Research Proposal
in the field of Biology

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I. Research Problem

Neutrophils are an incredibly important component of the innate immune response, and are often the first responders out of the inflammatory cell groups to inflamed sites. However, because of their capacity to secrete pro inflammatory cytokines, neutrophils can promote inflammation related diseases or tissue damage. The complex nature of these systemic interactions can often have severely deleterious results. An example of this is the interactions between neutrophils and red blood cells during Sickle Cell Disease, wherein neutrophils will often adhere to abnormal Red Blood Cells, primarily through the Macrophage-1 integrin, leading to significant blockages and vaso-occlusion. The resultant conditions may be life threatening, and are often the considered the main source of mortality in patients with Sickle Cell Disease. (Lard et al, 1999)(Zhang et al, 2015)

Recent studies into the non homogeneity of neutrophil behavior has yielded insight into the mechanisms through which neutrophils become increasingly functionally active over time, specifically through the activation of Toll-like Receptors and the presence of several microbiome derived molecules, including Peptidoglycan and Lipopolysaccharides (Zhang et al, 2015). (EXPAND HERE)

Relatively little is known about specific bacterial species that may promote the circulation and increased functional activation of neutrophils over time, and through which bacterially derived molecules the mechanism is initiated. It is known that the presentation of Peptidoglycan, which is present in both Gram positive and Gram negative bacterial groups, though significantly more so in the former category, increased aged neutrophil counts and activity in the bloodstream. Despite this foray, specific species and targeted groups have not been investigated and their role in the aging of neutrophils in circulation has yet to be defined.

Toll like receptors 2 and 4 are known to be necessary in the increased activation of neutrophils, and their removal has in turn reduced functional activation over time (Zhang et al, 2015). Moreover, while the administration of wide range antibiotics (ampicillin, streptomycin, metronidazole, and vancomycin), and the corresponding change in aged neutrophil count has been observed, the specific administration of particular antibiotics in order to preserve the commensal bacteria population while reducing overall neutrophil count has yet to be investigated.

Identifying these potential species and their characteristics will provide significant insight into more microbiome sourced mechanisms through which aging neutrophils are regulated, and provides significant opportunity for research into how modulation of the microbiome can result in positive implications for various inflammatory disease outcomes, including Sickle Cell Disease.

Because of the nature of the thesis project and its relatively short timeline, a specific species must be considered and evaluated for its effect on the aging of neutrophils. Segmented Filamentous Bacteria (SFB) provide themselves as an excellent candidate for study, because of their gram positive nature and thick Peptidoglycan walls, as well as their history in prompting immune responses in mice (Ivanov et al, 2010).

Based on the evidence that Peptidoglycan has increased aged neutrophil count in the blood stream, I hypothesize that the presence of Segmented Filamentous Bacteria, a gram positive commensal, will have an increased effect on the aging of neutrophils and their prevalence in the bloodstream.

To show this is possible, I have devised two independent, modular, and potentially parallel research aims. In the first of these, I must establish a positive relationship between the presence of SFB, and the increased functional activity of neutrophils in the bloodstream. Consistent with my hypothesis, I expect to see an increased activation of Toll like receptors when SFB populations are present versus in their absence. The second aim involves the administration of Vancomycin, an antibiotic known for its success against gram positive bacteria, to germ free mice that have had their microflora reinstated with a fecal transplant. Vancomycin has also shown particular success against SFB specifically (Klaasen et al, 1991), Here, I must show that there is a significant reduction in aged neutrophils present in the bloodstream after Vancomycin administration. If successfully established, these aims together validate the possibility that manipulation of the microflora can have a significant impact on the circulation of aged neutrophils, providing foundation for further studies into the subject.

II. Background

Neutrophils and the Innate Immune Response

Neutrophils are an abundant, short lived, highly mobile, type of granulocyte created in the bone marrow. Neutrophil lifespans can be anywhere from ten to ninety hours (Tak et al, 2013), and are normally found in the bloodstream. They are phagocytic, and typically arrive during the acute inflammatory phase, typically in response to factors like environmental stress or bacterial infection, traveling primarily through blood vessels following chemokine signals. If deployed and not presented with any immunological target or challenge, these neutrophils will in turn programmatically die as a result of caspase induced apoptosis within twenty four to forty eight hours (Luo et al, 2008).

The primary mechanisms through which Neutrophils combat extracellular microorganisms are phagocytosis and degranulation. As phagocytes, neutrophils ingest foreign particles, and are capable of doing this to a wide variety of microbes. Degranulation, in turn, allows the neutrophil to secrete various antimicrobial compounds from dedicated vesicles within the cell. Recently, it was also found that neutrophils are capable of expelling what are known as Neutrophil Extracellular Traps, or NETs (Brinkmann et al, 2004). These NETs are strands of DNA that function to trap and kill microbes, through the combination of various components (Urban et al, 2009).

Neutrophil activity, contrary to prior understanding, has been found to be relatively heterogenous in its character, specifically in their respective activation of the Mac-1 integrin. Neutrophils were found to have more inflammatory activity as a product of time, as well as less

expression of cell adhesion molecules that direct them to secondary lymphoid tissues, namely L-Selectin (Zhang et al, 2015)

Sickle Cell Disease and Vaso-Occlusive Crisis

Sickle Cell Disease is a series of blood disorders that leads to misshapen sickle like red blood cells as a result of a difference in the protein responsible for carrying oxygen in the blood, hemoglobin. The disease occurs as a result of the inheritance of irregular genes coding for hemoglobin, and requires inheritance from both parents. Carrying solely one copy of the gene doesn't result in symptoms. There are numerous complications that can result as a consequence of Sickle Cell Disease, even including changes in altitude or temperature. (Stuart et al, 2004)

A significant consequence of Sickle Cell Disease is the Vaso-Occlusive Crisis, which is the result of sickle cell shaped red blood cells that in essence obstruct each other and the capillaries that they are traveling in. This can lead to significant organ damage or necrosis, though the symptoms vary greatly as a result of numerous considerations about the specific situation in which the Vaso-Occlusive Crisis occurred. Vaso-occlusion of the spleen is particularly well known, as are occlusion of the liver, kidneys, and lungs (Anie et al, 2015).

Surprisingly, despite their importance in the innate immune response, neutrophils have been found to exacerbate the problem of Vaso-Occlusive crisis (Lard et al, 1999). Specifically, neutrophils have been found to adhere to sickle shaped red blood cells, contributing to the vaso occlusive crisis significantly, with interactions that sheared blood flow for several seconds (Turhan et al, 2002).

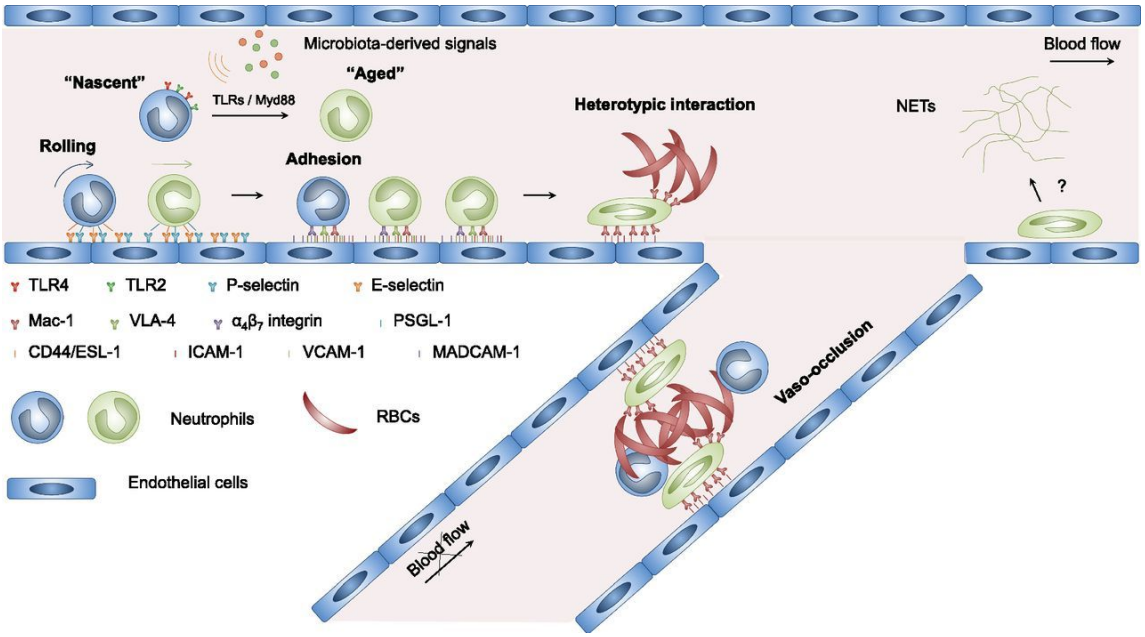

Figure 1: *The Vaso-Occlusion event is the result of several complex interactions between different cell types. Endothelial cells activate and recruit neutrophils, that then roll along the selectin molecules pictured. The activated Mac 1 integrins pictured adhere to the sickle cell shaped red blood cells, resulting in significant blockages. The aged subset of these neutrophils becomes increasingly activated by the signals that are derived from the microbiota, resulting in more Mac 1 integrin activation on the aged green subtype as compared to the nascent group, shown in blue.* (Zhang, 2016)

Segmented Filamentous Bacteria and Vancomycin

Segmented Filamentous Bacteria are known to be present in the gut microbiome of various animals, including fish and more notably, mice. They have been specifically shown to incite various immune responses in mice, including the induction of TH17 cells in the the gut (Ivanov 2010). SFBs are also Gram Positive, indicating they have a thick Peptidoglycan layer making up their outer membrane.

Vancomycin is a specific antibiotic, often used to treat infections of the skin and bone by gram positive bacteria. It has been shown to be particularly effective against SFB, even in lower doses than those which are normally administered (Klaasen et al, 1991). Vancomycin has an interesting method of action, as it works by interfering with cell wall structure and synthesis of Gram Positive Bacteria. However, this same effectiveness is mitigated when Vancomycin is used on Gram negative bacteria, because of differing mechanisms through which the bacteria synthesize their cell walls.

III. Experimental Design

Through the following experiments, I intend to investigate the role of gram positive bacteria on the increased functional activity of neutrophils over time, specifically through the administration and manipulation of SFBs. In addition to this, I will examine whether specifically targeted antibiotics will preserve portions of the microflora while also mediating the total amount of aged neutrophils in the bloodstream.

The rationale behind choosing SFBs lies in the fact that they have been observed to have immunomodulatory effects in various systems. SFBs have already been shown to be able to specifically induce TH17 cell presence in the gut, making it the first known commensal species that can affect the individual's immune fitness (Ivanov et al, 2010). On top of this, SFB are Gram Positive, with significantly thicker Peptidoglycan cell walls than Gram Negative species. Peptidoglycan was the primary ligand used to interact with the Toll Like receptors and increase neutrophil function over time. These details taken together make SFB a prime candidate for manipulation in order to incite neutrophil age related changes.

The rationale behind making use of Vancomycin for the second aim lies in its effectiveness in eradicating Gram positive populations, and also its history of success against SFBs in particular. SFBs was found to be particularly sensitive to the administration of Vancomycin, even at lower levels of administration than what is normally pursued (Klaasen et al, 1991).

The proposed procedures are designed to be run in parallel, making use of resources that are available to be executed upon during the nine month thesis period. Both experiments will make use of Taconic Germ Free mice, because of their accessibility and off the shelf nature. Specifically, the BALB/C breed will be the focus of both research aims, as it is widely used in immunology studies. Control groups will be made up of BALB/C mice as well as wild type mice.

Mice will be in the age range of six to eight months, and all mice will be kept in specific pathogen free conditions in order to maintain control over their exposure, and their food will also be autoclaved. Germ Free Mice will be maintained in sterile conditions with autoclaved food and water in order to maintain germ free conditions for control purposes and the trials themselves.

Aim 1: Establish the Effect of SFB on Aged Neutrophil Activity

This experiment's goal is to determine the role of SFB in modulating aged neutrophil activity. Aged neutrophils have been characterized as different from nascent and TNF-activated neutrophils in their downregulation of CD26L and upregulation of CXCR3 (Zhang, 2015). I will test whether the presence of SFB will confer an increased rate and presence of neutrophil aging. Information resulting from this test can help us determine whether specific bacterial populations are capable of modulating this aspect of neutrophil function.

To determine the information I'm seeking, I will inoculate Germ Free mice with SFB, specifically from SFB-mono mice. To inoculate the Germ Free group, I will collect fecal pellets from SFB-mono mice, and preserve them by freezing until immediately before administration is necessary. After homogenizing the pellets in water, I will administer via gavage to the Germ Free Mice, inoculating them with SFB. I will maintain control groups of Germ Free Mice and Wild Type Mice that are gavaged with water separately. This follows the model set forth by Ivanov and team in their investigation of SFB and TH17 cells (Ivanov, 2009).

After inoculation, SFB will be allowed to colonize for 4 weeks before. Donor neutrophils will be harvested from wild type donor mice, and marked with fluorescent microsphere beads that have been treated for 2 hours in Phosphate Buffered Saline. The beads will bind to Mac-1 integrins that are activated, quantified by the average number of beads bonded to each adherent neutrophil. The whole blood will be transfused to both the SFB colonized Germ Free Mice, as well as their control counterparts and Wild Type individuals. Multi Channel Fluorescent Microscopy will be used to detect and measure these neutrophils, as Mac-1 activation is understood to be representative of increased functional activity over time (Zhang et al, 2015).

Blood will also be drawn from the retro orbital plexus to be examined for leukocyte counts. Flow Cytometry will be used to sort the cells, with aged neutrophils gated by their CD62L-Lo CXCR4-Hi characteristic. Donor neutrophils will be tracked through the blood by their expression of CD45.1 and CD45.2. This allows for us to transfer neutrophils between mice and subsequently identify the donor neutrophils (Mobraaten, 1994). Aged neutrophil counts will be compared between the Germ Free, Germ Free with SFB, and Wild Type mice, in order to determine whether there is an increased rate of neutrophil aging. Mac-1 integrin activation will also be compared.

I expect to see an increased number of aged neutrophils in circulation within the Germ Free and SFB administered group versus the solely Germ Free group. Wild Type will be compared as well, to serve as a control, though it's aged neutrophil count may be higher because of other microbes in its system. I expect that the number will be higher in the SFB administered mice because of the presence of Peptidoglycan, which was shown to activate TLR2 receptors before (Zhang et al, 2015).

Aim 2: Establish the Effect of Vancomycin on Commensal Microbiota and Aged Neutrophil Count

This experiment's goal is to ascertain whether aged neutrophil count in the blood can be lowered while maintaining commensal gut microflora. I will be testing whether administration of a gram positive targeting antibiotic, Vancomycin, to germ free mice inoculated by a fecal transplant will result in an overall lowered aging of neutrophils when compared to those germ free mice that have not been given the antibiotic. The information derived and analyzed from this experiment could potentially give a foundational understanding in the impact and relationship that antibiotic administration has on increased functional activity in neutrophils.

To determine the information I'm seeking in this experiment, I will inoculate Germ Free Mice with microflora population through the administration of wild type mouse feces. 100 mg of mouse feces pellets will be suspended in Phosphate Buffered Saline, and filtered through a strainer in order to later administer the filtrate. I will then administer the filtrate to Germ Free mice via gavage, following the model set forth by Frenette and team (Zhang et al, 2015).

After inoculation, following the same procedure as above, the newly administered microflora will be allowed to propagate and colonize the mice for 4 weeks. There will also be control groups of Germ Free mice that have been colonized with the microflora, those that have not been colonized, as well as Wild Type counterparts. The trial group will then be administered a dose of Vancomycin of 1 gl^{-1}, every 4 weeks in their drinking water.

To ascertain the effect of microbiota depletion as a direct result of Vancomycin administration, I will conduct taxonomic microbiota analysis, using PCR amplification and sequencing of purified rDNA (Woo et al, 2008) (Zhang et al, 2015). The taxonomic results will be compared across the Vancomycin administered group, as well as the two control groups. After administration and analysis of microbiota, I will follow a similar procedure to administer and age neutrophils as was outlined in Aim 1. Donor neutrophils will be harvested from Wild Type Mice counterparts, and marked with micro fluorescent beads to facilitate Multi Channel Fluorescent Microscopy. The intention is to observe the beads adhering to activated Mac-1 integrins, in order to make a judgement as to whether increased functional activity is actually occurring or not.

The Whole blood will then be transfused to both the Germ Free Mice that have been administered with Vancomycin, their antibiotic free counterparts, and also to Wild Type Mice. Blood will then be drawn out of the retro orbital plexus to be examined for neutrophil counts, with the intention of quantifying how many aged neutrophils are present. Flow Cytometry will again be used to sort the cells, with aged neutrophils gated by their CD62L-Lo CXCR4-Hi characteristic. Aged neutrophil counts will again be compared to the Germ Free no antibiotic group and the Wild Type group as well.

In this case, I expect to see a significantly lower number of aged neutrophils in the blood of the Germ Free mice that were successfully administered with Vancomycin, because of Vancomycin potential to deplete SFB and other gram positive bacteria, and the resulting drop in Peptidoglycan. Since Peptidoglycan was the identified ligand for the TLR2 receptors activation, it follows that there should be less functional activation of the donor neutrophils over time.

IV. Data Analysis

Because of the data heavy nature of the potential results of our two aims, it is important to categorize and determine expected behavior and quantitative methods to be used. There won't be any statistical methods used to preset sample size in preparation for the experiments that make up the aims. Data values that will be accounted for in the experiments include Aged Neutrophil count as characterized by both Mac-1 activation via MFIM analysis and neutrophil count via Flow Cytometry. Since both Aims make use of similar procedures, we can expect that data will be processed in a similar manner, with similar outputs through the analyses mentioned above.

To analyze the microbiome data that emerges from the taxonomic analysis, the use of Qiime software and an OTU table can be made, with the family genus percentage derived from the OTU values. BIOM tables would be optimal software platform to analyze this data, making use of python frameworks like Scipy as well.

In regards to statistical methods, we can make use of t-tests to compare two separate groups, i.e. the germ free mice treated with Vancomycin and their untreated brethren. We can also make use of ANOVA tests to compare across groups, using the Anaconda computing toolset.

V. Research Limitations

There are some significant limitations in the proposed experimental procedures.

The first of these is that Vancomycin may in fact deplete some gram negative bacterial populations as well, which could deplete the microbiota present in the gut of the Germ Free mice that were administered with the antibiotic further than is desired in the procedure. For example, other populations of gram positive bacteria may be depleted rather than solely SFB. On top of this, while Vancomycin is not known to interfere extensively with gram negative bacteria as compared to other antibiotics, it still may exert some influence on certain species of gram negative coccus (Geraci, 1977). The effect would be even more significant with other antibiotics that have an effective range across both gram positive and negative bacterial variants, and Vancomycin was intentionally chosen to mitigate this limitation.

Another limitation lies in the effective colonization by multiple microflora species through gavage of fecal filtrate. Understanding the composition of microbiota within the fecal sample is necessarily controlled in some way in most experiments, and must also be controlled here. Collecting fecal samples from a single individual to be gavaged across the trial specimens may mitigate this limitation, or mapping through taxonomic analysis in some way.

Another method to mitigate the limitations that are present with Vancomycin administration is to acquire specific pathogen mice. Mice that are specifically lacking in SFBs

are available as Taconic breeds as well, allowing for easy access and fulfillment within the 9 month period.

VI. References

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