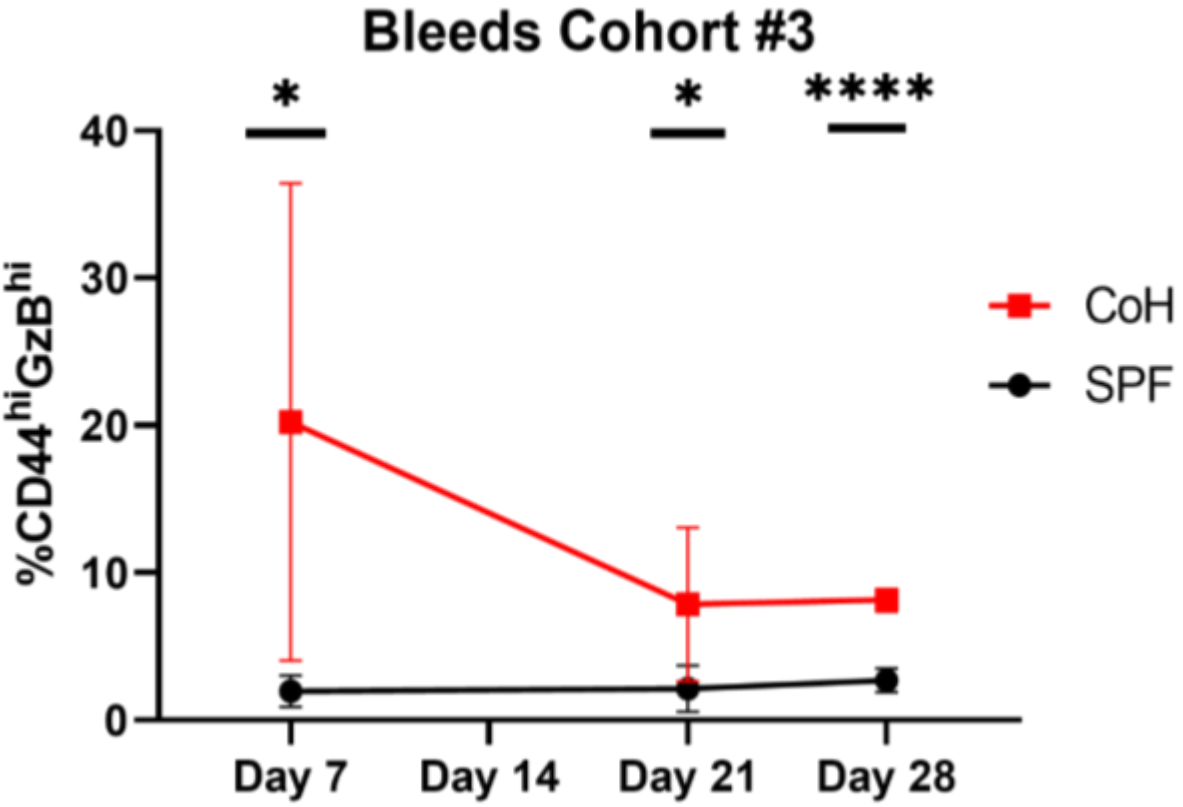

Figure 10: CoH mice develop more antigen-experienced and potentially more effective T cells.
Cohoused mice develop more antigen- experienced, effective $CD44^{hi}GrzB^{hi}$ CD8+ T cells over a cohousing period of 30 days, as seen in roughly weekly bleeds. Effector cells containing Granzyme B in blood sample of CoH mice spike initially and then level off after Day 21. 4-5 mice were bled for each group and each time point. Data are shown for Cohort #3 and have not been repeated yet.

cells in CoH mice compared to SPF controls initially, as seen on Day 7. Almost 20% of CD8+ T cells are positive for Granzyme B in CoH mice, which is a 10fold difference to SPF mice. The initial spike is probably seen due to a drastic shift of mice from a former clean, SPF environment to a cage with pathogen-infected PS mice. To combat infection of pathogens, the adaptive immune system rapidly upregulates effector CD8+ T cells to secrete Granzyme B and target intracellular pathogens such as viruses. After initial exposure, CD8+ T cells of CoH mice reduce production of Granzyme B over the next 7-14 days but retain a significantly higher percentage of about 4 times more $CD44^{hi}GzB^{hi}$ CD8+ T cells until Day 28, compared to clean SPF controls. As expected, due to the lack of pathogen exposure, no change in Granzyme B expression could be observed in SPF mice.

Overall, CoH mice develop significantly more antigen experienced and differentiated more $CD44^{hi}KLRG1^{hi}$ CD8+ T cells compared to SPF mice over a cohousing period of 30 days. Concurrently, more $CD44^{hi}$ CD8+ T cells CD8+ T cells of CoH mice also secrete significantly more Granzyme B compared to SPF mice at all bleed days. Due to the need of effector T cells following infection, production of Granzyme B spikes early, decreases over the first 21 days and levels off. This in contrast to a steady development of $CD44^{hi}KLRG1^{hi}$ CD8+ T cells observed in CoH mice.

MORE ANTIGEN EXPERIENCED, DIFFERENTIATED AND EFFECTIVE CD8+ T CELLS IN DIFFERENT ORGANS OF COH MICE

After a successful cohousing period of at least 30 days, mice were harvested at different time points to quantify subsets of CD8+ T cells in lymph nodes and spleens in addition to blood

samples. Similarly, as with the bleed days, antigen experienced and differentiated more $CD44^{hi}KLRG1^{hi}$ CD8+ T cells were quantified in Lymph nodes (LN), Spleen and blood of SPF and CoH mice on 3 different harvest days. Effector function of CD8+ T cells was quantified in spleen by examining the percentages of $CD44^{hi}GzB^{hi}$ CD8+ T cells there.

As shown in Figure 11 A-C, CoH mice had significantly more $CD44^{hi}KRLG1^{hi}$ CD8+ T cells across all 3 tissue samples. While percentages of more $CD44^{hi}KLRG1^{hi}$ CD8+ T cells were lower in lymph nodes and spleens of CoH mice compared to blood samples, they retained more antigen experienced and differentiated CD8+ T cells in other sites than the blood. In contrast, less than 2% of CD8+ T cells of SPF mice were $CD44^{hi}KLRG1^{hi}$ in all 3 tissues observed. This suggests that development of antigen experienced and differentiated CD8+ T cells in CoH mice due to microbial experienced is not only a transient change in CD8+ T cell subsets seen in the blood, but also visible in more permanent homing sites of T cells such as lymph nodes and spleen.

In line with the previous observations, spleens of CoH mice also contained a significantly higher percentage of effective $CD44^{hi}GzB^{hi}$ CD8+ T cells (Figure 11, D). While the overall percentages were decreased compared to blood samples, CoH mice had about 2.5 times more effector T cells than the SPF controls, as indicated by a higher percentage of $CD44^{hi}GzB^{hi}$ CD8+ T cells. Therefore, microbial experience such as in cohousing mice does not only cause a transient change in certain CD8+ T cell subsets. Instead, it permanently increases both $CD44^{hi}KLRG1^{hi}$ CD8+ T cells not just in the blood, but also in lymph nodes and spleens, as well as potentially increasing percentages of $CD44^{hi}GzB^{hi}$ CD8+ T cells in spleens of CoH mice versus SPF controls.

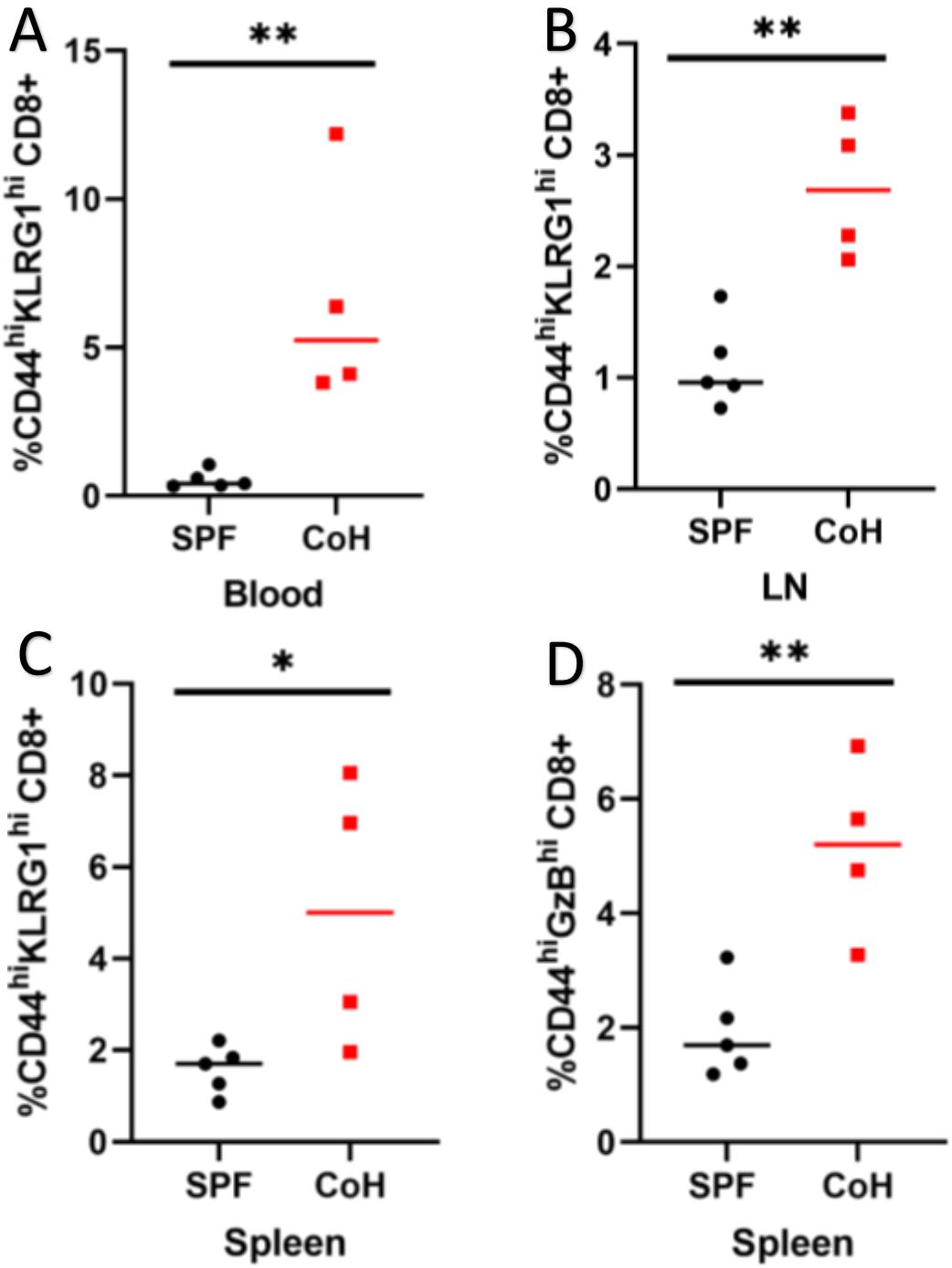

Figure 11: CoH mice have more antigen experienced, differentiated and effective CD8+ T cells in different organs.

A-C CoH mice have more antigen- experienced and differentiated $CD44^{hi}KLRG1^{hi}$ CD8+ T cells across different organs (blood, lymph nodes, spleen) compared to SPF mice when harvested. D CoH mice also have higher percentages of potentially more effective $CD44^{hi}GrzB^{hi}$ CD8+ T cells in the Spleen. Data are shown for 4-5 mice per group per tissue sample for Day 49 Harvest in Cohort 3. This has been repeated n=9 times, except for D.

SPLEEN CD8+ T CELLS OF COH MICE ARE NOT ACTIVATED MORE RAPIDLY AFTER IN-VITRO STIMULATION, BUT SECRETE SLIGHTLY MORE GRANZYME B

To examine the CD8+ T cell response in CoH versus SPF mice after activation, spleen CD8+ T cells were isolated and cultured in vitro for 3 days following each harvest. Separate culture plates for each day of culture analysis were coated using anti-CD28 and anti-CD3 antibodies and incubated at 37 C to eliminate the potential of contamination. 1 ml of supernatant was stored for ELISAs and the remaining supernatant and cell suspension was analyzed via flow cytometry using similar markers as on the harvests.

T cells upregulate CD69 immediately following stimulation and it is thus an excellent marker to quantify rapid activation of antigen experienced CD8+ T cells (Simms & Ellis, 1996). A representative gating strategy done in FlowJo is shown in Figure 17 in the appendix. Percentages of $CD44^{hi}CD69^{hi}$ CD8+ T cells were thus compared between SPF and CoH mice T cell cultures on Day 1 (early timepoint, about 16h after initiating of cultures) and Day 3 (late timepoint, about 56h after initiating) of in vitro T cell stimulation. T cell cultures done in previous cohorts established this timeline as the most promising.

We could not observe any significant difference in $CD44^{hi}CD69^{hi}$ CD8+ T cells in CoH compared to SPF mice on both days of in vitro stimulation (D35 Harvest). Individual variation in both SPF and CoH mice was relatively high on both time points. Interestingly, Day 3 did not yield much of a further increase in T cell activation, as average percentages of $CD44^{hi}CD69^{hi}$ CD8+ T cells remained the same in SPF and CoH mice and even decreased in cultures of other time points. In addition, CoH mice had slightly more effective CD8+ T cells after in vitro stimulation as

indicated by higher percentages of GzB^{hi} CD8+ T cells, but this was not significant. Figure 12 below illustrates the slight difference in spleen CD8+ T cells.

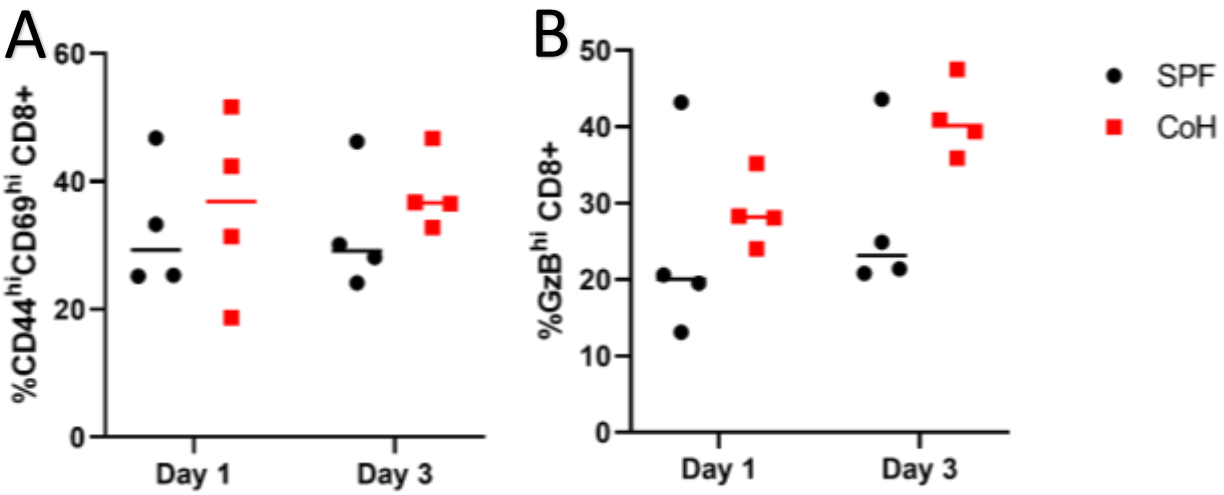

Figure 12 : CD8+ T cells of CoH mice secrete slightly more Granzyme B.

A T cells of CoH mice are stimulated more rapidly in vitro, as indicated by slightly higher frequencies of $CD44^{hi}CD69^{hi}$ CD8+ T cells cultured from spleens on both Day 1 and Day 3 of T cell in- vitro stimulation, but this was not significant. B CD8+ T cells of CoH mice also secrete slightly more Granzyme B, on average, than SPF mice, as seen by higher percentages of GzB^{hi} CD8+ T cells. Data are shown for 4 mice per group and per time point for Day 35 Harvest in Cohort 3. This has been repeated n=4 times.

In summary, we could observe some slight trends in activation and effector function of spleen CD8+ T cells in vitro. Both observations were not consistent across all cultures or individual samples and failed to reach significance.

TUMOR- INFILTRATING CD8+ T CELLS OF COH MICE ARE MORE ANTIGEN EXPERIENCED AND DIFFERENTIATED

B16 melanoma cells were injected into a subcohort of 5 SPF and CoH mice to examine the effects of microbial experience on tumor growth. Tumors were harvested after 11 days on Day 65 and tumor- infiltrating T lymphocytes were stimulated in-vitro analogous to spleen-derived T cells. Supernatant of tumor- infiltrating T cell cultures was analyzed on Day 1 and Day 3 to examine differences in T cell activation markers in cultures from SPF versus CoH mice

A significantly higher percentage of CD8+ T cells of CoH tumors was $CD44^{hi}KLRG1^{hi}$ compared to T cells of SPF tumors (Figure 13, A). While we had some individual variability in percentages of $CD44^{hi}KLRG1^{hi}$ CD8+ T cells in both groups, we observed similar results on two independent tumor cohorts (compare to Groeber, 2019). It suggests that this subset of T cells observed in significantly higher frequencies in CoH mice preferentially moves into tumor tissue as well. However, we cannot rule out that we observe more antigen- experienced and differentiated CD8+ T cells in tumor samples of CoH mice simply because CoH mice overall have higher numbers of these cells. To investigate T cell effectiveness, we also quantified percentages of $CD44^{hi}GzB^{hi}$ CD8+ T cells in tumor tissue, but saw no significant difference in CoH versus SPF mice tumor samples with a trend of more effective T cells in CoH mice (Figure 13, B).

We can conclude a significant difference in antigen experienced, differentiated $CD44^{hi}KLRG1^{hi}$ CD8+ T cells in CoH versus SPF tumors, but only saw a slight increase in $CD44^{hi}GzB^{hi}$ CD8+ T cells in tumor tissue when investigating effectiveness of the tumor-infiltrating lymphocytes.

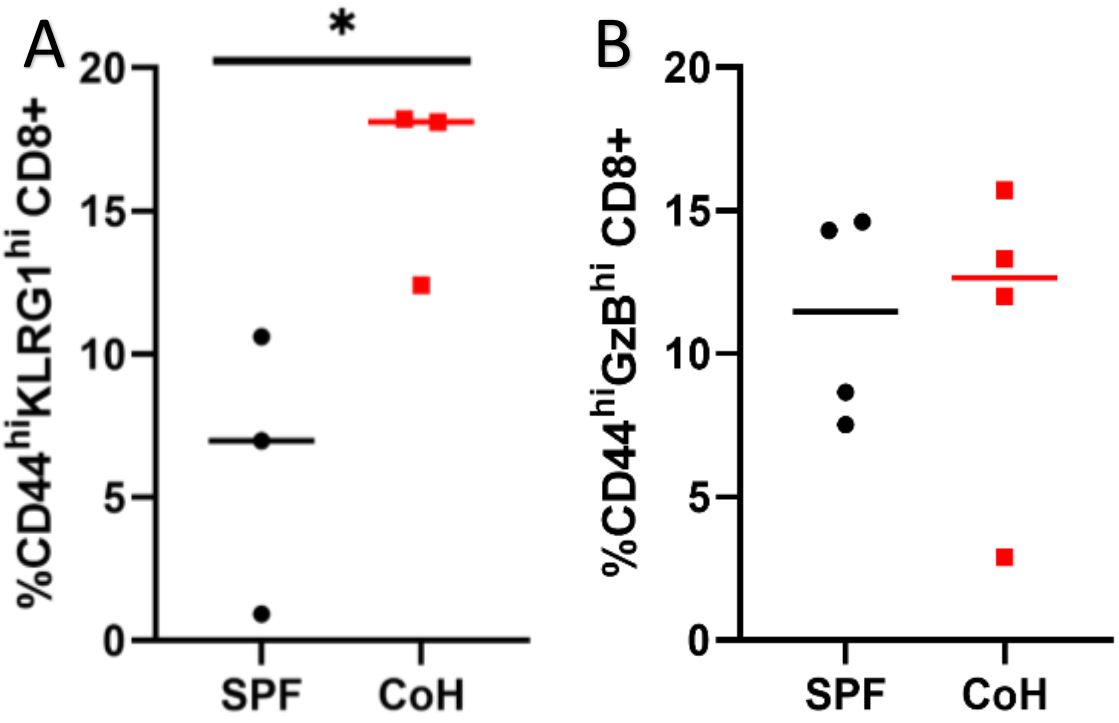

Figure 13: Tumor- infiltrating CD8+ T cells of CoH mice are more antigen experienced and differentiated.

A Tumors of CoH mice have significantly more $CD44^{hi}KLRG1^{hi}$ CD8+ T cells compared to tumors of SPF mice. Data are shown for n=3 mice per group of Cohort #1 and has been repeated n=1 time. B T cells of tumors in CoH mice not significantly more effective compared to SPF controls, as indicated by only marginally higher frequencies of $CD44^{hi}GzB^{hi}$ CD8+ T cells. Data are shown for 4 mice per group for Day 65 Harvest in Cohort #3. This has not been repeated yet.

TUMOR- INFILTRATING CD8+ T CELLS ARE MORE ACTIVATED AND DIVIDE MORE RAPIDLY IN COH MICE AFTER IN- VITRO STIMULATION

In line with the tissue observations on tumor-infiltrating lymphocytes, we also investigated potential differences in antigen experience, activation, and effectiveness of tumor-infiltrating lymphocytes isolated from harvested B16 melanoma tumors and then stimulated in vitro for 3 days, similar to spleen T cell cultures.

In contrast to spleen cultures, tumor- derived T cells of CoH mice were activated much more rapidly compared to SPF mice (Figure 14, A). Even on Day 1 about 16 hours after culture setup, there was almost a 2.5-fold difference in percentage of antigen experienced, activated $CD44^{hi}CD69^{hi}$ CD8+ T cells in tumor cultures of CoH mice versus controls. SPF mice cultures gained more $CD44^{hi}CD69^{hi}$ CD8+ T cells from Day 1 to Day 3 compared to CoH mice, but still had significantly less antigen experienced, activated CD8+ T cells in tumor cultures on Day 3 compared to CoH mice. On average, 53.5% of SPF were $CD44^{hi}CD69^{hi}$ CD8+ T cells on Day 3, which was comparable to the percentage of antigen experienced, activated CD8+ T cells of CoH mice on Day 1. In contrast, by Day 3, almost 3/4 of CD8+ T cells cultured from CoH mouse tumors had a $CD44^{hi}CD69^{hi}$ phenotype.

By staining T cells prior to in vitro activation with CFSE, a dye that gets 50% more diluted per cell division, we also examined percentages of divided CD8+ T cells in tumor cultures and compared these to divided CD8+ T cells of spleen cultures on Day 3. SPF mice had a higher percentage of divided spleen-derived, stimulated T cells on Day 3 compared to CoH mice (Figure 14, B), but not significantly more so. Interestingly, there were significantly more divided CD8+ T cells present in the supernatant of tumor cultures on CoH mice versus SPF mice (Figure 14, C).

CoH mice had over 25% more divided CD8+ T cells compared to SPF controls, pointing to an increased proliferation of CoH CD8+ T cells isolated from tumors compared to SPF controls. This was a striking difference especially because the frequency of divided spleen T cells was higher in SPF compared to microbially experienced CoH mice.

In summary, this suggests that microbial experience influences the anti-melanoma response in mice. There were significantly more activated $CD44^{hi}CD69^{hi}$ CD8+ T cells on both time points of T cell in vitro stimulation in CoH versus SPF mouse tumors. Additionally, CoH mice had a significantly higher percentage of divided CD8+ T cells present in tumor cultures on Day 3 compared to SPF mice, which was reversed when investigating frequencies of divided CD8+ T cells cultured from spleens. This seems to suggest that the higher frequencies of antigen experienced, differentiated, and effective CD8+ T cells observed in different organs of CoH mice might also get activated and proliferate more effectively in presence of cancer.

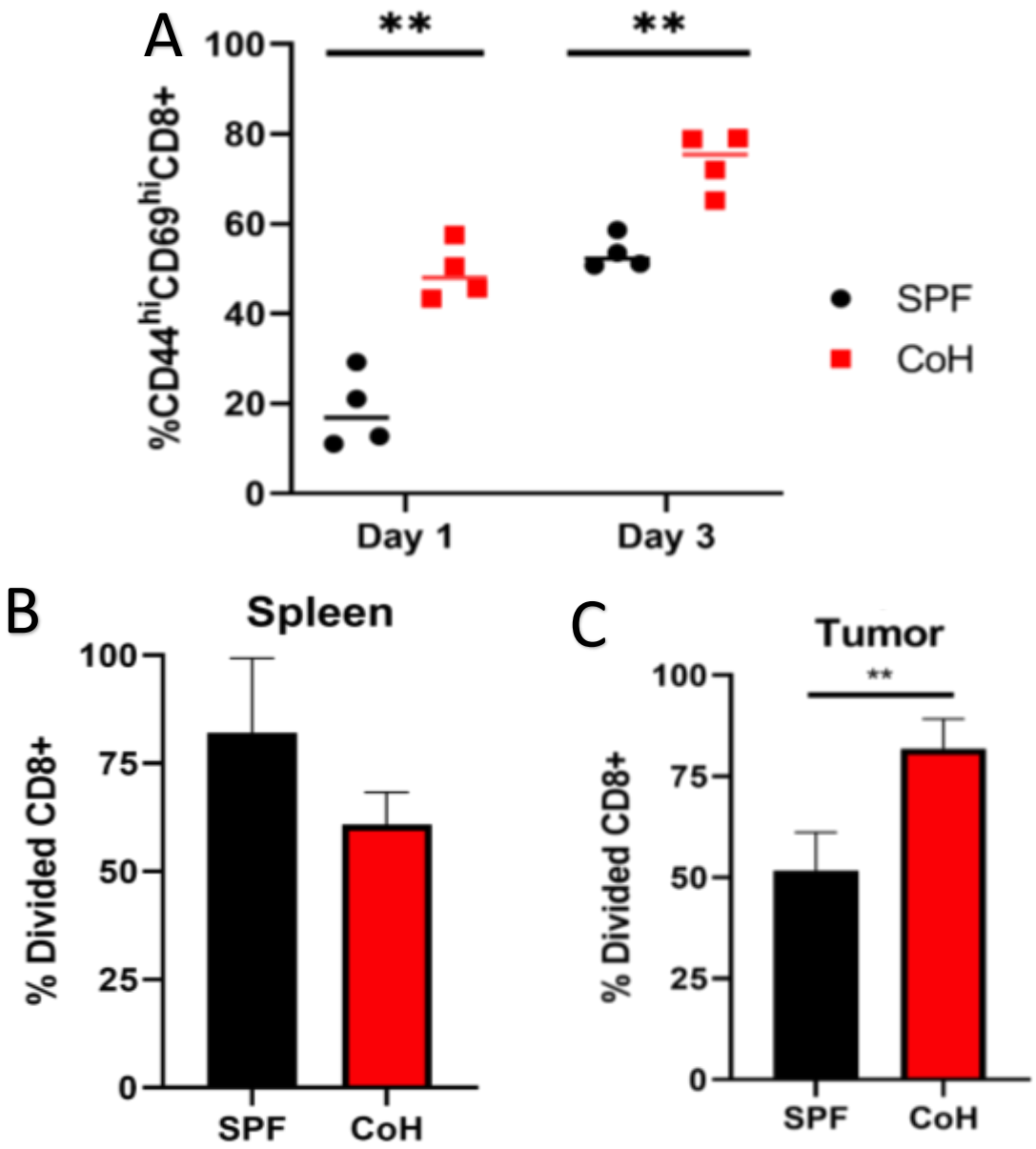

Figure 14: Tumor-infiltrating CD8+ T cells are more activated and divide more rapidly in CoH mice.

A T cells of CoH mice are stimulated more rapidly in vitro compared to SPF controls. There were significantly higher frequencies of $CD44^{hi}CD69^{hi}$ CD8+ T cells cultured from tumors of CoH mice on both Day 1 and Day 3 of T cell in- vitro stimulation. B Spleens of CoH mice have slightly less divided CD8+ T cells compared to spleens of SPF mice on day 3 of T cell in vitro stimulation. C Tumors of CoH mice have significantly more divided CD8+ T cells compared to tumors of SPF mice on day 3 of T cell in vitro stimulation. Cells were CFSE stained for the number of divisions and counted as "divided" beyond the first division peak CD8+ T cells. Data are shown for 4 mice per group on Day 3 of in vitro T cell stimulation on Day 65 Harvest in Cohort 3. This has not been repeated yet. Data are shown for 4 mice per group and per time point for Day 65 Harvest in Cohort 3. This has not been repeated yet.

CHAPTER 5: DISCUSSION

Mice with microbial experience are slowly being explored as a beneficial complementary mouse model for biomedical research in research facilities across the United States. Due to the fear of contamination of other laboratory animals and the risk of contracting zoonotic infections, safety standards usually remain stringent and make several other aspects of immunology and oncology research with these mice difficult, such as setting up T cell cultures or using cancer cell lines. Therefore, up to this date, no research on cancer models in microbially experienced mice has been reported and this thesis explored a novel impact of microbial experience on tumor development by injecting B16 melanoma cells into a subcohort of both cohoused and SPF mice and investigate the anti-melanoma CD8+ T cell response.

Interestingly, we could already see striking differences in antigen experienced and differentiated $CD44^{hi}KLRG1^{hi}$ CD8+ T cells in a small preliminary subgroup of cohoused mice. A trial B16 melanoma injection was done on 8 mice in our first cohort but could not be repeated in the second cohort. We followed up on these observations with a more extensive third cohort of 35 mice and analyzed the CD8+ populations of interest during the cohousing period, on harvest days, after in-vitro stimulation and in tumors of CoH mice compared to SPF controls.

We could repeat observations of cohort #1 and #2 successfully and incorporated Granzyme B as a marker that indicates the effector function of CD8+ T cells. In addition to a significant increase in $CD44^{hi}KLRG1^{hi}$ CD8+ T cells in CoH mice after 30+ days of cohousing, we observed a significant difference in potentially more effective $CD44^{hi}GzB^{hi}$ CD8+ T cells in CoH mice as well. These CD8+ T cells spiked early during cohousing on Day 7 and then seemed to decrease to an elevated level in CoH compared to SPF controls by day 21. In addition to differences in the

population percentages of GzB^{hi} CD8+ T cells, we could confirm differences in effectiveness by evaluating the mean fluorescent intensity of Granzyme B (MFI), which was also significantly higher in CoH mice. The MFI gives us a direct estimate of the amount of GzB produced in these T cells rather than just investigating percentages of GzB- positive T cells.

Unfortunately, due to clogging of our flow cytometer, we lack any data on the day 14 bleed. We aim to follow up with a more careful analysis of Granzyme B concentrations on the subsequent cohorts to precisely determine when Granzyme B increases and decreases during the 30- day cohousing period. Overall, differences in Granzyme B concentrations are in line with our hypothesis that CoH mice have a more experienced and thus effective CD8+ T cell response, indicating an overall more potent adaptive immune system. stimulated in vitro and demonstrated higher percentages of divided CD8+ T cells.

Unexpectedly, we report no significant differences in GzB^{hi} CD8+ T cells or MFI of Granzyme B on both days of T cell in vitro stimulation between CoH and SPF mice. While we saw an overall significant difference in $CD44^{hi}GzB^{hi}$ CD8+ T cells in spleens of harvested CoH compared to SPF mice, this trend was eliminated or partially reversed when spleen T cells were stimulated in vitro. So far, we have only been able to do few repeats of our in-vitro T cell experiments, and we hope to validate results and improve accuracy with future cohorts. Secondly, since we cannot strictly isolate just the $CD44^{hi}GzB^{hi}$ CD8+ T cells of both SPF and CoH mice to investigate in vitro stimulation specifically on this subset, we are not sure on whether our comparisons are accurate enough to detect any significant changes.

In addition to that, a more detailed analysis of Granzyme B frequencies on harvest and in T cell cultures is needed, since we observed a lot of variability in between different harvest

groups. This is probably due to spreading several pet store mice with potentially different pathogen burdens to our cages of CoH mice. Therefore, we cannot precisely control which pathogens infect which CoH mice. Since cages are pooled per harvest, CoH mice with variable microbial experience are analyzed together and compared as a group to SPF controls. While this is a condition that is difficult to eliminate, it also enhances the comparability of cohoused mice to human patients, since human individuals can have very variable exposure to microbes over the course of their life time, even if they are from the same location.

In future cohorts, we plan on weighing and measuring tumor size in mice to record any potential differences in growth due to microbial exposure. We hypothesized that tumor of CoH mice would be slightly smaller compared to SPF mice due to more activated, experienced, and differentiated CD8+ T cells observed. Due to the lack of accurate- enough instruments, we were not able to detect external differences in tumor development. Several studies on normal and microbially experienced lab mice have reported differences in size and growth of tumors due to microbial influence (Iida et al., 2013; Rosshart et al., 2017). Therefore, this is another area of further investigation of this project.

Additionally, in future cohorts, we aim to include a more thorough analysis of Interferon γ concentrations as an additional marker to characterize differences in effectiveness of CD8+ T cells, since Granzyme B has not been conclusive in Cohort #3. Due to time constrains and the effects of COVID-19, we could only do one pilot ELISA on IFNγ concentrations of 6 supernatant samples of a previous cohort. While we could see a slightly lower average concentration of IFNγ in SPF mice supernatant samples of Day 4 T-cell culture (preliminary data shown in Figure 18, appendix), this was not enough to detect significant differences between CoH and SPF due to a

relatively large individual variation. Since we have only tested supernatant samples of Cohort #1 so far via ELISA, we also cannot exclude a relatively high variability of samples due to it being our first cohort. Concentrations of IFNγ overall also fell into the higher end of the detection range of the ELISA test kit, as we used Day 4 as the second time point in the first T cell cultures of Cohort #1. Therefore, these first concentrations might already be too high to detect any meaningful differences between SPF and CoH T cells since stimulation had already been too long. In future analyses, we would characterize earlier T cell culture time points such as Day 1 and Day 3 to investigate differences or dilute supernatant samples 1:2 prior to testing.

To obtain more CD8+ T cells in tumors, we also aim to enrich for T lymphocytes in subsequent cohorts. On average, we only obtained percentages of about 1% of CD8+ T cells in tumor samples, due to large amounts of other non-immune related cells such as endothelial cells. By adding an enrichment protocol, we hope to increase the amount of tumor-infiltrating T cells and thus also increase accuracy of the subsets investigated.

We believe that repeats of our experiments will also help support our observations. We have seen significantly increased $CD44^{hi}KLRG1^{hi}$ CD8+ T cells in two independent cohorts of 3-4 mice per group so far but have not repeated other markers on tumor- infiltrating CD8+ T cell subsets included in this work. Subsequent tumor cohorts would be increased in size to account for possible excluded samples such as due to systemic dissemination of tumors due to imprecise B16 melanoma cell injections or deaths of mice after severe infections.

Lastly, while we aim to quantify T cell subsets equally in CoH and SPF mice and in T cell cultures, we unfortunately cannot stimulate specific subsets in vitro. Essentially, we cannot compare apples to apples, because we lack the supplies necessary to just stimulate

$CD44^{hi}KLRG1^{hi}$ CD8+ T cells in culture or isolate exclusively these T cells from spleen tissues and tumors for further analysis. While we only have limited markers available, we have chosen these carefully, since they have proven to be effective in earlier studies with CoH mice (Beura et al., 2016b). Future collaboration with more extensive labs in other research facilities such as the University of Arizona or the University of Minnesota could also be an option to increase the quality of our data.

In conclusion, we have successfully demonstrated that CoH, compared to SPF mice have more antigen experienced, differentiated, and effective CD8+ T cells across different organs. These CD8+ T cells develop during a cohousing period of 30+ days with pet store mice, which can successfully be done in a simple satellite vivarium. This leads us to mostly accept our Hypothesis 1 as stated in the introduction. More importantly, when injected with B16 melanoma, tumor-infiltrating CD8+ T cells in CoH mice are significantly more antigen-experienced and differentiated compared to SPF mice. They are activated more rapidly following T cell stimulation in vitro. Additionally, CD8+ T cells from tumors of CoH mice also show a significantly increased number of divided CD8+ T cells compared to SPF mice, suggesting that microbial experience also influences the functional properties of tumor- infiltrating CD8+ T cells specifically. This is in line with Hypothesis 2 a..

Various studies in the last years have demonstrated a previously underestimated impact of commensal microbes and exposure to pathogens in shaping the immune response to various immune related diseases and cancer. Microbial experience seems to influence the individual immune response in both mice and human patients (Beura et al., 2016b; Frankel et al., 2017; Iida et al., 2013; Rosato et al., 2019; Rosshart et al., 2017). With several successful

immunotherapeutic drugs being developed to treat cancer more effectively, it is necessary to have more accurate models to predict outcome of therapy based on the individual immune state of human patients. Due to their limited immune experience SPF mice fail to recapitulate many nuances of the adult human T cell response and likely the adaptive immune system as well. Adding CoH mice as an additional model in immune-related research could improve our understanding of the patient immune and anti-cancer response. While SPF mice have their place in basic biomedical and immunological research due to their ease in handling, cost-effectiveness and versatility, CoH mice could be implemented as an additional lab model to test vaccines and cancer-drugs before moving into human clinical trials. This could ultimately help reduce cost and enhance a more successful transition from bench to bedside. In summary, cohousing lab mice could be a valuable model to increase translation of cancer- and immune related research from lab to human patients.

	total SPF [%]	**total PS** [%]	**total CoH** [%]
Lymphocytic choriomeningitis virus	0.0	0.0	0.0
Mouse adenovirus 1 & 2	0.0	100.0	75.0
Mouse hepatitis virus	0.0	100.0	25.0
Mouse norovirus	0.0	50.0	50.0
Mousepox (Ectromelia virus)	0.0	50.0	50.0
Mouse parvovirus	0.0	100.0	100.0
MPV 1	0.0	50.0	0.0
MPV 2	0.0	0.0	0.0
MPV 3	0.0	100.0	100.0
MPV 4	0.0	0.0	0.0
Mouse rotavirus	0.0	0.0	0.0
Pneumonia virus of mice	0.0	0.0	0.0
Reovirus	0.0	0.0	0.0
Sendaivirus	0.0	100.0	0.0
Theiloviruses	0.0	100.0	100.0
Corynebacter bovis	0.0	0.0	0.0
Cilia-associated respiratory bacillus	0.0	0.0	0.0
Clostridium piliforme	0.0	50.0	0.0
Mycoplasma pulmonis	0.0	75.0	0.0
Mites	0.0	0.0	0.0
Pinworms	0.0	50.0	0.0
Aspiculuris tetraptera **(pinworm)**	0.0	50.0	0.0
Syphacia muris **(pinworm)**	0.0	0.0	0.0
Syphacia obevelata **(pinworm)**	0.0	0.0	0.0

Table 2: CoH mice get infected by various pathogens during cohousing, while SPF mice stay clean.

Pathogen testing by Charles River indicates that PS mice were infected with a variety of pathogens and confirmed that CoH mice acquired about half of them during a cohousing period of 30 days**.** The control group of SPF mice kept in the same satellite vivarium, in contrast, remain completely free of any pathogens that were tested for. Data are compiled for cohorts 1 and 2, fecal and blood samples were pooled from 2 cages for each group of mice for each sample (Groeber, 2019).

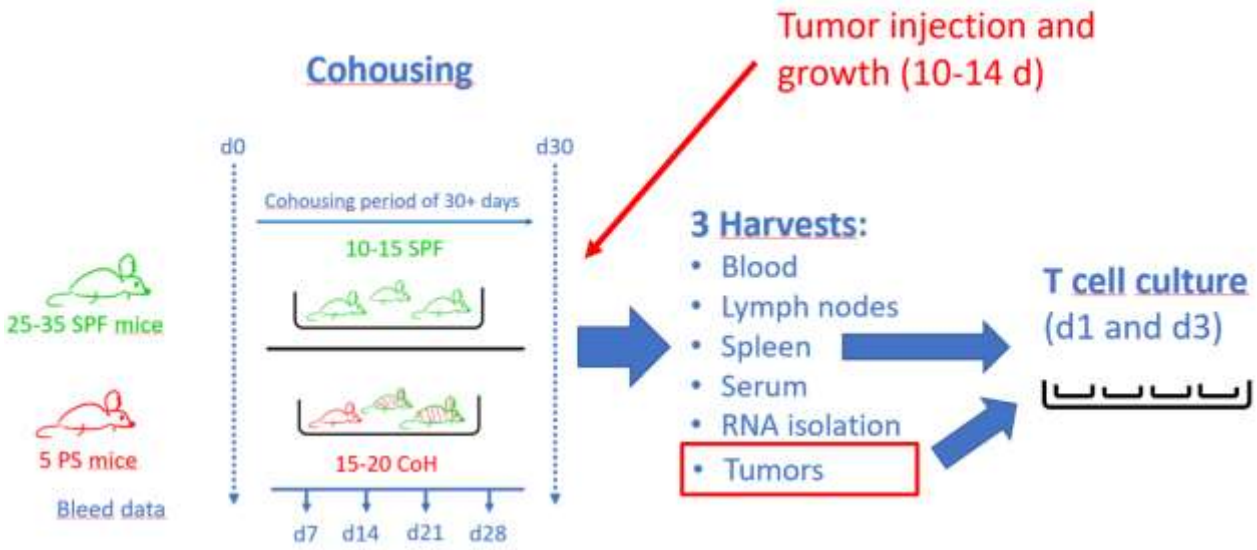

Figure 15: Experimental setup of our mouse cohorts

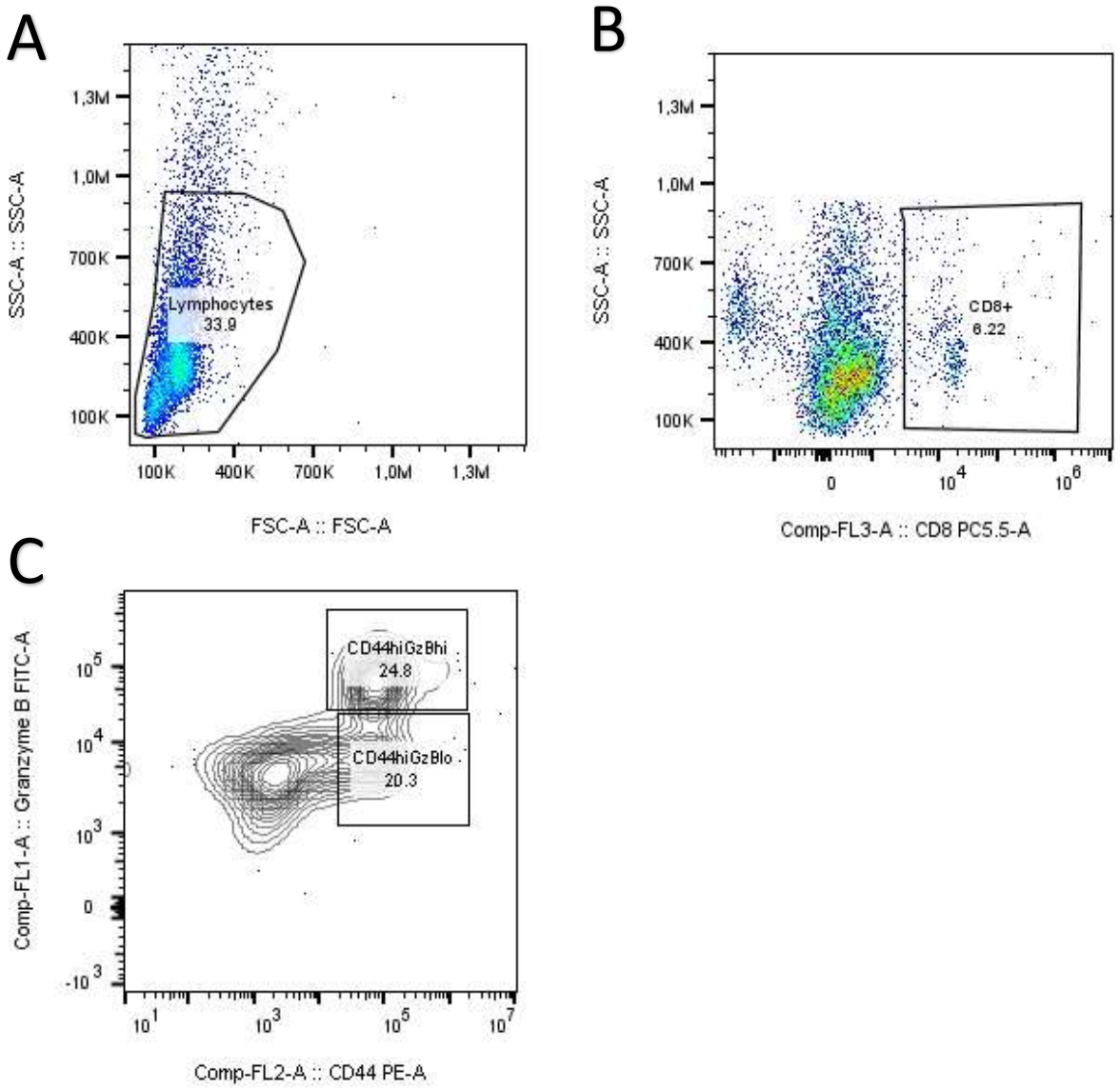

Figure 16: Gating strategy used to compare frequencies of CD44^{hi}GzB^{hi} CD8+ T cells.

Gating strategy employed in FlowJo to compare frequencies of CD44^{hi}GzB^{hi} and GzB^{lo} cells on weekly bleeds in both CoH and SPF mice. Lymphocytes were roughly gated off forward- and side scatter (A), and CD8+ cells were obtained using CD8 and side scatter (B). Out of the CD8+ T cells, those with CD44 expression were identified and separated into a GzB^{hi} and GzB^{lo} population, as seen in C. Geometric means were calculated on both CD44^{hi}GzB^{hi} and -GzB^{lo} cells as well to compare numbers of Granzyme B in between groups. Gates were identical for all blood samples per time point, and analysis was done similarly across all bleed days. CoH 3-1 is shown here as an example.

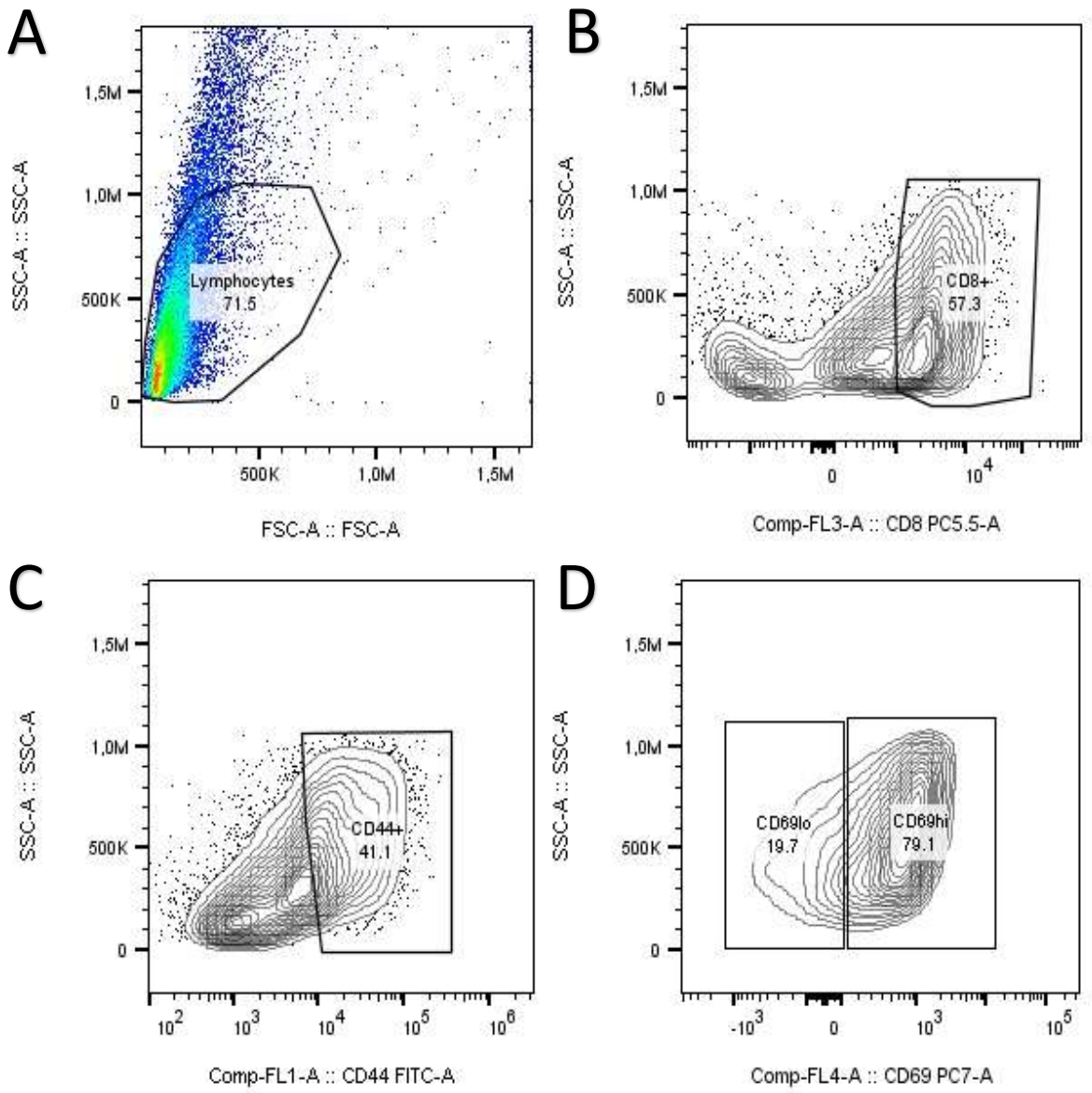

Figure 17: Gating strategy examine percentages of activated CD44^{hi}CD69^{hi} T cells.
Gating strategy employed in FlowJo to examine activated, antigen experienced CD8+ T cells after harvests and in-vitro stimulation. As seen in the bleed gating strategies, lymphocytes were roughly gated off forward- and side scatter (A) and CD8+ T cells were further gated off these lymphocytes (C). In D, CD8+ T cells were further separated into a CD44^{hi} (or CD44+) population and subsequently further divided into activated CD69^{hi} and inactive CD69^{lo} T cells. This was done identically for all samples per organ and time point, and gates were employed in a similar manner across different culture and harvest days. D65 Tu D3 Culture sample CoH 3-12 is shown here as a representative example.

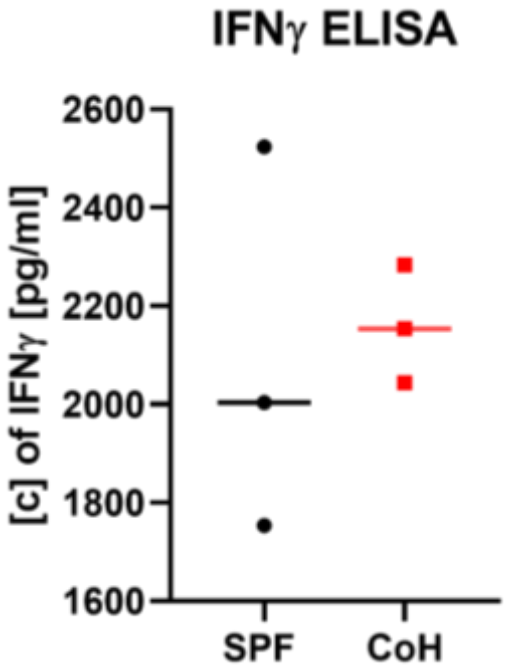

Figure 18: CoH mice have slightly higher concentrations of IFNγ.

The IFNγ concentration of supernatant samples collected after T cell in vitro stimulation from Cohort #1 were analyzed via ELISA. On average, CoH samples had slightly higher concentrations of IFNγ compared to SPF mice in this pilot test, but the difference observed failed to reach statistical significance. Supernatant samples of D60 Harvest of Cohort #1 from Day 4 of stimulation were analyzed in this ELISA, with n= 3 SPF and 3 CoH mice. This has not been repeated yet.

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