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Immune cell longevity and impact on microbial succession post-mortem

By

XINGZI WANG
THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

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in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

Abstract

The microbiome, the collection of the different microorganisms living in the human body, is a topic that connects the realms of disease, development, health, and forensics. The microbial communities within the human body are influenced by a variety of factors including, medical history, physiology, diet, hygiene, and other behaviors. Like a fingerprint, each person's microbiome is unique. In individuals who are immunocompromised, the microbiome can become imbalanced since bacterial colonies directly interact with an individual's immune system. The immune system therefore plays an important role in establishing the content of the microbiome. This relationship between the immune system and the microbiome continues post-mortem. After an individual dies, the subsequent death of immune cells allows the bacteria that make up the microbiome to expand. This bacterial expansion triggers decomposition active decay. Because of the predictable role of the microbiome in decomposition, measuring microbial succession has shown great promise in helping to estimate a more accurate post-mortem interval (PMI). Although progress has been made in refining microbe-based calculations of PMI, immune system function at the time of death has not been closely examined as a factor that can impact the postmortem microbial succession estimation. The goal of this study is to explore the pattern of postmortem immune cell death, and how the death of immune cells impairs the accuracy of estimating PMI. Viability of intestinal immune cell subsets at different post mortem intervals were analyzed so that future studies can analyze how immune cell survival impacts bacterial succession. Ultimately, the goal of this research is to illuminate how immune status at the time of death can impact post-mortem microbial succession.

Introduction

Postmortem Interval (PMI) -

When human remains are found, one of the first questions an investigator is "when did this happen?" Providing an accurate estimation of the post-mortem interval (PMI), which is an estimation of the length of time between an individual's death and the discovery of the body, is essential when investigating cases involving deceased individuals (Shrestha & Krishan, 2020). Establishing an accurate PMI can help accept or reject an alibi, corroborate witness statements, reconstruct the events since death, and generate investigative leads (Metcalf et al. 2013).

A major obstacle to calculating an accurate PMI is the variability of individual factors. The living environment, body temperature, the type of clothing worn, circumstances of death, body storage, and sample preservation. All of which contribute to unpredictable changes in decomposing patterns. Since forensic scientists still do not fully understand the role each variable plays in the processes of decomposition, generating a uniform formula to calculate a PMI is difficult to achieve (Metcalf et al. 2013). For these reasons, forensic scientists and pathologists combine different approaches in order to estimate an accurate PMI. Some of these approaches include observing the degrees of decomposition in a certain environment or the developmental stages of arthropods on the decedent.

Postmortem Decomposition-

To predict PMI, a forensic specialist needs to first understand the processes and stages of decomposition. Decomposition begins immediately after death. Cells within the body begin to go through autolysis due to a lack of blood circulation, deficiency of oxygen and nutrient supply, and release of macromolecules. Then, the bacteria present in the GI tract spreads through intestinal barriers to various tissues or lymph nodes, causing the breakdown of cells and organs.

This process is called putrefaction. The putrefactive growth of bacteria will release bodily gases and cause internal bloating. Fermentation, the process of producing alcohol due to bacterial activity, will also occur after the putrefaction stage, which can cause further change to the internal environment of the body. Finally, the tissues lose their original shape and undergo liquefaction (DeBruyn and Hauther 2017).

Postmortem microbial succession-

Postmortem microbiological screening is a newer tool that is being developed to estimate the time since death. By examining certain microbial markers in a deceased person's body, a forensic analyst can precisely determine the time since death. However, the microbial environment varies among individuals. Studies have shown the potential importance of researching the relation of the microbiome to human development, health, and disease (Heimesaat et al. 2012). One of the most defining factors that shape a person's microbial profile is the condition of the immune system. The immune system plays an important role in establishing a person's microbiome population, which ties it to forensic estimations of postmortem interval.

The microbiome is the combination of bacteria, viruses, fungi, and other microbes living in and on the human body. Human-associated microbiomes connect the realms of disease, development, health, and forensics. The microbial environment within the human body can be determined by a variety of factors like diet, substance use, hygiene, etc. Like a fingerprint, each person's microbiome is unique to them the result of individual biology, experiences and behaviors. In death investigation, the decomposition events are driven by postmortem microbial activity. Therefore, microbes serve as important physical evidence in the field of forensics and anthropology connecting an individual to their environment even after death. They can serve as a factor to calculate PMI, trace evidence on a body's surface, provide soil profiles, and locate

hidden graves.

Microbial Forensics-

Before the development of DNA sequencing technologies, the use of microbes in forensic science was limited by difficulty in identifying specific types of microbes in the human body. More recently, however, forensic analysts can use DNA sequencing to characterize the microbial communities and began to explore the pattern of postmortem growth and changes in microbial communities (Metcalf et al. 2017).

Different studies have demonstrated the potential of tracking microbes in forensic science, specifically in helping to calculate more accurate PMI. In 2012, Heimesaat et al. measured the number of bacteria in intestinal and extra-intestinal organs (mesenteric lymph nodes (MLNs), spleen, liver, kidney, and cardiac blood) group at different time points after death. They provided the changes of main intestinal bacteria and observed several findings. First, the number of bacteria Enterococci significantly increased from 1 hour to 72 hours postmortem. Second, the Lactobacilli number increased from 0 to 15 minutes, after 24 hours the loading number of Lactobacilli is are higher than the loading number before 24 hours. Third, the numbers of the obligate anaerobic Gram-negative species increased faster between 24 and 72 hours postmortem than before 1 hour postmortem. Fourth, the bacteria Clostridia first declined until 30 minutes postmortem, and then increased until 6 hours postmortem, a second decrease pattern was observed from 6 hours to 24 hours postmortem; followed by that, the number of Clostridia increased to the maximum at 72 hours postmortem. The cardiac blood samples are tested for bacteria at 72 hours postmortem, and the research team find among all cardiac blood samples, 100% were culture-positive for E. coli, 75% were culture-positive for *Escherichia coli*, and 62.5% were culture-positive Lactobacilli (Heimesaat et al. 2012).

By monitoring changes in microbial composition in a decomposing mouse corpse for 48 days, the research of Metcalf et al. in 2013 showed there are specific bacterial phylum and families that dominate in each stage of decomposition. The trajectories of dominating microbial families in each stage are very similar and predictable. Thus, Metcalf et al. could generate a microbial clock for the first 72 hours after death, which reflects the types of postmortem microbial communities in each stage of decomposition and their changes over time. This microbial clock can be used as a scale to estimate the accurate PMI through the microbial evidence (Metcalf et al. 2013).

In 2016, Burcham et al. identified particular bacterial species that can serve as a biological marker to assist the determination of PMI. By tracking the transmigration of major bacterial species with fluorescently labeled proteins from 1h to 60 days after death, the authors' findings complemented previous research that the abundance of *Staphylococcus aureus* KUB7 and *Clostridium perfringens* WAL 14572 have the power to track the PMI (Burcham et al. 2016).

In 2017, DeBruyn and Hauther did a further screening of the entire bacterial community of the human gut. Despite considerable variation among individuals, the research identified consistent changes in microbial communities over the period of 30 days after death. Their research showed that diversity and richness of bacteria groups changed from early to a late stage of decomposition. The microbial environment including bacteria communities and non-bacterial components in all stages of decomposition were identified through DNA sequencing and were quantified with qPCR. In the early stage of decomposition, the bacterial diversity is relatively high. In contrast, the microbial communities in the late stage of decomposition decreased in their diversity, but the complexity of the major bacteria groups increased. The overall changes in bacterial diversity and complexity can serve as a forensic indicator and demonstrate the power of

microbiology in the field of forensics (DeBruyn and Hauther 2017).

Immune system

Microbial colonization is influenced by the body's immune system, which is responsible for fighting off infectious diseases. In the GI tract, the immune system and the gut microbial community maintain a mutual relationship by regulating the response to pathogens together. (Lambring et al. 2019). However, the role of the immune system as a factor that can impact the postmortem microbial succession estimation has not been closely examined in previous forensic studies. The immune system can shift patterns of microbe growth and succession in a living person's body. The immune system also evolves and changes with microbial community by helping them maintain certain tolerance to antigens that are not very harmful. (Belkaid, 2014) Microbial structural diversities are observed within individuals who are immunocompromised, but the effects of immune disorder on microbial community are still not utilized.

The immune system, as the defense of the human body, includes the physical barriers and immune cells. The fast response of the immune system is controlled by the innate immune cells. Innate immunity can detect an antigen within hours and minutes. After the antigen passed the epithelial barrier of the body chemical mediators recruit innate immune cells. The phagocytic cells, including macrophages, granulocytes, and dendritic cells, detect inflammatory inducers and produce mediators such as cytokines. The mediators can either directly eliminate antigens, or act on other target tissues to induce an immune response. Innate immunity cannot closely discriminate between specific antigens and does not generate long-term immunity. If the innate immune system is not sufficient to control the infection, the adaptive immune response will take effect. In contrast to innate immunity, adaptive immunity takes longer to respond, and cells of adaptive immunity provide long-lasting, antigen-specific immunity. (Janeway et al.) The immune

system therefore is a highly complex system with many contributors, stimuli and regulators, tuned to maintain the integrity of body in its environment and maintain it in homeostasis.

Immune Cells

The cells of the innate immune system travel in the body through blood, lymph, and other body fluids. The phagocytic cells are sensor cells that can detect antigens and initiate immune responses. There are three types of phagocytic cells: macrophages, granulocytes, and dendritic cells. Macrophages have a longer life span and they can produce inflammatory mediators to induce inflammation, which helps recruit other immune cells to the site of infection. Macrophages can also help directly kill the pathogens after the cells are activated. Granulocyte is the collective term for neutrophils, eosinophils, and basophils. Neutrophils are the most abundant type of granulocytes; they can secret enzyme and antimicrobial substances to fight bacterial infection in intracellular vesicles. Neutrophils take care most of the infection in the body. Eosinophils and basophils are less abundant granulocytes involved in the allergic inflammatory process. Similar to neutrophils, eosinophils can also secret enzymes to destroy pathogens. In contrast, they are a little larger than neutrophils, which make them possible to digest large parasites. Natural killer (NK) cells are large lymphocytes that can kill other cells, which made them similar to the B and T cells of adaptive immunity. However, unlike the B cells and T cells, the effect of NK cells is not antigen-specific, and do not produce long-term immunity. (Janeway et al. 2017)

The immune cells that composed the adaptive immunity are B cells and T cells. B cells and T cells all originated in the bone marrow but mature in different sites, B cells in the bone marrow and T cells in the thymus. Both types have receptors to help them specifically recognize

various antigens. B cells recognize and bind to the free antigens in the extracellular spaces of the body, then they secret antibodies. The antibodies can also bind antigens with their antigen recognition sites. The binding neutralizes antigens to prevent them from binding to normal cells, marks them, and lets other phagocytic cells such as macrophages, which circulate in the blood, help destroy them. Instead of directly binding to the antigens in the extracellular space, T cells deal with the body's cells that have antigens present. T cells recognize specific antigens by detecting peptide fragments of antigens on the surface of the infected host cells. Those peptide fragments are delivered to the cell surface by proteins named MHC molecules located in the host cells. (Janeway et al. 2017)

Factors that influence the immune system-

The immune system closely relates to a person's medical history and is linked to major diseases such as type 1 diabetes and colorectal cancer. The immune system can shape the microbial environment within a person's system by their symbiotic relationship (Lambring et al. 2019). Therefore, when microbial succession gains more tractions as an important tool to calculate PMI, forensic scientists need to pay more attention in the relationship of microbes with the immune system. The condition of the immune system before the time of death is important to provide a more accurate PMI estimation.

Previous researchers have provided PMI calculations by analyzing the change in microbiome community after death. However, a forensic analyst still needs to consider the change in the original microbial species present in a body prior to death caused by a shift in the human body environment before death. A known immune profile, or a detailed analyzation of immune response, of a deceased person will give forensic scientists more background information to predict the original microbial species within the body before death; and thus,

forensic scientists can choose the best microbial community as a biological indicator of PMI (Lambring et al. 2019).

Intestinal immune system and microbiome interactions-

Burcham et al. discussed the microbial communities within a person's body can be very dynamic. The bacterial community present at the time of death relies completely on the nutrients and surfaces areas provided by a living body. The body environments and sources are maintained and utilized by the person's immune system. After death, the number of living immune cells within a body starts to deplete. Then, without the regulation of the immune system and blood circulation, the microbial colonization expands and digests the body. By making the whole body a nutrient source, the growth of bacteria is out of the control of immune system. The number of bacteria elevate explosively. The bacteria start to transmigrate from their original places to other organs (Burcham et al. 2016).

In the research of Heimesaat et al., innate and adaptive immune cells were quantified postmortem in the intestines by immunohistochemistry staining. They find that the number of caspase3+ non-viable cells increased significantly after 3 hours postmortem and until 24 hours postmortem. The neutrophilic granulocytes numbers remained unchanged until 3 hours, and increased four times at 12 and 14 hours while the number of T cells start to decrease significantly. The B cell number was halved at 24 hours postmortem, which is not a markable change compare to the rapid decreasing of T cells. Heimesaat et al. claim that the change in the immune system can significantly affect the body environment, and thus alter the microbial succession, result in the stage of putrefaction (Heimesaat et al. 2012).

Flow cytometry is used to identify immune cell types according to their size and phenotype at single cell level and quantify a specific cell type using antibody cell staining as surface marker.

It is highly effective and can deal with a large number of cells and parameters in a short time, which makes flow cytometry a powerful tool for immune cell researches (Pitoiset, 2018). In the current study, flow cytometry was used as a quantitative approach to measure post-mortem immune cell viability. Additionally, multicolor flow cytometry will allow for a comprehensive analysis of immune cell subsets which will expand upon the immunohistology work by Heimesaat, et al, which was limited to T cells, B cells, and neutrophils.

The purpose of my project is to calculate how long immune cell subsets survive postmortem. My hypothesis is that following the death of an individual, immune cells do not die immediately and that immune cell longevity depends on the physiological location of the cell.

Materials and Methods

1. Experimental Design and Sample Collection

A total number of sixteen 8 to 12 -week-old C57BL/6 mice were housed in a vivarium at University of California, Davis is used in this project. All procedures were carried out following protocols approved by the Institutional Animal Care and Use Committee (Protocol #22324 The Role Of The Immune System Post-mortem In Microbial Composition Post-mortem). Mice were are euthanized with carbon dioxide at 0, 3, 24, and 72 hours postmortem, small intestinal lamina propria, spleen, and bone marrow were excised. Four mice were analyzed at each time point, they are stored at room temperature in a hood of Ehrlich Lab at Environmental Toxicology Department, University of California, Davis.

Immune Cell Analysis (Flow Cytometry)

Single cell suspensions were made from the lamina propria, spleen, and bone marrow using previously established methods (Kahalehili et al, 2021, Ehrlich, et al, 2014). Flow cytometry is used to determine the abundance of viable immune cells in the small intestine for each group and time point. The cells were first labeled with viability dye 780, and then stained with fluorescent dye labeled antibodies to identify specific immune cell populations（Pitoiset, et al., 2018）.

The antibodies used to identify immune cell populations are listed in Table I and include markers for CD19 (B cells), CD11b (macrophages), CD45 (pan-leukocyte), CD3 (T cells), CD11c (dendritic), Ly6G (granulocytes), Siglec-F (eosinophils), NK1.1/CD49b (natural killer cells). The bone marrow cells were only stained with the marker CD45 to identify all immune cells prior to differentiation. These markers and corresponding immune cells were evaluated at time point 0-, 3-, 24-, and 72-hours postmortem under flow cytometry to visualize the patterns of immune cell depletion and changes in immune cell populations over time postmortem. Representative flow cytometry plots and gating strategy is shown in Figure 1.

Table 1: List of antibodies used to detect immune cells

Dyes	Concentration (mg/uL)	Markers	Antibody Clone	Cells Represent
APC	0.2	CD19	eBio1D3 (1D3)	B cells
AF700	0.2	CD11b	M1/70	Macrophages
PE-Cy7	0.2	CD45	30-F11	Pan Leukocyte
e450	0.2	CD3	17A2	T cells
BV510	0.2	CD11c	N418	Dendritic
FITC	0.5	Ly6G	Gr-1, RB6-8C5	Granulocytes

Percp710	0.2	Siglec-F	1RNM44N	Eosinophils
PE	0.2	NK1.1/CD49b	NKR-P1C, Ly-55, PK136	Natural Killer (NK) cells

Statistical Analyses

Flow cytometry data was analyzed using Cell Expert Software (Beckman Coulter), and data were plotted in GraphPad Prism. A one-way ANOVA with Tukey's ad hoc test was used for statistical analysis. A p-value of less than .05 was considered statistically significant.

Results

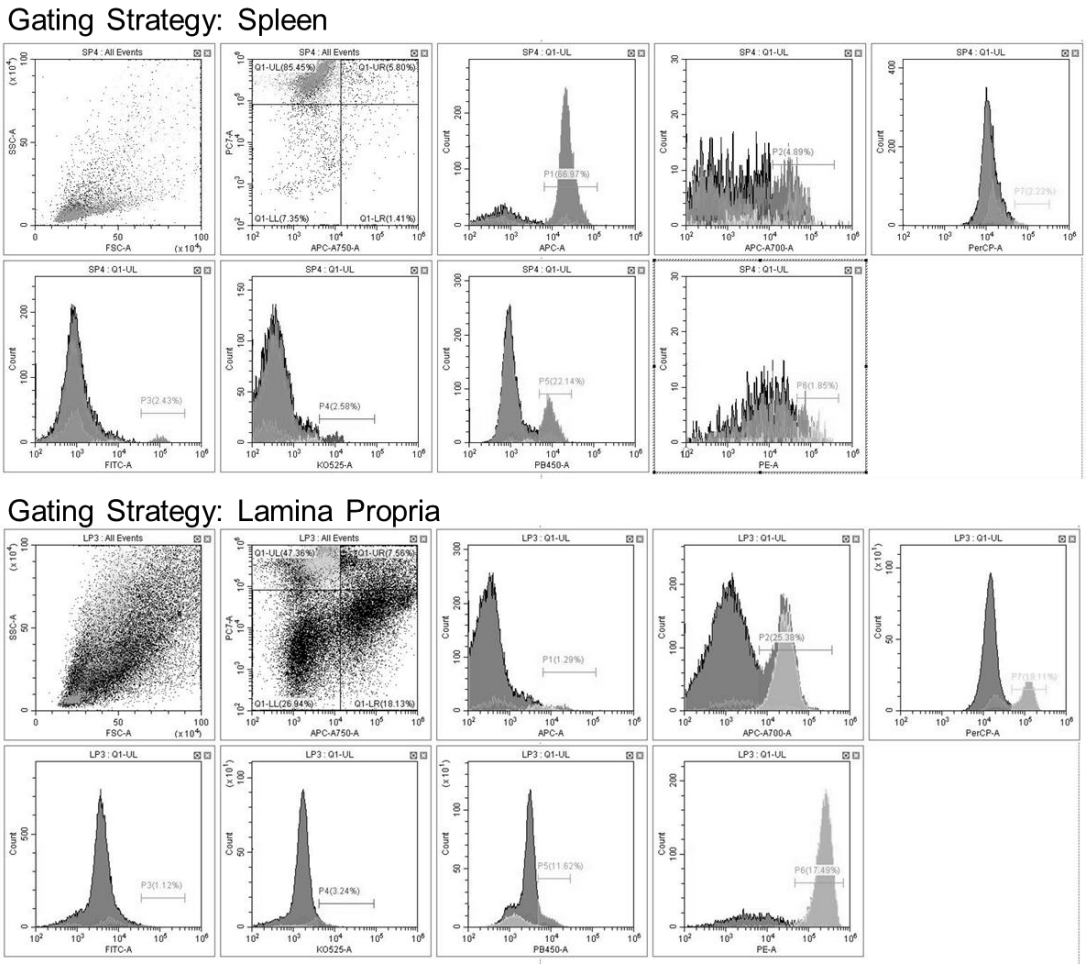

Figure 1: Gating strategy for immune cell analysis. The top plots show an example gating strategy for spleen, the bottom plots show an example gating strategy for lamina propria. X-axis represents florescence intensity, and Y-axis represents cell counts.

Leukocytes remain viable for the first three hours and dramatically die off by 24 hours.

To identify the longevity of immune populations postmortem, cells were isolated from the bone marrow, spleen, and lamina propria at 0, 3, 24, and 72 hours post-mortem. These tissue sites were selected because of their importance related to immune system generation and function. The bone marrow is the primary site where immune cells are generated. The spleen is a secondary lymphoid organ, where blood gets filtered and differentiated leukocytes are abundant.

The lamina propria of the small intestine is where microbes must overtake immune cells to initiate decomposition postmortem.

Cells were then stained for CD45, a pan-leukocyte marker expressed on all white blood cells along with viability dye. In bone marrow, the percentage of viable immune cells remained stable for the first 3 hours, and then decreased to near zero by 24 hours postmortem. In the spleen, the percentage of viable immune cells decreased slightly (about 5%) in the first 3 hours postmortem, and most cells died off at 24 hours. In contrast to the spleen and bone marrow, most immune cells died in the first 3 hours in the lamina propria (Figure 2).

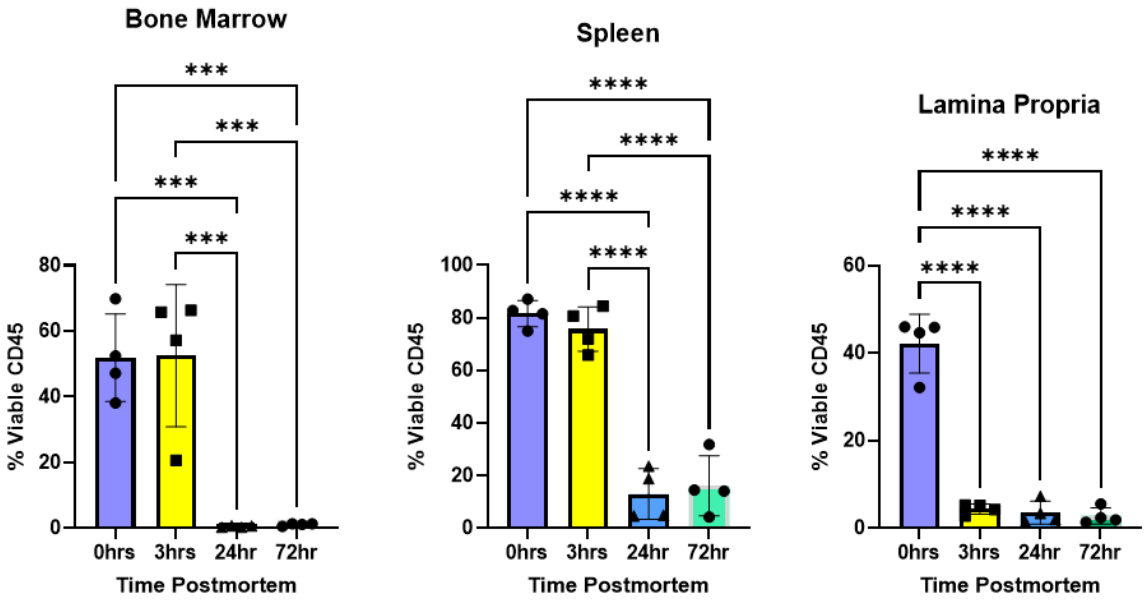

Figure 2: Survival of leukocytes post mortem in lamina propria, spleen, and bone marrow. Cells were stained with viability dye and anti-CD45 and the percentage of viable CD45+ cells is displayed. The proportion of viable leukocyte population was determined at 0, 3, 24 and 72 hours post-mortem. N=4 mice/time point. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

B cells die off rapidly in the spleen, but represent an increasingly large percentage of viable CD45+ cells in the lamina propria.

B cells are lymphocytes that produces antibodies. In the spleen, the percentage of B cells gated on both viable immune cells and total cells showed a rapid decrease from 3 to 24 hours postmortem. In lamina propria, the percentage of viable B cells remained stable over the 72 hour period, and therefore represented an increasingly large fraction of the viable immune cells over time (Figure 3).

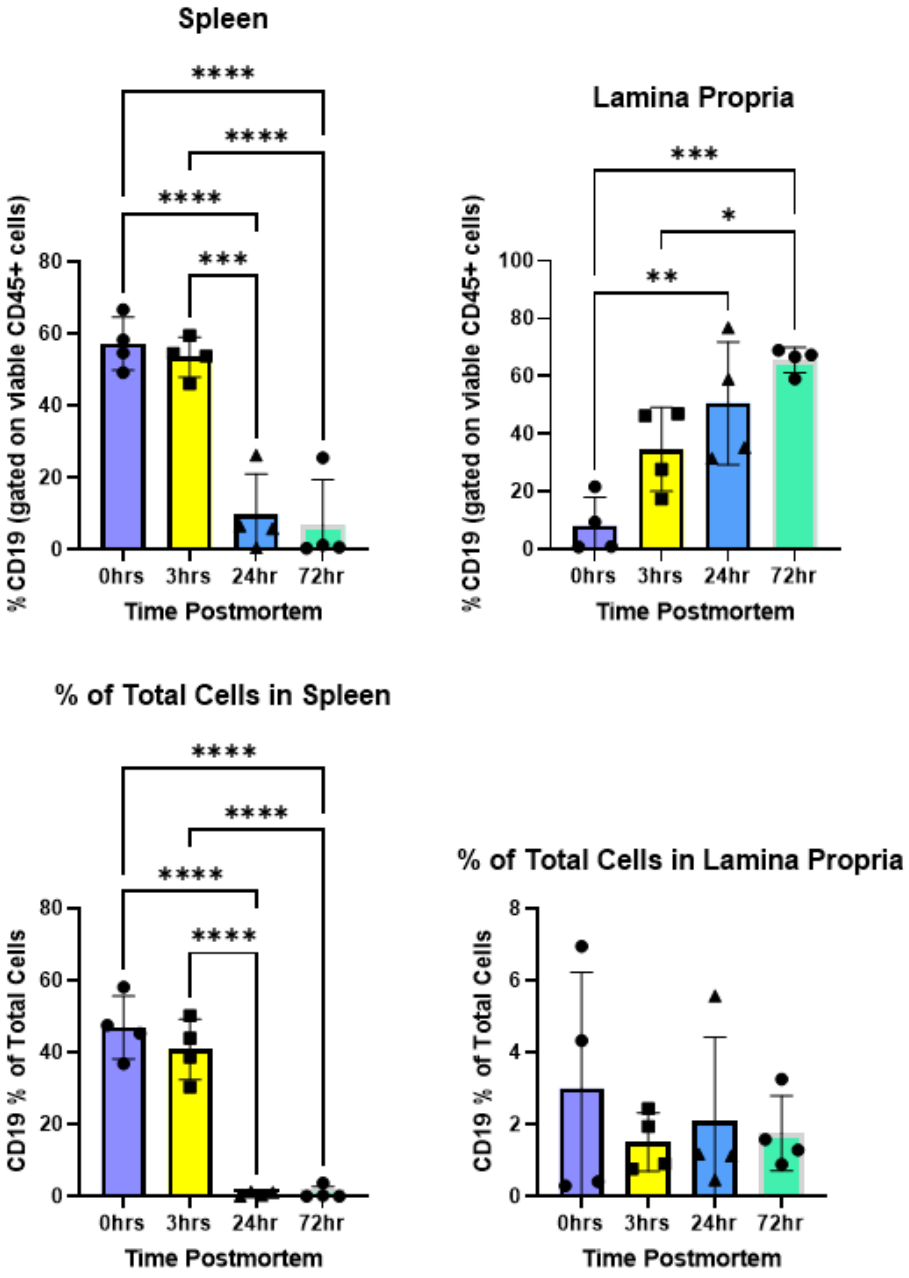

Figure 3: Survival of CD19+ B cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-CD19 the percentage of CD19 + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+CD19+ are displayed in the lower plots. N=4 mice/time point. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

T cell viability reflects the overall viability of immune cells in the spleen and lamina propria.

T cells are lymphocytes that respond to antigen specific stimuli and can either directly kill or aid in the immune responses of other cell types. In both the spleen and the lamina propria, the portion of viable immune cells that were T cells remained relatively stable throughout the 72 hours period, and therefore largely reflected the pattern of overall immune cell death shown in Figure 4.

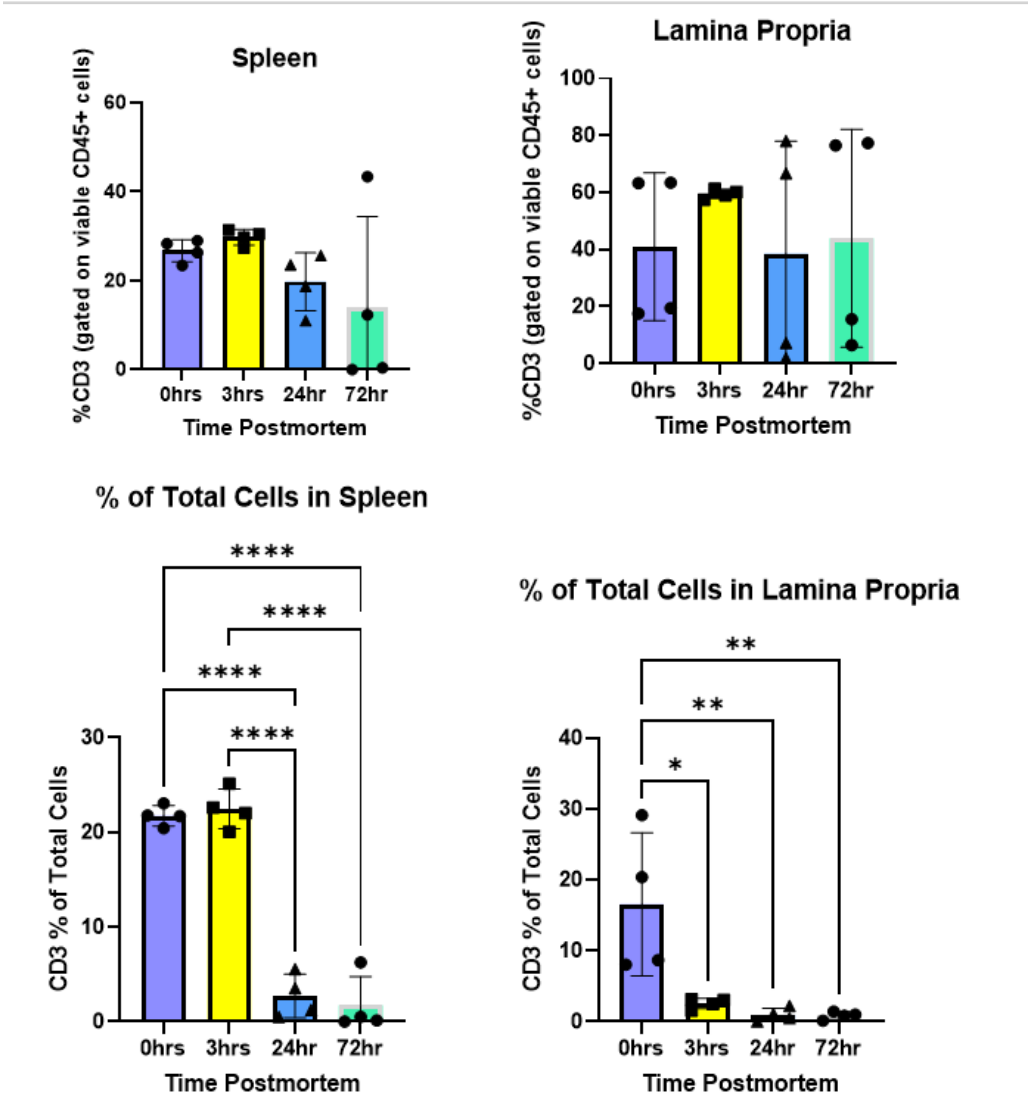

Figure 4: Survival of CD3+ T cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-CD3 the percentage of CD3 + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+CD3+ are displayed in the lower plots. N=4 mice/time point. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

The proportion of macrophages that make of viable immune cells initially increases at 3 hours.

Macrophages are phaogyctic innate immune cells that can produce a variety of inflammatory mediators. In the spleen, the percentage of macrophages in total cells drops rapidly after 3 hours postmortem. In the lamina propria, the percentage of macrophages gated on viable immune cells increased from 0 to 3 hours and remains high at 24 hours post mortem. The percentage of lamina propria viable macrophages in total cells significantly decreased from 0 to 3 hours postmortem and remains about the same amount from 3 to 24 hours (Figure 5).

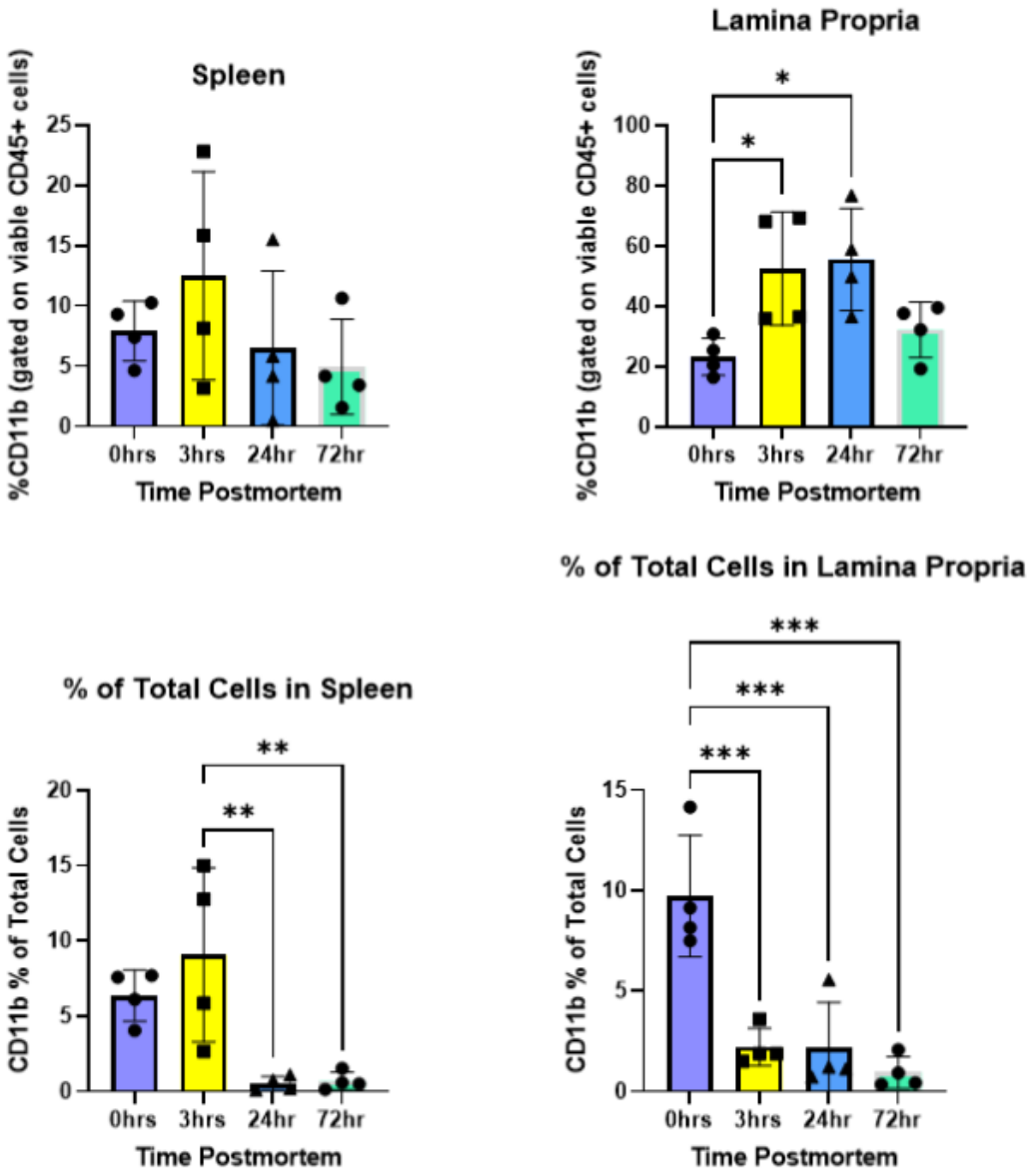

Figure 5: Survival of CD11b+ Macrophages cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-CD11b the percentage of CD11b + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+CD11b+ are displayed in the lower plots. N=4 mice/time point. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Decline of dendritic cell viability is modest over 72 hours

Dendritic cells are phagocytic cells that present antigen to T cells. No significant changes were observed in dendric cells' postmortem longevity. The data are shown in Figure 6.

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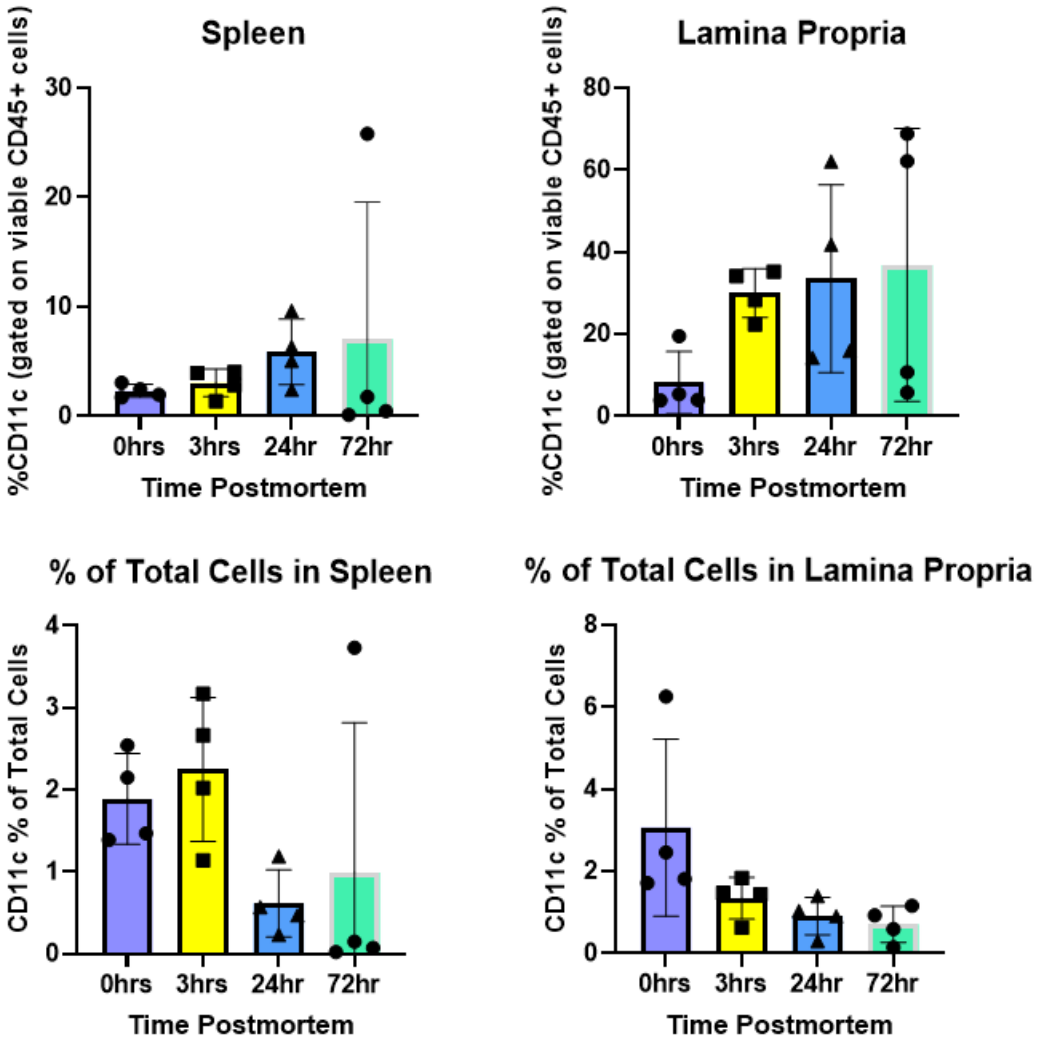

Figure 6: Survival of CD11c+ Dendritic cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-CD11c the percentage of CD11c + cells

gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+CD11c+ are displayed in the lower plots. N=4 mice/time point.

Granulocytes die off after 3 hours postmortem in spleen

Granulocytes are white blood cells, that include neutrophils, eosinophils, and basophils. They circulate with blood and kill pathogens. The number of granulocytes percentage in spleen total cells shows no significant change in the first 3 hours postmortem, but it decreases significantly by the 24-hour time point (Figure 7).

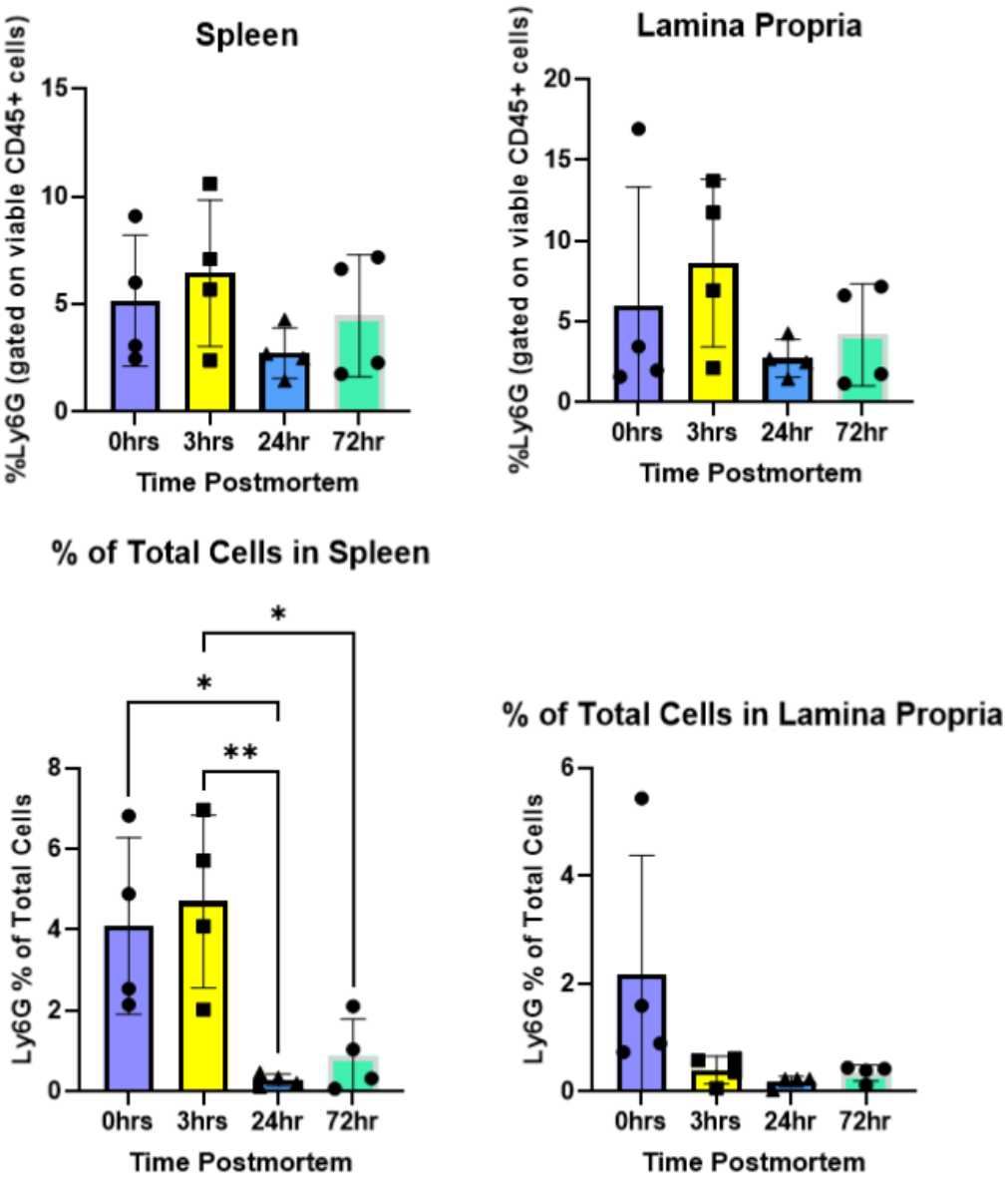

Figure 7: Survival of Ly6G+ Granulocytes post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti- Ly6G the percentage of Ly6G + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+ Ly6G are displayed in the lower plots. N=4 mice/time point. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

Eosinophils percentage of total cells in lamia propria decrease sharply at 3 hours.

Eosinophils are one type of granulitic cell that is involved in the allergic inflammatory process. In the spleen, eosinophils represented a large percentage of the viable cells at 24 and 72 hours, although the data was variable across replicates. Similarly, eosinophils were overrepresented among viable immune cells in the lamina propria post-mortem (Figure 8).

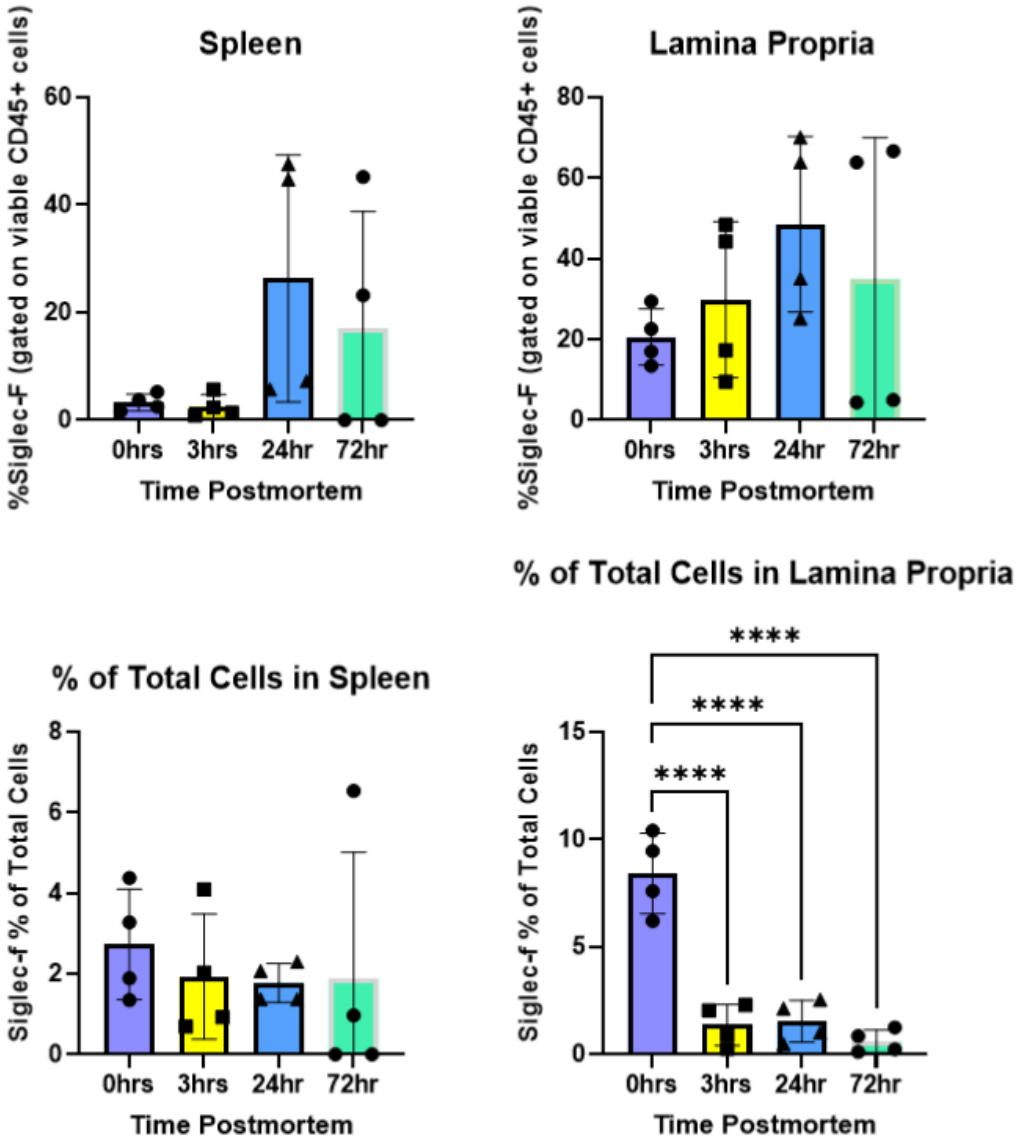

Figure 8: Survival of + Eosinophils cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-Siglec-F the percentage of Siglec-F + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that

are viable CD45+ Siglec-F are displayed in the lower plots. N=4 mice/time point. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Natural Killer (NK) cells percentage of total cells decreases after 3 hours in spleen, but it decreases in the first 3 hours in the lamina propria.

Natural killer (NK) cells are large lymphocytes that can directly fight off infections although their response is not antigen-specific and do not form long-term immunity. In the spleen, NK cells remained viable until after 24 hours post-mortem. In lamina propria, the proportion of viable NK cell was remained relatively stable, and followed a similar pattern of cell death as overall CD45+ immune cells (Figure 9).

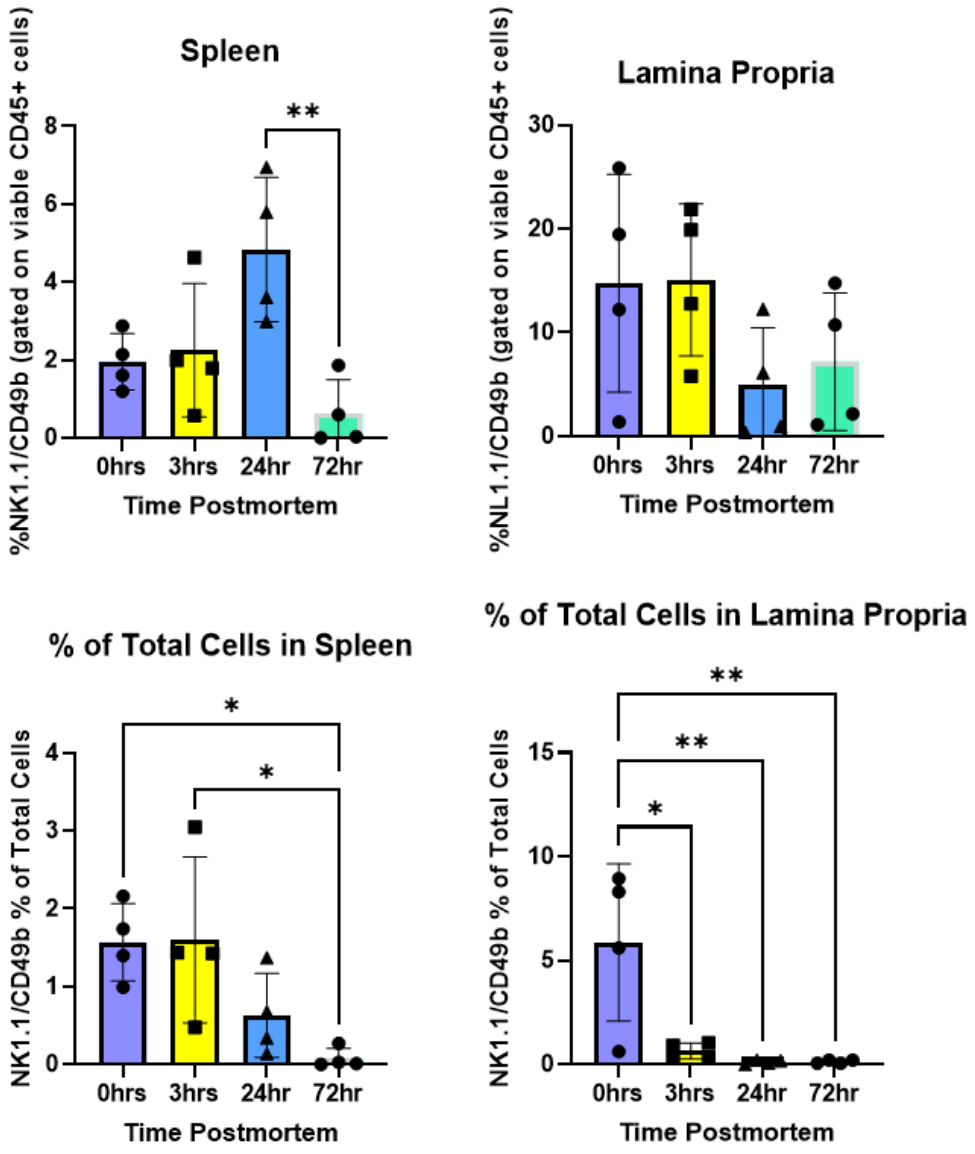

Figure 9: Survival of NK1.1/CD49b+ NK cells post mortem in lamina propria and spleen. Cells were stained with viability dye and anti-CD45 and anti-NK1.1/CD49b the percentage of NK1.1/CD49b + cells gated on viable CD45+ cells are displayed in the upper plots, and percentage of total cells that are viable CD45+ NK1.1/CD49b are displayed in the lower plots. N=4 mice/time point. *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.

Discussion

The overall goal of this research project is to determine how the number and viability of immune cells change postmortem. Since the immune cell activity after death are intrinsically related to microbial succession, identifying how long immune cells survive postmortem, and if there is preferential immune cell longevity can help uncover the events that lead to microbial-driven decomposition overtime.

According to the results of viable CD45+ cells, the immune cells in lamina propria dies out faster than in bone marrow and spleen. Among the cell percentages of total cells, most of the immune cells (B cells, T cells, macrophages cells, dendritic cells, eosinophils cells, and NK cells) in spleen that are alive at 0 hours postmortem start to die off between the period of 3 to 24 hours postmortem, and in lamia propria many immune cells (T cells, macrophages cells, eosinophils cells, and NK cells) dies out during 0 to 3 hours postmortem. Therefore, the immune cells live shorter in lamina propria than in spleen in the postmortem environment. This could relate to the sudden growth of microbiomes in intestines after death.

Of the total cell percentage, T cells dies out fast during the first 3 hours postmortem in lamina propria, while no significant changes in B cells observed in lamina propria. This observation is consistent with the findings of Heimesaat et al. that T cells are more affected by the postmortem conditions than B cells in small intestine. I also found the percentage of B cells of viable immune cells are decreasing in spleen but increasing in lamina propria, which could indicate B cells depletes slower in the postmortem environment than the other types of immune cells in lamina propria.

The percentage of viable immune cells that were macrophages cells increased between 0 to 3 hours, and the number remained high at 24 hours postmortem in lamina propria. NK cells percentage for viable immune cells does not decrease until the 24 to 72 hours period in spleen.

The postmortem longevity pattern of those two cells can be further studied, and they have potential to be used as postmortem biomarkers.

Initially, the goal of my project was to study the role of immune system in decomposition and how it affects the postmortem microbial succession. I performed a preliminary study using two groups of mice at four time points (0, 3, 24, and 72 hours postmortem). Among the two group of mice, one group of C57BL/6 mice were administered intraperitoneal injections with busulfan of 40 mg/kg dose to deplete the immune (following a protocol described in Montecino-Rodriguez et al, 2020). The second group of mice was administered a vehicle control. I expected the busulfan would deplete bone marrow hematopoietic progenitors, and subsequently result in immunodeficiency. Unfortunately, these initial studies determined that peripheral immune cell depletion were unsuccessful. To quantify immune cells, I stained cells from the small intestine with viability dye and antibody biomarkers (as described in the methods). Flow cytometry was used to determine the abundance of viable immune cells in the small intestine for each group and time point. I also collected small intestinal contents for the purpose of identifying microbial populations by qPCR. However, when I analyzed the samples that were supposed to be immunocompromised and compared them to the samples of control mice, I found that busulfan failed to deplete all of the immune cells. It is possible that busulfan treatment did not impact the resident immune cells in the intestine that have a slower rate of turnover (Chen et al., 2021). Furthermore, reduced bloating in the intestine of the busulfan treated mice suggested that busulfan had bactericidal properties, limiting utility for postmortem microbial succession studies. The data presented in this thesis, therefore focused on the immune cell longevity aspect of my project.

However, going forward there are several alternate approaches that could be used to identify how the immune system impacts microbial succession. To further study how postmortem immune cell longevity can impact microbial succession, immune-deficient mice (NOD/SCID mice or irradiated mice) could be used instead of chemical destruction of immune cells. Initial studies could be performed to see how immunodeficiency affect postmortem decomposition followed by studies analyzing microbial succession.

Future studies could also analyze additional time points. If the immune cell viability is measured at additional time points between 3 and 24 hours, the trend of depletion can be more precisely defined the impact of immune cells longevity on the change in intestinal microbial communities could also be compared in future research to see how the immune status affects the microbial postmortem clock. The study could contribute to a more accurate estimation of PMI.

In conclusion, this research project identified the pattern of immune cell longevity in bone marrow, spleen, and lamina propria postmortem and found that most immune cells were depleted during the first 3 hours in the intestine, and between 3 and 24 hours in spleen and bone marrow. The differential ability of immune cells to survive (B cells, macrophages, and eosinophils having the longest longevity), may reflect their ability to adapt to a hypoxic environment. The fact that most immune cells in lamina propria, the site of most interactions of immune cells with microbiomes, dies in the first three hours could be a key factor allowing for microbial translocation and bloating during the early decomposition stage.

Supplemental Figures: Percent of total viable cells

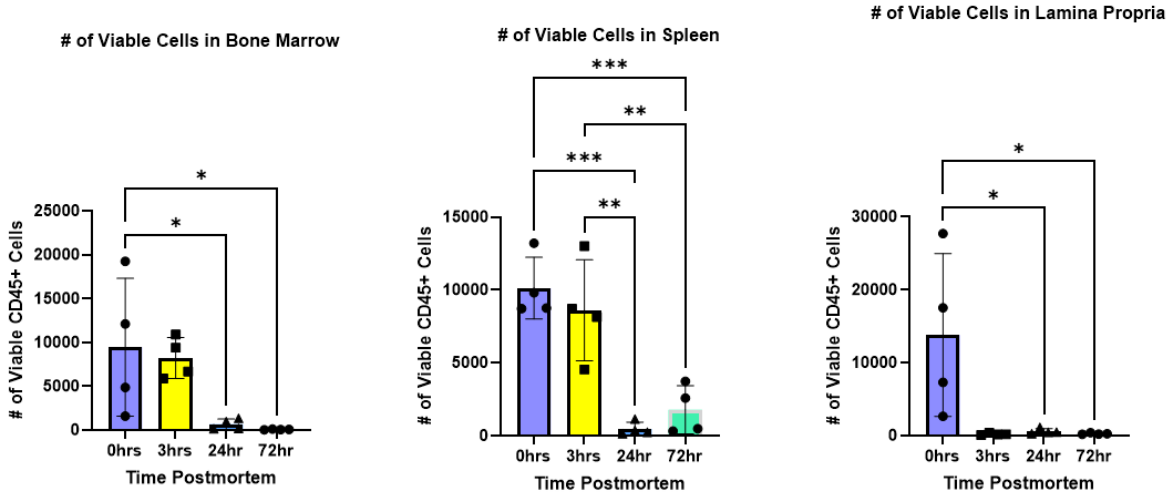

Figure 10: Numbers of viable CD45+ pan-leukocyte cells difference btw % and total number in supplemental section; follow the same pattern as the percentage of viable cell subsets gated out of total cells. Note exceptions to that.

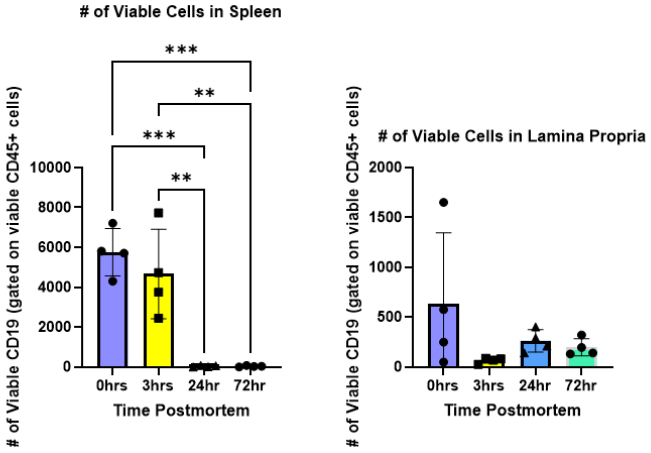

Figure 11: Numbers of Viable CD19+ B Cells

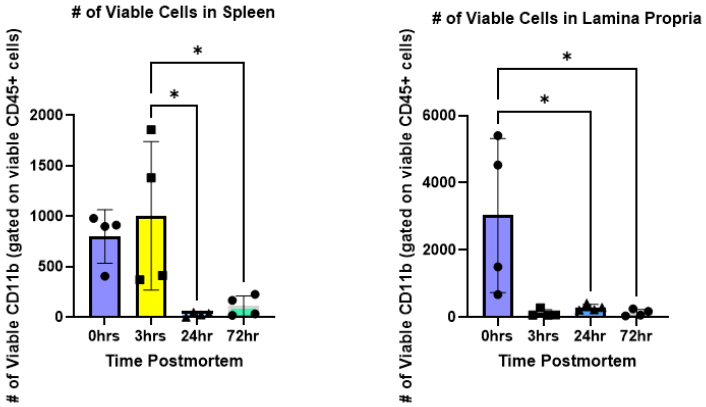

Figure 12: Numbers of Viable CD11b+ Macrophages Cells

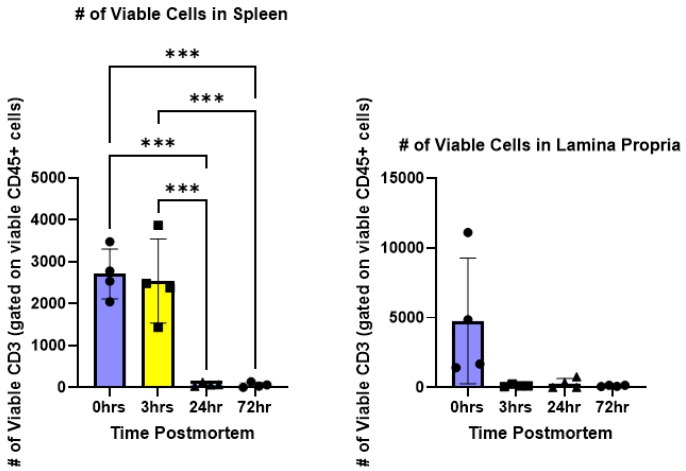

Figure 13: Numbers of Viable CD3+ T Cells

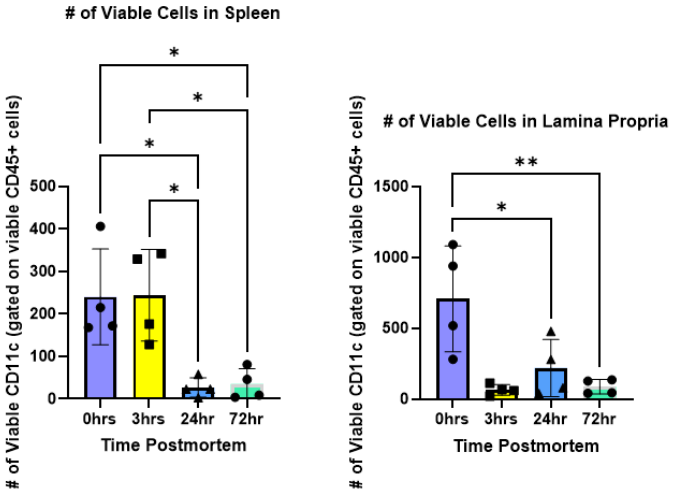

Figure 14: Numbers of Viable CD11c+ Dendritic Cells

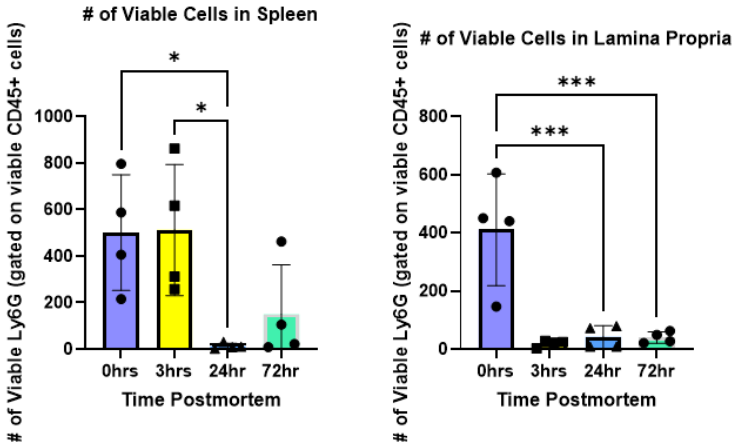

Figure 15: Numbers of Viable Ly6G+ Granulocytes Cells

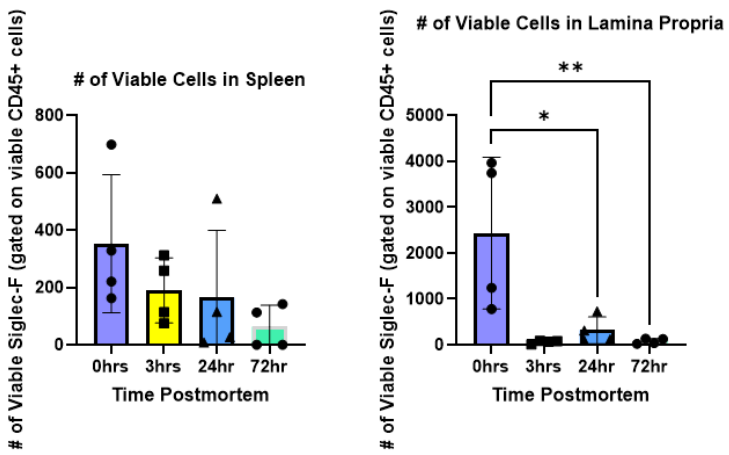

Figure 16: Numbers of Viable Siglec-F+ Eosinophils Cells

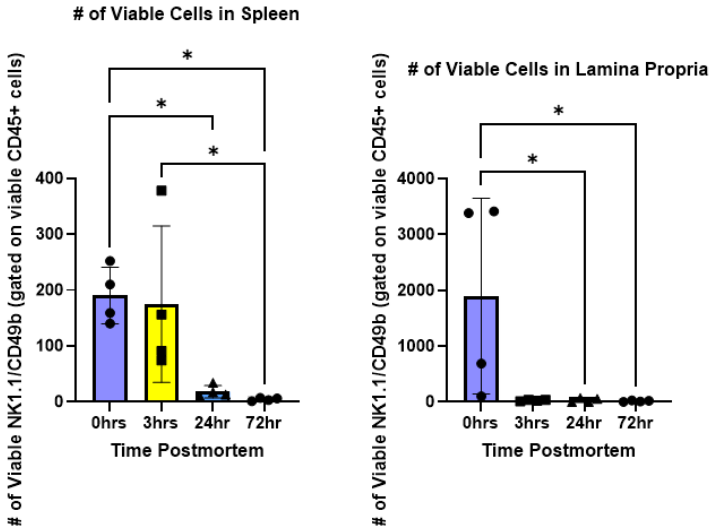

Figure 17: Numbers of Viable NK1.1/CD49b+ NK Cells

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