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Evaluating the microbial experience influence on anti-tumor immune response

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Abstract

Despite the steady increase of hygienic standards, the CDC continues to report an increase of immune-mediated diseases such as allergies and asthma. Human avoidance of microbial exposure and subsequently less experienced immune systems may be the cause for this increase. We investigated if increased microbial exposure results in increased immunity to cancer (B16 melanoma) by measuring activated lymphocytes between two groups of C57Bl/6 mice: specific pathogen free (SPF) mice (which had little microbial exposure) and cohoused (COH) mice (which were exposed to numerous microbes). Previous research shows that the CoH mouse model mimics a human adult's immune system, while the SPF mouse model mimics that of a human infant's. Activated lymphocyte levels were quantified with weekly bleeds using antibody staining flow cytometry. Anti-tumor response was evaluated through multiple harvests in which blood, lymph nodes, spleens, and tumors were collected and analyzed with flow cytometry. CoH mice were expected to have heightened levels of activated lymphocytes and an anti-tumor response superior to the SPF mice. The CoH mice did gain microbial experience and showed in a higher quantity of activated CD8+ T cells. Preliminary results suggest that this may have resulted in an increased anti-tumor response and slowed cancer proliferation.

Introduction

The immune system is a complex network including physical barriers (the first line of defense), the innate system (the second line of defense), and the adaptive system (the third line of defense). Each has its own functions and components, but all aim to protect the body from both itself and from potentially harmful non-self cells (Sompayrac, 2008). Considering that failure of the immune system makes the body vulnerable to pathogens and immune-mediated diseases (such as asthma, allergies, and cancer), an extensive understanding of immunity is imperative to advancements in human medicine.

In an effort to protect our health, hygienic standards have been steadily increased throughout history. Reminders are everywhere: hand sanitizer is a staple in public places, signs remind people to wash their hands and cover their cough, and high-touch surfaces are regularly disinfected. Despite these efforts, the CDC reports a steady, rising trend in immune-mediated diseases, such as allergies and asthma.

Studies, such as one utilizing an Amish community in Indiana and a Hutterite community in South Dakota, suggest that this rise could be caused by the modern tendency to avoid microbial exposure. The Amish and Hutterite communities are less avoidant of microbial exposure than the majority is, and researchers studied them to evaluate how heightened microbial exposure has influenced their immune system development. The Amish practice traditional, small-scale farming that results in a high level of microbial exposure. The Hutterites practice more industrial, regulated farming, which results in less microbial exposure than the Amish, but more exposure than the majority of the human population. They found that the Amish had extremely low percentages of children affected with asthma when compared to both the general population and even to the Hutterite population. They thought this to be the result of Amish

children being exposed to traditional farming, and an increased level of microbes, from a very early age. The early exposure strengthened their immune systems and asthma did not develop (Stein et.al, 2016).

The objective of this study was to determine if microbial exposure leads to a superior anti-tumor immune response. This was evaluated by measuring levels of activated lymphocytes and analyzing the anti-tumor response between immune systems in mice with little or extensive microbial experience. We utilized a unique mouse model that includes both specific pathogen free (SPF) and pet store (PS) mice for our research. SPF mice have been inbred in order to be genetically identical and are housed in exceptionally sanitary conditions, which means that they have had little to no exposure to microbes; therefore, their immune system more so mimics the immune system of a human infant than of a human adult (Beura et.al, 2016). PS mice, however, have been exposed to a myriad of pathogens. This means that the immune system of a PS mouse more closely resembles the immune system of an average adult human (Beura et.al, 2016). We cohoused PS mice with a group of SPF mice for a minimum of 28 days, during which many microbes were acquired by the SPF mice (called cohoused; CoH). We compared activated lymphocyte levels of the SPF mice to the CoH mice, both pre- and post-melanoma injection. The study was conducted in an indoor, light-controlled and temperature-controlled environment.

Methods

We obtained forty C57Bl/6 SPF mice from Charles River Laboratories and five mice from a local pet store and took preliminary blood and fecal samples before beginning the cohousing process. We then divided the mice into nine cages, four containing SPF mice only (five mice in each), and five containing the cohoused mice (four SPF and one pet store mouse in

each). The mice were kept in an isolated room with lights on a 12-hour timer and were separated by a shelf with SPF mice on the top and cohoused mice on the bottom.

We conducted weekly retro-orbital bleeds of both SPF and CoH mice under anesthesia for twenty-eight days of cohousing the mice. After thirty days of cohousing, we began harvests that included blood, lymph node, spleen, and tumor collection of both SPF and CoH mice (Table 1). We processed the tissue samples via filtration with 70 um filters. Day 30 (spleen) and Day 34 (spleen and tumor) harvests included anti-CD3/anti- CD28+IL2 cultures for *in vitro* T cell stimulation. All samples were antibody stained and quantified using flow cytometry.

For tumor-inclusive harvests, the mice received a subcutaneous injection of B16 melanoma cells in both flanks ten days prior to the harvest. Upon collection, cell counts of the tumor samples were taken. Tumor cells harvested on day 48 were enriched for T cells using a MojoSort Isolation Kit.

Data collected by flow cytometry was analyzed by Flow Jo (Treestar) and Graphpad Prism.

Table 1: *Harvest Schedule*

Cohousing day	Samples collected	T cell culture (Y/N)
30	Lymph nodes, spleen, blood	Y
34	Lymph nodes, spleen, blood, tumor	Y
41	Lymph nodes, spleen, blood	N
48	Lymph nodes, spleen, blood, tumor	N

Results

Fecal and blood analysis at Charles River Laboratories showed that the pet store mice successfully exposed the cohoused mice to microbes, while the SPF mice remained unexposed (Table 2). Our analysis of retro-orbital bleeds over the cohousing period showed that a higher quantity of activated CD8+ T cells were present in the cohoused, microbially experienced mice than in the SPF mice (Figure 1).

On average, the tumors harvested from the cohoused mice contained fewer live tumor cells than the tumors harvested from the SPF mice. This data was analyzed to be insignificant with a T-test (Figure 2), however, an increase in experimental size may further clarify the results. KLRG1 is expressed on activated CD8+ T cells, and indicates potentially potent anti-tumor CD8 cells capable of tumor destruction. We discovered that the frequency of $KLRG1^{hi}$ CD8+ T cells may be higher in tumor cells of cohoused mice than in tumor cells of SPF mice, suggesting a superior anti-tumor response; although this data was also analyzed to be insignificant with a T-test (Figure 3), increasing our experimental size may help resolve whether there are indeed any significant differences. Increased levels of Granzyme B, which is released by T cells to cause apoptosis in target cells, suggests an active immune response; however, analysis of Granzyme B^{hi} CD8+ T cell quantities between cohoused and SPF tumor cells was mostly inconclusive at this time (Figure 4).

Table 2: *Charles River Laboratories Pathogen Testing*

Viruses	SPF Mice Day 0	SPF Mice Day 30	Pet Store Mice Day 0	Cohoused Mice Day 30
Rotavirus (EDIM)	-	-	-	-
Mouse Hepatitis Virus	-	-	+	+
Murine Norovirus	-	-	+	+
Mouse parvovirus type 1	-	-	+	-
Mouse parvovirus type 3	-	-	+	+
Sendai virus	-	-	+	-
Mousepox (Ectromelia virus)	-	-	-	-
Lymphocytic Choriomeningitis virus	-	-	+	+
Mouse adenovirus 1 and 2	-	-	+	+
Pneumonia virus of mouse	-	-	-	-
Reovirus	-	-	-	-
Cilia-Associated Respiratory Bacillus	-	-	-	-
Mycoplasma pulmonis	-	-	+	-
Clostridium piliforme	-	-	+	-
Pinworm	-	-	+	-
Mites	-	-	-	-

Figure 1: Retro-orbital bleeds show an increase in activated CD8+ T cells in cohoused mice over time

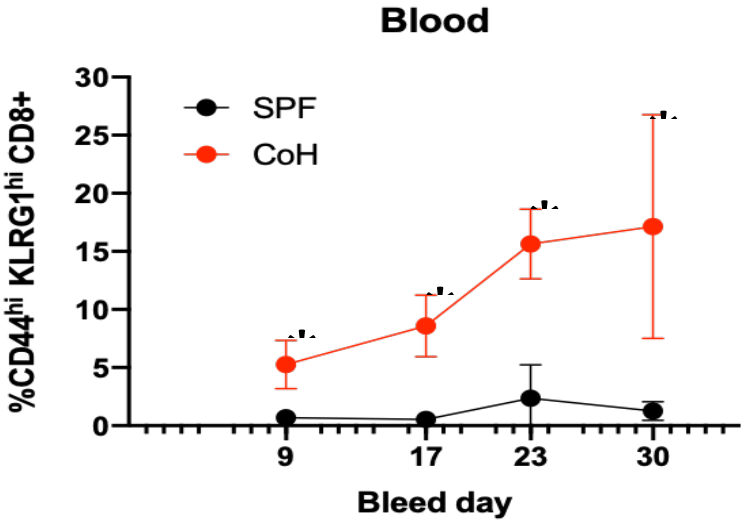


Figure 2: Quantities of live tumor cells are lower in cohoused mice than in SPF mice

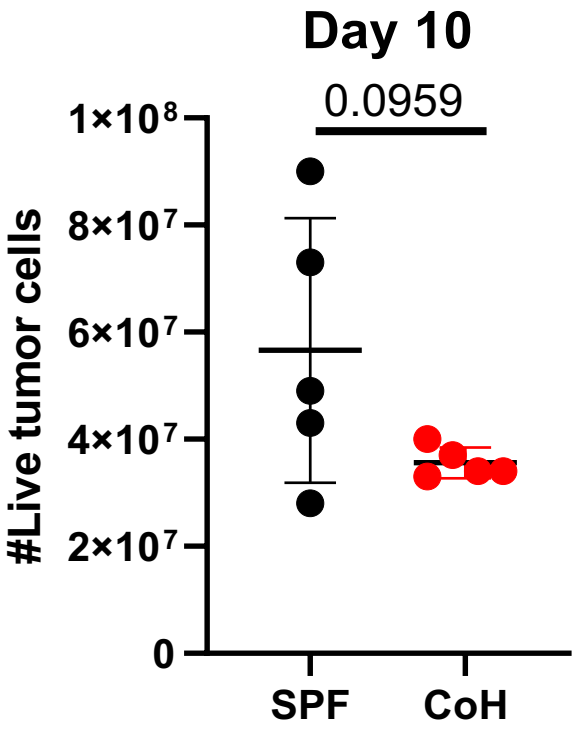


Figure 3: Frequency of KLRG1^{hi} CD8+ T cells in SPF mice vs day 48 cohoused mice in T cell enriched tumor samples

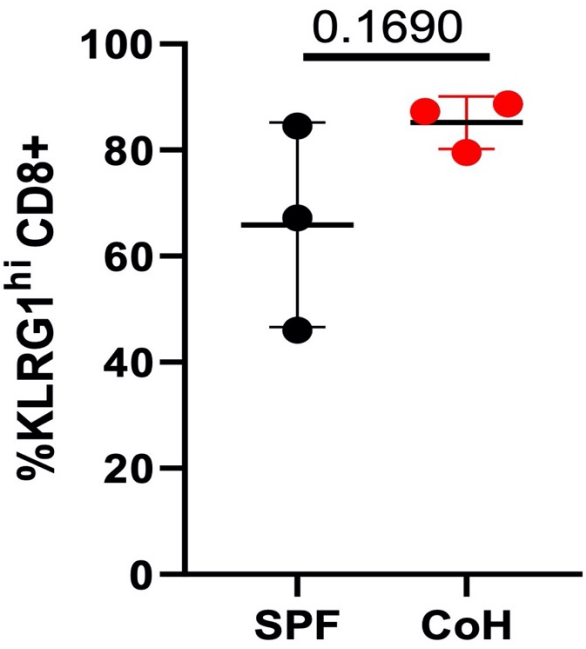
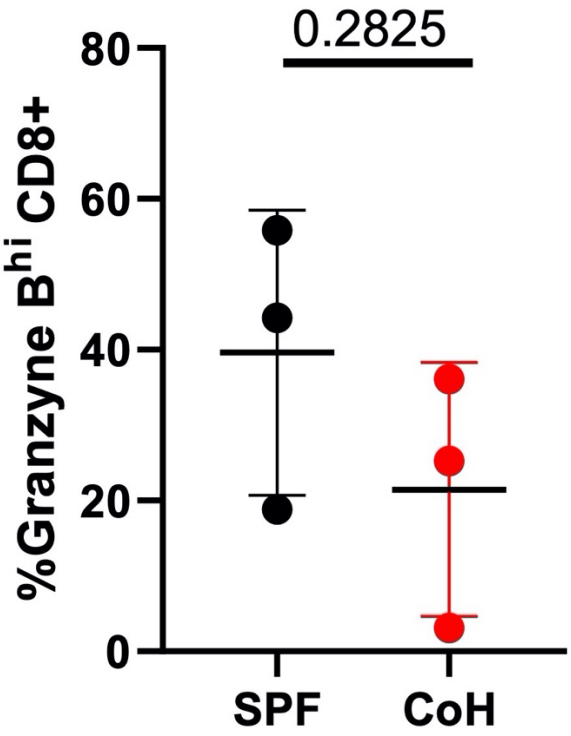


Figure 4: Frequency of Granzyme B^{hi} CD8+ T cells in SPF mice vs day 48 cohoused mice in T cell enriched tumor samples



Discussion

We discovered that the CoH mice were exposed to several viruses from the PS mice and gained microbial experience. This allowed us to test responses on a more human adult-like immune system than traditional mouse models without sacrificing genetic consistency. We found that the CoH period (and subsequent microbial exposure) changed the immune system of the mice; They gradually showed a higher percentage of experienced ($CD44^{hi}$ $KLRG1^{hi}$) CD8+ T cells than the SPF mice throughout the cohousing period. In their study, Beura et.al. (2016) showed similar results; the mice were microbially experienced using the same model. Given these results, it does appear that the immune system of a CoH mouse provides better testing opportunities when studying adult human applications than does the immune system of an SPF mouse (Beura et.al., 2016).

Our preliminary results show a trending lower number of live tumor cells in the CoH mouse tumors than in the SPF mouse tumors, which suggests that a more experienced immune system may result in a superior anti-tumor response. The microbial experience that the CoH mice gained resulted in a higher quantity of activated CD8+ T cells, which may have assisted in a more prompt immune response and ensured that the growth of the tumor was slowed. This data was analyzed to be insignificant using a T-test, however this may be due to the small sample size.

Again, likely due to small sample sizes, the T cell enriched samples (MojoSort) also resulted in data that was analyzed to be insignificant with a T-test. However, the overall trends show that activated CD8+ T cell percentages may be higher in tumors from CoH mice compared to SPF mice; this is especially evident in the analyzed percentages of $KLRG1^{hi}$ CD8+ T cells.

Conversely, the Granzyme B^{hi} CD8+ T cells showed the opposite trend, which was unexpected and will need further investigation with larger sample sizes and additional cohorts of mice.

Further studying of this model of CoH mice is necessary to fully determine their value to the field and their superior representation of the adult human immune system. Their use in continued research regarding the effect of microbial experience on the immune system's response to cancer and other human diseases is important to the understanding of the body's response to disease and distinguish true pathogen from harmless allergen and self. This understanding is crucial to the development of future medical practices and treatments for many immune-related diseases, such as allergies, asthma, autoimmunity, and cancer.

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