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CD 8 MEMORY CELL LOCALIZATION IN BONE MARROW

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ABSTRACT

Immunological memory is one of the most important attributes of the vertebrate adaptive immune system and is also one of the least understood components. The generation and maintenance of CD8 T memory cells are important processes that provide long term immunity and protection against recurrent infections. These memory cells have been demonstrated to preferentially reside in the bone marrow of mice; however the localization within the bone marrow is poorly understood. Bone marrow is a highly structured organ, and understanding the localization of memory T cells may lead to key insights about what kind of signals are maintaining these cells. All immune cells begin in the bone marrow as hematopoietic stem cells. Some of these stem cells leave the bone marrow and home to the thymus, to develop into naïve T cells. Once presented with antigen in the periphery, the T cell becomes activated. Some cells proliferate into terminal effector cells, fighting off an infection then dying, while others differentiate into long-lived memory T cells. Central memory T cells are characterized by expression of CD45RO, CD62L, and CCR7, at first populating lymph tissue and eventually coming to rest in the bone marrow. These cells also exhibit enhanced ability to clear a recurrent infection. The fine anatomy of murine bone marrow is complex - the spaces in the spongy bone matrix are packed with hematopoietic stem cells and stromal cells. This work proposed in this thesis will attempt to visualize memory T cell localization in the bone marrow through the use of cellular staining followed by confocal microscopy.

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Chapter 1

Introduction

Brief History

Up until the mid 1960s, the role of the thymus and associated lymphocytes was a mystery, and some scientists even went so far as to hypothesize that the organ was vestigial. Of course the thymus is an essential part of immunologic protection and T-cell generation (the "T" derived from thymus). It was a fortuitous exploration of leukemia genesis and the effect of thymectomy that discovered thymectomized mice were much more susceptible to infections and paved the way for further studies of thymus derived lymphocytes[1]. Not to say humanity hasn't actively been taking advantage of immunological memory since the advent of vaccination in 1796, an innovation published by Edward Jenner. Since then, vaccination has successfully eradicated smallpox, a virus that had been the scourge of humanity for centuries. Without immunologic memory generation, vaccines would be rendered useless.

The Immune System

The immune system is the defense system that stands between an organism and a veritable host of pathogens eager to invade and cause disease. It's a system that has to be capable of recognizing and destroying or repelling foreign agents, from viruses, to bacteria, to helminthes, and everything in between. Immune systems have been observed in a wide variety of organisms, although lower animals generally have more rudimentary immunological mechanisms. There are two branches of the immunes system, the innate and the adaptive. Generally, jawed fish and evolutionarily successive vertebrates have both the innate and adaptive systems, while preceding chordates and non-vertebrate animals only exhibit the innate response.[2] These two components work in congress to identify foreign organisms, clear infections, and prevent auto-reactive

immune responses. In addition, the adaptive immune system generates immunological memory, which wards off subsequent disease by rapid clearance of the pathogen.

The Innate Immune System

The innate immune system is the first line of defense against invading pathogens after they've overcome the physical and chemical barriers presented by the epithelia and stomach acid, respectively. It is a swift damage control measure for infections, but also functions as an eventual activator of the adaptive immune system. The innate response is immediate and depends upon complement activation and non-specific recognition of pathogenic agents. Complement is activated by foreign carbohydrates expressed on pathogens, inducing opsonization and inactivation or phagocytosis of the target by the cells of the innate immune system. The innate immune cells are not capable of specific recognition of particular pathogens, but can distinguish self from non-self, and respond accordingly. These cells are macrophages, neutrophils, natural killer cells, basophils, eosinophils, and monocytes[3].

The mechanism by which foreign agents are identified by innate immune cells is simple pattern recognition receptors, which enables them to recognize a broad range of pathogens. The molecules recognized are common among bacteria, viruses, and fungi, but are not found on human cells, avoiding the issue of autoimmunity. This simple recognition is effective, but the innate immune system rarely clears an infection independently of the adaptive immune response. Recognition of complement opsonized pathogens and particles is also an important component of the innate immune system. Pathogen recognition induces cytokine release, which attracts other immune cells to the site of infection, as well as vasodilation. Intracellular pathogens can be controlled via NK cells inducing apoptosis of infected cells. Extracellular pathogens can induce phagocytosis by macrophages and other phagocytic cells, which then digest the pathogen[3].

The activation of the adaptive immune response is accomplished by recognition of antigen in the context of the major histocompatibility complex (MHC). All host cells

constitutively express MHC I, which will exhibit antigens from endogenous pathogens if the cell is infected. Professional antigen presenting cells (APC) express MHCII, which contain antigens from exogenous pathogens the APC has phagocytized. These cells are macrophages and dendritic cells, the latter straddling the line between the innate and adaptive immune system. Once a macrophage digests a pathogen, the molecular fragments (antigen) can be processed and packaged in a major histocompatibility complex, which can be recognized by specific cells of the adaptive immune system, which in turn activates them. Dendritic cells sample the peripheral environment of the body in order to take up and present antigens to adaptive immune cells[4]. The cytokines expressed by innate cells also impact adaptive immune cells profoundly, in a wide variety of ways.

The Adaptive Immune System

The adaptive immune system is much more finely tuned response than its innate predecessor. The cells that mediate the adaptive response, dubbed lymphocytes, are generated in the bone marrow from multipotent hematopoietic stem cells and differentiate into a library of adaptive immune cells. The adaptive immune system depends upon the recognition of antigens associated with specific antigens, which tailors the immunological response to best clear the infection. The initiation of the adaptive response occurs when a pathogen is detected and digested by a dendritic cell, which then migrates to the closest lymph nodes and presents the antigens acquired from the pathogens in the context of MHC I and MHC II. The lymph node is populated by naïve, inactive T-cells and B-cells, and each cell has a MHC receptor that will only bind to the specific antigen-MHC II complex presented by the dendritic cell. Upon binding its specific antigen and coreceptor, also found on APCs, the cell becomes activated and undergoes clonal expansion[5].

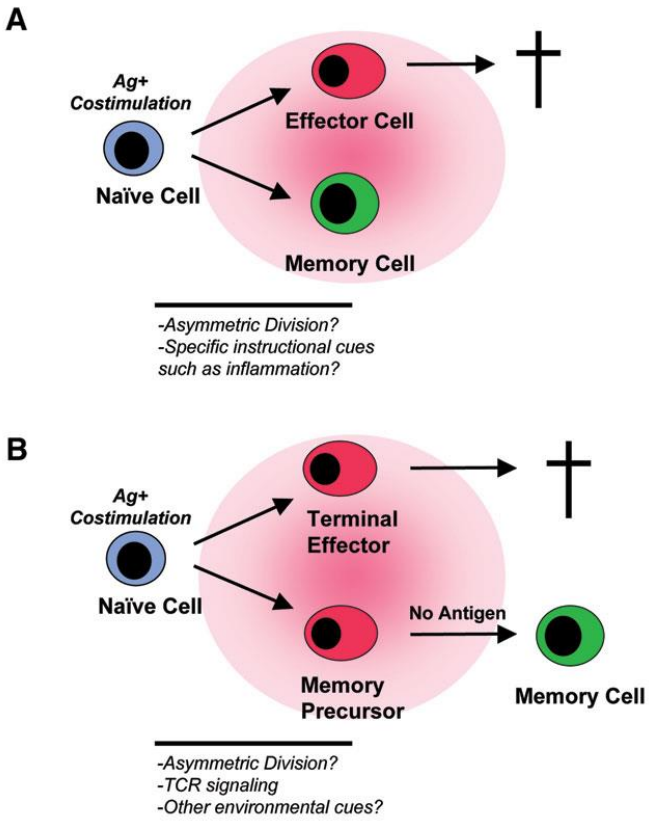
B-cells mature and differentiate into plasma effector cells, remaining in the lymph nodes for the duration of infection and producing antigen specific antibodies. Antibodies opsonize

targets via antigen recognition, which enhances phagocytosis by innate immune cells. B cells are the mediators of humoral immunity.

Effector T-cells

Unlike B-cells, T-cells mature in the thymus, hence the "T". The maturation process in the thymus is important because it eliminates auto-reactive cells, inducing apoptosis if self-antigen is recognized during development. The thymus provides the appropriate environment for differentiation as well. The T cells differentiate into a variety of subtypes, including CD4 and CD8 T-cells. The former express CD4 protein, and are dubbed helper T-cells. These cells enhance the adaptive immune response by contributing to the maturation and class switching process of B cells, an important component of humoral immunity. They also contribute to the activation and proliferation of CD8 T cells. CD8 T-cells produce cytotoxic proteins that directly induce apoptosis of infected cells when triggered by the appropriate antigen in the context of MHC I. The process is tightly controlled, and limits bystander damage. T-cells are the mediators of cellular immunity[6].

Figure 1) Model of Memory Generation Illustration of different hypotheses regarding the point at which memory differentiates and what may prompt the process[7]. (Adapted from "CD8 T-Cell Memory Differentiation during Acute and Chronic Viral Infections." Kalia, et al. Figure 2.)



Memory T cells

Initial infection will induce all of

the maturation processes described above, but only a fraction of the T-cell population generated will endure after the pathogen has been cleared. There are two hypothesized models as to how memory cells differentiate. Most of the cells differentiate into terminal effector cells, but a subset of cells differentiate into long term memory cells via one of two hypothesized models illustrated in figure 1. This memory population is characterized by an increased frequency of antigen specific precursors when compared to a naïve population, a faster response with increased cytotoxic protein generation, and antigen independent homeostatic maintenance. T cell memory is further delineated into central memory and peripheral memory branches. The former is maintained largely in lymphoid tissues, and express CD44, CD62L, and CD127[7]. These cells can differentiate into effector cells upon antigenic stimulation, but possess no effector capabilities themselves. The peripheral memory cells exhibit limited effector capabilities.

Cellular Localization Mechanisms

Chemotactic localization of immune cells is a well-documented phenomenon. T cell maturation involves migration from first the bone marrow to the thymus, then through lymph tissues, until activation homes the cells to the site of infection. This is accomplished via the appropriate expression of membrane bound receptors and their complementary ligands in each respective tissue. Receptor-ligand interaction is essential for the survival of CD 8 cells prior to activation, as well as in integral element of homeostatic maintenance of CD 8 memory cells. The signals that induce central memory CD8 T cell maintenance are IL-15 and IL-7, which interacts with respective receptors on the cell surface and promote survival[8]. Logically, memory cells with associate with tissues that express these signals.

Tissues of the Immune System

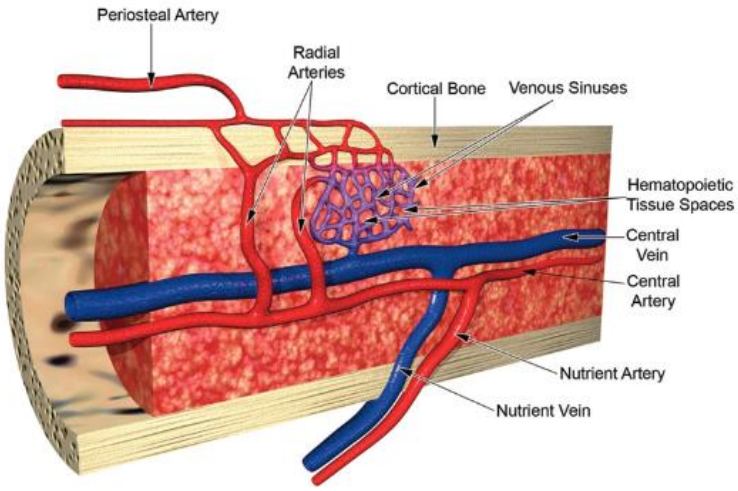
The organs that compose the immune system in most mammals include the spleen, the thymus, the bone marrow, and various lymph nodes throughout the body. The spleen is essentially a very large lymph node. Lymph nodes are involved in active surveillance of blood,

and will activate the adaptive immune system if an infection is detected. The bone marrow is essentially the nursery of all adaptive immune cells, and provides an appropriate environment for T and B cell precursors[3]. The bone is also an important site for memory maintenance, hence the focus of this thesis[9]. The thymus's role in T cell maturation was described above, and is the only tissue not collected and analyzed for this project.

Murine Bone Marrow Anatomy

Normal bone marrow anatomy is a highly compact and structured organ. It is the site of hematopoiesis, and is densely packed with hematopoietic stem cells, as well as megakaryocytes which bud platelets, another essential component of the blood[10]. These cells are lined with venous sinuses that supply blood as well as provide a route for mature cells to enter the blood stream and migrate to the appropriate area of the body. Lymphocytes also employ these sinuses if an infection is detected. Red marrow is responsible for hematopoiesis, which gives it the characteristic color, while yellow marrow is composed of stromal cells that produce fat tissue[11].

Figure 2) Normal murine bone marrow anatomy. The tissue is densely packed, and highly structured. Lymphocytes may aggregate around venous sinuses, an appropriate environment to respond to infection[11]. (Adapted from "Normal Structure, Function, and Histology of the Bone Marrow." Travlos. Figure 5.)



Chapter 2

Materials and Methods

Generation of memory mice

The mice used in this experiment were Thy1.2 C57/Black 6 mice and Thy1.1 P14 mice. Latter are CD8 T cell transgenic mice with an exclusive TCR specificity for the GP33-41 epitope of the Lymphocytic Choriomeningitis Virus (LCMV). The P14 transgenic model allows for the generation of large numbers of memory cells, compared to the endogenous frequency, which is critical for the adoptive transfer experiments proposed in this study. The different Thy 1 marker allow for differentiation of adoptively transferred donor cells from the endogenous recipient cells. The 100,000 CD8 cells were adoptively transferred from P14 to C57 Bl6 mice, which were then challenged with $2x10^5$ PFU LCMV. Memory formation typically occurred ~30 days post infection. Spleen, inguinal lymph nodes, and femur bones were collected from three mice in total, one memory mouse, one naïve mouse, and one mouse described in the following section regarding CMTMR and CFSE transfer.

Flow cytometry

Flow cytometry operates on the principle of fluorescently labeling cells with markers indicative of the maturity and function of individual cells in a population. Cells are counted and identified by individual cell passage through a laser and measuring the subsequent fluorescent emission. By analyzing blood samples, immune characteristics of individual mice can be extrapolated from the cell populations detected. Cell populations indicative of immune memory express CD127 hi, CD44 hi, CD2L hi, and KLRG1 lo. The stains used were for the markers CD8, CD127, CD44, Thy1.1, Thy1.2, and KLRG1.

Column Purification and CMTMR and CFSE Transfer

Negative CD8 T cell column purification was used to purify, isolate, and transfer CD8 T-cells from a naïve P14 mouse (Thy 1.2/Thy1.1) and two P14 chimeric memory mice (Thy 1.1/Thy 1.1). The process is summarized in figure 3.

The first step of this process was sacrificing the three mice and collecting their spleens. The memory mice spleens were pooled and processed together. The spleens were homogenized by mashing them between two glass slides then incubated for 1 minute with 1 ml .83% NH_4Cl in order to lyse the red blood cells. The cells were then resuspended in 14 ml RPMI + 1% Fetal Bovine Serum (RPMI 1%) and spun down at 1200 rpm for 10 minutes at 4^oC. The cells were then resuspended in 2 ml RPMI 1%. The splenocytes were counted to determine concentration and a 200 ul sample was collected at this point to perform a precolumn stain for purity comparison. The cells were then filtered, spun down again, and resuspended in 2% FBS in PBS. The volumes were dependent upon cell concentrations, with a desired final concentration of 100 x 10^6 cell/ml. The memory cell solution was 1.415 ml and the naïve solution was .530 ml. The volumes were then transferred to two separate 5 ml tubes for the negative selection process, which was accomplished with the use of an EasySep CD8 negative selection kit[12]. 50ul/ml of Normal Rat Serum was added to both tubes. Kit provided 50ul/ml CD8 enrichment cocktail #1 was then added, and incubated for 30 minutes at 4^oC. After incubation, the tubes were filled with 2% FBS in PBS and spun down at 1200 rpm for 10 minutes at 4^oC then resuspended to 100 x 10^6 cell/ml. Kit provided cocktail#2 was added at 100ul/ml and incubated for 15 minutes at 4^oC. This binds to any cells other than CD8 T cells, prepping them to bind to the ferromagnetic nanobeads. After vortexing well, the kit provided nanomagnetic beads were added at 100ul/ml, binding to all non CD8 T cells. No vortexing was done after addition of the beads to prevent cell loss. The 5 ml FACS tubes were then filled with 2% FBS in PBS to make both total volumes 2.5 ml. The tubes were then placed in prechilled magnets and incubated for 5 minutes on ice. During incubation, any cells bound to the ferromagnetic beads are posited on the walls of the tube, leaving only CD8 T cells remaining in

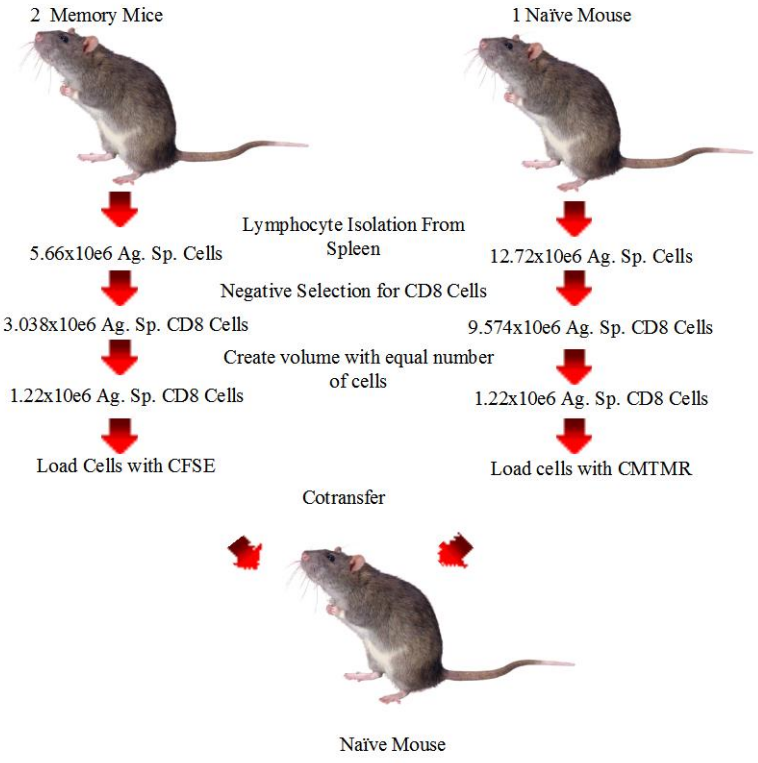
solution. After incubation, the solutions were decanted into separate 15 ml tubes. The magnetic incubation was repeated twice more with fresh 2.5 ml 2% FBS in PBS volumes in order to completely wash the beads of any residual CD8 cells. 50ul of solution from both the naïve and memory tubes were taken at this point for post-column purity comparison. The purity comparison can be found in Figure 5 in the result section.

Once isolated, the naïve and memory CD8 T cells were incubated with CMTMR and CFSE, respectively. These amine-reactive dyes are membrane permeable and bind to various amine residues on intracellular proteins with minimal toxicity to the cells, allowing the cells to be tracked in vivo. The loading was accomplished by first diluting CFSE or CMTMR stock to a 1:500 concentration in RMPI. Then a 50:50 dye solution to cell solution was mixed and incubated for 7 minutes on ice under aluminum foil to prevent photobleaching. $1/10^{th}$ volume FBS was then added to the volume, and incubated for an additional three minutes. The solution was then spun down at 1000 rpm for 1 minute and washed with RPMI + 10% FBS and spun down again at the same speed and time. The cells were counted in between spins and finally resuspended at 8 $x10^6$ cells/ml. An equal volume of both the CMTMR and CFSE loaded cells were then mixed. The final cell solution was transferred intravenously to a naïve Thy 1.2/Thy 1.2 homozygous C57/Bl6 mouse and the cells were incubated in the mouse for 48 hours. The mouse was sacrificed and the spleen, inguinal lymph nodes, and femurs were collected.

Embedding and Cross Sectioning of Soft Tissues

The soft tissues collected and embedded for this project were spleens and inguinal lymph nodes. Once extracted from the mouse, it was a simple process of placing the tissues in OCT medium liquid medium in Cryostat cassettes, then storing them at -80°C until solidified. Once solidified, the tissues were cross-sectioned using the Shandon Cryostat Microtome. Cross sections were 10 nm thick. The samples were collected on charged electronegative slides and stored at -80°C[13].

Figure 3) Negative Selection for CD8 T cells followed by loading cells with CMTMR and CFSE, and adoptive transfer to a recipient mouse. Each step of the process whittled down the lymphocytes in the solution, increasing purity of CD8 cells. An equal number of CMTMR and CFSE labeled cells were transferred to the recipient mouse. The cell numbers were determined by performing cell counts in between each step, and purity of CD8 cell concentration was determined via Flow cytometry preceding loading the cells with CFSE or CMTMR. Volumes for various reagents were determined based on cell concentration.



Paraffin Embedding and Cross Sectioning of Femurs

The processing of femurs was more complicated due to the inability to cross section hard tissue directly. Each femur was fixed in 4% PFA for three days, and then decalcified in 0.5M EDTA solution for eleven days, until the bone was bendable without brittleness. The bone was then dehydrated and paraffinized using the Shandon Citadel 2000 Paraffin Infiltration unit, using the animal tissue settings (program C). Once paraffinized, the femurs were embedded in the center of paraffin cassettes using a Shandon Histocenter II embedding unit. They were then cross sectioned using a Shandon Finesse Paraffin Microtome. The cross sections were 10 nm thick and were collected on charged electronegative slides. The samples were then rehydrated using a Shandon Gemini Varistainer set to the immunohistochemistry program, and stored at -80°C.

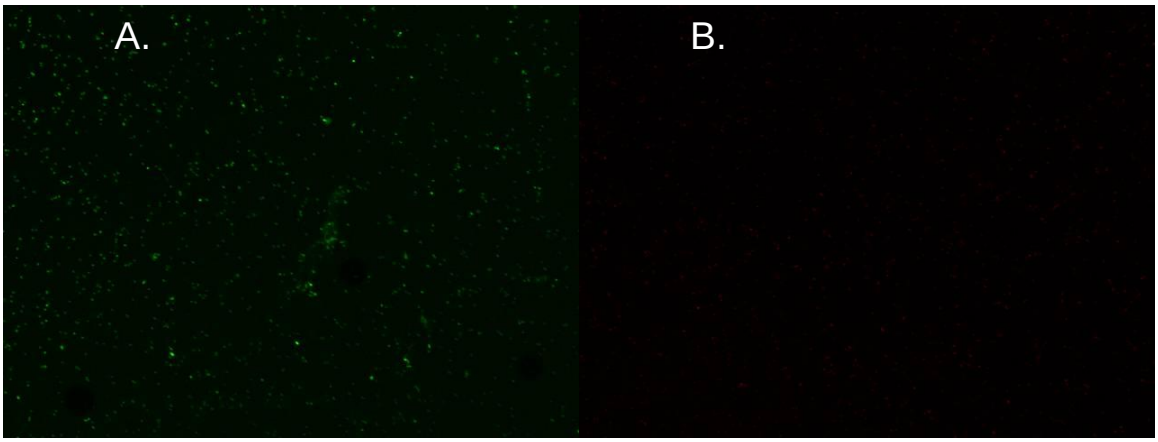
Section Staining

The cryostat slides were fixed in acetone for ten minutes, and allowed to dry. Blocking was accomplished through the incubation of the samples with 200 µl (100 µl for lymph nodes) 5% Normal Mouse Serum (NMS) for 20 minutes in the dark, followed by a 200 µl 1XPBS wash. The samples were then incubated for 10 minutes in the dark at room temperature with 2 drops of reagent A (strepavidin), followed by four PBS washes. Another 10 minute incubation with 2 drops of reagent B (biotin) followed by four PBS washes. The preceding two steps were done using the Invitrogen Avidin/Biotin Blocking Kit[14], and concluded the blocking steps of the staining process, which prevents non-specific binding of antibodies. The femur cross sections were incubated with a 50% trypsin solution for 15 minutes at 38^{o} C, an antigen retrieval step necessary for paraffinized samples. After that, the surface tension of the samples was reduced via incubation with TBS and .025% 100x triton solution for two 5 minute intervals. Blocking was accomplished via a 2 hour incubation in 10% NMS and 1% BSA TBS solution. Incubation with antibodies for both soft tissue and bone cross sections were an hour, at 1:50 and 1:25 concentration for CD8 Brilliant Violet 421 and Thy1.1 Alexa 488, respectively. Blanks were also created to serve as a control to calibrate the microscope setting.

Confocal Microscopy Analysis

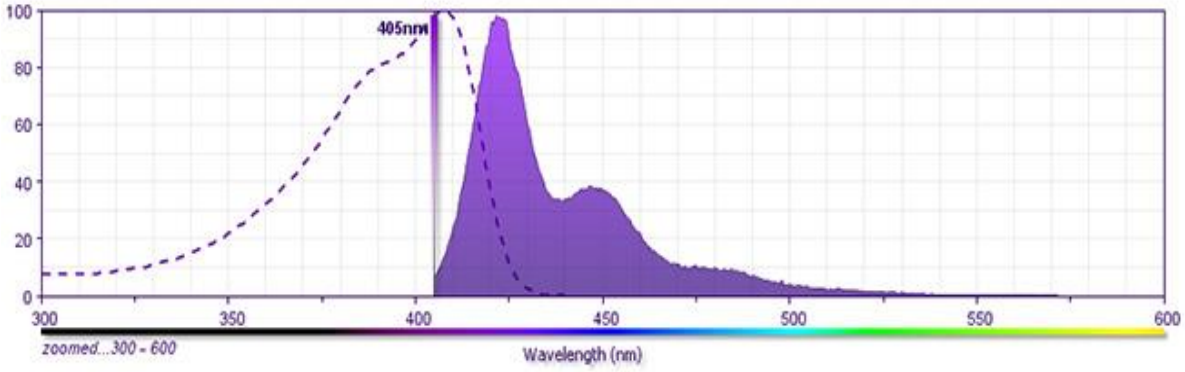
Confocal microscopy operates on the same principle as Flow cytometry, but instead of cell counting, the fluorescence is recorded via a microscope and provides images of the labeled cells within the context of tissue instead of population percentages. Different colored lasers are used to excite the labeled cells and the resultant emissions are captured and translated into images. There were two setting used in this project. Each time images were collected, the laser setting were adjusted to minimize autofluorescence. Positive controls were used to ascertain that the stains were working, and blank slides were used to control for autofluorescence.

Figure 4) Positive controls for CMTMR and CFSE. The controls were made using the CMTMR and CFSE incubation procedure detailed in the materials and methods section applied to a cell smear. The levels of fluorescence in these samples should be much more concentrated than those observed in the tissue.



The stains used directly on the cross sections were Alexa 488 Thy1.1 conjugate and a Brilliant Violet CD8 conjugate. The blue and violet laser channels used were A488 and DAPI, respectively. There was not a specific BV421 channel, but DAPI has been demonstrated to excite the fluorochrome. BV421 excitation and emission values are illustrated in figure 4. There is little to no overlap between the stains, so they should not interfere with one another with regard to signal detection. CFSE and CMTMR also exhibit no overlap in excitation and emission.

Figure 5) Emission and Excitation Spectra for Brilliant Violet 421. Excitation with the violet laser excites the fluorochrome, with peak excitation at 405 nm represented by the dashed line. The solidly filled line is the emission spectra that results from excitation, which is measured by the confocal microscope[15]. (Adapted from BD Bioscience Multicolor Flow Cytometry Spectrum guide[16].)



For the CFSE and CMTMR images, there lasers used were blue and green for the respective dyes. For both sets of image collection an additional transmitted light image in order to provide context for where in the tissue fluorescence was detected. All images were collected at 10x or 40x in order to attempt to capture the structure of the tissue as a whole. All stains and associated excitation/emission values can be found in Table 1.

Table 1) Stains used to visualize cells in tissues. The Thy1.1 and CFSE stains overlap and the same laser was used to excite both, but since they were never applied to the same sample it did not impact the image acquisition process. Otherwise none of the stains overlap in excitation or emission spectra.

Fluorochrome	**Conjugate**	**Excitation**	**Emission**
BV421	**CD8**	**405 nm**	**421 nm**
A488	**Thy1.1**	**495 nm**	**519 nm**
CFSE	**N/A**	**494 nm**	**521 nm**
CMTMR	**N/A**	**541 nm**	**565 nm**

Chapter 3

Results

Flow Cytometry Data

Figure 6) Flow Cytometry to Estimate the Purity of Naïve and Memory cells following Negative Selection Procedure. Below are pre negative selection column and post negative selection column comparison for both the naïve (A) and memory cells (B). More cells were collected from the memory mice spleens, but CD8 T cells made up a higher percentage of the naïve mouse cell population, exhibited by the pre-column peaks. The density of points reflects the relative amount of cells collected from the spleens, with the naïve cell sample having a noticeably higher purified CD8 cell count. The percentage of CD8 T cells in the naïve solution went from 24.2% pre purification to 95% post purification. In the memory cell solution, the CD8 percentage went from 4.08% pre purification to 88.3% post purification. Negative selection purification is generally considered successful if it is around 90% and for the purposes of this project, 88.3% was an acceptable purification.

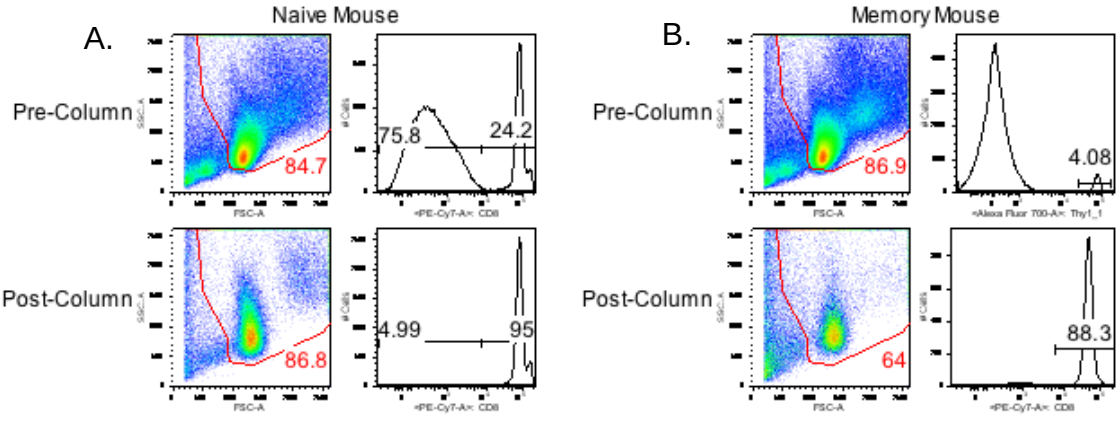
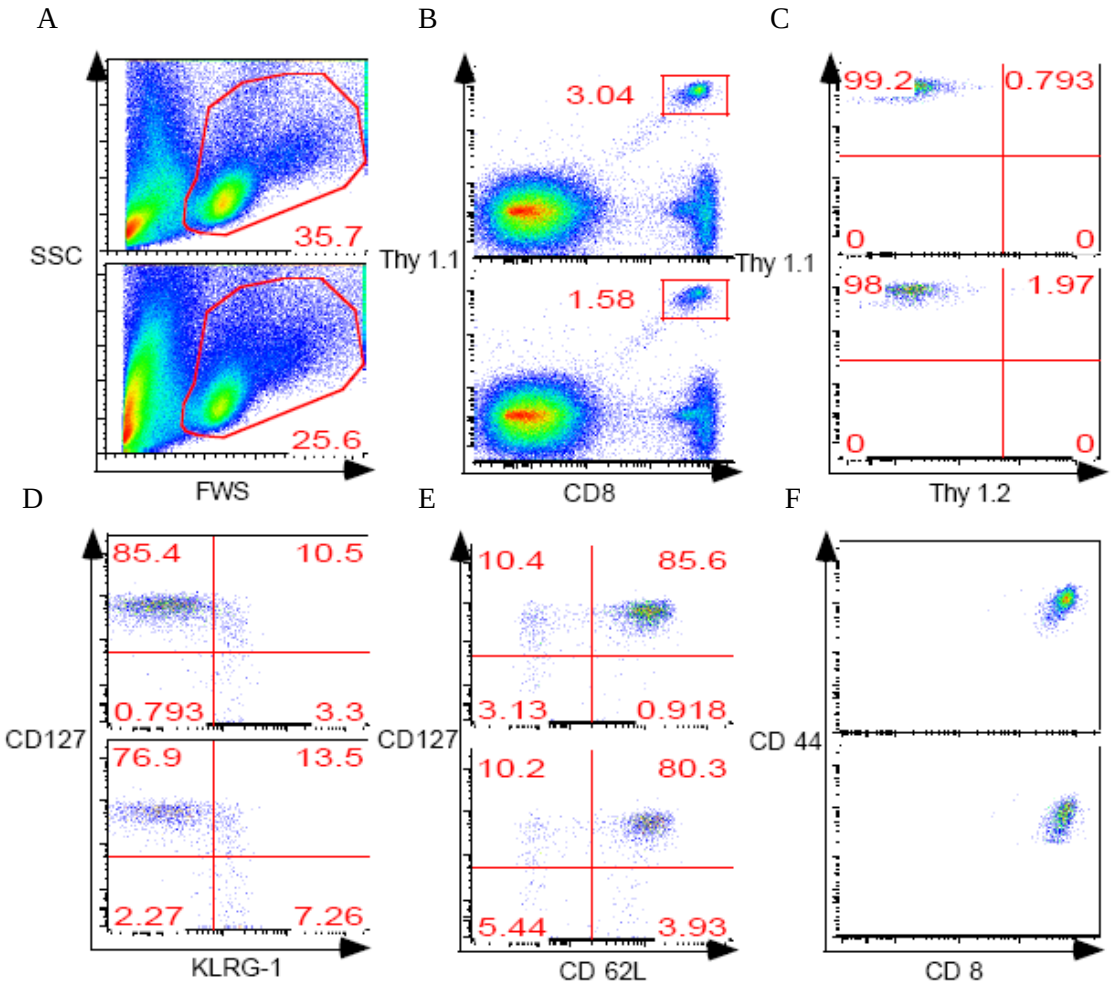


Figure 7) Flow Cytometry to Analyze the Phenotype of Memory Cells from the Two Donor Mice. Graph A represents the live cells detected by the forward scatter and side scatter. Those cells were gated to produce graph B which represents the Thy1.1 and CD8 double positive cells, which are the CD8 cells derived from the P14 mouse as indicated by the Thy1.1 marker. Those cells were then gated to produce graph C which reconfirms the Thy1.1 P14 derived cells and identifies them as Thy1.2 negative. The cells gated in graph B were analyzed again to produce graph D which represents the CD127 positive and KLRG1 negative cells, both hallmarks of memory cells. The cells gated in graph B were analyzed again to produce graph E which represents the CD127 and CD62L double positive cells, also hallmarks of memory cells. Graph F was also created using the gated population from graph B and represents the CD8 and CD44 double positive cells, confirming that the cells are indeed antigen-experienced memory cells.



Spleen Memory CD8 Localization

Figure 8) Confocal Images of CFSE and CMTMR Loaded Spleen Sections. A and B are CFSE and CMTMR images of the same section, as are C and D. The brighter spots in the CFSE images are CD8 cells loaded with CFSE. Both fields were collected near the edge of the spleen. The images collected of the CMTMR dye were very dim, and there did not seem to be any signal independent of the CFSE signals. All images were collected at a magnification of 10x.

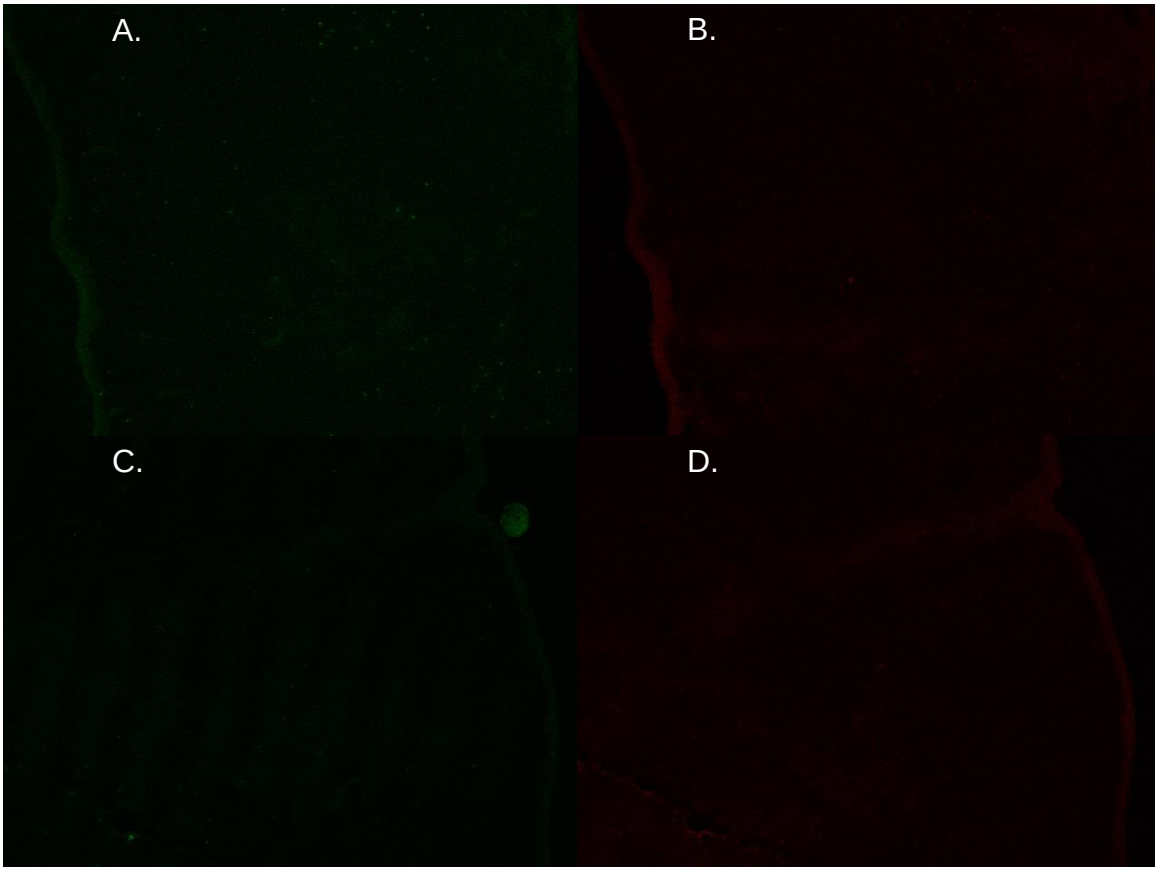
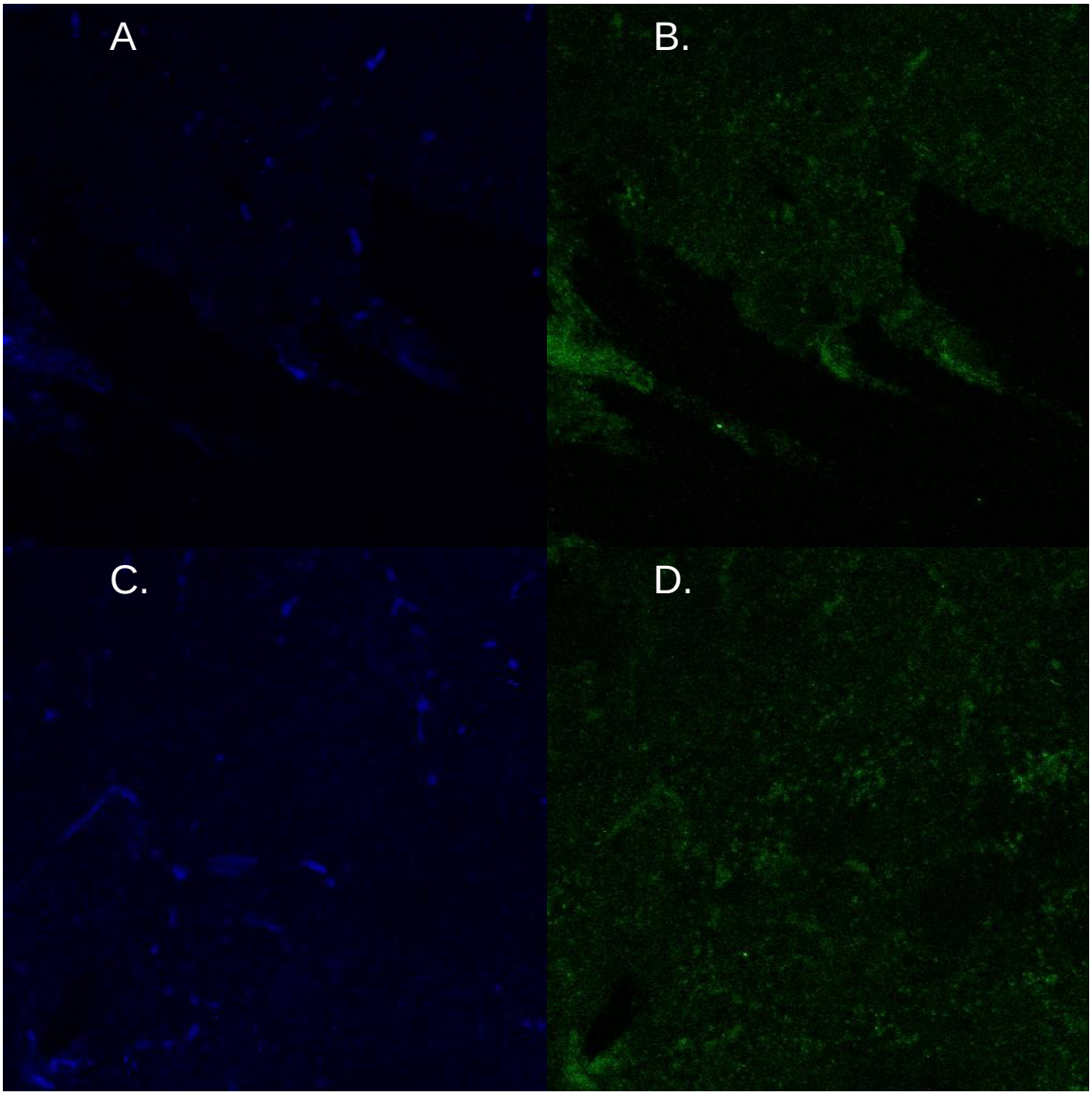


Figure 9) Confocal Images of Stained Spleen Sections. The sections were from different memory mice. A and C are CD8 stained and B and D are Thy1.1 stained. The vacuolation in the bottom left corner of B and D may be an artery or vein. Side by side images are from the same field of tissue, with CD8 on the left column and Thy1.1 on the right column. All images were collected at magnification of 10x. The Mouse that the sections were taken from was a memory mouse.



Memory CD8 Localization in Bone Marrow

Figure 10) Confocal Image of CFSE and CMTMR Loaded Bone Marrow Section. Side by side images were collected from the same field of tissue, with CFSE on the left column and CMTMR on the right column. A and B were collected at a magnification of 10x and C-F were collected at a magnification of 40x. The higher the magnification, the more signal is captured, so the 40x images are brighter. There appears to be aggregation of CFSE loaded CD8 T cells from the memory mouse around venous sinus structures in images C and E. The CMTMR images did not detect fluorescence of the naïve CD8 cells.

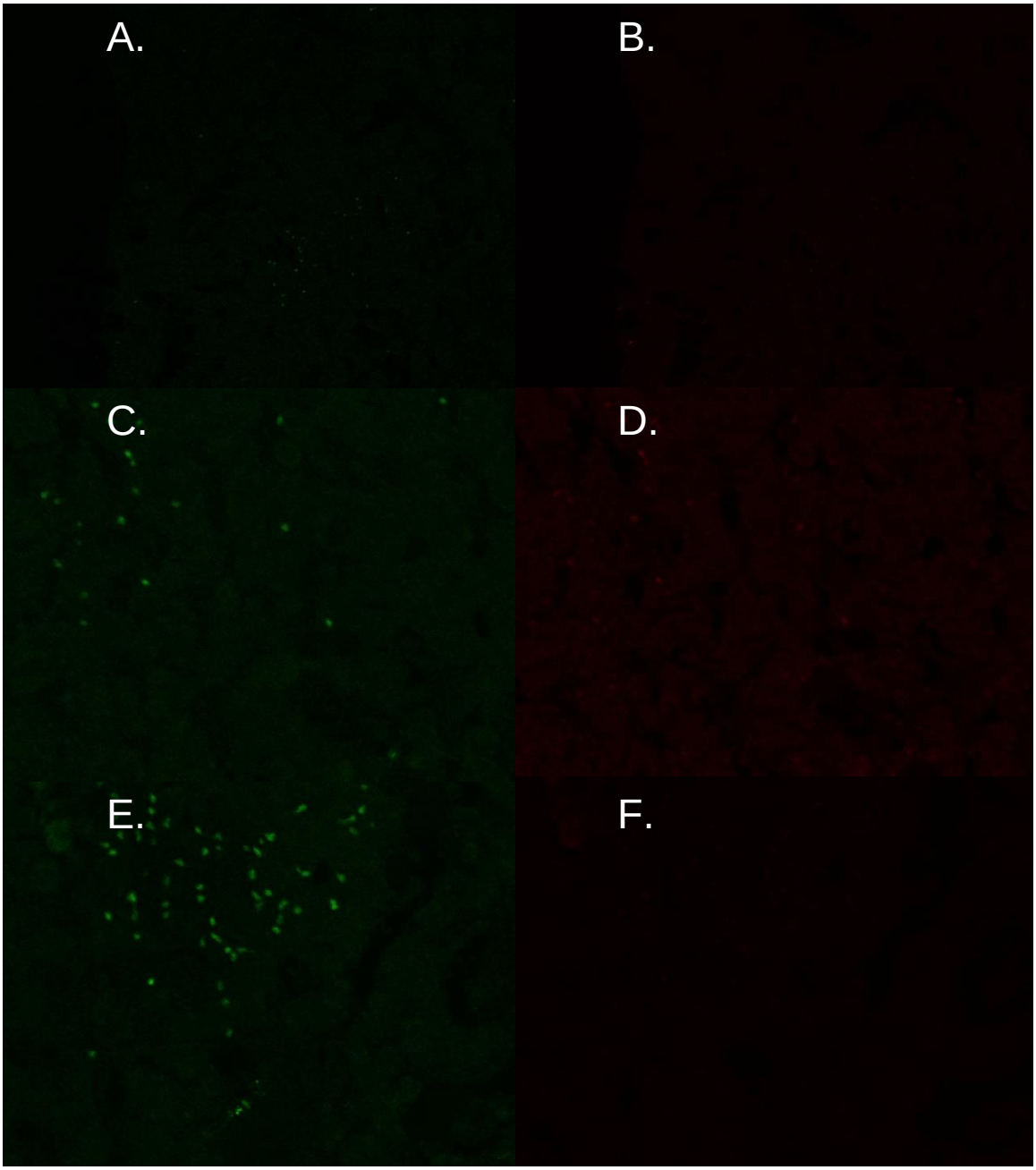
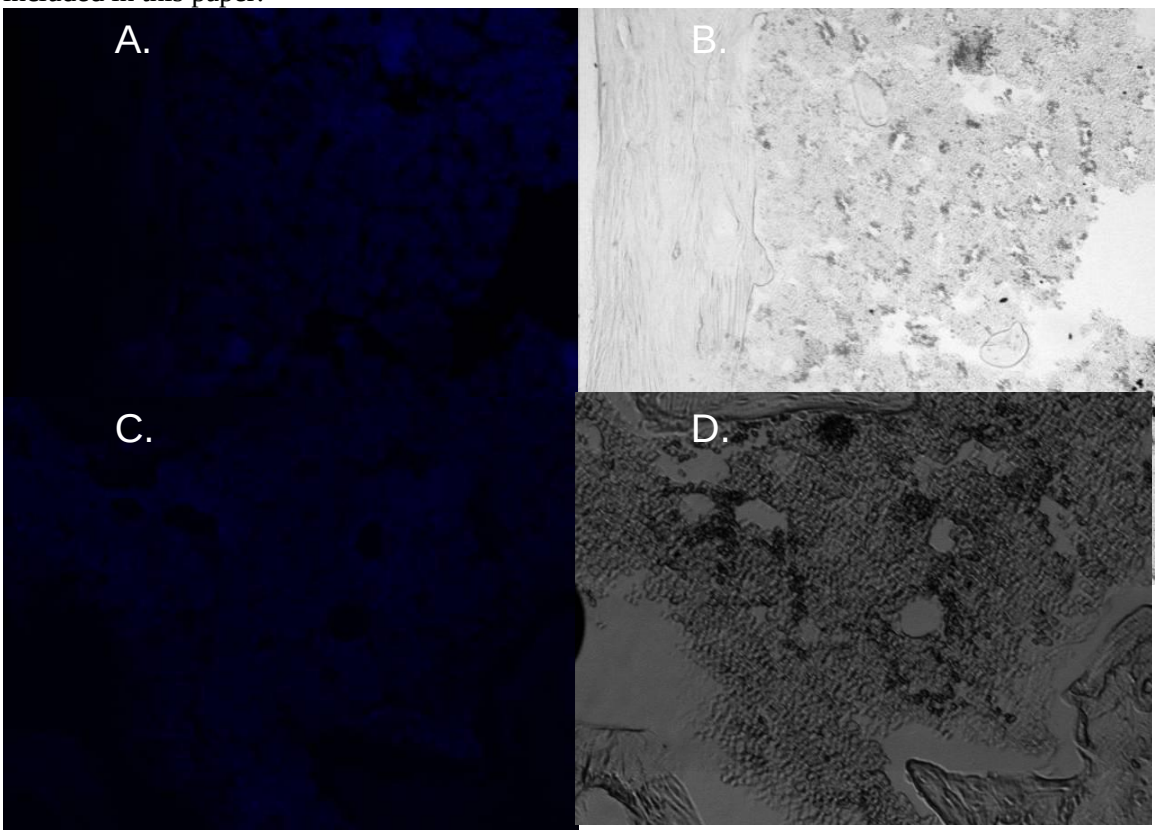


Figure 11) Confocal Images of Stained Bone Marrow Section. The images side by side are of the same field of tissue with CD8 stain on the left and the transmitted image on the left. All images were collected at 10x magnification. The Thy1.1 channel was completely dark, and was not included in this paper.



Chapter 4

Discussion and Conclusion

Visualization of CD8 T cells from memory mice in the context of bone marrow was accomplished, but the clarity of the images and the secondary stains were less successful. As a result, the information that can be extrapolated from the project is limited. The images in some cases were dim and difficult to make out the fluorescent signals, which may be rectified with further manipulation of the microscope setting.

The CFSE bone images showed the transferred memory cells well, and demonstrated that there may be some aggregation of memory CD8 T cells around the venous sinuses in the bone marrow. The sinuses would expose them to circulating antigens, and allow them to respond in a timely manner to secondary infections. This localization may also be due to interaction with other immune system cells that propagate homeostatic proliferation via the expression of IL 15, like macrophages. The CFSE spleen images exhibit a more scattered CD8 distribution.

The CMTMR loaded cells were not recognizable in the images and the Thy1.1 staining of the bone was also unsuccessful. In order to fully visualize the naïve localization, an option moving forward would be to stain the sections from the CFSE/CMTMR experiment with A488 Thy1.1 and A647 Thy 1.2. The former would fluoresce with the green laser, the latter with the red laser. The naïve mouse cells were heterozygous for Thy1.1 and Thy1.2, the memory mouse cells were Thy1.1 homozygous, and the recipient mouse cells were Thy1.2 homozygous. There would be confusion of signals with the CFSE, but the cells that fluoresce both green and red would have to be CD8 cells transferred from the naïve mouse.

Another step that could be taken to test the impact of pariffinization of hard tissues would to employ a different hard tissue sectioning protocol. It may be advantageous to embed the

femurs in OCT medium directly, avoiding the dehydration and rehydration steps, which may reduce the ability of the antibodies to bind to their respective markers, even after trypsin mediated antigen retrieval. That may improve signal strength as well, thereby increasing image clarity.

In conclusion, while the images collected are not perfect, they do exhibit some aggregation of memory CD8 cells within the bone marrow. Further exploration of the cellular environment can be conducted using the methods suggested above.

REFERENCES

1. Miller, J. f. a. p. 2004. Events that led to the discovery of T-cell development and function – a personal recollection. *Tissue Antigens* 63: 509–517.
2. Charles A Janeway, J., P. Travers, M. Walport, and M. J. Shlomchik. 2001. Evolution of the adaptive immune response.
3. Murphy, K., P. Travers, M. Walport, and C. Janeway. 2012. *Janeway's Immunobiology*, 8th ed. J. Scobie, E. Lawrence, I. Moldovan, G. Lucas, B. Goatly, S. Huish, and M. Toledo, eds. Garland Science, New York, NY.
4. Steinman. R.M. Dendritic cells:versatile controllers of the immune system (Lasker Award commentary). Nat. Med., 13:1155-1159, 2007.
5. Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Chapter 24, The Adaptive Immune System. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21070/
6. Sheridan, B. S., and L. Lefrancois. 2011. Regional and mucosal memory T cells. *Nat Immunol* 12: 485–491.
7. Kalia, V., S. Sarkar, and R. Ahmed. 2000. CD8 T-Cell Memory Differentiation during Acute and Chronic Viral Infections.
8. Rubinstein, M. P., N. A. Lind, J. F. Purton, P. Filippou, J. A. Best, P. A. McGhee, C. D. Surh, and A. W. Goldrath. 2008. IL-7 and IL-15 differentially regulate CD8+ T-cell subsets during contraction of the immune response. *Blood* 112: 3704–3712.
9. Todd C. Becker, Shana M. Coley, E. John Wherry and Rafi Ahmed. Bone Marrow is Preferred Site for Homeostatic Proliferation. *J Immunol* 2005; 174:1269-1273.5
10. Lei Ding & Sean J. Morrison. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*. **495**, 231–235.March 2013.
11. Travlos, G. S. 2006. Normal Structure, Function, and Histology of the Bone Marrow. *Toxicol Pathol* 34: 548–565.
12. EasySep™ Mouse CD8+ T Cell Enrichment Kit. For Immunomagnetic isolation of untouched mouse CD8+ T cells. Catalog #: 19753.
13. Immunohistochemistry Frozen Protocol. Novus Biologicals. <http://www.novusbio.com/support/support-by-application/immunohistochemistry-frozen/protocol.html>
14. Invitrogen Avidin/Bioting Blocking Kit. Used to block endogenous biotin or biotin-binding activity in tissue sections. Catalog #: 00-4303
15. Chattopadhyay, P. K., B. Gaylord, A. Palmer, N. Jiang, M. A. Raven, G. Lewis, M. A. Reuter, A. K. M. Nur-ur Rahman, D. A. Price, M. R. Betts, and M. Roederer. 2012. Brilliant violet fluorophores: A new class of ultrabright fluorescent compounds for immunofluorescence experiments. *Cytometry Part A* 81A: 456–466.
16. Multicolor Flow Cytometry. BD Biosciences. <http://www.bdbiosciences.com/research/multicolor/spectrumguide/>

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