EXPRESSION PROFILING OF CRTAM IN CD8 T CELLS DURING ACUTE VIRAL INFECTION

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ABSTRACT

During previous experiments of acute viral infection with Lymphocytic Choriomeningitis Virus, it was discovered through genome-wide microarray analysis that CRTAM mRNA was being significantly upregulated in CD8 T cells at the peak of the effector response as compared to naïve and memory CD8 T cells. This led us to ask the question - what are the CRTAM expression kinetics early after an acute viral infection and the relative tissue association for this upregulation. It was also thought that CRTAM could play a role in the differentiation of CD8 T cells into memory CD8 T cells. The experiments began with a screening of P14 chimeric transgenic mice at three time points (Naïve, day 8, and day 30) for upregulation in the expression of CRTAM. It was found that, indeed, CRTAM was differentially expressed in day 8 CD8 T cells in comparison to naïve and memory CD8 T cells. But only a very small subset of the population was expressing CRTAM at elevated levels. This led to the next experiment to test for CRTAM upregulation earlier in the time course of infection. Day 3 was selected as a time point that represents an early phase for CD8 T cell priming in this system. Again, CRTAM was upregulated on a small subset of the population but it was being expressed anywhere between half to a full logarithmic difference in comparison to the unactivated endogenous population of CD8 T cells. This was relatively early in the disease time course so the next experiment was to check expression over three days with strong controls on either end of the disease time course. Days 3, 4, and 5 were selected. Naïve and memory P14 chimeric mice were used as controls. Expression of CRTAM in CD8 T cells remained high in a small subset of the population, even more so at day 5 post-infection. However, day 5 samples showed higher percentage of the CD8 T cell population expressing CRTAM in higher quantities. It was clear that a small subset of the CD8 donor population was expressing CRTAM at higher levels than the rest of the population over the course of T cell proliferation, activation, and effector differentiation – day 3 through day 8. The
focus was switched to see if the CRTAM +ve subset grew in size or intensity depending on the tissue location of CD8 T cells in the body. To determine this, CD8 T cells in the spleen, blood, liver, and lymph nodes of day 5 mice were stained for CRTAM. It was discovered that the lymph nodes showed much higher expression than the rest of the tissues. This matches previous research that ties CRTAM to retention of CD8 T cells in the lymph node for late phase activation through interactions with Necl-2. Overall, the experiments revealed an interesting subpopulation of CD8 T cells that express CRTAM in much higher quantities. But, the function of that subpopulation, whether it is to facilitate retention in the lymph nodes or whether there is no nominal function, is yet to be understood completely. The best procedure to follow these experiments is to isolate this subpopulation and stain it for more characteristics to understand why it expresses CRTAM in such high quantities and what the subpopulation contributes to the process of disease eradication in the body.
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Chapter 1

Introduction

This thesis is designed to communicate the procedure that I followed, the results that I found, and place all of this into the context of Immunology. This is done in multiple parts. This introduction will serve to describe the current understanding of immunology, moving from a macro to micro context, and place the study of CRTAM in that context. Following the Introduction, the Results section will explain what the four experiments revealed. The methods and Materials will be described afterward, starting with information about the techniques used and then finishing with a description of experimental design. Finally, the Conclusion will speak about the implications of the research and new directions that the research could take.

Introduction to Immunology

The human body is an amazing piece of evolutionary machinery. Over the millions of years that humans have been evolving, they have developed incredible ways to adapt to the world around them. One of the most amazing adaptations has been the immune system. Simply, the immune system is the collection of organs and processes that protect the body from foreign pathogens and prevent reinfection from previously encountered pathogens. Without this system to protect the human body, even the simplest infections, viruses, or fungi would run rampant, infecting and destroying cells. Humans cannot survive without the immune system.

The immune system can be divided into two broad categories: the innate immune system and the adaptive immune system.
The innate immune system is, as its name suggests, innately prepared to tackle any infections. The innate immune system is made up of several components including antibacterial proteins and several cell types. These cells include neutrophils, eosinophils, basophils, mast cells, phagocytes, Natural Killer (NK) cells, and dendritic cells (Murphy, 2012). All of these cells recognize foreign objects through a range of standard mechanisms. The innate immune system is meant to handle, at an elementary level, most infections that the body encounters and, if the infection is serious enough, stave off serious infection until the adaptive immunity can respond. The drawback to the innate immune system is that it is indiscriminate in its specificity of recognition and tends to be broader. More importantly, it becomes exhausted easily (Murphy, 2012). If it were the only source of protection, any reoccurring infection or serious infection would be able to outlast it.

The adaptive immune system compliments the innate immune system as its opposite and is unique to vertebrates. This paper will focus on the adaptive immune system. Where the innate system is given a broad set molecular recognition techniques, the adaptive immune system creates specific cells for recognition and destruction of the specific pathogen. Cells in the adaptive immune system go through a process called clonal selection and deletion (Murphy, 2012). In this process, cells are selected based on their ability to recognize specific antigens related to the virus, bacteria, or fungus. All cells that do not recognize the specific antigen are not selected, and moreover, if any cell targets the body, it is killed. This process occurs in the bone marrow and thymus, long before the adaptive cells are exposed to the rest of the body. This process ensures that all adaptive cells leaving the immune system will target the intended antigen and will not attack the human body. In the adaptive immune system, there are two major classifications of cells: T cells and B cells (Murphy, Basic Concepts of Immunology, 2012). Generally, T cells are the cells that attack any foreign antigen while the B cells moderate response where necessary and augment the response by creating secondary proteins such as antibodies.
The innate immune response and the adaptive immune response are joined together by a particular group of cells, commonly called Antigen Presenting Cells (APCs). These cells ingest a foreign object, process it, and then place the antigen on a presenting molecules. These cells then move to germinal centers in lymph nodes and activate the adaptive immune system by docking with T cells and B cells through a receptor process (detailed in later sections). These APCs alert the adaptive immune system that something is wrong and present the system with the most crucial information: the recognizable moiety that the adaptive cells can recognize. APCs also stimulate response by emitting chemokines\(^1\) and cytokines\(^2\) to help elicit a greater response (Murphy, 2012). The most common APC is the dendritic cell. APCs act as the first line of defense, consuming bacteria and foreign antigens or consuming dead cells. They then use presentation molecules to coat their surface in proteins comprised of the foreign antigen (Murphy, T Cell-Mediated Immunity, 2012). Once the APC ingest and present the antigens, they relocate from the site of infection to the lymph node where they come in contact with naïve T cells waiting for activation. Without APC activity, the adaptive immunity would not be triggered and the secondary responses and immunological memory would not be created.

In the immune system, there are two sides: central immune system and the peripheral immune system. The central immune system is comprised of organs where lymphocytes are generated. This includes organs such as the thymus and bone marrow. Peripheral tissues include the lymph nodes, the spleen and any mucosal tissues. This is where naïve, effector, and memory lymphocytes are maintained. In this paper, the focus will be on the peripheral adaptive immune system, as the cells and protein in question appear after activation after cells have left the central system (Murphy, 2012).

---

1. Signaling proteins involved heavily in chemotaxis, or the moving of cells to a location.

2. Broad category of signaling proteins that cause varying effects in cells
The T cell

The focus of this research is the CD8 T cell. In this section, the T cell, its development and its function will be discussed.

T cell Morphology

The T cell is one of the two cell lines that provide cell mediated immune response. There are two distinct types of T cells and they are determined simply by the difference in surface receptor proteins. There is the CD8 T cell and the CD4 T cell. On the T cells surface is a complex called the TCR or the T Cell Receptor. This complex is made of several components. First is the receptor itself. This is what interacts with the antigen being presented by an infected cell. Then there are several co-receptors, which without the cell will not create an effective response to a presented antigen. It is these two co-receptors – CD4 and CD8 – which create the fundamental difference between CD4 T cells and CD8 T cells. CD4 T cells have the CD4 co-receptor while the CD8 T cells have the CD8 co-receptor (Murphy, The Development and Survival of Lymphocytes, 2012). Each of these receptor complexes, along with co-stimulatory molecules, binds to a presentation molecule on an infected cell (Kaech, Wherry, & Ahmed, Effector and Memory T Cell Differentiation: Implications for Vaccine Development, 2002). This surface protein complex is called the Major Histocompatibility Complex (MHC). There are two classes of MHC molecules that can be expressed by infected cells: MHC Class I and MHC Class II. MHC Class I molecules present peptides that are about 8 to 10 amino acids long and present exclusively to CD8 T cells. MHC Class II molecules can bind peptides that are 13 amino acids or larger and
bind exclusively to CD4 T cells. The expression of the MHC molecules is expressed differently across varying cell types (Murphy, The Development and Survival of Lymphocytes, 2012). Since MHC Class I molecules are recognized by CD8 T cells, whose function involves cytotoxicity (discussed later), almost all cell types throughout the body can express this molecule. MHC Class II functions to activate CD4 T cells, or helper cells; therefore, APCs, macrophages, and B lymphocytes can phagocytose bacteria or an infected cell and represent the antigens on its cell surface to help activate other areas of the immune system.

T cells provide a variety of effector functions in the body. They are most notably recognized in cytotoxicity. Most APCs, innate immune cells, and antibodies are fairly good at recognizing foreign objects and removing them from the system. However, many viruses and bacteria can infect cells in the human body. At this point, the above mentioned cells prove relatively useless because the foreign pathogens are hiding in a sense, protected behind the cell membranes of cells our body cannot attack. This is where T cells come into play. They can recognize changes in the human cell’s surface characteristics and, if the cell is showing the characteristics of infection, can begin the process of apoptosis (Russell & Ley, 2002).

T cell Development

This section will detail the stages of T cell development from the naïve stage to the effector stage and finally into the memory stage, will describe each stage, and will discuss each of the markers expressed during each stage.

T cell development begins in the bone marrow. There, multipotent hematopoietic stem cells differentiate into progenitor cells (Funk, Kincade, & White, 1994). These cells migrate quickly into the thymus and become thymocytes. It is within the thymus where all major development events occur. Upon arrival to the thymus, the progenitor cells receive stimulation
from stromal cells via the Notch pathway (Murphy, The Development and Survival of Lymphocytes, 2012). This causes the progenitor cells to begin differentiation specifically into T cells. Notch pathway activation is maintained throughout T cell development. After about a week of differentiation, the T cells begin a period of extreme proliferation. During this period, approximately $5 \times 10^7$ cells are produced daily. Of the cells produced, however, only 2% to 4% leave the thymus as mature T cells (Shortman, Egerton, Spangrude, & Scollay, 1990). This low rate of mature cells is a result of the many development steps that the cells go through while in the thymus that prevent auto-immune responses, or nonspecific responses.

During the proliferative stage, the progenitors begin expressing CD3 on the cell surface. At this time, the cells are not expressing CD4 or CD8 so, the cells are called “double negative” at this time. DN cells begin to divide into two distinct populations: $\alpha:\beta$ T cells and $\gamma:\delta$ T cells. The $\gamma:\delta$ T cells are exported to the periphery. The $\alpha:\beta$ T cells develop CD4 and CD8 receptors and as a result, enter the “double positive” phase. T cells in the double positive phase make up most of the thymocytes. This phase is broken into two classes: large active and small resting. During the large active stage, the T cells proliferate and bridging between TCR’s occurs as rearrangement of variable regions occur in the TCRs (Murphy, The Development and Survival of Lymphocytes, 2012). After proliferation ceases, the double positive cells become small resting and can have one of two fates. If the small resting T cell cannot recognize self-peptides and MHC molecules, they will die as they cannot pass positive selection. This is where a majority of T cells die, as they express low levels of self-recognizing TCRs. The other fate is that the T cell can recognize self-peptides and MHC molecules. It is there for positively selected and begins to mature by producing more TCRs (Hogquist, et al., 1997). Once maturation continues, the T cell enters the “single positive” stage and selects either the CD4 or the CD8 molecule and exclusively expresses that selected molecule on the cell surface. This is when the T cell can be differentiated as either a CD4 T cell or a CD8 T cell (Singer, Adoro, & Park, 2008). During and following this maturation
process, both cell types undergo negative selection. This process ensures all cells that respond to self-antigens or produce an autoimmune response. Only between 2% and 4% of the total thymocyte population make it to maturation (Kishimoto & Sprent, 1997). Once the T cells have matured, they leave the thymus and migrate through chemotaxis to the lymph nodes where they will undergo activation by APCs (Murphy, T Cell-Mediated Immunity, 2012).

Once T cells have matured, they are still considered “naïve” or unactivated. They have not yet encountered an antigen and have yet to express effector functions. The naïve cells migrate to the lymph node where they wait to be activated (Kaech, Wherry, & Ahmed, Effector and Memory T Cell Differentiation: Implications for Vaccine Development, 2002). As stated earlier, APCs will come into the lymph node, baring the antigens of the invading pathogen on their cell surfaces. The T cell’s TCR will dock with the APC’s MHC I complex and the TCR will recognize the intended target molecule (Belz, Carbone, & Heath, 2002). Immediately, the T cell population will become activated and will enable effector functions. Specific proteins (to be discussed later) keep the T cells in the lymph node while they proliferate to provide a strong adaptive response. Finally, the activated T cell population egresses from the lymph node and flows into the body. Using the TCR, the T cells find human cells expressing MHC I and initiate the apoptosis Fas pathways within the cell to start targeted cell death (Russell & Ley, 2002). This response peaks for several days and works until there is bacterial or viral clearance. Then the T cell population begins to decline. This happens as effector cells die off. Finally, the populations levels off and T cell death ceases. The remaining 5 to 10% of the cells differentiate into memory T cells (Kaech, Hemby, Kersh, & Ahmed, 2002). These cells are what provide long lasting immunity to the body. The cells maintain both effector functions and act as stem cells, producing more T cells if another pathogen with the same antigen appear in the system again. These cells create immunity to diseases for years.
Disease Time Course

The disease time course begins with infection. Immediately, in the site of infection, reactions begin to occur. Macrophages and other innate immune cells immediately begin to consume bacteria and infected cells. As they do this, they release cytokines and chemokines that attract more cells to fight the infection. Meanwhile APCs begin their journey to the lymph nodes. By day 2 and 3, T lymphocytes have been activated and are proliferating and moving throughout the body to destroy any antigens that they encounter (Cui & Kaech, 2010). B cells act as activators, enhancing the immune response provided by NK cells and T cells. By day 8, there is a robust effector response to the virus or bacteria.

By day 8, viral and bacterial titers are almost completely diminished while T cell response has peaked. After day 8, the T and B cell response and population decreases in strength until about day 30, where it plateaus. The remaining population, about 5 to 10% of the original population, differentiates into memory T and B cells. These provide the longer lasting protection known as immunological memory. It is within this time course that we look to characterize where CRTAM is expressed.
The CRTAM Protein

The CRTAM protein is expressed in CD8 T cells and is the focus of this research because there is still much to be learned about the full function of this protein. Previous experiments had tertiary results that showed CRTAM mRNA expression was up for day 8 effector CD8 T cells. The reason for this was not known, and resulted in this paper and its experiments. Before going into this, the following section will describe the current knowledge about the CRTAM protein.

Figure 2 – Role of CRTAM in the retention of T cells in the Lymph Node – (Takeuchi, et al., 2009)

CRTAM is a cytotoxic and regulatory T cell molecule that passes through the cell membrane as a transmembrane protein. It has V and Cl-like Ig domains. Activation causes up-regulation of CRTAM in CD4+ and CD8+ T cells. CRTAM expression is restricted only the CD4 and CD8 T cells and all double negative subpopulations.

It has many functions in both CD4 and CD8 T cells. CRTAM interacts with NK cells causing their cytotoxicity to increase as well as causing an increase NK mediated rejection of tumors that are expressing CADM3. In in-vitro experiments, it has been discovered that CRTAM promotes IFNγ, IL22, and IL17 secretion by CD8 T cells (Yeh, Sidhu, & Chan, 2008). CRTAM is found to bind to a specific molecule: Necl-2 (CADM1). Necl-2 is CRTAM’s ligand and is
expressed on CD8+ dendritic cells that are found in the lymph nodes. This interaction retains the
T cells in the lymph nodes where they can proliferate during late phase activation (Takeuchi, et
al, 2009). This allows for a greater amount of CD8 T cells egressing from the lymph nodes. The
CRTAM/Necl-2 interaction plays a part in retention of cells in the thymus as well.

CRTAM−/− mice3 show a decrease in thymic growth, show defective thymocyte
maturation, and reduced protective immunity to viral infections. T cells show increased receptor-
mediated proliferation but produce noticeably lower levels of IFNγ, IL22, and IL17; and are
unable to construct cytoplasmic multi-module scaffold proteins tied to cellular polarization.
Finally, without CRTAM, the number of CTL’s in the lymph node is decreased as it serves as a
retention factor.

CRTAM was selected as a target molecule for several reasons. First, previous
experiments within this lab have shown that mRNA for CRTAM expression was substantially
upregulated for day 8 effector T cells. In previous experiments performed by this lab, CRTAM
was a secondary target molecule and through those experiments the following data were revealed.

![Raw mRNA Intensity Values of CRTAM from Affymetrix Microarray](image)

**Figure 3 – Affymetrix Microarray data of CRTAM mRNA expression**

3 This indicates a knockout mouse where the mouse is bred not to express CRTAM
Figure 3 depicts the raw mRNA intensity from naïve, day 8, day 16, and day 30 mouse samples. This suggests that day 8 effector T cells saw a marked increase in the expression of the mRNA tied to CRTAM expression and production. These mRNA data were purified by FACS. Naïve P14 mice received no infection, while day 8, 16, and 30 all received infection from LCMV. Following purification by FACS, total RNA was isolated using a miRNeasy kit. The samples were then analyzed using an Affymetrix Microarray. Based on these results, the lab determined that CRTAM could be a very interesting target molecule. Curiosity as to why CRTAM was significantly upregulated in day 8 CD8 T cells led to the following experiments. Also, the relatively high expression of CRTAM mRNA in day 16 and day 30 in relation to naïve samples also drew interest to see if CRTAM played a role in the differentiation of effector T cells into memory T cells.

Another reason CRTAM was selected as a target molecule was due to the lack of knowledge surrounding this protein. Current understanding of CRTAM is still expanding and all of its effects and mechanisms are not fully understood. CRTAM expression and function in CD4 T cells is better understood than it is in CD8 T cells. By focusing on expression patterns in CD8 T cells, a better picture of the scope of CRTAM’s function can be developed. The origins of the set of experiments were to see precisely where CRTAM was expressed and if, at any point, it was expressed differentially. The purpose was to see if there was any relationship between CRTAM and memory T cells, and more broadly, where CRTAM showed up in the time course of infection and immune response. The hypothesis was that at some time point, particularly close to the memory phase, CRTAM would be upregulated in the T cell population and would be expressed in higher amounts in a subset of the population. In the following paper, the experiments and the results from those experiments are discussed to reveal what was discovered about the expression of CRTAM.
Chapter 2

Results

In the following section, the results of the four experiments performed to determine if CRTAM is differentially expressed at a certain point in the CD8 T cell response to LCMV infection. The section opens up with a description of the major analytical tool used followed by the results broken down by experiment. For all information about Method and Materials, see Chapter 3.

Analysis by FlowJo

FlowJo is a program that is used to analyze the acquired results from fluorescently labeled cells on a per cell basis. To begin, compensation controls are created to correct for a specific fluorochrome’s emission into detectors other than its own. Additionally, the fluorescent labels can autofluoresce\(^4\), which creates background noise (see flow cytometry section for full details). Once the voltages for each channel have been set and the compensation matrix has been calculated, the cell population can be analyzed more accurately. A specific cell type is usually identified by plotting two labeled proteins against each other on the graphs. Gates are used to focus in on a particular group of cells so that further analysis can be created. Calculations such as percentages, means, and medians can also be calculated and added to the graphs. A standard statistic used throughout this paper is MFI or Mean Fluorescent Intensity, which shows the fluorescent intensity of a particular fluorochrome on a per cell basis. This is helpful in identifying

\(^{4}\) Emitting fluorescent light without stimulation, or not under normal circumstances
the populations that are expressing a protein at a higher level. After the samples have been acquired, the compensation matrix is checked manually via FlowJo and populations are analyzed by gating target cell types. All samples are set to have the same compensation matrix and gates. From there, a layout for all samples can be created for presentation. For examples, see appendix B.

**Experimental Results**

**Experiment 1: Broad Screen**

The purpose of this first screen was to get a sense if there and where in the time course of infection CRTAM is upregulated. This involved sampling naïve, day 8, and day 30 (post-infection with LCMV<sub>Arm</sub>) mouse blood with a cellular stain for CRTAM. Staining protocols and flow cytometry are covered in the Methods and Materials section.

The sequential gates were created in the following order: Live cell population → CD8 T cell population → Antigen specific CD8 T cell population → Donor antigen specific CD8 T cells → CRTAM<sub>High</sub> donor antigen specific CD8 T cells. Once these gates were established, the donor population was paired with CD44 and CD62L to show and activation had occurred. Finally, a histogram was created to show the expression of CRTAM in the donor antigen specific CD8 T cell population. This was performed for the naïve control blood sample, the day 8 effector blood sample, and the day 30 memory blood sample.
Figure 4 – Kinetics of CRTAM and Granzyme B expression in CD8 T cells during the course of acute LCMV infection

Figure 5 shows the donor antigen specific CD8 T cell and the CRTAM specific gates as well as histograms of Granzyme B expression. This is done to show the difference between the naïve, effector, and memory populations. CRTAM+ gates (middle) show the percentage of CRTAM+ cells of donor CD8 T cells (left). The bar chart indicates there is a larger number of CRTAM-expressing CD8 T cells during the effector phase. However, the population of cells that were CRTAM$_{\text{High}}$ was very small. The next logical step was to find a time point in the infection where the cells were CRTAM$_{\text{High}}$ compared to naïve mice and were expressed in a larger portion of the subset. To do this, earlier time points were selected as a target for future experiments. It was determined that not many important development time points would occur between day 8 and day 30. One time point to explore would be the memory differentiation stage, but isolating that time point would require an extensive time. Also, the decrease in expression of CRTAM
between effector and memory stages suggested that there would be no useful time points to
explore. It was decided to focus on differential expression before day 8 as more developmental
events occurred between that time point and initial infection.

**Experiment 2: Day 3 Time Point**

Day 3 post-infection was selected as a starting factor as days 1 and 2 were too close to
infection. Day 3 would begin to see the activation of T cells. To ensure a thorough analysis, three
mice were used and both blood and spleen samples were taken. Spleen samples were run three
separate ways: cellular stain only (CS), intracellular stain only (ICS), and both cellular and
intracellular stains (CS+ICS). Blood samples were run with both cellular and intracellular stains.
Cell transfers, sacrificing protocols, staining protocols, and flow cytometry are covered in the
Methods and Materials section.

The CRTAM\(^{\text{High}}\) population was gated as follows: Live cell population \(\rightarrow\) CD8 T cell
population \(\rightarrow\) Antigen specific CD8 T cell population \(\rightarrow\) Donor antigen specific CD8 T cells \(\rightarrow\)
CRTAM\(^{\text{High}}\) donor antigen specific CD8 T cells. Once these gates were established, the donor
population was paired with CD44 and CD62L to show activation had occurred. Finally, a
histogram was created to show the expression of CRTAM in the donor antigen specific CD8 T
cell population versus the endogenous antigen specific CD8 T cell population. This was
performed for three day 3 blood and spleen samples, and to both CRTAM staining groups.
Figure 5 – Mean Fluorescence Intensity (MFI) of cell surface (CS) and intracellular (ICS) CRTAM expression in CD8 T cells at day 3 post-infection

The top histograms in Figure 6 show the CRTAM MFI of unactivated, endogenous CD8 T cells (grey) and donor CD8 T cells (black). The bar graph below shows the MFI of CRTAM. It clearly shows that the subset of CD8 Donor cells were expressing CRTAM at a half to a full logarithmic difference as compared to the endogenous, unactivated CD8 T cell population. The results showed that a small subset of day 3 donor antigen specific CD8 T cells were differentially expressing CRTAM at a logarithmic difference versus the control inactivated, endogenous CD8 T cell population. This occurred in both blood and spleen. There was concern that using the endogenous, unactivated CD8 T cell population (CD44lo) was not providing completely accurate results as a true naïve control would give the best comparison, so in future experiments, it was decided a full naïve control would be used. Also, with differential expression at day 3, new time points could be added to explore how far CRTAM expression lasted in the activation and response of CD8 T cells. However, only a small subset of the population was expressing CRTAM in high amounts. This experiment established that between the time point of day 3 and day 8,
CRTAM was upregulated in a small subset of the T cell population. However, it was not clear whether that population size increased at any point. This led to the next experiment.

**Experiment 3: Day 3, 4, and 5 Time Points**

This experiment was set up to focus on three time points post-LCMV infection: Day 3, 4, and 5. For each time point, two P14 mice were created. To show comparisons with true controls, both a naïve P14 mouse as well as a memory P14 mouse were sacrificed. Blood and spleen samples were taken from each mouse. Considering results from the day 3 experiment showed no difference in cellular versus intracellular staining of CRTAM, only a cellular CRTAM stain was applied to test for the target protein. Cell transfers, sacrificing protocols, staining protocols, and flow cytometry are covered in the Methods and Materials section.

Due to the small frequency of CRTAM+ CD8 T cells, cell staining was carried out with three replicates. The donor CD8 T cell population was gated as described before. Once these gates were established, the donor population was paired with CD44 and CD62L to show and activation had occurred. Finally, a histogram was created to show the expression of CRTAM in the donor antigen specific CD8 T cell population. This was performed for two sets of data: blood and spleen samples.
Figure 6 – Spleen: CRTAM expression kinetics early during an effector CD8 T cell response

Figure 6 shows flow plots of donor antigen specific CD8 T cells in spleen. The percent shows percent of CRTAM+ cells of total cells. The bar graph shows the percent of total T cells that are CRTAM+. The results from the blood samples showed that CRTAM expression was not differential between most of the samples for any of the time points and the naïve control and the memory control; day 3 blood samples actually showed no donor CD8 T cells (data not shown). This occurred because there was too small of a population to show up on analysis.

The results from the spleen samples showed that a small subset of the CD8 T cells from day 4 and especially day 5 were expressing CRTAM at higher levels than the naïve or memory control. In the case of day 5 mice, both samples were showing about half a logarithmic difference greater than the controls. Furthermore, the day 5 samples were showing a larger amount of CRTAM\textsuperscript{High} cells. This was the second result that showed a small yet distinguishable subpopulation expressing CRTAM at a higher level than the rest of the population of CD8 T cells. Relatively conclusively, it could be suggested that high expression of CRTAM occurred in only a small subset of spleen and blood samples. Based on previous research, CRTAM is used to
retain T cells in the lymph nodes. To this point, tissues outside of the blood and spleen had been excluded, suggesting a fourth experiment.

**Experiment 4: Day 5 Full Tissue Analysis**

Based on experimental data from previous research, CRTAM is used as a retention protein for late phase activation in the lymph nodes (Takeuchi, et al., 2009). The T cells are kept in the lymph node to induce proliferation for a strong, late-phase response. It was hypothesized based on these findings that the small subset population expressing higher levels of CRTAM was the original donor population that adhered to the lymph nodes during activation. This prompted us to analyze CRTAM expression not only blood and spleen but also lymph nodes and liver. Lymph node and liver were added to show the site of activation and the primary site of infection, respectively. Day 5 was selected due to its statistically higher percentage of CRTAM$^{\text{High}}$ cells. A B6 naïve mouse was also included as a control. All samples were processed according to the procedures in the Methods & Materials section.

The donor CD8 T cell population was gated as previously described and a histogram was created to show the expression of CRTAM in the donor antigen specific CD8 T cell population. This was performed for all sets of data: blood, spleen, lymph nodes, and liver samples.
Figure 7 – Expression of CRTAM (in MFI) in lymphoid and non-lymphoid CD8 T cells at day 5 post-infection

The top histograms show the expression of CRTAM in the CD8 antigen specific donor population. Each histogram is paired with the MFI of CRTAM. The bottom bar graph summarizes the top figure and displays the CRTAM expression across the tissues. From the experiment, it was determined that the donor CD8 T cells in the lymph nodes showed a higher expression of CRTAM on day 5 post infection. Donor CD8 T cell populations in blood, spleen, and liver did not show differential expression of CRTAM. This directly correlates to the previous research that suggested Necl-2 and CRTAM interact to retain T cells in the lymph node to aid late-phase activation and proliferation.
Chapter 3
Methods and Materials

In the following section, the methods used as well as the experimental designs will be detailed. The first sections detail the process of chimeric mouse creation and the use of mice as a model as well as Flow Cytometry and Fluorescent Staining. Following these introductory descriptions, the experimental designs of the four experiments performed will be described.

Chimera Mice and the Mouse Model

The Mouse Model

The mouse is the most common model used for immunological research for several reasons. First, it shares a very close similarity to humans in several key organ systems, most notably the immune system, the endocrine system, the cardiovascular system, the nervous system, and the skeletal system. Because both humans and mice share a complex innate and adaptive immune response, mice are used as a model for how a human would potentially react (Spencer, 2012).

More importantly, much like humans, mice can develop similar diseases and conditions such as diabetes, cancer, and meningitis. Because the mouse model proceeds through almost identical phases of infection, response, recovery, and long lasting immunity, they are well fitted to represent a human system in similar circumstances (Spencer, 2012).

Mice can also easily undergo genetic engineering to produce particular strains of mice. This could include knockout mice strains, where a particular protein is either downregulated or not expressed at all; or immunocompromised mice (nude and SKID mice), where either the
removal of an organ system, a necessary protein, or the knockdown of a cell type interferes with the ability of the mouse to have a functional immune system.

Finally, mice are cheap and easy to maintain, easy to breed, and, due to high breeding rates, can produce many replicates within a given experiment. The ability to clone the mice also ensures that experiments are completed with less variability. This allows more models to be used to validate a particular result and add statistical significance to any findings.

**Lymphocytic Choriomeningitis Virus (LCMV)**

The virus used in this experiment and typically in the most of these types of experiments is LCMV$_{Arm}$. This is the Armstrong 53b strain of the Lymphocytic Choriomeningitis Virus. This virus is used in mouse models because it causes an acute viral infection, which shows a strong immunological response in mice and is a good model for an acute viral infection. It can be added to many different reagents to induce an infection in the mouse model. It has a low chance of infection in humans. Most importantly, there is a high rate of viral clearance in mice. This means that the mouse can be infected and the progression from naïve to effector to memory can be documented without concern that the mouse will die from infection.

**P14 Chimeric Mouse**

C57BL/6 wildtype mice were used as the subjects of the following experiments. If a P14 mouse was used as the model, the overwhelming response from antigen specific T cells could have overloaded the mouse’s system, in effect, killing it. But, by creating a chimeric mouse using a B6 mouse and transferring in low doses of P14 transgenic GP33-41 antigen specific Thy 1.1 cells, the response to infection with LCMV could easily be tracked by staining for Thy 1.1.
Specifically, $1 \times 10^6$ splenocytes from thy 1.1+ P14 mice bearing the D<sup>b</sup>GP33 specific TCR were transferred into C57BL/6 wildtype mice to create the chimera mouse model.

To create the above mouse model, a Thy 1.1+ P14 mouse was sacrificed by CO<sub>2</sub> followed by cervical dislocation. The spleen was then harvested and prepared and counted according to the section on splenocyte isolation below. From the sample that remained of the isolated cells, a dilution was made to create and aliquot of $1 \times 10^6$ cells to be transferred into the B6 mouse. The dilution was filtered to ensure that no foreign objects obstructed the blood vessels of the recipient mouse. The cells were adoptively transferred intravenously into the tail vein of the recipient B6 mouse.

**Cell Isolations**

To accurately count and sort the cells of interest, they must first be isolated from the mouse, the organ, and the tissues. This process is completed in the isolation procedures that follow. These processes take the raw sample in question, whether blood, spleen, liver, or lymph node, and isolates a large amount of the cells by removing extraneous cells that would cloud up or distort readings by a flow cytometer. In the following subsections, the processes for PBMC, Splenocyte, Liver, and Lymph Node isolations are detailed out. All information was taken directly from standard lab protocols.

**PBMC Isolation**

Blood was harvested from the mouse via a capillary from the orbital sinus. The sample was placed in 0.5 mL of 4% sodium citrate (dissolved in deionized water). This is used as an anticlotting agent. 2 mL of RPMI media (dissolved in DW) supplemented with 2% FBS was
added to the test tube. The sample was then vortexed. Using a Pasteur pipette (9”), histopaque-1077 was inserted at room temperature under the blood sample (underlay). The volume of the histopaque-1077 was anywhere from 1/3 to ½ the volume of the blood/RPMI mix above it. The samples were placed in the centrifuge and spun down for 20 minutes, at 20°C at 2000 RPM with no brake. After the sample was spun down, a Pasteur pipette (5 ¾”) was used to siphon the PBMC (blurry, fuzzy layer) above the interface and transfer the PMBCs into a separate tube with 2 mL of RPMI media supplemented with 2% FBS. When transferring between tubes, both were labeled identically to ensure there are no confusion or cross-contamination. After the transfer into the second tube, the sample was then spun down again for 10 minutes at 2000 RPM at 4°C. Upon completion of the spinning, it was confirmed that a pellet formed. The supernatant was poured off and the pellet was disturbed by dragging the tube along a test tube rack. After the pellet was disturbed, the sample was resuspended in 200 µL of FACS buffer. The sample was then put on ice until it was ready to be plated.

**Splenocyte Isolation**

Spleen samples arrived whole, or cut in half, on ice and stored in 4 mL of R1 supplemented with 2% Fetalplea. The sample was then vortexed and the 4 ml of R1 and the spleen samples were poured into a small petri dish. The rough sides of two glass slides were used to gently crush the spleen by rubbing action, releasing the splenocytes. The slides were rinsed with R1 using a 5 mL syringe. Without drawing up the white stroma, the mixture was siphoned up with the same syringe and placed into the original tube. The dish was rinsed again with R1 and the rinse liquid was siphoned up, avoiding the stroma. The tube of the released splenocytes was then spun down for 10 minutes at 1200 RPM at 4°C with brakes. The supernatant was poured off and the pellet was disturbed by running the tube along a peg rack.
After the cell were disturbed, 1 mL of 83% ammonium chloride was added to the tube for precisely 1 minute. This is used to destroy any RBCs mixed in the splenocytes population. After the timer was started, the sample was vortexed to ensure all cells in the sample are exposed to the ammonium chloride. After 1 minute, the tube was filled to the top with R1 and inverted several times to wash the cells and dilute the ammonium chloride to end the lysing process. The sample was then spun down for 10 minutes at 1200 RPM at 4°C with breaks. The sample’s supernatant was then poured out and the pellet was disturbed by dragging the tube along a tube rack. The cells were re-suspended with R10. The cells were then ready to be counted for transfer or plated for screening or staining.

The process of counting cells is used when a specific number of cells are needed to be transferred into a mouse. This is done by taking trypanblu/PBS solution and making a 1/100 dilution of the splenocytes. 198 µL of the trypanblu solution is mixed with 2 µL of cells. 10 µL of this solution is taken and placed on a hemocytometer. The hemocytometer is placed under a microscope and the live cells are counted. Trypanblu stains the inside of dead cells while remaining only on the outside of living cells. Therefore live cells resemble halos while dead cells appear as full circles. Once the number of cells from each of the four quadrants have been counted, they are averaged and multiplied by the dilution factor (100) and then that number is multiplied by 10,000. This gave the number of cells per mL. This was multiplied by total volume of the cell sample and this resulted in the total cell number. Based on this, a dilution was created to be transferred into B6 WT mice.

Following counting and screening, the remaining cells are diluted and then are ready for use in transferring or for resuspension in FACS for staining.
Liver Isolation

Following profusion and harvest from the mouse, the liver was placed in about 20 mL of RPMI + 2% FBS on ice until it was ready to process. The liver sample was dumped into a cell strainer. Using the end of a syringe plunger, the liver was crushed and rinsed through the strainer with RPMI + 2% FBS. This process took about 5 minutes or until all that remained was a whitish connective construct. The cells were spun down at 2,000 RPM for 10 minutes at room temperature. The cells were resuspended in 5 ml of room temperature 44% Percoll (in RPMI). The cell mixture was transferred into a 15 mL conical tube. 3 mL of room temperature 67% Percoll (in PBS) was then underplayed using a 9” glass Pasteur pipette. The cells were then spun down at 2,000 RPM for 20 minutes with no brakes at 20°C. After spinning, the fat was removed and discarded using a plastic Pasteur pipette. Following the fat removal, the lymphocytes at the interface were removed using a 5 ¾” Pasteur and added to a tube with 10 ml of RPMI + 2% PBS. The lymphocytes were then spun down at 2,000 RPM for 10 minutes. The supernatant was discarded and the cells were washed once in RMPI + 2% PBS. They were finally resuspended in 500 µL RMPI + 2% PBS. They were then counted and finally plated.

Lymph Node Isolation

Spleen samples arrived whole, or cut in half, on ice and stored in 4 mL of R1 supplemented with 2% Fetalplea. The sample was then vortexed and the 4 ml of R1 and the spleen samples were poured into a small petri dish. The rough sides of two glass slides were used to gently crush the spleen by rubbing action, releasing the splenocytes. The slides were rinsed with R1 using a 5 mL syringe. Without drawing up the white stroma, the mixture was siphoned up with the same syringe and placed into the original tube. The dish was rinsed again with R1 and
the rinse liquid was siphoned up, avoiding the stroma. The tube of the released splenocytes is then spun down for 10 minutes at 1200 RPM at 4°C with brakes. The supernatant was poured off and the pellet was disturbed by running the tube along a peg rack.

The sample was then spun down for 10 minutes at 1200 RPM at 4°C with breaks. The sample’s supernatant was then poured out and the pellet was disturbed by dragging the tube along a tube rack. The cells were re-suspended with R10. The cells were then ready to be counted for transfer or plated for screening or staining.

**Fluorescent Staining and Flow Cytometry**

Cellular stains are used to detect the presence or absence of surface proteins on the surface of a cell. These surface proteins act as markers to indicate which stage a cell is in or what its current state of action may be. A staining agent is comprised of two parts: the antibody and the fluorescent label. The antibody is a Y shaped protein structure. The pronged end has variable regions that allow that end of the antibody to recognize and attach to a target assay. In this case, the target assay is a particular surface protein. The single end of the antibody is a constant region where various effector molecules can be attached. In this case, the effector molecule is a fluorescent label which, when stimulated by a laser, emits a certain light frequency which is then read by a flow cytometer. For each cellular marker, there is a specific protein that targets it and there can be several different varieties of fluorescent labels used depending on the target, the cell, and the stage at which the cell is in its life cycle.

Intracellular staining works in a very similar way except that all for the proteins being stained are within the cellular matrix inside the cell. This used more to show the turnover rate of a particular protein. The surface stain may show very little of a particular protein, but when the inside of the cell is stained, there is a much higher concentration of the protein, indicating that the
protein is coming to the surface and leaving at a high rate. Intracellular stains can also be used to detect proteins that will not appear on the surface of the cell, for example Granzyme B will not appear on a T cell’s surface, but instead will be transported via vesicle. Only intracellular stains will detect this and other non-surface proteins.

Once the cells have been labeled and washed, they will be counted and sorted using flow cytometry. This is a technique used to determine specific qualities about a population of cells. A sample of cells will be drawn into the flow cytometer and, using a laser, the fluorochromes attached to the proteins of interest will be stimulated and then categorized by their label. The data is then compiled and the user can create plots of the presences of certain markers versus other markers. For example, a user can plot the CD8 marker versus the CD44 marker to see which of the CD8 T cell population is high and which is low. The machine used for the following experiments’ analyses was a 16 channel flow cytometer.

**Cellular Markers**

In the cellular and intracellular stains, specific markers were targeted because the presence or absence of these markers help to distinguish certain cell populations from other ones. By selecting these markers, the donor versus endogamous CD8 T cell populations could be distinguished and the expression of CRTAM in each population could be measured. Below is a list of the markers used followed by an explanation of the creation of the staining panels used.

**CD8**

This marker is what distinguishes the two major types of T cells. This surface protein can be found as part of the TCR complex and is a co-receptor for T cell activation. Its absence will
cause an anergic reaction from the T cell. This marker is used to differentiate CD8 T cells from all other PBMCs.

**CD44**

CD44 is a surface protein that CD8 T cells produce once that have been activated. It is used as a maker to differentiate the population of activated cells from those that are not as it is high in effector and memory T cells but low in naïve T cells (Kalia, Sarkar, & Ahmed, 2010).

**CD127**

CD127 is the IL15 α-receptor. IL15 is a homeostasis molecule that tells the cell to continue surviving. This marker is therefore used to distinguish effector populations from naïve and memory populations. Both naïve and memory populations will express CD127 in high amounts on the cell surface while the effector population will have low or no expression (Kalia, Sarkar, & Ahmed, 2010).

**CD62L**

CD62L is an L-selectin that is used for homing naïve T leukocytes to lymphoid tissue. Tissue close to the lymphoid tissue contain P and E selectins which interact to bring the T cells to the correct area. Following activation, the L-selectins disappear on the cell’s surface. Therefore, CD62L is used to distinguish between naïve, unactivated T cells and activated T cells. Much like CD 127, CD62L’s expression is high in naïve and memory cells but is low or nonexistent in the effector population (Kalia, Sarkar, & Ahmed, 2010).
**Granzyme-B**

Granzyme B is a molecule present in CTLs that is used in the process of killing infected target cells. Granzyme B activates the Caspase 3 which in turn activates a nuclease downstream which degrades the DNA in the nucleus and causes the cell to undergo apoptosis. This was selected as a marker because it is only present in activated effector T cells. It is used to distinguish the Granzyme B high population, which is comprised of effector T cells. The low populations would be made up of naïve and memory cells.

**CRTAM**

This is the major experimental protein of the study. All cells were stained for this to determine the levels at which it was expressed on the cell’s surface or internally.

**KLRG-1**

KLRG-1 is a surface protein that is used to differentiate effector populations from naïve and memory. Typically, the effector populations express high levels of KLRG-1 while naïve and memory T cells express low levels. (Kalia, Sarkar, & Ahmed, 2010)

**Thy1.1**

Thy 1.1 is a surface protein that characterizes P14 CD8 T cells. The cells that are tagged with Thy 1.1 are antigen specific against LCMV, as they are derived from cells that have been exposed to the virus before. This is used to differentiate the donor population of CD8 T cells from the endogenous population of CD8 T cells.
Cellular Stains

Once cells have been processed and have been suspended in FACS, they are ready to be plated. Based on the size of the pellet created in the bottom of the test tubes, between 50 µL, 60 µL, or 70 µL of the cells from the test tube will be transferred into a labelled U-bottom 96 well plate. Bring the well up to 200 µL by adding the appropriate amount of FACS based on the initial amount of cells that were put into the well. The plate was spun down for 2 minutes at 1800 RPM at 4°C. Following this, the bottom of the plate was checked to ensure a pellet formed. The media was then flicked off and the top of the plate was dabbed on a paper towel to remove residual media without flipping the plate back over. The plate was then covered again and tapped lightly to resuspend the cells.

The staining process began by adding 50 µL of the FACS mixed with the staining panel antibodies into each well that needed cellular staining. CRTAM was stained for using CRTAM PE in a 1:100 dilution. A multichannel pipette was used to mix each well. The plate was then left to incubate at 4°C in the dark for 45 minutes. Following incubation, 150 µL of FACS were added to each well to bring the total volume of each well up to 200 µL. The plate was then spun down for 2 minutes at 1800 RPM at 4°C. The media was flicked and wiped away and the pellet was resuspended. The plate was then washed two more times each time with 200 µL of FACS with spinning down after each wash at the above time, speed, and temperature.

Intracellular Stains

This procedure usually follows the cellular staining process, but does not have to if the sample being stained is only receiving intracellular stain. In the cases that this occurred, the
sample was left to sit in 50 µL of FACS, free of cellular staining antibodies, while the cellular stain took place. Otherwise, the intracellular samples were treated the same as the cellular stains.

The plate was spun down for 2 minutes at 1800 RPM at 4°C. The media was flicked, the plate was wiped of excess media, and cells were resuspended. To fix the cells and make the cell membrane more porous, 70 µL of 1XCytofix/CytoPermit was added to each well. The plate was left to incubate for 20 minutes at 4°C in the dark. Following incubation, 130 µL of 1XPerm/Wash buffer (10X diluted with DW) was added to each well to bring the total volume in each to 200 µL. After fixing and permeabilizing the cell membrane, Perm/Wash was used to wash the cells. The plate was then spun down for 2 minutes at 2200 RPM at 4°C. The media was flicked and wiped away and the cells were resuspended. The cells were washed two more times; each time they were washed in 200 µL of Perm/Wash and spun down for 2 minutes at 2200 RPM at 4°C.

After the cells have been spun down after the last wash, 50 µL of the Perm/Wash with the intracellular cellular staining antibodies were put into each well. The plate was then left to incubate for 45 minutes at 4°C under dark conditions. Following the staining, 150 µL of Perm/Wash was added to each well. The plate was then spun down for 2 minutes at 2200 RPM at 4°C. This was followed by two more washes with 200 µL of Perm/Wash and two more spin cycles for 2 minutes at 2200 RPM at 4°C. After this, there were two more washes each with 200 µL of FACS and two more spin cycles at the above specifications.

If the same was acquired that day, then the cells were resuspended in 100 µL of FACS and 100 µL of 4% paraformaldehyde (PFA) dissolved in PBS. If the sample was going to be acquired the following day, then the FACS/PFA solution mixed in each well was left on for 300 minutes at 4°C in dark conditions. After the incubation, the cells were washed twice in 200 µL of FACS and spun down twice for 2 minutes at 2200 RPM at 4°C. The sample was then stored in dark conditions at 4°C in 200 µL of FACS. The most a sample had to wait before acquisition was three days and this occurred only with early screening data.
Flow Cytometry

Flow cytometry is a technique, performed by a flow cytometer, which uses laminar flow and laser excitation to stimulate fluorochromes attached to antibody-labelled cells to sort and count cell populations. Cells are forced through a tube using laminar flow, which causes the cells to pass through a laser beam one cell at a time. The fluorochromes on the antibodies on the cell are excited and fluoresce. Sensitive multiplier tubes pick up this fluorescence as well as scattered light and collect information on the size and granularity of the cell as well as the expression of the surface protein based on intensity of the stain. Once the cell is counted, the mixture is atomized so that one cell is in one droplet and is into the cell sorter. There, charged plates diverge the cells into a given channel, set by particular voltages. Each channel can correspond to a particular marker or label. Once the cells have been sorted, graphs plotting one particular marker versus another can be populated. This raw data that is produced can be analyzed by FloJo to compensate for background noise, to create photo gates that focus on a specific population, and finally to create plots that show a particular population versus the target protein of interest – in this case CRTAM.

Experiment 1: Broad Screen

To begin, a broad screen was performed. The purpose was to identify if effector or memory populations were expressing high levels of CRTAM against a naïve population as a control. This experiment was also used to perfect isolation and staining techniques. This first screen focused on three groups of mice: naïve, day 8, and day 30 mice. The naïve had not yet been infected and acted as a control. Day 8 mice are considered to be expressing effector cells and adaptive response should be robust by this time point. It was therefore selected to represent
the effector stage. By day 30, all effector cells have died off or differentiated into memory T cells. Therefore, this time point was selected to represent the memory T cell population.

This first screen used blood samples only. There was a single mouse per experiment/control group. A single capillary of blood was taken from each mouse and processed based on the procedures detailed out in the PBMC isolation section.

**Experiment 2: Day 3 Time Points**

This time point experiment was the first cell transfer experiment performed. Three B6 mice each had 1 million mature CD8 T cells transferred in from the spleen of a P14 Thy 1.1 mouse. They were given less than 24 hours for the transfer to acclimate and then the mice were all infected with LCMV at the same time. Three days later, the mice were sacrificed via CO$_2$ anesthesia and cervical dislocation. Spleen and blood samples (cardiac puncture) were acquired and processed following the protocols on PBMC and splenocyte isolations. The spleen samples were stained three separate ways: cellular stain only, intracellular stain only, and both cellular and intracellular stains. Blood samples were given cellular and intracellular stain. CRTAM was stained for both cellularly and intracellularly. Controls for CRTAM expression were taken from the inactivated CD8 T cell populations instead of a naïve mouse.

**Experiment 3: Day 3, 4, 5 Time Points**

The experimental design for this experiment was run similarly to the previous three experiments. Three experimental groups were set up with two control groups. Each experimental group was composed of two chimera mice prepared according to the protocol described in the Chimera Mouse section above. The first experimental group was exposed to LCMV for 3 days...
before they were sacrificed. The second experimental group was exposed to LCMV for 4 days and the third was exposed for 5 days. The first control group was a naïve P14 mouse, and the second was memory P14 mouse⁵. To ensure uniformity, all mice were sacrificed on the same day. This meant a staggered infection regiment. First, all mice from all experiment groups had 1 million cells transferred into their thymus. Day 5 mice were infected within 24 hours of the transfer. Day 4 mice were infected 48 hours after transfer. Day 3 mice were infected 72 hours after thymocyte transfer. The naïve and memory mice were both sacrificed with the experimental groups. Mice were sacrificed via CO₂ anesthesia and cervical dislocation. Blood was collected via cardiac puncture and the spleen was removed and placed into 4 mL or RPMI. Only a cellular CRTAM stain was used in this experiment. Blood and spleen samples were processed and then stained and analyzed by flow cytometry. For full descriptions of the procedures, please read above sections.

**Experiment 4: Day 5 Full Tissue Analysis**

Following experiment 3, a final experiment was constructed to test if the subpopulation of the donor antigen specific CD8 T cells were accumulating in a particular tissue in the body. The experiment consisted of an experimental group of two P14 day 5 mice and a single control B6 naïve mouse. The day 5 P14 mice were created by transferring 1 million Thy 1.1 P14 CD8 T cells purified from the spleen of a P14 mouse. Within 12 hours after the cell transfer, the mice were infected. After five days of infection, all mice were sacrificed by CO₂ anesthesia followed

⁵ At least 30 days post infection
by cervical dislocation. To do full tissue analysis, blood, spleen, liver, and lymph nodes samples were collected. A cellular CRTAM stain was used. All samples were processed, stained, and acquired using the techniques described in above sections.
Chapter 4

Conclusion

Based on the results, it can be concluded that CRTAM is upregulated between days 3 and 8 on a small subset of the population of donor antigen specific CD8 T cells. In the samples where this occurred, there was anywhere from a half to a full greater logarithmic difference between the expression of CRTAM in the time point sample versus the naïve or memory control. There was a significantly higher population of population of CRTAM$^{\text{High}}$ Donor T cells in the lymph nodes, supporting previous research suggesting CRTAM and Necl-2 helped to retain T cells for proliferative events. The population difference was around half a logarithmic difference higher compared to CRTAM$^{\text{High}}$ populations in the liver, blood, or spleen.

This research could be furthered by isolating the CRTAM$^{\text{High}}$ T cells and further characterizing any distinguishing proteins and DNA/RNA structures to discover how and why they form. This could lead to the discovery of a smaller subset of T cells that specialize in aiding late phase activation, retention, and proliferation of T cells. Expansion of the experiment could include time points such as days 2, 6, 7, 9, 10, 15, 20, and 25. The purpose of this would be to determine the exact expression timeline of CRTAM. This could help determine if CRTAM has a role in the differentiation of effector T cells into memory T cells.
Appendix A

Glossary

1. Chemokines – Signaling molecules that cause cells to move up the chemokine gradient to the location of the chemokine’s production.

2. CTL – (Cytotoxic T Lymphocyte) is a type of T cell that uses killing pathways, such as the Granzyme B pathway, to kill infected cells within the body.

3. Cytokine – signaling molecule which causes effects in target cells.

4. Effector – activated by contact with APCs; fulfilling roles in cytotoxicity, etc.

5. IFN-γ – a cytokine tied to activation of macrophages in the innate and the adaptive immune response.

6. IL-17 – pro-inflammatory cytokine

7. IL-22 – inflammatory cytokine used for signal transduction and activation of intracellular kinases.

8. Intracellular – inside of the cell.

9. PBMC – (Peripheral Blood Mononuclear Cells) are the major constituent that make up blood. Within this broad category of cells includes lymphocytes; the target of this paper.

10. Memory – long lived effector cells that provide immunological memory and act a stem cells for cells in case of reinfection by the antigen specific pathogen.

11. MFI – (Mean Fluorescence Intensity) shows the average fluorescent activity in a given channel. This indicates usually where the most activity falls.

12. Naïve – uninfected by LCMV; not activated by antigen.
Appendix B

Raw Data Figures

Figure 8 – Exp 1 Raw Data
Figure 9 - Exp 2 Raw Data
Figure 10 - Exp 3 Raw Data PBL
Figure 11 - Exp 3 Raw Data SPL
Exp 4: Day 5 Tissue Analysis

Figure 12 - Exp 4 Raw Data
BIBLIOGRAPHY


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