Visualizing CD8$^+$ T cell responses to foreign and self-antigen

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i
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Dedication

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Abstract

CD8+ T cells can recognize any infected cell of the body, making them essential in the immune response against intracellular pathogens. A critical function of CD8+ T cells is the directed killing of target cells through cytolysis. This mechanism is dependent on direct cell-cell contact, which makes the migratory capacity of CD8+ T cells paramount to their successful immune response. The small intestine (SI) is the biggest mucosal surface between the host and the environment. The immune system in this compartment must actively eliminate infection while maintaining tolerance to normal flora, self, and food-antigen. Using two-photon laser scanning microscopy, I evaluated foreign-and self-specific CD8+ T cell motility in the SI. I found that foreign-antigen specific CD8+ T cell behavior varied throughout infection, and was independent of the $\alpha_E$ integrin. Interestingly, self-specific CD8+ T cells were initially reactive to self-antigen in vivo but this behavior was altered after further tolerance induction. These studies inform our understanding of the requirements for effective CD8+ T cell immunosurveillance in the SI. I also evaluated what characterizes and contributes to a self-specific CD8+ T cell response to protein in the SI. Using a heterologous prime-boost-boost (HPBB) approach, I generated functional self-specific CD8+ T cells. This response matured throughout boosting, showing the potential of self-specific CD8+ T cells. I also used HPBB to evaluate foreign-antigen specific CD8+ T memory development and showed that the time interval between each boost impacts CD8+ T cell memory longevity.

vii
# Table of Contents

Acknowledgements...........................................................................................................i

Dedication.............................................................................................................................vi

Abstract................................................................................................................................vii

List of Figures.........................................................................................................................xii

1 Introduction.........................................................................................................................1

1.1 CD8+ T cell response of the adaptive immune system.................................................2
  1.1.1 Naïve CD8+ T cell specificity and activation.......................................................2
  1.1.2 CD8+ T cell effector functions and memory formation.........................................5
  1.1.3 Heterologous prime-boost strategies to generate antigen-specific CD8+ T cells..........................................................8

1.2 CD8+ T cell motility and tissue surveillance.................................................................10
  1.2.1 Basic cell movement..............................................................................................11
  1.2.2 T cell migration in secondary lymphoid organs..................................................12
  1.2.3 T cell migration in nonlymphoid tissue................................................................14
  1.2.4 Assessing immune cell motility in situ affords insight into disease pathogenesis.................................................................................................................................16
  1.2.5 Immune cell migration in the small intestine.......................................................17

1.3 Self-specific CD8+ T cell responses.............................................................................20
  1.3.1 Central and peripheral mechanisms maintaining T cell tolerance to self-antigen..........................................................20
  1.3.2 Low avidity self-specific T cells generate immune responses..........................24
  1.3.3 Enhancing the self-specific T cell response to tumor antigen..........................25
  1.3.4 Generating a self-specific T cell response to protein in the small intestine............26

2 Shortened intervals during heterologous boosting preserve memory CD8+ T cell formation but compromise longevity.........29
2.1 Introduction................................................................................................................30

2.2 Results........................................................................................................................33

2.2.1 Short intervals between heterologous boosts generate large numbers of Ag-specific CD8+ T cells.................................................................33

2.2.2 Ag-specific CD8+ T cells generated with short HPBB migrate to and populate multiple non-lymphoid tissues.........................................................35

2.2.3 Ag-specific CD8+ T cells have similar functionality regardless of immunization interval length.................................................................38

2.2.4 Ag-specific CD8+ T cells generated through short-boosting display a memory phenotype..................................................................................42

2.2.5 Short-boosted Ag-specific CD8+ T cells display altered metabolic properties compared to long-term boosted cells........................................44

2.2.6 Ag-specific CD8+ T cells generated through short boosting contract without stabilization, despite displaying a memory phenotype........47

2.3 Discussion..................................................................................................................48

2.4 Materials and methods..............................................................................................54

2.4.1 Mice and infections.................................................................................................54

2.4.2 Intravascular staining, isolation of lymphocytes and flow cytometry.....................55

2.4.3 Recall and protection assays...................................................................................57

2.4.4 Metabolic assays.....................................................................................................58

2.4.5 Statistical analysis....................................................................................................59

3 Visualizing CD8+ T cell dynamics in the small intestine...........................................60

3.1 Introduction................................................................................................................61

3.2 Results........................................................................................................................63

3.2.1 Ag-specific CD8+ T cell motility in the small intestine is multifarious during the course of infection...............................................................63

3.2.2 Tissue micro-architecture dictates CD8+ T cell motility in the small intestine.................................................................................................67

3.2.3 CD8+ T cell locomotion in the small intestine is independent of αE integrins.................................................................................................70
3.2.4 Resident memory CD8+ T cell migration is locally restricted........73

3.2.5 Self-specific CD8+ T cells are arrested in the presence of cognate antigen.................................................................76

3.3 Discussion..................................................................................................................................................79

3.4 Materials and methods.................................................................................................................................84
  3.4.1 Mice and infections.................................................................................................................................84
  3.4.2 Tissue harvesting and processing.........................................................................................................85
  3.4.3 Flow cytometry and peptide stimulation.............................................................................................86
  3.4.4 Tissue freezing, immunofluorescence and microscopy.....................................................................87
  3.4.5 Photoconversion of OT-I Kaede CD8+ T cells....................................................................................88
  3.4.6 Two-photon laser scanning microscopy and analysis........................................................................88
  3.4.7 Statistical analysis...............................................................................................................................89

4 Repeated antigen stimulation generates functional self-specific CD8+ T cells.........................................................90

4.1 Introduction................................................................................................................................................91

4.2 Results......................................................................................................................................................94
  4.2.1 Repeated antigenic stimulation generates a large, functional population of self-specific CD8+ T cells..................................................................................................................94
  4.2.2 Self-specific CD8+ T cells exhibit a canonical memory phenotype................................................97
  4.2.3 Self-specific CD8+ T cells are undetectable in the small intestine...................................................98
  4.2.4 Self-specific CD8+ T cells bear TCR of low affinity compared to foreign antigen-specific T cells.........................100
  4.2.5 Kb-SIINFEKL-specific CD8+ T cells form OVA bearing mice can be detected at earlier time points with SIINFEKL peptide stimulation........................................................103
  4.2.6 Kb-SIINFEKL-specific CD8+ T cells from I-FABP OVA mice are polyclonal..........................................105
  4.2.7 Kb-SIINFEKL-specific CD8+ T cells can be detected in OVA bearing mice using tetramer stabilization and enrichment techniques..............................................................108

4.3 Discussion...............................................................................................................................................109

4.4 Materials and methods.............................................................................................................................116
4.4.1 Mice and infections.................................................................116
4.4.2 Tissue harvesting and processing.................................117
4.4.3 Flow cytometry and peptide stimulation..............118
4.4.4 Naïve CD8+ T cell tetramer enrichment..............119
4.4.5 BrdU experiments...............................................................120
4.4.6 Statistical analysis.................................................................120

5 Conclusions..................................................................................121

5.1 The potential of self-reactive CD8+ T cells.........................122
  5.1.1 Self-reactivity: A blessing in disguise?......................122
  5.1.2 Heterologous prime-boost-boost in practice........125

5.2 CD8+ T cells: the shape shifters of the immune system.......126
  5.2.1 If T cells could talk...........................................................127

References....................................................................................129
List of Figures

2.1 Short-boosting intervals generate large numbers of Ag-specific CD8+ T cells
.................................................................................................................................34

2.2 Short-boosted Ag-specific CD8+ T cells migrate to and populate multiple non-lymphoid tissues....................................................................................................................36

2.3 Ag-specific short-boosted CD8+ T cells in certain non-lymphoid tissues display a resident memory phenotype........................................................................................................38

2.4 Short-boosted Ag-specific CD8+ T cells express granzymes B and protect against viral infection.......................................................................................................................39

2.5 Short-boosted Ag-specific CD8+ T cells are functional........................................40

2.6 Short-boosted Ag-specific CD8+ T cells have a memory phenotype.....................43

2.7 Short-boosted Ag-specific CD8+ T cells exhibit altered metabolic function........46

2.8 Short-boosted Ag-specific CD8+ T cells do not form stable memory....................48

3.1 Ag-specific CD8+ T cell motility varies during LCMV infection..........................66

3.2 Tissue architecture dictates CD8+ T cell motility in the small intestine............69

3.3 CD8+ T cell motility in the small intestine is independent of CD103.....................72

3.4 Memory CD8+ T cells in the small intestine have narrowed migration..............75

3.5 Self-specific CD8+ T cells in the small intestine are slowed in the presence of autoantigen.................................................................77

4.1 HPBB generates functional self-specific CD8+ T cells................................................96

4.2 Self-specific CD8+ T cells exhibit a memory phenotype..........................................99

4.3 Kb-SIINFEKL-specific CD8+ T cells in I-FABP OVA mice have TCR of lower affinity compared to B6.................................................................101

4.4 Self-specific CD8+ T cells which are undetectable by tetramer can be visualized with peptide stimulation.................................................................105

4.5 Self-specific CD8+ T cell Vβ clonality is distinct among mice............................107

4.6 Naïve Kb-SIINFEKL-specific CD8+ T cells in I-FABP OVA mice.........................109
Chapter 1

Introduction
1.1 **CD8\(^+\) T cell response of the adaptive immune system**

The immune system is composed of the innate and adaptive responses, which work together to protect the body from invading pathogens. Cells of the innate immune system, like dendritic cells (DCs), recognize broad components of microbes, become activated, and rapidly respond to infection. Activated innate immune cells signal to the adaptive immune system that there is an ongoing infection. In contrast to innate cells, cells of the adaptive immune system, like CD8\(^+\) T cells, have highly specialized receptors that recognize components of particular pathogens, making them “experts” at eliminating the microbe they are specific for. When adaptive immune cells recognize their cognate ligand, they expand in number and elicit effector functions to clear infection. Following pathogen clearance, many of the responding adaptive immune cells die, but a pool of memory cells remains to fight a potential re-infection. Understanding how effective adaptive immune memory responses are generated is a goal of vaccination.

1.1.1 **Naïve CD8\(^+\) T cell specificity and activation**

Pathogens are infectious agents such as bacteria, viruses, parasites and fungi that can cause disease. The immune system’s function is to combat any potential pathogen the host encounters. For this reason, cells of the adaptive immune system possess receptors that bind to specific components originating from a particular pathogen. CD8\(^+\) T cells are cells of the adaptive immune system
that express T cell receptors (TCRs) that recognize specific protein fragments, called peptides, in the context of major histocompatibility complex I (MHCI) \textsuperscript{1,2}. All nucleated cells of the body express MHCI, and this is a way for infected cells to present microbial peptides to CD8\textsuperscript{+} T cells, so that T cells specific for that microbe can detect infection and contain spread of disease \textsuperscript{3–7}. For this reason, CD8\textsuperscript{+} T cells are essential in the host-defense against intracellular pathogens, like viruses.

CD8\textsuperscript{+} T cell receptor generation occurs in the thymus during T cell development, where the TCR genes undergo random rearrangement facilitated by recombination-activated gene (RAG) enzymes \textsuperscript{8–10}. Because it cannot be predicted what infections a host may acquire, the immune system must compromise the numerical quantity of any one given cell receptor specificity to ensure a greater diversity of T cells overall \textsuperscript{11,12}. For example, only a few CD8\textsuperscript{+} T cells out of the millions present in the host may have a receptor that recognizes a component of the chickenpox virus, while another few T cells have a receptor specific for a part of the measles virus. Previous to coming in contact with a cognate peptide:MHCI expressing cell, the CD8\textsuperscript{+} T cell is considered to be functionally naïve, or antigen in-experienced. In mice, it has been predicted that the naïve precursor frequency of any given CD8\textsuperscript{+} T cell specificity is 10-1000 cells total \textsuperscript{11,12}.

With such a small number of cells expressing a certain TCR, it is difficult to imagine that T cells are able to “find” the infected cell they may be specific for, as the entire host would be difficult for 10 cells to survey. To account for this, the
immune system ensures that naïve T cells come in contact with the antigen that they are specific for by restricting their circulation through secondary lymphoid organs (SLOs), including the blood, lymphatic system, and spleen\textsuperscript{13,14}. During an infection, components of the invading microbe called pathogen-associated molecular patterns (PAMPs) engage pattern recognition receptors (PRRs) on DCs, which induce drastic morphological changes and activation of DCs \textsuperscript{15}. The major function of DCs is to phagocytose and process antigen into peptide fragments to present on MHC \textsuperscript{16,17}. Antigen-bearing DCs, as well as free antigen, can travel from the site of infection through lymphatics to the lymph node where they are then centralized with naïve CD8\textsuperscript{+} T cells coming from the circulation \textsuperscript{18,19}. Concentration of both T cells and antigen-bearing DCs in the lymph node increases the chances that a naïve T cell will meet its appropriate antigen-presenting cell (APC). Once a CD8\textsuperscript{+} T cell comes in contact with an APC expressing the peptide:MHCI complex its TCR is specific for, the CD8\textsuperscript{+} T cell becomes activated. This is the first step of a three-signal process required for complete differentiation into an effector cell. After TCR engagement, the CD8\textsuperscript{+} T cell must receive co-stimulation through CD28 binding to CD80/86 molecules on the DC for optimal proliferation and IL-2 production \textsuperscript{20–24}. Finally, cytokines from surrounding cells, such as IL-12 and IFN-\(\alpha/\beta\), bind to receptors on the T cell, providing signals for maturation into a functional effector T cell \textsuperscript{25–27}. Directly following activation, CD8\textsuperscript{+} T cells undergo massive clonal proliferation and epigenetic modifications that result in the T cells becoming more efficient responders to infection \textsuperscript{28,29}. 
1.1.2 CD8+ T cell effector functions and memory formation

Once a CD8+ T cell has become activated by a DC, that CD8+ T cell no longer requires co-stimulation to act and is able to perform several important effector functions to eliminate infected cells. Upon ligation with a target cell, CD8+ T cells secrete cytotoxic granules that instigate programmed cell death of the target cell. These granules consist of perforin and granzymes, which are complexed with serglycin and are stored in an active form within the CD8 T cell.[30,31] Perforin directs the entry of the granule contents into the cytosol of the infected target cell, where granzymes, which are serine proteases, are able to activate a proteolytic caspase cascade which degrades cellular DNA.[32] This process is highly controlled, with only target cells bearing the specific antigen the CD8+ T cell TCR recognizes being killed, while bystander cells are left healthy. This prevents massive tissue damage to the host. CD8+ T cells also release the cytokines, such as IFNγ, which increase expression of MHCI and peptide loading proteins to optimize the likelihood that T cells will be able to recognize their target cell.[33]

Since T cells are dependent on contacting their target cell to facilitate cytotoxicity, it is vital to their success that they gain entry to tissues to access cells at the infection site. To this end, CD62L, a ligand allowing access to SLOs, is decreased so activated CD8+ T cells can no longer circulate through lymph nodes.[34,35] Instead, expression of the integrin very late antigen-4 (VLA-4) is upregulated, which binds to vascular cell adhesion molecule-1 (VCAM1), a vascular endothelium adhesion molecule that is highly expressed at sites of...
inflammation. Cell-adhesion molecules, such as lymphocytes function associated antigen-1 (LFA-1, also known as CD11a), CD2, and CD44 are also increased, helping to facilitate tissue entry\textsuperscript{36–38}. Certain integrins allowing specific tissue access may be imprinted on CD8\textsuperscript{+} T cells depending on the site of activation\textsuperscript{39}. For example, cells activated in the mesenteric lymph node that drains the small intestine (SI) will upregulate $\alpha_4\beta_7$ in response to retinoic acid, which is in high abundance in this compartment. This integrin binds to mucosal vascular addressin cell adhesion molecule (MAdCAM-1) on vascular endothelial cells of the SI and is required for entry into this area\textsuperscript{40,41}.

After the infection is cleared, much of the expanded population antigen-specific CD8\textsuperscript{+} T cells eventually dies, but a portion is retained as long-lived memory T cells\textsuperscript{42}. This remaining population is substantially larger than the previous naïve pool, and can respond rapidly to re-infection with the same pathogen\textsuperscript{43–45}. Memory T cells are better equipped to fight infection than naïve T cells because they have pre-synthesized cytotoxic granules, are more readily proliferative, and do not need priming in LNs to start responding. There are many types of memory T cells that vary in their anamnestic response and migration patterns\textsuperscript{46}. This was largely defined in seminal work published by Sallusto and Lanzavecchia nearly two decades ago that found rather than a monolithic memory cell pool, there was indeed a diverse population of cells each possessing distinct functional properties\textsuperscript{47,48}. Central memory T cells (T\textsubscript{CM}), regain expression of CD62L, and circulate through the spleen and lymph nodes following primary infection clearance. Effector memory T cells (T\textsubscript{EM}) maintain
circulation through non-lymphoid tissues (NLT) and are able to kill more rapidly than T_{CM}. Recently, an additional subset of memory T cells has been shown to reside long-term in tissues without re-circulating through other areas. These tissue-resident memory cells (T_{RM}) are able to rapidly quench invading pathogens at the site of infection^{49,50}.

Research is currently ongoing to understand how each flavor of memory T cell is generated^{51}. An important study out of the Busch lab showed that a single adoptively transferred naïve CD8^{+} T cell was able give rise to both effector T cells and disparate memory T cell subsets^{52}. This suggests that naïve T cells have the potential to become both effector and memory T cells, and there are not distinct populations within naïve T cell precursors that are destined to become singly effector or singly memory T cells. Other studies have showed roles for both inherent cell properties, such as TCR affinity, and environmental cues, such as cytokines, for the generation of distinct memory cells subsets, but much still remains to be discovered in this area^{46,53-55}.

More is known regarding how a CD8^{+} T cell survives the contraction phase of the effector response and goes on to become a long-lived memory cell. Memory cell potential does not appear to be equal in all effector cells. In model systems measuring the antigen-specific response to lymphocytic choriomeningitis virus (LCMV) or Listeria monocytogenes (Lm), a portion of T cells that expresses CD127, CD27, and anti-apoptotic marker B cell lymphoma-2 (BCL-2), lacked killer cell lectin-like receptor G1 (KLRG1) were shown to be intrinsically better at persisting than other cells^{56,57}. The transcription factor
eomesodermin (Eomes) has also been deemed important, as CD8+ T cells lacking Eomes are unable to generate a canonical long-lived memory cell phenotype of CD122, CD62L, CXCR3, and CXCR4 expression \(^{58,59}\). Our understanding of the impact of immunometabolism on long-term T cell survival is also burgeoning. Effector T cells use aerobic glycolysis to rapidly synthesize lipids needed for the tremendous bioenergetic demands of clonal expansion and cytotoxic molecule production \(^{60,61}\). During memory generation, T cells switch to mitochondrial oxidative phosphorylation, which helps CD8+ T cells survive the changing nutrient availability and tissue microenvironment after infection clears \(^{62–65}\). In studies inhibiting glycolytic metabolism, CD8+ T cell memory was enhanced \(^{63,66}\). These observations indicate that the bioenergetic capacity of a CD8+ T cells can influence its fate after pathogen clearance.

Memory formation is the biological foundation of vaccination, which aims to generate a memory immune response specific for a particular pathogen. Therefore, if a person encounters the pathogen they are vaccinated against, memory cells will rapidly respond and prevent illness.

### 1.1.3 Heterologous prime-boost strategies to generate antigen-specific CD8+ T cells

Arguably no other biomedical development has had a larger impact on human health and the prevention of disease than vaccination. Due to vaccination, diseases that once caused life-long deleterious effects or death, such as polio, measles and smallpox, are now considered diseases of the past. The
protection afforded by the vast majority of licensed vaccines is antibody based, whereby neutralizing antibodies are able to rapidly contain invading microbes before they are able to enter host cells. There are still many serious infections, such as HIV, tuberculosis, and malaria, for which there is not an effective vaccine.

As CD8+ T cells are key players in the immune response to intracellular infection, successful vaccination against these diseases may require an efficacious CD8+ T cell response.

Many strategies to generate CD8+ T cells use a prime-boost approach. Initial stimuli, such as DNA or an infectious vector containing a desired antigen, are used to expand or “prime” a particular population of antigen-specific CD8+ T cells. Typically, at a memory time point after priming, this antigen-specific CD8+ T cell population is enlarged numerically, or “boosted” with an infectious vector containing the same antigen used to prime the response. A system using heterologous prime-boost-boost (HPBB) has been shown to generate large numbers of highly functional CD8+ T cells that survive long-term. Here, the host is exposed to three different microbial vectors containing a common protein or epitope to stimulate antigen-specific CD8+ T cells of interest.

While HPBB generates protective and long lasting CD8+ T cells, the time interval between each boost is at least several weeks to months, leaving the host vulnerable to infection during time to vaccine completion. This may not be ideal in situations, such as a fast-moving outbreak, where protection needs to be achieved quickly. We have also learned from licensed vaccines that require boosting, this elongated time frame can result in lack of compliance and failure to
complete vaccination regimens. Taken together, these concerns suggested that perhaps a decreased total immunization time may be beneficial in designing an optimal CD8+ T cell vaccine. However, it was not known the impact shortened boost intervals would have on CD8+ memory T cell formation and function. It was possible that curtailed rest periods between inflammatory events might be detrimental to the response, causing defective effector functions and leading to CD8+ T cell exhaustion. In my thesis work, I sought to examine the impact of two-week intervals between HBPP on the generation and function of memory CD8+ T cells. My studies demonstrated that while brief intervals between HPBB were able to generate functional CD8+ T cells, this came at the cost of generating long-term memory. We believe that dissecting how optimal memory CD8+ T cells are generated and how they migrate in tissues to survey for disease will better inform vaccine design against many recalcitrant pathogens.

1.2 CD8+ T cell motility and tissue surveillance

CD8+ T cells are dependent on directly contacting other cell types for their activation, effector function, and anamnestic response. A cornerstone in a successful CD8+ T cell immune response and containment of pathogens is the ability to migrate within lymphoid organs and tissues to seek out and destroy infected or cancerous cells. T cells must act quickly; adjusting their migration to their surroundings, and crossing many diverse tissue barriers, making them one of the most adaptable and exploratory cell types in the body. As motility is
intimately linked to effective CD8+ T cell surveillance, evaluating locomotive behavior in situ is paramount to fully comprehending how an immune response is mediated within non-lymphoid tissues (NLTs). This has further implications for effective therapy and vaccine design aimed at generating protective immune responses within the tissues.

1.2.1 Basic cell movement

General motility is largely controlled by the extremely coordinated process of actin polymerization within the cell cytoplasm. In response to extracellular cues, the actin network within the cell remolds. This leads to cell protrusions, called lamellipodia, which allow for the cell to assume directionality. Lamellipodia are also important for environmental probing, cell body translation, and extravasation through tissues. The trailing edge of the cell, or filopodia, contains bundles of actin filaments that are coupled to the motor protein, myosin. Myosin slides along actin fibers, and contracts the cell body, driving the cell forward.

Cells are able to quickly sense and respond to changing environments through surface receptors that are attached to the cytoskeleton and molecular motor proteins. These include cell adhesion molecules (CAMs), such as cadherins, members of the immunoglobulin super family, selectins, and integrins. These molecules facilitate binding to other cells or extracellular matrix proteins, such as proteoglycans, collagens, and multi-adhesive matrix proteins,
like fibronectin. Cells are also able to sense chemokines, and follow them along a concentration gradient, known as chemotaxis.

1.2.2 T cell migration in secondary lymphoid organs

Our understanding of CD8+ T cell motility in situ has burgeoned with the development of two-photon laser scanning microscopy (TPLSM). Maria Goeppert-Mayer first proposed the idea of two-photon absorption in her thesis dissertation in 1931. Essentially, a fluorophore is excited by near simultaneous absorption of two-photons near infrared wavelength, providing nearly half each of the energy that is required to achieve full fluorescence emission. Fluorescence is then restricted to the focal spot formed by the microscope objective, which gives an inherent optical sectioning effect. Lower energy wavelengths endure less light scattering, enabling deeper penetration of tissue and resulting in negligible photodamage or photobleaching. Successive images are acquired by recording the fluorescence signals at sequential focal planes every 10-30 seconds for periods that can last up to a few hours. These data are then compiled into a time-lapse movie.

In the last decade, multiple groups have used TPLSM to characterize the motility of naïve T cells in SLOs. In the diffuse cortex of the lymph node, naïve T cells have a stochastic migration pattern and move rapidly at 10–12 µm/min, supporting their pro-migratory search for antigen loaded-APCs. This behavior is not dependent on integrins, but rather, is guided by the fibroblastic
reticular cell network and follicular dendritic cells in the lymph node cortex \(^{82,86,87}\). It has also been shown using pertussis-toxin treatment that g-protein coupled receptor (GPCR) signaling of G\(\alpha_i\) isoforms is important for maintenance of naïve T cell velocity and tight turning angles in the lymph node \(^{88–90}\). Among the pertussis-toxin sensitive receptors, CCR7 showed a significant role, and studies using CCR7 knock out models showed a substantial reduction in T cell velocity \(^{88–91}\). Accordingly, due to disparate chemokine expression and structural differences within the lymph node, there are also regional differences in T cell motility. In the follicle edge, T cell motility is non-random while in the subcapsular sinus, T cells are much slower than in the diffuse cortex \(^{86,92}\). T cells in the medullary region are also slower, moving at 6\(\mu\)m/min \(^{93,94}\).

When T cells encounter cognate antigen presented by DCs in the lymph node, stable contacts are formed and can last for hours \(^{84,95,96}\). As T cells are inherently motile, this suggested that a stop signal was necessary for long-term interactions with DCs. Early studies investigating this concept in the 1990s showed that TCR engagement resulted in T cell arrest due to actin remodeling of microtubules near proximal to the contact site \(^{97,98}\). Later studies using TPLSM in in vivo models showed that calcium flux correlates with cell arrest upon antigen encounter, whereby cells with the highest calcium levels have the lowest velocities \(^{99,100}\).

After T cell activation in the LN, T cells undergo a period of proliferation and differentiation. Here, activated T cells gain effector functions and specific-
tissue homing receptor expression that facilitates access to NLTs via locally inflamed and resting endothelium.

### 1.2.3 T cell migration in nonlymphoid tissues

Peripheral tissues, particularly mucosal sites, are constantly exposed to potentially infectious microorganisms. T cells surveying NLTs for infection and cancer must be able to migrate through diverse tissue architecture and adapt to their environment. Like in the lymph node, T cell motility in NLTs relies heavily on cell intrinsic, chemical, and environmental cues for guidance.

Considerable research has examined the effect of TCR signaling on T cell motility during the course of infection or disease in NLTs. In skin, using an ear transplant model, both CD4+ and CD8+ T cells were arrested within skin allografts, but not isografts, showing antigen-specific interactions. Furthermore, CD8+ T cells were associated with dead or dying cells inferring a local cytotoxic T cell response. In experiments examining *Toxoplasma gondii* parasitic infection in the brain, CD8+ T cells had reduced velocity and accumulated near parasites. Other studies using cancer models showed that CD8+ T cells initially arrested on tumor cells bearing cognate antigen but after tumor reduction, T cells accelerated. The increase in T cell velocity is presumably needed for antigen specific CD8+ T cells to locate any remaining tumor antigen. The behavior of T cells initially arresting and regaining acceleration once antigen is cleared has been observed in many infectious models and tissues. In influenza A (IAV) infection in the trachea, CD8+ T cells were slowest during
the acute phase of viral infection, but transiently increased velocity on day 9, after infection starts to clear\textsuperscript{111}. Similar phenomena have been observed in IAV infection in the lung\textsuperscript{112}. These studies provide insight into the effector T cell behaviors in response to cognate antigen in NLT. While the field is growing and much has been learned regarding T cell motility at early time points after infection, more research into memory time points is necessary to fully evaluate CD8\textsuperscript{+} T cell surveillance in NLTs.

In addition to cognate antigen, the environmental architecture of tissues can also play a role in governing T cell motility\textsuperscript{103,113,114}. T cells engage with many components of the extracellular matrix, which can be altered during infection. Pathogens can cause global environmental changes altering chemokine expression or disrupting the stromal network impairing T cell surveillance\textsuperscript{115–117}. In the course of \textit{Toxoplasma gondii} infection, fibrous networks in the central nervous system are altered. T cells were able to crawl along the fibrous networks similarly to what has been observed in the lymph node stromal scaffold and did so in a CCL21 guided manner\textsuperscript{110}. Altered T cell motility is also observed in different locations during \textit{Mycobacterium bovis} liver infections. Inside the granuloma, T cell migration is slower and more constrained than compared to T cells in the surrounding sinusoidal network\textsuperscript{118,119}. Additional studies inspecting T cell motility in the skin have also showed architectural influences on motility. In the epidermal layer, CD8\textsuperscript{+} T cells take on an almost dendritic cell like morphology, abandoning their typical amoeboid shape, and are trapped within
the constraints of the contiguous epidermal cells. This restricted CD8+ T cell motility to two-dimensions \(^{49,120,121}\).

Through these and many other studies, it has become appreciated that lymphocyte motility in the SLOs and NLTs is context dependent. This speaks to the vast adaptability of T cells and demonstrates their plasticity in mounting immune responses in varying tissues.

### 1.2.4 Assessing immune cell motility in situ affords insight into disease pathogenesis

Many infectious microbes have evolved to manipulate T cell motility to escape detection and increase spread of disease. Evaluating T cell motility in the tissue during infection has also led to many important discoveries about pathogen immune evasion \(^{101,104,122-124}\). For example, in chronic infection with LCMV, there is prolonged immobility of antigen-specific CD8+ T cells \(^{125}\). In other research evaluating parasite infection, large amounts of parasites present in cysts were undetected and ignored by migrating T cells \(^{109}\). These studies reveal how chronic infection persists in the presence of an active T cell response.

Evaluating cell behavior using TPLSM analysis has also helped to pinpoint cause of disease in some instances. Functional loss of the small adapter protein, signaling lymphocyte activation-associated protein (SAP), in T cells results in lack of germinal center responses in mice and X-linked lymphoproliferative disease development in humans \(^{104,126}\). Imaging studies indicated that the immunodeficiency arose from a shortened duration of contact between B cells
and SAP-deficient CD4+ T cells. This brief interaction was insufficient to provide CD4+ T cell help to B cells for germinal centers formation 127. This study unveils the critical need for stable contacts between cells to facilitate immune functions. Multiple studies indicate that the induction of programmed cell death protein-1 (PD-1) expression on CD8+ T cells overrides cell arrest in response to cognate-antigen 128,129. When self-specific CD4+ T cell responses to islet antigen were evaluated, PD-1 blockade restored normal motility in tolerant T cells and facilitated tissue destruction 128. This study directly demonstrates in vivo evidence for self-reactive side effects that are observed following treatment with checkpoint blockade.

The ability to directly visualize immune cells in vivo has provided insight into normal immune cell migration and function as well as into key components of host-pathogen relationships. Understanding the quantitative parameters that control effective surveillance of NLTs may give insight into how disease dissemination is thwarted. Such findings are pivotal in designing therapeutics and preventative measures for disease where initial containment is critical for protection.

1.2.5 Immune cell migration in the small intestine

The SI represents the biggest mucosal interface between the host and the outside environment. It is the site of many primary infections with the potential for dissemination and, therefore, understanding immune cell surveillance in this compartment is of great interest. Many elegant studies have used TPLSM to
better understand DC motility and luminal sampling in the SI\textsuperscript{130–133}. During the steady state, lamina propria (LP) DCs are sessile, yet actively extend and retract dendrites to probe for antigen\textsuperscript{133}. McDole et al., identified that goblet cells facilitate luminal soluble antigen delivery to DCs in the SI through goblet-cell-associated antigen passages\textsuperscript{132}. It has also been observed that \textit{Salmonella} infection can recruit CD\textsubscript{103}\textsuperscript{+} dendritic cells to the epithelium to sample antigens for presentation in the mesenteric lymph node\textsuperscript{130}.

The SI mucosa has been charged with the task of maintaining tolerance to normal flora and food antigens, while simultaneously preventing infectious pathogen dissemination\textsuperscript{134}. As such, CD\textsubscript{8}\textsuperscript{+} T cell motility in this compartment might be quite different than in other NLTs. Additionally, the gut presents a rather unique tissue architecture for T cells to navigate, consisting of densely packed layers of longitudinal and circular muscle, and the loose connective tissue of the LP in the villi and crypts. A major goal of my thesis work was to understand CD\textsubscript{8}\textsuperscript{+} T cell motility in the SI and to decipher how CD\textsubscript{8}\textsuperscript{+} T cells are able to adequately survey and contain potential systemic threats.

While little is known about antigen-specific CD8\textsubscript{αβ}\textsuperscript{+} T cell responses in the SI, other types of T cell responses have been characterized. CD8\textsubscript{αα}\textsuperscript{+} γδ T cells, which constitute a very large population of intraepithelial lymphocytes (IELs), are very motile and able to migrate between the epithelium and LP of the villi. This migration was dependent on expression of the tight junction protein, occludin, by CD8\textsubscript{αα}\textsuperscript{+} γδ T cells\textsuperscript{135}. Recently, Sujino et al., found similar dynamic
patterns in CD4+ T cells in the SI, which attributed the transcription factor T helper inducing POZ-kruppel factor (ThPOK), to CD4+ T cell maintenance in the epithelial layer 136.

Studies investigating Lm infection of the SI indicate resident CD8+ T cells are required for protection against disseminated disease 137. This and other findings show the importance for CD8+ T cell mediated responses in situ, but antigen-specific CD8+ T cells are relatively rare in this tissue. At a memory time point following LCMV infection, there are only six antigen-specific CD8+ T cells per 1000 nucleated cells 138. Is such a small frequency of cells able to sufficiently sample this large expanse of tissue for infection? For successful pathogen containment, what type of CD8+ T cell “coverage” is necessary in the SI? Studies evaluating CD8+ T cell motility here will inform future therapies targeted at generating tissue resident CD8+ T cells in the SI. A major aim of my thesis work was to determine the mobility requirements for maintaining adequate CD8+ T cell inspection of this expansive tissue. As this likely required a highly mobile and carefully orchestrated CD8+ T cell response, I sought to evaluate both pathogen and self-specific CD8+ T cell locomotion at several time points during an immune response. This work showed a disparate migratory behavior pattern of CD8+ T cells throughout the course of infection and antigen clearance.
1.3 Self-specific CD8+ T cell responses

Nearly 100 years ago, the German immunologist and Nobel laureate, Paul Ehrlich, proposed the idea of “horror autotoxicus” or “the horror of self-reactivity” and theorized that self-specific immune cells would not be produced to protect the body from self-harm. It is now well understood that this is not the case, and adaptive immune cell rearrangement is completely random, affording a vast repertoire of specificities, including those for self-antigen. While the immune system has put in place mechanisms to delete self-specific cells or render them non-responsive, it is evident that this process is not complete. Nearly 24 million Americans suffer from autoimmune disease mediated by self-reactive responses. Comprehending what characterizes and contributes to an autoreactive response has implications for targeted treatment of autoimmune disease. Furthermore, many tumors express self-antigen, and thus safely harnessing mechanisms of tolerance reversal for cancer immunotherapy may be beneficial.

1.3.1 Central and peripheral mechanisms maintaining T cell tolerance to self-antigen.

CD8+ T cells recognize peptide in the context of MHCI, which is expressed on all nucleated cells of the host. This ensures that CD8+ T cells have the potential to recognize any cell in the body that is infected with a pathogen. While this is an effective way to detect intracellular pathogens, it comes with a risk of T
cells responding to uninfected, healthy cells, which present self-protein in MHC molecules. Because of the ability of CD8+ T cells to directly kill target cells, self-specific CD8+ T cells have the potential to cause immense tissue damage. The immune system has mechanisms that aim to eliminate the activity of self-reactive T cells.

Once T lymphocyte progenitors leave the bone marrow and migrate to the thymus, they undergo maturation and TCR rearrangement. Cells that are double positive for CD4 and CD8 molecules are then tested for their ability to bind self-MHC. Those that bind to self-peptide:MHC are positively selected and become single-positive for CD4 or CD8. This process is followed by negative selection, where T cells that bind to self-peptide with high affinity are eliminated through clonal deletion. Medullary thymic epithelial cells (mTECs) facilitate this process by presenting a vast array of tissue-specific self-antigens (TSAs) as controlled by the master transcription factor autoimmune regulator (AIRE). CD4+ T cells with high-affinity for self-peptide:MHC may also undergo clonal diversion, where they are diverted to T regulatory cells during selection. T regulatory cells express the transcription factor forkhead box P3 (Foxp3) and function to suppress an immune response. While central tolerance is exceptionally effectual, it is impossible for all tissue antigens to be presented in the thymus, and both developmental antigens and food antigens are not presented to maturing T cells. There are also low affinity self-reactive T cells that may not induce negative selection. Therefore, some self-reactive T cells survive
central tolerance. For this reason, there are mechanisms to dampen and induce tolerance in autoreactive cells that exit into the periphery.

Peripheral tolerance mechanisms are crucial to controlling self-specific T cell reactivity and work to prevent cells from responding to their cognate antigen. These include anergy induction, cell deletion, and cell suppression. Anergy ensues when T cells do not receive co-stimulation and signal 3 cytokines upon activation. Anergic cells are essentially dormant and are unable to proliferate or secrete cytokines in response to antigen stimulation. Immature DCs that lack co-stimulatory molecules can induce anergy in antigen-specific CD8+ T cells, and are therefore termed tolerogenic DCs. This occurs during an inappropriate immune response, such as to self or food antigen, where inflammatory cues for the APC to mature and express co-stimulatory molecules are not present. Recently, a population of lymphoid stromal cells have been shown to express TSA both in an AIRE-dependent and independent manner and may contribute to peripheral tolerance.

In another mechanism, autoreactive T cells that are chronically engaged with peptide:MHC may die through activation induced cell death (AICD) that is governed through Fas:Fas ligand and BCL-2-interacting mediator of cell death (Bim)-mediated apoptosis. Further repression of self-reactivity is driven by T regulatory cells, which secrete high levels of IL-10 and transforming growth factor-β (TGFβ) that dampen T cell proliferation by blocking IL-2 and downregulating MHC molecule expression. Another barrier to self-reactivity is ignorance of self-specific T cells to their self-ligand based on their restricted
migration through SLOs. This is further imposed by the failure of certain T cells to respond without additional help from other immune cells\textsuperscript{147}.

Central and peripheral tolerance mechanisms are not always completely effective and lapses in tolerance mechanisms do occur. The frequent occurrence of T cell mediated autoimmune diseases indicates that induction and maintenance of tolerance can fail. How disease develops is still an active area of study but breakdown of tolerance mechanisms, as well as the contribution of genetic susceptibility and environmental factors, are at the core of this research. It is becoming more appreciated that an immensely diverse repertoire of self-specific T cells exists both in healthy humans and mice. Early studies identifying T cell epitopes able to bind to myelin basic protein showed that these T cells were present in healthy adults\textsuperscript{160}. This was more recently demonstrated by Mark Davis’ group, when they identified a plethora of CD8\textsuperscript{+} T cells specific for self in the blood of healthy human donors that were numerically equal to naïve foreign-antigen specific cells\textsuperscript{161}. Anergic melanocyte-specific T cells were also detected in healthy individuals and were kept in check by T regulatory cells, making the melanocyte-specific T cells functionally invisible in the periphery\textsuperscript{162}. Mouse studies have also identified a peripheral self-specific T cell population. When T regulatory cells are temporally depleted from mice, nearly 4\% of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells expanded indicating the high numbers of normally suppressed cells\textsuperscript{163}. Checkpoint blockade using antibodies to block inhibitory receptor signaling through PD-1/PD-L1 and cytotoxic T-lymphocyte-associated protein-4 (CTLA-4) have great promise in treating cancers; however, a common side effect of these
treatments is autoimmune disease. This additionally provides proof of a self-reactive T cell pool in the periphery.

1.3.2 Low avidity self-specific T cells generate immune responses

As central tolerance deletes T cell clones that bind to self-ligands with high-affinity, T cells that escape this process are the responsibility of peripheral tolerance mechanisms, and are likely of low affinity. An important question then becomes are these low-affinity clones capable of mounting an immune response? If so, what are the requirements for those cells to respond to self-antigen?

Several groups have addressed these questions.

In one study, researchers crossed mice expressing chicken ovalbumin (OVA) protein in pancreatic β cells (Rat-insulin promotor, Rip-mOVA) to Vβ5 mice. Vβ5 mice express only the β5 chain of the TCRβ repertoire, which skews CD8+ T cells to recognize the SIINFEKL epitope of OVA. The resulting mice had a detectable polyclonal population of CD8+ T cells specific for ‘self’ that were of heterogeneous affinity. Following expansion by vaccination with Lm-OVA, CD8+ T cells infiltrated the pancreatic islets and mice developed high blood sugar, indicative of diabetes. By comparing the functional avidity of self-specific CD8+ T cells to nonself-specific T cells, the authors determined that high affinity self-specific CD8+ T cells had been efficiently removed from the repertoire, and low affinity clones were becoming effector cells and causing autoimmunity in these mice. In a follow-up study, authors showed that low avidity CD8+ T cells in the...
endogenous repertoire of Rip-mOVA mice could be expanded and cause diabetes
166.

In an elegant study published by Ed Palmer’s group, the optimal T-cell
receptor affinity for inducing autoimmunity was investigated by using Rip-mOVA
mice expressing variant OVA proteins in pancreatic β cells that were above and
below the threshold of affinity needed for negative selection in the thymus 168.
Mice expressing antigens that were just above the threshold for negative selection
exhibited the highest risk for developing autoimmune disease, demonstrating
that self-reactive T cells with affinity around the threshold needed for deletion by
central tolerance were able to escape and created an increased chance of
developing autoimmunity 168.

These studies indicate the importance of investigating how low avidity
self-specific CD8+ T cell responses are generated to better target therapies for
autoimmune disease. These findings may also have implications for cancer
immunotherapy, where expanding self-specific CD8+ T cells to tumor antigen
may have a beneficial effect.

1.3.3 Enhancing the self-specific T cell response to tumor antigen

High numbers of tumor infiltrating CD8+ T cells have been associated with
better cancer prognosis 171. Many tumor antigens are non-mutated proteins also
expressed in normal tissues. Exploiting self-specific CD8+ T cells that have
escaped negative selection to destroy tumor cells has been a goal of cancer
immunotherapy 172,173.
Expanded low avidity self-specific CD8$^{+}$ T cells against tumor antigen have the capacity to reduce tumor burden$^{174,175,175-177}$. Early studies showed that a self-specific T cell response to a ubiquitously expressed self-antigen could be enlarged by vaccination against this protein. Vaccinated mice were able to control tumor size more readily than control mice, indicating a role for self-specific CD8$^{+}$ T cells$^{175}$. Recently, using a DNA prime Ad5 boost against a colorectal self-antigen, researchers were able to show increased survival to tumor challenge attributed to a polyclonal CD8$^{+}$ T cell response$^{177}$. In a therapeutic study, adoptively transferred naïve self-specific CD8$^{+}$ T cells specific for a brain-tumor antigen were able to inhibit tumor progression in advanced stage disease after expansion with prime-boost infections$^{176}$.

These studies show the promise of harnessing self-specific CD8$^{+}$ T cell effector responses for tumor reduction. More investigation into the potential reactivity and maturation of self-specific CD8$^{+}$ T cells is needed to fully understand how these T cells can be safely and effectively expanded for cancer immunotherapy. Furthermore, it will be crucial to explore whether these responses can be improved upon, as only modest tumor reduction was observed in the above-mentioned studies.

1.3.4 Generating a self-specific T cell response to protein in the small intestine

The SI is a unique tissue in which to study self-specific responses because it actively maintains tolerance to normal flora and food antigen$^{134}$. Somewhat
paradoxically, it must also recognize and respond to pathogenic assault. Therefore, tolerogenic pressure to self-protein in this compartment may be different than in other tissues.

Many autoimmune diseases, both local and systemic, affect the small bowel \(^ {178}\). While CD8\(^+\) T cells have been highly implicated in disease progression, CD8\(^+\) T cell responses to gut proteins have been difficult to assess because an exact self-antigen responsible for disease development has yet to be identified \(^ {179}\). To address this, our laboratory has developed a mouse model that allows for the tracking of self-specific CD8\(^+\) T cell responses to a model antigen in the SI \(^ {180-182}\). In this mouse, OVA is under the control of the intestinal fatty acid binding protein promoter (I-FABP OVA), which restricts OVA expression to mature enterocytes of the SI \(^ {180}\). Under normal conditions in I-FABP OVA mice, endogenous OVA-specific cells are not detectable, indicating tolerance is maintained to this protein. This is not due to inaccessibility of antigen to naïve T cells, as adoptively transferred transgenic CD8\(^+\) T cells specific for the SIINFEKL epitope of OVA (OT-Is) readily proliferate in I-FABP OVA mice \(^ {181-183}\).

Vaccinating I-FABP OVA mice with multiple vectors containing OVA resulted in the generation of a self-specific CD8\(^+\) T cell response. Using this system of tolerance reversal, I explored what characterizes and contributes to an endogenous CD8\(^+\) T cell response against self-protein in the SI to dissect what is required for the generation of an autoreactive T cell population here. My studies showed that the self-specific CD8\(^+\) T cell pool was responsive to antigen, and increased in functional avidity over the course of tolerance reversal. These
studies reveal the great potential of self-reactive CD8+ T cells, and could have broader implications for many types of autoimmune disease and cancer.
Chapter 2

Shortened intervals during heterologous boosting preserve memory CD8+ T cell function but compromise longevity

2.1 Introduction

Vaccine strategies that are able to generate high frequencies of memory CD8+ T cells may be essential to prevent or limit infections by pathogens such as HIV, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, hepatitis C virus and influenza viruses, where humoral immunity may not be sufficient for protection \(69,184-188\). Memory CD8+T cells are poised to perform a diverse set of effector functions upon Ag re-encounter. Central memory T cells (T\textsubscript{CM}) proliferate rapidly after recall, while effector memory cells (T\textsubscript{EM}) are capable of rapid degranulation and cytokine release \(48,189,190\). Tissue resident memory (T\textsubscript{RM}) CD8+ T cells are positioned at the frontlines and contain invading pathogens by inducing an anti-viral state to prevent systemic infection \(49,50\). These diverse functional properties of memory CD8+T cells make them an important component of the immune response and a critical cell type elicited by certain vaccines.

Heterologous prime-boost-boost (HPBB) strategies have been shown to generate large quantities of Ag-specific CD8+ T cells that rapidly develop into memory CD8+ T cells, which remain stable and contract very little over time, accelerating protection upon pathogen re-encounter \(71,72\). This is a multi-step immunization scheme, where the host is exposed to different vaccine vectors containing a common protein or epitope to stimulate CD8+ T cells of interest \(70,191\). While this method generates large numbers of long lasting memory CD8+ T cells, the time intervals between each boost are at least 28 days long and can be
as long as six months. This may not be ideal in situations where protection needs to be achieved quickly, such as during an outbreak. We have learned from current licensed antibody based vaccine regimens that lengthy intervals between boosts can contribute to poor vaccine compliance\textsuperscript{192,193}. In fact, nearly 28% of children surveyed did not comply with their vaccination schedule\textsuperscript{194}. Other studies showed that vaccine non-compliance can be as low as 30% in older children and adult populations\textsuperscript{195}. This lack of compliance and failure to complete vaccination regimens are acknowledged to contribute to increased infection and disease incidence for many pathogens, which can be controlled by efficient vaccination. It is therefore of interest to develop vaccine strategies that decrease total immunization time, yet generate protective immunity.

We sought to identify methods to more rapidly generate protective memory CD8\textsuperscript{+} T cells. This has been explored in a system where primary FluMist responses were boosted 7 days later with recombinant \textit{Listeria monocytogenes} (LM), yielding protection against lethal influenza challenge\textsuperscript{196}. Wong et al., have demonstrated protection against a bacterial challenge by boosting primary LM responses 7 days later with a heterologous vector\textsuperscript{197}. Interestingly, rapid boosting has also shown to improve survival from tumor challenge using a vesicular stomatitis virus (VSV)-human dopachrome tautomerase (hDCT) prime followed by an adenovirus-hDCT boost within as little as 4 days\textsuperscript{198}. Additional studies show that CD8\textsuperscript{+} T cell immunization in settings of low inflammation results in rapid development of memory phenotype CD8\textsuperscript{+} T cells, which respond within days to boosting and protect against microbial challenge\textsuperscript{199,200}. 

31
While the above studies demonstrate that shortening boosting intervals can generate protective CD8+ T cells, direct comparisons between short and long-term boosting efficacy remain to be extensively explored. It is unknown how the longevity of memory CD8+ T cells is affected when using short-boosting regimens. Therefore, in this study we shortened boosting intervals between three sequential, non-cross-reactive vectors to examine how this impacts CD8+ T cell phenotype, effector function, quantity, location and longevity. We found that short HPBB results in large numbers of Ag-specific CD8+ T cells that are as protective and functional as T cells generated using longer intervals between boosts. Interestingly, while CD8+ T cells generated using shortened boost intervals express canonical memory markers, they fail to survive long-term and continue to gradually contract over time. This correlates with differences in metabolic activity at early memory time points following the tertiary boost. These results reveal that short-boosting intervals can generate effector Ag-specific CD8+ T cells that are comparable in measures of standard function and protection against challenge to long-term boosted CD8+ T cells. However, brief boosting intervals come at the cost of compromising memory T cell longevity. This suggests that while short-boosting is useful for establishing protection rapidly, additional measures, such as future boosts, may need to be implemented to prevent contraction of the short-boosted CD8+ T cell memory population.
2.2 Results

2.2.1 Short intervals between heterologous boosts generate large numbers of Ag-specific CD8+ T cells

To test the ability of short heterologous prime-boost-boost (HPBB) intervals to generate a high number of Ag-specific CD8+ T cells, three replicating vectors encoding OVA were administered to mice 14 days apart (Figure 2.1A). Mice were sacrificed at days 7 and 14 following 1° (VSV-OVA), 2° (VSV-OVA + LM-OVA), or 3° (VSV-OVA + LM-OVA + VV-OVA) vaccinations and the frequency and numbers of Kb-SIINFEKL-specific CD8+ T cells were evaluated in peripheral blood lymphocytes (PBL), spleen and small intestinal intraepithelial lymphocytes (IEL) (Figures 2.1B-F).

The frequency of Kb-SIINFEKL-specific CD8+ T cells in PBL at day 7 increased after each vaccination, on average from 9.5% of total CD8+ T cells at 1° to 31% at 2° and 51% after 3° (Figures 2.1B, D). An increase in Ag-specific CD8+ T cell frequency at this time point was also noted in spleen throughout the vaccination regimen (Figure 2.1B). Notably, Kb-SIINFEKL-specific CD8+ T cells increased more robustly in PBL than spleen with each boost (Figure 2.1B). By day 14, a decrease in Kb-SIINFEKL-specific CD8+ T cells was observed relative to the percent of cells present at day 7 after 1° or 2° boosting events (Figures 2.1B-C). Enumeration of total numbers of Kb-SIINFEKL-specific CD8+ T cells in the spleen revealed that this shortened boosting strategy induced large numbers of Ag-specific CD8+ T cells (Figure 2.1E).
Figure 2.1 Short-boosting intervals generate large numbers of Ag-specific CD8+ T cells. (A) Short-boosting immunization regimen. Boosts occurred 14 days apart. (B, C) Peripheral blood lymphocytes (PBL), spleen, and small intestinal intraepithelial lymphocytes (IEL) were analyzed (B) 7 days or (C) 14 days after 1º, 2º or 3º boosting. Plots are gated on CD8+ T cells. (D) Percent of CD44+ Kb-SIINFEKL+ CD8+ T cells in PBL at day 7 (black) and 14 (white) after 1º, 2º or 3º boosting. (E-F) Number of CD44+ Kb-SIINFEKL+ CD8+ T cells in spleen (E) and IEL (F) at day 7 (black) or day 14 (white) following 1º, 2º or 3º boosting. Data are representative of 2 experiments, N=3 mice per experiment.

Indeed, nearly 6 x 10^6 cells were present at day 7 following 3º boost and at day 14 there was no evidence of contraction. Ag-specific CD8+ T cells in the IEL
also expanded after immunization (Figure 2.1F). While there was a loss of K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells in this compartment between days 7 and 14 after 3\textdegree boosting, heterologous boosting increased the numbers of Ag-specific CD8\textsuperscript{+} T cells approximately 5.5-fold when compared to cell numbers at day 14 after the 1\textdegree vaccination step. In conclusion, the immunization regimen with short intervals was able to generate abundant Ag-specific CD8\textsuperscript{+} T cells.

### 2.2.2 Ag-specific CD8\textsuperscript{+} T cells generated with short HPBB migrate to and populate multiple non-lymphoid tissues

We evaluated whether T cells generated following short-boosting intervals could access non-lymphoid tissues (NLT) and establish broadly distributed memory. At least 95 days after 3\textdegree immunization, K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells were enumerated in spleen, inguinal lymph nodes (IgLN), IEL, lung, liver, female reproductive tract (FRT) and salivary gland (SG) (Figures 2.2A-B). In these tissues, Ag-specific CD8\textsuperscript{+} T cells were readily detected, indicating efficient seeding of K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells to NLT, as described previously \textsuperscript{201}. The frequencies of Ag-specific CD8\textsuperscript{+} T cells in most NLT were greater than those in the secondary lymphoid organs: \textasciitilde40\% of CD8\textsuperscript{+} T cells were specific for the OVA vaccine insert. In PBL, \textasciitilde30\% of CD8\textsuperscript{+} T cells were K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells (Figure 2.2A).
Figure 2.2 Short-boosted Ag-specific CD8+ T cells migrate to and populate multiple non-lymphoid tissues. Splenocytes, inguinal lymph nodes (IgLN), peripheral blood lymphocytes (PBL), small intestinal intraepithelial lymphocytes (IEL), lung, liver, female reproductive tract (FRT) and salivary gland (SG) lymphocytes were isolated at least 95 days after 3° infection. (A) Plots are gated on CD8+ T cells. (B) Quantification of Kb-SIINFEKL+ CD8+ T cells as in (A). (C-D) Mice were injected with anti-CD8α Ab i.v. to identify the precise localization of T cells within the tissue parenchyma (I.V.-) or within the tissue vasculature (I.V.+). (C) Representative flow plots gated on CD44+ Kb-SIINFEKL+ CD8+ T cells. (D) Quantification of Kb-SIINFEKL+ CD8+ T cells as in (C). Data are representative of 2 experiments, at least N=3 mice per experiment except IEL and SG where is N=3 total.

Contact between CD8+ T cells and infected targets is essential for efficient pathogen control. Thus, the location of Ag-specific T cells within tissue parenchyma or tissue vasculature was determined by using a previously described i.v. Ab staining technique (Figure 2.2C)\textsuperscript{202}. This technique selectively labels cells in the vasculature of a tissue by i.v. injecting fluorochrome-labeled Ab. Cells that are in the tissue parenchyma are not accessed by Ab and will remain unlabeled. At least 95 days after 3° boosting, Kb-SIINFEKL-specific CD8+ T cells
in the spleen were distributed evenly between white pulp and red pulp at a 1:1 ratio (Figures 2.2C-D). As expected, the distribution of cells in the IgLN was biased to the i.v. Ab negative compartment and in the PBL was biased to the i.v. Ab positive compartment. Ag-specific CD8+ T cells in the lung and liver were predominantly found in the vasculature, while Kb-SIINFEKL-specific CD8+ T cells in the FRT were evenly distributed between i.v. Ab positive and i.v. Ab negative fractions (Figures 2.2C-D).

Intravenous Ab negative cells in IgLN had high expression of CD62L while cells in the spleen, IEL, lung, liver, FRT and SG were CD62L low (Figure 2.3A). While most of Kb-SIINFEKL-specific CD8+ T cells in secondary lymphoid organs (SLO) were CD103 and CD69 negative, the majority of cells in the IEL and SG were positive for both of these markers. Ag-specific CD8+ T cells in the i.v. Ab negative fraction in the FRT displayed bimodal expression of CD69 but were not CD103 positive (Figure 2.3B-C). In summary, CD8+ T cells activated after the short-boost immunization scheme penetrated multiple NLTs and exhibit phenotypes consistent with T_{EM} and T_{RM} cells.
Figure 2.3 Antigen-specific short-boosted CD8+ T cells in certain non-lymphoid tissues display a resident memory phenotype. (A-C) Phenotyping of splenic, inguinal lymph node (IgLN), peripheral blood lymphocytes (PBL), small intestinal intraepithelial lymphocytes (IEL), lung, liver, female reproductive tract (FRT) and salivary gland (SG) cells isolated at least 95 days after 3° infection. Histograms are gated on CD44+ Kb-SIINFEKL-specific CD8+ T cells in the tissue parenchyma (I.V.-, black, solid line) and tissue vascular (I.V.+, dark grey, dotted line.) CD44 low CD8+ T cells from spleen (solid, light grey, filled) are shown as a non-antigen experienced control. Data are representative of 2 experiments, at least N=3 per experiment, except IEL and SG where is N=3 total. Cd62L data are representative of 1 experiment of at least N=3.

2.2.3 Ag-specific CD8+ T cells have similar functionality regardless of immunization interval length

We went on to assess the functionality of the CD8+ T cells generated after short-boost vaccination compared to cells produced after longer immunization intervals. Granzyme B expression was evaluated at day 5 after 3° immunization and compared between short- and long-boosted T cells. On a per cell basis, granzyme B production was similar in both populations of Ag-specific CD8+ T cells indicating equal ability to kill target cells (Figure 2.4A). At this same time
point, the protective capacity of effector CD8\(^+\) T cells generated by short- and long-boosting was evaluated by quantifying vaccinia virus titers. In spleen, viral titers in long- and short-boosted mice were not detectable, while infected naïve mice, which do not have K\(^b\)-SIINFEKL-specific memory CD8\(^+\) T cells, had a significantly higher viral load (~10\(^4\) PFU) (Figure 2.4B). In the ovaries, a primary site of vaccinia virus replication, there was a significant decrease in viral titers in both long-boosted mice and short-boosted mice, as compared to titers in naïve, infected animals (Figure 2.4C).

**Figure 2.4 Short-boosted Ag-specific CD8\(^+\) T cells express granzyme B and protect against viral infection.** (A) Splenocytes were isolated at day 5 post 3\(^{\circ}\). Granzyme B expression is shown on CD44\(^+\) K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells from short-boosted mice (black, solid line) and long-boosted mice (grey, dashed line); grey filled histogram indicates gating on CD44 low CD8\(^+\) T cells. (B) Spleen and (C) ovaries from 3\(^{\circ}\) short-boosted, 3\(^{\circ}\) long-boosted or 1\(^{\circ}\) VV-OVA challenged mice were isolated at day 5 after VV-OVA to determine vaccinia virus titers. Data are representative of 2 experiments, N=3-4 mice per experiment. Graphs show mean +/- SEM. * = p<0.05.

To evaluate Ag-specific T cell function after the final boost, K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells were examined for cytokine production following SIINFEKL peptide re-stimulation at day 14 after 3\(^{\circ}\) short and long HPBB. Ag-specific short-boosted CD8\(^+\) T cells were able to produce interferon-\(\gamma\) (IFN\(\gamma\)) and tumor
necrosis factor-α (TNFα) in response to SIINFEKL peptide at a similar capacity as long-boosted T cells (Figure 2.5A). Importantly, equal numbers of Ag-specific T cells are generated, regardless of boosting history and all of these cells produced IFNγ cytokine (Figures 2.5A-B).

Figure 2.5 Short-boosted Ag-specific CD8⁺ T cells are functional. (A) Splenocytes from short-boosted or long-boosted mice were stimulated at day 14 after 3⁰ HPBB with or without SIINFEKL peptide and examined for TNFα and IFNγ production. Plots are gated on CD8⁺ T cells. (B) Kᵇ-SIINFEKL tetramer⁺ CD8⁺T cells (black) and IFNγ-producing CD8⁺ T cells (white) were enumerated in spleen at day 14 post 3⁰. Data are representative of 2 experiments N=1-5 mice per experiment. (C) Splenocytes were isolated 32 days post 3⁰ from short- and long-boosted mice, enriched for CD8⁺ T cells and equal numbers of Kᵇ-SIINFEKL-specific CD8⁺ T cells were transferred to naïve CD45.1 mice. New hosts were infected with VSV-OVA 1 day after transfer. 6 days after infection, the number of donor⁺, Kᵇ-SIINFEKL⁺ CD8⁺ T cells was enumerated in spleen. Data are representative of 1 experiment, N= 6-7. (D) Splenocytes were isolated at day 95 post 3⁰ from short-boosted mice and ≥95 days post 3⁰ from long-boosted mice, enriched for CD8⁺T cells and sorted for Kᵇ-SIINFEKL-specific CD8⁺ T cells. Either 2 x 10³ or 2 x 10⁵ Kᵇ-SIINFEKL-specific CD8⁺ T cells were transferred to CD45.1 mice. One day after transfer, recipients were infected with VSV-OVA and donor CD45.2 Kᵇ-SIINFEKL-specific CD8⁺ T cells from short-boosted (solid line) and long-boosted (dotted line) mice were measured in the blood at the indicated time points. Gray lines are low cell number transfers and black lines are high cell number transfers. At all time points, there is no significant difference in tetramer⁺ % between short- and long-boosted cells at low or high numbers of transferred cells. (E) Recipient mice from (D) were sacrificed at day 22

40
post infection and donor Kb-SIINFEKL-specific CD8+ T cells from short-boosted (black) and long-boosted (white) mice were enumerated from spleen. Data in (D) and (E) are representative of 1 experiment N=at least 4.

To investigate if short-boosted T cells are able to proliferate upon recall as well as long-boosted T cells, 32 days after 3°, equal numbers of Kb-SIINFEKL-specific memory CD8+ T cells from short- or long-term boosted mice were transferred into naïve CD45.1 recipients, which were then infected with VSV-OVA. Enumeration of donor Kb-SIINFEKL-specific CD8+ T cells at day 6 after infection revealed that both populations expanded similarly regardless of previous boosting history, indicating similar proliferative potential between these cells (Figure 2.5C).

To determine if the proliferative potential of short-boosted T cells was maintained until a late memory time point, we also evaluated the ability of Kb-SIINFEKL-specific CD8+ T cells to expand upon recall at least 95 days after 3°. Either 2 x 10³ or 2 x 10⁵ Kb-SIINFEKL-specific CD8+ T cells from short-boosted or long-boosted mice were transferred to naïve CD45.1 recipients and infected 1 day later with VSV-OVA. The two different input numbers of cells were chosen to eliminate the impact of transferring large numbers of cells on individual T cell functions. We tracked donor Ag-specific CD8+ T cells in the blood of recipient mice following infection to determine the timing of peak expansion. Whether a low or high number of cells were transferred, short-boosted T cells expanded as well as long-boosted T cells upon recall. The peak of expansion was similar for both populations of transferred cells, although lower cell number inputs resulted
in a delayed response (Figure 2.5D). At all time points, there was no significant difference between the short- and long-boosted cell populations. 22 days later, Ag-specific CD8+ T cells were enumerated in the spleen (Figure 2.5E). The number of donor short-boosted and long-boosted Kb-SIINFEKL-specific CD8+ T cells in mice receiving low numbers of cells was not statistically different. Similarly, donor short-boosted and long-boosted Kb-SIINFEKL-specific CD8+ T cells seeded at high numbers were also not statistically different, but more cells were recovered with high transfer. Taken together, both short and long immunization schema-derived Ag-specific CD8+ T cells displayed similar functionality.

2.2.4 Ag-specific CD8+ T cells generated through short-boosting display a memory phenotype

HPBB with long-boost intervals results in stable CD8+ T cell memory that contracts very little over time.71,72 It might be expected that shortening the immunization intervals would result in a terminally differentiated population of cells, due to an increased inflammatory environment and more frequent Ag contact. To evaluate whether short-boosted Ag-specific CD8+ T cells could differentiate into classical memory T cells, Kb-SIINFEKL tetramer+ cells were evaluated for canonical memory markers at day 30 after 3° short-term and long-term boosting. Memory precursor effector cells (MPECs), effector CD8+ T cells which give rise to memory T cells, are defined by expression of the IL-7 receptor α-chain, CD127, and lack of killer cell lectin-like receptor subfamily G member 1
(KLRG1) expression \[^{56,57}\]. At day 30 after 3\(^{\circ}\), approximately half of the short-boosted K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells are positive for CD127, but not KLRG1, thus displaying a classic MPEC phenotype (Figures 2.6A-B). This is a significantly higher percentage of MPECs than in long-boosted T cells, as the majority of long-term boosted K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells (~70\%) are positive for both CD127 and KLRG1 (Figures 2.6A,C). Per cell expression of CD127, as measured by geometric mean fluorescence intensity (GMFI), indicated similar levels of CD127 on short- and long-boosted Ag-specific CD8\(^+\) T cells (Figure 2.6D).

**Figure 2.6 Short-boosted Ag-specific CD8\(^+\) T cells have a memory phenotype.** (A) Visualization and (B-C) quantification of KLRG1 and CD127 expression on CD44\(^+\) K\(^b\)-SIINFEKL tetramer\(^+\) CD8\(^+\) T cells from short-boosted (black) or long-boosted (white) mice at day 30 post 3\(^{\circ}\) immunization. Plots are gated on CD44\(^+\) K\(^b\)-SIINFEKL tetramer\(^+\) CD8\(^+\) T cells from splenocytes. (D) Expression of indicated proteins on CD44\(^+\) K\(^b\)-SIINFEKL tetramer\(^+\) CD8\(^+\) T cells from short-boosted (black, solid line) and long-boosted (dotted, grey line) mice; grey filled histograms indicate expression on CD44 low CD8\(^+\) T cells. Data are representative of at least 2 experiments, N=2-5 mice per experiment. Graphs show mean +/- SEM. ***p=0.0001, and **** p <0.0001.
Other canonical memory markers such as the IL-15 receptor β chain, CD122, the IL-2 receptor α chain, CD25, and a member of the tumor necrosis factor receptor superfamily, CD27, were all similarly expressed between short-boosted and long-boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells. The expression of the transcription factor T-bet was also similar on short- and long-boosted T cells. While Eomes protein appears decreased on short-boosted Ag-specific T cells in comparison to long-term boosted cells, this is not statistically significant when comparing GMFI (data not shown). Bcl-2, an anti-apoptotic marker, is comparable in short- and long-term boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells. Despite shortening the time between immunizations to 2 week intervals, short-boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells were not PD-1 high, indicating that short HPBB does not drive a phenotype associated with T cell exhaustion (Figure 2.6D). These data demonstrate that shortening the gap between immunizations did not alter the acquisition of canonical memory markers by Ag-specific CD8\textsuperscript{+} T cells.

2.2.5 Short-boosted Ag-specific CD8\textsuperscript{+} T cells display altered metabolic properties compared to long-term boosted T cells

The metabolic activity of memory CD8\textsuperscript{+} T cells has been linked to their function and survival\textsuperscript{61,63–66,203}. Oxidative phosphorylation has been shown to be a dominant metabolic pathway for memory CD8\textsuperscript{+} T cells\textsuperscript{204,205}. Compared to naïve and effector T cells, memory T cells maintain a significantly higher spare
respiratory capacity (SRC), which is important to their establishment and rapid recall response to reinfection. SRC is the reserve capacity of cells to make energy in response to increased stress or work. Since both short-boosted and long-boosted T cells displayed phenotypic markers associated with long-term memory, we investigated whether short- and long-boosted CD8+ T cells exhibited similar metabolic signatures. To evaluate this, the oxygen consumption rate (OCR), an indicator of oxidative phosphorylation, of Ag-specific CD8+ T cells under different cell stressors was measured in purified Kb-SIINFEKL-specific CD8+ T cells at days 30, 51 and 69 following the 3° boost using the Seahorse platform (Figure 2.7). This assay uses compounds that specifically target electron transport chain components to disrupt cellular respiration. OCR was measured at basal conditions and after the addition of each of the indicated metabolic inhibitors to assess metabolic function. Oligomycin and rotenone addition measure ATP-linked respiration and non-mitochondrial respiration, respectively. The addition of FCCP, which uncouples the electron transport chain, allows for measuring maximum OCR (Figure 2.7A). From this, SRC can be calculated, which is the corrected basal OCR subtracted from corrected maximum OCR. Our analysis revealed reduction in maximal OCR in short-boosted Kb-SIINFEKL-specific CD8+ T cells compared to long-boosted T cells at day 30 (Figure 6). By day 51 after 3°, short-boosted Ag-specific cells still exhibited lower maximal OCR compared to long-boosted Kb-SIINFEKL-specific CD8+ T cells, but this difference was not statistically significant (Figures 6B-C). At day 69 after 3° boosting, there was no metabolic differences observed between short- and long-
boosted Ag-specific CD8+ T cells (Figures 2.7B-C). At all time points, basal OCR and SCR was not statistically different between short- and long-boosted Kb-SIINFEKL-specific CD8+ T cells although SCR was decreased in comparison to long-boosted T cells at day 30 and 51 (Figures 2.7A,C and data not shown). In summary, short-boost-derived memory CD8+ T cells exhibited metabolic differences as compared to long-boosted cells, but only at earlier memory time points.

Figure 2.7 Short-boosted Ag-specific CD8+ T cells exhibit altered metabolic function. CD44+ Kb-SIINFEKL-specific CD8+ T cells were purified at day 30, day 51 and day 69 after 3° short or long HPBB via flow sorting. Oxygen consumption rate (OCR) was measured under basal conditions and after the addition of the indicated mitochondrial inhibitors. Oligomycin is an inhibitor of ATP-synthase and measures ATP-linked respiration; FCCP uncouples electron transport and allows for measuring maximal OCR. Rotenone addition permits measuring non-mitochondrial respiration.
(A) Shows raw data from Seahorse platform at day 30 and represents the effects of mitochondrial inhibitors on OCR of short-boosted (black line) and long-boosted (grey line) of K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells. Raw data from day 51 and day 69 are not shown. (B) Corrected maximal OCR and (C) spare respiratory capacity (SRC) for short-boosted (black) and long-boosted (white) K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells at the indicated time points. Data for day 30 are representative of 2 experiments, N=2-6 mice per experiment. Data for day 51 represent 1 experiment, N=2-3 mice. Data for day 69 represent 1 experiment, N=3 of 2-9 mice pooled for short-boost and N=3 either single or 2 mice pooled for long-boost. Graphs show mean +/- SEM. *p=0.0154. Short- and long-boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cell OCR cannot be compared across time points because varying cell densities were used on different days.

2.2.6 Ag-specific CD8\textsuperscript{+}T cells generated through short-boosting contract without stabilization, despite displaying a memory phenotype

Metabolic differences between short-boosted and long-boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells at early memory time points indicated potential disparities in the ability of short-boosted T cells to establish stable memory. To evaluate whether short-boosting and long-boosting vaccine regimens result in the formation of memory CD8\textsuperscript{+} T cells of similar quantity and longevity, K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cell responses were evaluated in the spleen over 134 days following 3° vaccination (Figure 2.8). At an effector time point after 3° boost (days 5-14), numbers of Ag-specific CD8\textsuperscript{+} T cells generated by short- or long-boosting intervals were not statistically different with ~8 x 10\textsuperscript{6} and 1 x 10\textsuperscript{7} cells, respectively (Figure 2.8B). Long-term boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells were stably maintained after 3°, at least for 117 days, which was the last time point. In contrast, K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells generated with short-boosting intervals contracted significantly by day 30 after 3° vaccination and this population continued to decline over 134 days. The number of Ag-specific CD8\textsuperscript{+}
T cells enumerated at 134 days after short-boosting was nearly 12-fold lower than cells at an effector time point, whereas long-boosted K\textsuperscript{b}-SIINFEKL-specific CD8\textsuperscript{+} T cells showed little contraction (Figure 2.8B). Thus, short-boosted CD8\textsuperscript{+} T cells are not stably maintained in the absence of Ag compared to cells generated by longer intervals between immunizations.

**Figure 2.8** Short-boosted Ag-specific CD8\textsuperscript{+} T cells do not form stable memory. Splenocytes were isolated at indicated times after 3\textsuperscript{rd} from short-boosted and long-boosted mice. (A) Plots are gated on CD8\textsuperscript{+} T cells. (B) CD44\textsuperscript{+} K\textsuperscript{b}-SIINFEKL\textsuperscript{+} CD8\textsuperscript{+} T cell numbers were enumerated in short-boosted (black) and long-boosted (white) mice at indicated time points. Data are representative of at least 2 experiments, N=2-10 mice per experiment, except day 117 where N=3. Graphs show mean +/- SEM. *p= 0.0174, **p= 0.0023, and *** p = 0.0001.

**2.3 Discussion**

CD8\textsuperscript{+} T cells contribute to protection against many infections and efficacious CD8\textsuperscript{+} T cell memory may be particularly important to eliminate certain pathogens. Vaccine strategies should generate high quality memory CD8\textsuperscript{+} T cells that can rapidly purge the offending organism from the host and ideally, vaccination would afford almost immediate protection. Heterologous prime-boost-boost (HPBB) is a multi-step immunization scheme, which has the
remarkable feature of forming high quantities of stable memory CD8+ T cells. However, boosting intervals are long, meaning that vaccination is not complete for several months. Thus, in this study we investigated how the quality, quantity and location of Ag-specific CD8+ T cells is affected by decreasing the time between boosting events. It has been previously demonstrated that expression of CCL21, the ligand for CCR7, is downregulated during different microbial infections, including lymphocytic choriomeningitis virus (LCMV), LM and VV. This leads to altered migration and priming of T cells. Chemokine downregulation is transient and, by day 14 after infection, CCL21 levels are back to pre-infection concentrations. Therefore, we chose 14 days as the resting period between boosting events to allow animals to regain some immune homeostasis. We show that condensing the intervals between boosting results in functional and protective Ag-specific CD8+ T cells in 4 weeks.

We discovered that Ag-specific CD8+ T cell numbers early after each vaccination step increase within PBL, spleen and IEL. Indeed up to 62% of CD8+ T cells were Kb-SIINFEKL-specific in PBL by day 14 after the final boost. Short HPBB also led to memory T cell populations in the lungs, liver, FRT, and SG that comprise up to 40% of CD8+ T cells. Thus, short HPBB can generate large populations of Ag-specific CD8+ T cells throughout the host.

A concern was that this curtailed immunization schedule may have induced a state of exhaustion or non-responsiveness in Kb-SIINFEKL-specific CD8+ T cells. However, the 3° recall was robust and short-boosted T cells were able to protect against infection as efficiently as long-boosted T cells. All Kb-
SIINFEKL-specific CD8\(^+\) T cells at day 14 post 3\(^o\) produced cytokines and were not PD-1 high, other indicators that these T cells were not exhausted. Furthermore, Ag-specific CD8\(^+\) T cells generated through short-boosting have similar proliferative capabilities compared to long-term boosted T cells. Therefore, the 14-day intervals between immunizations did not induce functional defects associated with overexposure to an inflammatory microbial environment. This may be expected, as there appears to be a window of opportunity for the induction of exhaustion, which necessitates prolonged exposure to this environment. When CD8\(^+\) T cells specific for chronic LCMV clone-13 are transferred at day 8 after infection to a naïve, uninfected new host, certain hallmarks of T cell exhaustion are prevented\(^{207}\).

Many infections begin at mucosal surfaces. CD8\(^+\) T cell mediated killing requires direct contact with target cells and vaccines must position CD8\(^+\) T cells at the site of infection. The recently described CD8\(^+\) T cell memory subset, tissue resident memory T cells (T\(_{RM}\)), are uniquely positioned to act as immediate sentinels of re-infection, setting up an anti-microbial state\(^{49,50}\). As precise localization of T cells within tissue compartments can be complicated by vascularization, we used an intravascular staining technique to discriminate cell localization between the tissue parenchyma or tissue vasculature\(^{202}\). We found that short-term HPBB drives T cell migration to the parenchyma in multiple non-lymphoid tissues (NLT), which are populated for at least 95 days after the last immunization. The CD62L low phenotype of these cells indicates that the majority of cells in the tissue generated from short-boosting are likely T\(_{EM}\) cells,
which is in line with what has been shown for HPBB with long-boosting intervals in secondary lymphoid organs \cite{208}. However, a significant fraction of cells in the IEL and SG are CD69 positive and CD103 positive suggesting that they are T_{RM} \cite{138,209,210}. Generating CD8{\textsuperscript{+}} T cells with a T_{RM} phenotype may be particularly important for vaccination, as this cell type has been shown to be critical in providing protection during re-infection \cite{49,50,211}. It was recently shown that CD8{\textsuperscript{+}} T cell quantity has been underappreciated, particularly in NLT, due to sub-optimal tissue disruption and lymphocyte isolation techniques and that CD103/CD69 phenotyping may not label all T_{RM} \cite{138}. Thus, it is likely that the numbers of K{\textsuperscript{b}}-SIINFEKL-specific CD8{\textsuperscript{+}} T_{RM} cells are higher than what we have observed herein.

Formation of a stable population of memory CD8{\textsuperscript{+}} T cells is important for a vaccine to achieve long-term protection. CD127 expression on Ag-specific CD8{\textsuperscript{+}} T cells has been shown to predict memory precursor effector cells (MPECs), which go on to become memory T cells \cite{57}. MPECs have also been shown to lack expression of KLRG1 \cite{56}. Given the brevity of rest periods between immunizations, short-boosted CD8{\textsuperscript{+}} T cells would have been predicted to be terminally differentiated, but based on phenotype, these appear to be optimal memory CD8{\textsuperscript{+}} T cells. In fact, a greater percent of short-boosted tetramer{\textsuperscript{+}} CD8{\textsuperscript{+}} T cells were positive for CD127 and negative for KLRG1, as compared to long-boosted T cells. Therefore, it was surprising to discover that the short-boosted K{\textsuperscript{b}}-SIINFEKL-specific CD8{\textsuperscript{+}} T cells continuously eroded over a 134-day timespan. It might have been expected that short-boosted T cells would display a short-lived
effector cell phenotype of KLRG1 positive, CD127 negative, but this was not the case. Interestingly, exhausted CD8+ T cells, which have a loss of memory potential, lack expression of KLRG1 during chronic LCMV clone 13, indicating that absence of KLRG1 expression is not always associated with T cell longevity.

T-bet and Eomes have been shown to impact CD127 and KLRG1 expression in effector CD8+ T cells, where high Eomes and low T-bet expression induces a KLRG1 low, CD127 high population of CD8+ T cells that is maintained into memory. However, in our study, expression of T-bet and Eomes was similar between short- and long-boosted populations despite their disparate longevity. Short-boosted Ag-specific CD8+ T cells also express similar levels of CD27, CD122, CD25 and Bcl-2 as compared to long-boosted T cells, indicating that they are capable of receiving survival signals via these molecules, but they fail to be maintained equally. This incongruity suggests that there are other factors, contributing to the decay of short-boosted CD8+ T cells that can overcome a signature indicative of long-lived memory T cells.

The metabolism of CD8+ T cells is important in memory cell function and survival. Oxidative phosphorylation supports the metabolic needs of memory CD8+ T cells, while glycolysis supports effector T cells. To this end, the spare respiratory capacity (SRC) of memory CD8+ T cells has been shown to be greater than that of naive and effector CD8+ T cells and is thought to regulate memory CD8+T cell formation and functionality. Therefore, it was surprising, given the continuous contraction of short-boosted CD8+ T cells, that
SRC was not statistically different between short- and long-boosted T cells. However, short-boosted Ag-specific CD8+ T cells have a significantly lower maximal oxygen consumption rate (OCR) compared to long-boosted T cells at day 30. This might have deleterious effects on longevity, even though recall responses were not affected. Perhaps shortening boost intervals does not give ample time for cells to become bioenergetically stable between immunizations. This is supported by the recent finding that the mitochondrial function of CD8+ T cells increases with time after infection 213. One interpretation is that short HPBB generates a mixed population of cells differing in their mitochondrial capacity.

Less metabolically fit Ag-specific CD8+ T cells may dominate the response early after 3° immunization and rely more heavily on glycolysis during steady state conditions. As the inflammatory environment subsides and growth factors are reduced, these cells may preferentially die over time leaving a more stable memory cell population to survive long-term. This could explain the reduced, but not significant, SRC of short-boosted Kb-SIINFEKL-specific CD8+ T cells at day 30 and 51 after 3°, whereby some T cells are able to function at higher SRC than others. Indeed, the contraction of short-boosted Ag-specific CD8+ T cells slows substantially around day 60 after 3°. This slowed contraction may be explained by the identical metabolic activity of short- and long-boosted Kb-SIINFEKL-specific CD8+ T cells observed at day 69 after 3°.

Interestingly, studies show that T cells that take up high amounts of glucose exhibit the molecular profile of short-lived effector cells 66. We show here a different phenomenon: metabolic alterations of T cells that display a memory
phenotype, but do not form stable numbers of memory T cells. Further investigation of the metabolic pathway and energy consumption by CD8\(^+\) T cells generated through short-boosting would be needed to reveal if metabolism is directly contributing to memory T cell contraction. Regardless of metabolic activity and continued contraction, short-boosted T cells are able to respond to infection at any time point measured after 3\(^\circ\) comparably to long-boosted mice. This further supports our conclusions that functionality is not compromised following short-boosting, even at late memory time points.

Our study shows that shortening the boosting intervals during HPBB will generate Ag-specific CD8\(^+\) T cells that are functional and protective. While these CD8\(^+\) T cells gradually decay in number, they are still able to proliferate and respond to infection comparably to long-boosted T cells. Despite protracted contraction, the population of short-boosted CD8\(^+\) T cells remaining after 3\(^\circ\) is still greater than the population remaining after 1\(^\circ\) and 2\(^\circ\) boosts, indicating that short HPBB is still advantageous over single boosts. Overall, shortening boosting intervals preserves the function of Ag-specific CD8\(^+\) T cells, despite ongoing contraction, and could be useful in situations where protection needs to be achieved rapidly.

2.4 Materials and Methods

2.4.1 Mice and Infections

C57BL/6J and \(Ptprc_a\) \(PepC_b\)/BoyJ (CD45.1) mice were purchased from
The Jackson Laboratory (Bar Harbor, ME) or The National Cancer Institute (Frederick, MD). To generate short-boosted mice, animals were primed with 1 x $10^6$ PFU VSV-OVA i.v. (1°), rested for 14 days, boosted with 1 x $10^4$ CFU LM-OVA i.v. (2°), rested for 14 days, then given an additional boost with 2 x $10^6$ PFU vaccinia virus (VV)-OVA i.v. (3°)\textsuperscript{214–216}. Long-term boosted mice were generated with this same regimen, but with at least 28 days rest between each boost. All mice were used in accordance with the National Institutes of Health and the University of Minnesota Institutional Animal Care and Use Committee guidelines.

2.4.2 Intravascular staining, Isolation of lymphocytes and Flow cytometry

To discriminate between CD8\textsuperscript{+} T cells in tissue parenchyma versus tissue vasculature, i.v. injected Ab was used as previously described\textsuperscript{202}. Briefly, 3\(\mu\)g of anti-CD8\textalpha Ab (53-6.7, Biolegend, San Diego, CA) was injected i.v. and allowed to circulate for three minutes prior to mouse sacrifice.

Organs were harvested and digested as previously described\textsuperscript{138}. For isolation of small intestinal IELs, Peyer's patches were removed, the small intestine was cut longitudinally and then laterally into small pieces. Pieces were incubated for 30 minutes with stirring at 37°C with 0.154mg/mL dithioerythritol (Sigma-Aldrich, St. Louis, MO) in 10% HBSS/HEPES. FRT, lung and SG tissues were cut into small pieces in RPMI 1640 containing 5% FBS, 2 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2} and 0.5mg/mL type IV collagenase for FRT (Sigma-Aldrich, St. Louis, MO)
or 100 U/mL type I collagenase for lung and SG (Worthington, Lakewood, NJ) and incubated for 1 hr at 37°C with stirring. After enzymatic digestion, the remaining tissue pieces were mechanically disrupted using a gentleMACs dissociator (Miltenyi Biotec, San Diego, CA). The liver was mechanically dissociated by pushing the tissue through a 70µm-cell strainer. Single cell suspensions of IEL, FRT, lung, liver and SG were further separated using a 44/67% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient. Spleen and lymph nodes (LN) were dissociated mechanically. Splenocytes and blood were treated with ACK lysis buffer to lyse red blood cells.

The following antibodies were used for flow cytometry: anti-KLRG1 (2F1), anti-Eomes (Dan11ma), anti-T-bet (4B10), anti-CD44 (IM7), anti-CD122 (TMB1), anti-CD27 (LG.7F9), anti-CD69 (H1.2F3) (all from eBioscience, San Diego, CA), anti-CD8α (53-6.7, eBioscience, Biolegend, San Diego, CA), anti-CD103 (M290), anti-CD25 (PC61), anti-Bcl-2 (Bcl-2/100) and anti-CD127 (SB/199) (BD Biosciences, San Jose, CA). Cell viability was determined using Ghost Dye™ Red 780 (Tonbo Biosciences, San Diego, CA). Kb-SIINFEKL-specific CD8+T cells were identified using H-2Kb tetramers made in house containing the SIINFEKL peptide (New England Peptide, Gardener, MA). The BD Biosciences intracellular kit for cytokine staining and the eBioscience FoxP3 kit for transcription factor staining were used in accordance with manufacturer’s directions. Peptide stimulation was performed as previously described. Briefly, splenocytes were plated in RPMI 1640 containing 10% FBS, 1x NEAA, 2mM L-glutamine, 1mM
sodium pyruvate, 1x penicillin/streptomycin and 0.05mM β-mercaptoethanol and incubated with 1μg/mL SIINFEKL peptide and 1μg/mL GolgiPlug (BD Biosciences, San Jose, CA) for four hours at 37°C. Cells were washed and stained with fixable LIVE/DEAD aqua dead cell stain (Life Technologies, San Diego, CA) before surface and intracellular staining. Samples were acquired on an LSRII flow cytometer (BD Biosciences, San Diego, CA).

2.4.3 Recall and Protection Assays

For transfer and recall experiments, CD8+ T cells were enriched from splenocytes of CD45.2 mice at day 30 or ≥95 after 3° using an EasySep CD8+ T cell isolation kit (STEMCELL Technologies, Vancouver, Canada). For early memory transfers, 2.5 x 10^5 K^b-SIINFEKL-specific CD8+ T cells were transferred i.v. to CD45.1 mice. For late memory transfer experiments, cells were flow sorted and either 2 x 10^3 or 2 x 10^5 K^b-SIINFEKL-specific CD8+ T cells were transferred i.v. to CD45.1 mice. In all cases, recipient mice were infected i.v. with 1 x 10^6 PFU of VSV-OVA the day after cell transfer.

To determine the ability of T cells to protect upon re-infection, 3° short-boosted and long-boosted mice were generated as described above and sacrificed five days after infection with 2 x 10^6 PFU VV-OVA i.v. (the 3° boost). Ovaries and spleen were harvested, homogenized and viral loads were analyzed by plaque assays on 143B cells, as previously described. Naïve mice were infected with 2 x 10^6 PFU VV-OVA i.v. as a control.
2.4.4 Metabolic Assays

CD8+ T cells were enriched using an EasySep CD8+ T cell isolation kit (STEMCELL Technologies, Vancouver, Canada) and Kb-SIINFEKL-specific CD8+ T cells were purified from the resulting population using flow cytometric sorting. At day 30, day 51 and day 69 following 3° short- or long-boost, Ag-specific CD8+ T cells were plated at 2-8.5 x 10^5 cells/well (cell numbers were kept similar within experiment) in non-buffered, sodium bicarbonate free, RPMI 1640 containing 25mM glucose, 2mM L-glutamine and 1mM sodium pyruvate. For day 69 experiments, cells were pooled from 2 to 9 short-boosted mice or up to 3 long-boosted mice. Oxygen consumption rate (OCR) was evaluated using the Seahorse XF-24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA). The non-corrected basal OCR was directly measured, followed by the addition of 0.0063mM oligomycin (Sigma-Aldrich, St. Louis, MO) to evaluate ATP production. Non-corrected maximum OCR was examined by addition of 0.015mM fluoro-carbonyl cyanide phenylhydrazone (FCCP) (Sigma-Aldrich, St. Louis, MO) and non-mitochondrial respiration was measured following the addition of 0.019mM rotenone (Sigma-Aldrich, St. Louis, MO). Corrected basal OCR, corrected maximal OCR and spare respiratory capacity (SCR) were calculated as shown earlier. Briefly, to calculate corrected basal OCR and corrected maximum OCR, non-mitochondrial respiration was subtracted from basal and maximum OCR. SCR was determined by subtracting corrected basal OCR from corrected maximum OCR.
2.4.5 Statistical Analysis

An unpaired two-tailed Student’s $t$-test was performed when groups were compared using Prism (GraphPad Software Inc.). For all analysis, $p$ values of less than 0.05 were considered significant and were indicated by asterisks (*).
Chapter 3

Visualizing CD8$^+$ T cell dynamics in the small intestine
3.1 Introduction

A critical function of CD8+ T cells is the highly specific killing of target cells through directed cytolysis. As cognate antigen is initially rare, the scanning behavior of naïve CD8+ T cells in secondary lymphoid organs (SLO) is paramount to mounting a successful immune response. Equally important is the motility of effector and memory CD8+ T cells within nonlymphoid tissues (NLT) that have the heavy burden of surveying a vast array of cell types in a great expanse of tissue to rapidly detect and eliminate potential invading pathogens or transformed cells. Dissecting the motile behavior of CD8+ T cells during steady state and infection may give insight into the requirements for efficient disease surveillance and could potentially inform vaccine development and treatments for recalcitrant pathogens requiring CD8+ T cell responses for protection.

The small intestine (SI) is the biggest mucosal interface between the host and the outside environment. As such, it is the site of many primary infections with the potential for dissemination and therefore immune cell surveillance in this compartment is of great interest. Furthermore, the SI must maintain tolerance to normal flora and food antigens, while simultaneously preventing infectious pathogen dissemination. Therefore, T cell motility in this compartment might be quite different than other NLTs devoid of microbial and food antigen. Our knowledge of T cell motility in situ has grown with the development of two-photon laser scanning microscopy (TPLSM). Studies evaluating CD8αα intraepithelial lymphocytes (IELs) in the SI showed that these...
cells were highly motile during steady state and were able to migrate between the epithelial and lamina propria (LP) of the villi. Other investigation focused on CD4+ T cells showed Th17 CD4+ T cells and FoxP3+ T regulatory cells were similarly motile in the SI. Recently, Sujino et al., found that while FoxP3+ T cells typically reside in the LP, they are able to migrate to the epithelial layer upon loss of the transcription factor ThPOK. To our knowledge, motility of CD8αβ+ T cells in the SI has not been explored.

Resident memory (T_{RM}) CD8+ T cells in the SI contribute to disease prevention and dissemination of pathogens. While they are important for pathogen containment, and maintaining immune homeostasis, T_{RM} are relatively rare, and only six antigen-specific CD8+ T cells per 1000 nucleated cells were observed at a memory time point after LCMV infection. This suggests that CD8+ T cells may be highly motile to adequately inspect for infection or disease in the SI. We used TPLSM to dissect antigen-specific CD8+ T cells responses in the SI during and after viral infection. While antigen-specific CD8+ T cells are initially slowed after infection, these cells accelerated upon antigen clearance. At memory time points, antigen-specific T_{RM} retained a slightly reduced speed but continued to survey the epithelium and LP of the villi. The physical location of CD8+ T cells within the tissue seemed to dictate their mobile behavior. Although the majority of T_{RM} upregulated CD103 at stable memory, their migration was independent of CD103 interactions. We also examined self-specific CD8+ T cell responses to protein in the SI, as this tissue is under constant tolerogenic
pressure. We found that while self-specific CD8+ T cells did not respond to antigen *ex vivo*, they were still capable of surveying the tissue comparably to foreign-antigen specific CD8+ T cells at early time points after tolerance induction. However, as tolerance was established, self-specific CD8+ T cells lost their ability to arrest upon antigen encounter. These studies broaden our knowledge of effective CD8+ T cell immunosurveillance in the SI.

### 3.2 Results

#### 3.2.1 Antigen-specific CD8+ T cell motility in the small intestine is multifarious during the course of infection.

To explore foreign-antigen specific CD8+ T cell behavior in a mouse infection model in the SI, we used the well characterized lymphocytic choriomeningitis virus (LCMV). LCMV is a natural mouse pathogen and infects cells via the cell surface receptor, α-dystroglycan, which is widely expressed in the SI in both the smooth muscle and epithelium of the crypts and villi. As such, the SI is abundantly infected with LCMV after single systemic infection. To track LCMV-specific CD8+ T cell responses *in situ*, we crossed ubiquitin-green fluorescent protein (GFP) mice to mice expressing a transgenic T cell receptor (TCR) that recognizes the gp33 epitope of LCMV (P14-GFP CD8+ T cells). Naïve P14-GFP CD8+ T cells from these mice were transferred into B6 mice that were subsequently infected with LCMV (Figure 3.1A). To verify that P14-GFP CD8+ T cells could be observed in the SI, tissue was harvested on days 5, 8, and 30.
following LCMV infection. At all time points, P14-GFP CD8+ T cells were readily detectable by flow cytometry in both the epithelium and LP of the SI (Figure 3.1B). For TPLSM, a piece of explanted jejunum was imaged (see methods section). Prior to imaging, mice were injected with Hoescht stain that allows better visualization of intestinal architecture. The SI was imaged from the serosal side. Using this approach, we were able to penetrate the tissue up to ~200μm and could clearly visualize P14-GFP CD8+ T cells in both the crypts of Lieberkühn (from herein referred to as crypts) and villi (Figure 3.1C). At days 5, 8, and 30 after LCMV infection, P14-GFP CD8+ T cells in the villi were motile in all three dimensions (Figure 3.1D, supplementary movies 3.1, 3.2). At day 5 post LCMV, effector P14-GFP CD8+ T cells were moving at a speed of ~5μm/minute. By day 8, P14-GFP CD8+ T cells had accelerated to ~8μm/minute, which coincides with the known antigen clearance of LCMV (Figure 3.1E, supplemental movie 3.1). The vast majority of P14 CD8+ T cells in the SI at a memory time point after LCMV infection are considered resident, and do not recirculate through the host. The speed of tissue T_{RM} in the SI was 3μm/minute slower than P14-GFP CD8+ T cells at day 8 after infection, and was comparable to that of day 5 effector P14-GFP CD8+ T cells (Figure 3.1E, Supplemental movie 3.2). However, the scanning behavior of memory P14-GFP CD8+ T cells was also unique to earlier time points in that the cells exhibited a wider median turning angle (the angle a cell deviates through time) and a smaller confinement ratio (displacement divided by path length) in comparison to P14-GFP CD8+ T cells at day 5 and day
8 post infection (Figure 3.1F-G). This difference in behavior between memory and effector time points is also seen in flower plots (tracks set to same origin) that indicate day 30 P14-GFP CD8+ T cells have shorter path lengths than compared to P14-GFP CD8+ T cells at day 5 and 8 after LCMV infection (Figure 3.1H).
Figure 3.1 Antigen-specific CD8+ T cell motility varies during LCMV infection. 

A) Naive P14-GFP CD8+ T cells were transferred to B6 mice and infected with LCMV 1 day later. B) Intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated from the small intestine (SI) at the indicated time points and evaluated for Thy1.1+ GFP+ CD8+ T cells (P14-GFP CD8+ T cells). Plots were gated on CD8+ T cells. C) Prior to imaging, B6 mice were injected with Hoescht stain (dark blue) to visualize intestinal architecture and location of P14-GFP CD8+ T cells (cyan) within the tissue. Two-photon laser scanning microscopy (TPLSM) orthogonal views of villi and crypts are shown. D) Tracks of P14-GFP CD8+ T cells were measured using Trackview software. E) Mean track speed (μm/min) for Thy1.1+ GFP+ CD8+ T cells (P14-GFP CD8+ T cells) at Day 5, Day 8, and Day 30. F) Median turning angle (°) for Thy1.1+ GFP+ CD8+ T cells (P14-GFP CD8+ T cells) at Day 5, Day 8, and Day 30. G) Confinement ratio for Thy1.1+ GFP+ CD8+ T cells (P14-GFP CD8+ T cells) at Day 5, Day 8, and Day 30.
The last panel shows perspective surface rendering of crypts and villi. **D)** Imaris generated cell tracks (orange) of TPLSM of P14-GFP CD8+ T cells (cyan) in the villi (outlined in yellow dashed lines) of the jejunum at the indicated time points after LCMV infection in orthogonal (top) or perspective (bottom) presentation. **E)** Speed **F)** median turning angle **G)** Confinement ratio of days 5 (magenta), 8 (blue), 30 (green) P14-GFP+ CD8+ T cells in the villi after LCMV infection. **F)** Flower plot analysis of (D). Scale bars are 20μm and bar graphs show SD. A Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison’s test was performed to evaluate statistical significance. *indicates a p value of 0.02, **<0.008, ****<0.0001. Data are representative of 3 experiments totaling at least 4 mice.

### 3.2.2 Tissue micro-architecture dictates CD8+ T cell motility in the small intestine

Stromal cells and the extracellular matrix (ECM) can affect interstitial T cell migration in the tissues. Inflammation from infection or injury can cause re-organization of proteins within the ECM, altering T cell behavior. Therefore, we wondered if CD8+ T cell motility in the SI was impacted by the immediate environment.

The SI can be divided into 4 layers: the serosa, muscularis externa, the submucosa, and the mucosa (Figure 3.2A). The outermost layer of the SI is the serosa, which is a smooth membrane consisting of a thin layer of connective tissue. Adjacent to the serosa is the muscularis externa composed of both longitudinal and circular smooth muscle that facilitates peristalsis. The submucosa, a dense layer of irregular connective tissue, separates the muscularis externa from the mucosa, and contains the blood vessels, lymph vessels, and nerves that support the SI. The muscularis mucosae is the first part of the mucosa from the serosal side and lies below the LP. The LP is composed of
loosely connective collagen and is separated from the lumen by a single layer of columnar epithelial cells that are organized into villi and crypts (Figure 3.2A). To evaluate CD8+ T cell *in situ* behavior in the different layers of the SI, we adoptively transferred P14-GFP CD8+ T cells to mice and 1 day later infected with LCMV. CD8+ T cell behavior was recorded on days 5 and 8 after infection. The serosa and both longitudinal and circular muscle of the muscularis externa were clearly distinguishable by Hoescht stain (Figure 3.2B). In the serosa and longitudinal muscle, P14-GFP CD8+ T cells were elongated and migrated in a direction parallel to myocytes. In the less dense circular muscle, T cells maintained an amoeboid shape similar to that observed in the villi (Figure 3.2B). Cell tracks in the outer layers of the SI at both day 5 and day 8 indicated that P14-GFP CD8+ T cells had shorter path lengths than in the villi, and that their migration was restricted to x-y dimensions with very few cells showing any motile behavior in z-direction (Figure 3.2C, supplementary movie 3.3). By day 8 after infection, the vast majority of T cells in this portion of the SI had contracted (Figure 3.2C, supplementary movie 3.3). At both time points, P14-GFP CD8+ T cells evaluated in the muscularis externa had a wide median turning angle and a low confinement ratio indicating confined motility (Figure 3.1E-G). In contrast, P14-GFP CD8+ T cells in the crypts were more motile, exhibit longer path lengths and greater velocity at both day 5 and day 8 post-infection (Figure 3.1D-E,
supplementary movie 3.4).

A.

![Image of histological section with labeled structures]

B.

<table>
<thead>
<tr>
<th>Serosa</th>
<th>Muscularis externa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Longitudinal muscle</td>
</tr>
</tbody>
</table>

![Image showing tissue staining and cellular distribution]

C.

![Image showing cellular tracks over time]

D.

![Image showing cellular tracks over time]

E.

![Graph showing mean track speed over time]

F.

![Graph showing median turning angle over time]

G.

![Graph showing confinement ratio over time]
Figure 3.2 Tissue architecture dictates CD8⁺ T cell motility in the small intestine. A) Histological section of SI jejunum showing serosa, muscularis externa, submucosa, and mucosa as depicted. Architecture is visualized by staining E-cadherin (magenta) Collagen IV (yellow) and CD31 (cyan). Scale bar is 75µm. B) P14-GFP CD8⁺ T cells were transferred to B6 and infected with LCMV. Five days post-infection, P14-GFP CD8⁺ T cells (cyan) in the serosa (left) muscularis externa (right two panels) were imaged using TPLSM. Hoescht stain (dark blue) was injected prior to imaging. C) Imaris generated cell tracks (orange) of TPLSM P14-GFP CD8⁺ T cells (cyan) in the combined muscularis externa and serosa at days 5 (left) and 8 (right) after LCMV infection. D) Same as in (C) but in crypts of SI. Scale bars are 30µm. E) Mean track speed F) median turning angle G) confinement ratio of P14-GFP CD8⁺ T cells at days 5 (magenta) and 8 (blue) after LCMV infection the indicated locations of the SI. Bar graphs show SD. A Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison’s test was performed to evaluate statistical significance. ****indicates a p value of <0.0001. Data are representative of 3 experiments totaling at least 3 mice.

P14-GFP CD8⁺ T cell locomotion was restricted to the LP and epithelium of the crypts, often orbiting around the entire planar section of the crypt. P14-GFP CD8⁺ T cells were never observed traversing the luminal area. While the velocity of P14-GFP CD8⁺ T cells in the crypt increased at day 8, the turning angle and confinement ratio was not different between days 5 and 8 after LCMV, similar to observations in the villi (Figure 3.1, Figure 3.2E-G, supplemental movie 3.4). There were too few cells present in the muscularis externa and crypts at memory time points for analysis with TPLSM. Taken together, this detailed characterization of P14-GFP CD8⁺ T cells in the SI showed their reliance on immediate tissue architecture to adopt their migratory pattern.

3.2.3 CD8⁺ T cell locomotion in the small intestine is independent of αE integrin.

Cellular adhesion molecules, like integrins, are important for facilitating T cell extravasation into SLO and NLTs. In some cases, integrins have shown to
be important for T cell motility within tissue \(^{80,227–229}\). Both intraepithelial lymphocytes (IEL) and LP lymphocytes (LPLs) express the \(\alpha_E\) integrin, CD103, which binds to E-cadherin \(^{138,209,230}\). At day 5 following LCMV infection, \(~35\%\) of P14 CD8\(^+\) T cells in both the epithelium and LP were CD103\(^+\) (Figure 3.3A-B). This frequency of CD103 expressing P14 CD8\(^+\) T cells increased to nearly \(~80\%\) of IELs and \(50\%\) of LPLs by day 8 post LCMV and by day 30 most T\(_{RM}\) in both compartments expressed CD103 (Figure 3.3A-B). To assess if CD103 was playing a role in governing CD8\(^+\) T cell movement in the SI, we crossed P14-cyan fluorescent protein (CFP) mice to P14 mice that lacked CD103. We adoptively transferred equal numbers of P14- GFP CD103\(^+/+\) (wild type) CD8\(^+\) T cells and P14-CFP\(^+\) CD103\(^-/-\) CD8\(^+\) T cells (CD103KO) to B6 mice and infected them with LCMV. Behavior of each transferred population was evaluated at various time points after infection. In the crypt and villi at days 8 and 14 after infection, WT P14-GFP CD8\(^+\) T cells and CD103KO P14-CFP CD8\(^+\) T cells displayed similar migratory behaviors (Figure 3.3C-F, supplementary movie 3.5, 3.6). We hypothesized that perhaps motility was more dependent on CD103 at a memory time point, but at day 30 in the villi, no changes in motility were observed between CD103 sufficient and deficient P14 CD8\(^+\) T cells (Figure 3.3 E-F, supplementary movie 3.6). We also failed to detect differences in median turning angle and confinement ratios (data not shown). These data conclusively show that P14 CD8\(^+\) T cell motility in the SI during LCMV infection does not require 

71
the integrin, CD103. Similar results were observed with experiments evaluating the need for β1 integrin (CD29) (data not shown).

Figure 3.3 CD8⁺ T cell motility in the small intestine is independent of CD103. A) Phenotypic analysis of P14 IELs (top) and LPLs (bottom) at day 5, 8, and 30 after LCMV infection. Plots are gated on P14 CD8⁺ T cells. B) Frequency of CD103⁺ IELs (top) and LPLs (bottom) as in (A). C) Naïve wild type (WT) P14-GFP and CD103KO P14-CFP CD8⁺ T cells were transferred to B6 mice that were infected with LCMV one day later. Imaris generated cell tracks (WT-orange, CD103KO-magenta) of TPLSM of P14-GFP CD8⁺ T cells (cyan) and P14-CFP CD103⁻/⁻ CFP CD8⁺ T cells (magenta) in the crypts at the indicated time points after infection. Scale bars are 20µm. D) Mean track speed of P14 CD8⁺ T cells in (C) at day 8 (blue) and day 14 (purple), where filled circles are WT and open circles are CD103⁻/⁻. E) As in E but in the villi. F) Mean track speed of P14 CD8⁺ T cells in (E) at days 8 (blue), 14 (purple), and 30 (green) after LCMV infection where filled circles are WT and open circles are CD103⁻/⁻. Scale bars are 30µm. Bar graphs
show SD. A Kruskal-Wallis one-way ANOVA with Dunn’s multiple comparison’s test was performed to evaluate statistical significance. ** indicates a p value of 0.006. Data are representative of 2 experiments totaling at least 4 mice.

### 3.2.4 Resident memory CD8\(^+\) T cell migration is locally restricted

We found that memory P14-GFP CD8\(^+\) T cells in the SI move at 5\(\mu\)m/minute (Figure 3.1). Even though this speed is modest compared to CD8\(^+\) T cells in LNs, in a span of 25 hours it implies a single CD8\(^+\) T cell traveling in a straight line can cover up to 7.2mm. However, our imaging showed that CD8\(^+\) T cell motility was relatively confined (during a 30-40 minute imaging window) at day 30 after LCMV infection, and suggested that the distance CD8\(^+\) T cells could travel might be restricted. We found similar results using a different viral infection, where memory OT-I CD8\(^+\) T cells, which recognize the K\(^b\)-SIINFEKL epitope of Ovalbumin (OVA), had slowed motility at day 30 post VSV-OVA (data not shown). To assess the distance traveled by memory CD8\(^+\) T cells in the SI, we took advantage of the Kaede fluorescent reporter mouse, which during steady state emits GFP but upon short exposure to violet light can be photoconverted to emit red fluorescent protein (RFP)\(^{231}\). Here, by detecting change in fluorescence, one can track cells from a site of origin\(^{232}\). We crossed Kaede mice to OT-I mice and the resulting OT-I Kaede CD8\(^+\) T cells were adoptively transferred to B6 mice that were subsequently infected with vesicular stomatitis virus (VSV)-OVA. To verify specific conversion of OT-I Kaede CD8\(^+\) T cells, a piece of the SI was explanted and imaged before and after photoconversion from the serosal side of the tissue. Conversion of OT-I Kaede CD8\(^+\) T cells was
observed in both the crypts and the villi of the SI and was highly specific to OT-I Kaede CD8+ T cells, as other cells in the tissue were not affected by photoconversion (figure 3.4A). For tracking converted OT-I Kaede CD8+ T cells in vivo, a small piece of the ileum was surgically exposed and subjected to excitation with 405nm light at three different spots for 2.5 to 10 minutes. Following conversion, the tissue was replaced in the mouse peritoneal cavity, and 24 hours later harvested for evaluation of the presence of photoconverted OT-I Kaede CD8+ T cells in the different parts of intestine. As expected, photoconverted OT-I Kaede CD8+ IELs and LPLs (Kaede-red) were present in the ileum of mice exposed to the light but remained undetectable in sham surgery controls (Figure 3.4B C-D). In the jejunum and duodenum of photoconverted animals, only few (<1%) of converted OT-I Kaede CD8+ T cells were detected (Figure 3.4B-D). Negligible photoconverted OT-I Kaede CD8+ T cells were detectable in the mesenteric lymph nodes (MNL), spleen, and peripheral blood lymphocytes (PBL) (Figure 3.4D). These data suggest that memory CD8+ T cells in the SI survey relatively narrow regions.
Figure 3.4 Memory CD8+ T cells in the small intestine have narrowed migration. OT-I Kaede CD8+ T cells were transferred to B6 mice and infected 1 day later with VSV-OVA. A) Photoconversion of OT-I Kaede CD8+ T cells in the crypt (left panels) and villi (right panels) of the ileum 30 days after VSV-OVA infection. B) Flow cytometric analysis of photoconverted OT-I Kaede IELs (Kaede-red) in the indicated parts of the intestine 30 days after VSV-OVA infection. C) Same as B but OT-I Kaede LPLs. D) Frequency of photoconverted (Kaede-red) IELs and LPLs in B and C as well as in the mesenteric lymph node (MLN), spleen, and peripheral blood lymphocytes (PBL). All flow plots were gated on OT-I CD8+ T cells. Bar graphs show SEM. Data are representative of 3 experiments totaling at least 6 mice.
3.2.5 Self-specific CD8\(^+\) T cells are arrested in the presence of cognate antigen

In many tissues, self-specific CD8\(^+\) T cells exhibit altered motility in comparison to foreign antigen-specific CD8\(^+\) T cells and that this may be a mechanism of peripheral tolerance\(^{128,129}\). As the SI is a primary site of infection, it needs to maintain active immune surveillance; however, it must also curtail inappropriate immune responses to self-protein, normal flora, and food antigen. We wondered whether the motility of self-specific CD8\(^+\) T cells would be impacted by the presence of autoantigen in the SI. To examine this, we used mice which expressed OVA under the control of the intestinal fatty acid binding promoter (I-FABP OVA); Here, OVA is expressed as a self-protein in the mature enterocytes of the SI\(^{180–182}\). When OT-I CD8\(^+\) T cells were adoptively transferred into I-FABP OVA mice, large numbers of OT-I CD8\(^+\) IELs and LPLs were detected in the SI at both day 5 and day 10 following transfer\(^{181,182}\), Figure 4.5A, C). The intraepithelial compartment was more densely populated by OT-I CD8\(^+\) T cells compared to the LP, indicating self-specific OT-I CD8\(^+\) T cells were able to localize to areas where their cognate autoantigen was present. This is in contrast to the P14 CD8\(^+\) T cell/LCMV system, where the opposite was observed (Figure 3.1). OT-I CD8\(^+\) T cells in I-FABP OVA mice were unresponsive to antigen stimulation \textit{ex vivo} at both time points after transfer and the vast majority failed to make cytokine in response to SIINFEKL peptide (Figure 3.5B, D). To study the \textit{in situ} behavior of this self-specific CD8\(^+\) T cell population, we transferred naïve OT-I-GFP CD8\(^+\) T cells to I-FABP OVA mice. While OT-I CD8\(^+\) T cells did not
respond to antigen *in vitro, in vivo* they displayed slow migration phenotype at day 5 following transfer and moved at a speed of ~4µm/minute (Figure 3.5 E-F, supplementary movies 3.7).

**Figure 3.5** Self-specific CD8$^+$ T cells are slowed in the presence of autoantigen. OT-I CD8$^+$ T cells were transferred to mice expressing OVA in the SI (I-FABP OVA) and evaluated at day 5 and day 10 after transfer. A) Flow cytometric analysis of OT-I IELs (top) and LPLs (bottom) in I-FABP OVA mice at days 5 and 10 after transfer. Plots are gated on live lymphocytes. B) Cytokine production after SIINFEKLI peptide stimulation *ex vivo* of OT-I IELs and LPLs at the indicated time points after transfer to I-FABP OVA mice. Plots are gated on CD8$^+$ T cells. C)
Quantification of OT-I IELs (left) and LPLs (right) at day 5 (magenta) and day 10 (blue) after transfer to I-FABP OVA mice. **D** Percentage of cytokine producing OT-I IELs (left) and LPLs (right) after SIINFEKL peptide stimulation *ex vivo* at days 5 (magenta) and day 10 (blue) after transfer to I-FABP OVA mice. **E** OT-I-GFP CD8+ T cells (cyan) were transferred to I-FABP OVA mice and evaluated in the villi using TPLSM at day 5 (left most panel) and 10 (right most panel) after transfer. Anti-Kb-SIINFEKL antibody was administered to I-FABP OVA mice 5 days after OT-I GFP CD8+ T cell transfer (middle panel). Imaris generated cell tracks are in orange. **F** Mean track speed

Anti-Kb-SIINFEKL antibody was used to dissect whether this slow speed of OT-I CD8+ T cells was dependent on interactions with autoantigen 111. Upon blocking of Kb-SIINFEKL binding, OT-I CD8+ T cells accelerated to ~5.5μm/minute, suggesting that self-specific CD8+ T cells were actively interacting with self-antigen in the SI (Figure 3.5, E-F, supplementary movie 3.7). At day 10 after OT-I-GFP CD8+ T cell transfer to I-FABP OVA mice, OT-I-GFP CD8+ T cells were nearly ~2 fold faster than day 5 OT-I-GFP CD8+ T cells (Figure 3.5, E-F, movie not shown). While average speed was different at day 5 in comparison to day 10 OT-I-GFP CD8+ T cells, the median turning angle and confinement ratio remained unchanged between the groups (Figure 3.5, G-H). Similarly, anti-Kb-SIINFEKL blocking antibody did not alter these motility measurements (Figure 3.5, G-H). These data indicate the ability of self-specific CD8+ T cells to interact with self-antigen in the SI and this interaction can modulate T cell migration.
3.3 Discussion

CD8+ T cell activation and effector functions are dependent on directly contacting other cells. This is important for restraining cytotoxicity to infected and transformed cells, but imposes a requirement for CD8+ T cells to be highly motile in their quest for target cells. The development of TPLSM has led to a better understanding of how CD8+ T cells migrate deep inside SLOs and NLTs, and has also provided insight into disease pathogenesis and immune control of infection. Through these studies, it has become appreciated that lymphocyte migration in different tissues is variable and suggests the need for CD8+ T cell mobility to adapt to environmental cues.

The SI is a challenging tissue for CD8+ T cells to traverse. In the mouse, it spans ~30cm but the surface area is extensively increased by the presence of villi and crypts that facilitate maximal nutrient absorption. This poses a large burden of surveillance on CD8+ T cells to adequately scan the SI for infected and cancerous cells. As the largest mucosal surface of the body, the SI is often the site of primary infection, and encounters an onslaught of microbes daily. In addition to sustained inspection and elimination of pathogens, the SI, somewhat ironically, must also maintain tolerance to normal protein and food antigens. This constant balance between activation and suppression of the immune response makes the SI one of the most dynamic immune environments in the host. Evaluating CD8+ T cell locomotion in the SI may reveal how effective immunosurveillance of this tissue is managed. Additionally, these studies could
inform vaccine development and therapy targeting diseases and cancers that require CD8+ T cells for protection.

During the early effector response to LCMV infection (day 5) we found that P14-GFP CD8+ T cells in the crypt and villi of the jejunum moved at ~5µm/minute. It has been shown in many model systems that CD8+ T cells slow and arrest following TCR engagement with cognate antigen. Therefore, this slowed T cell motility at early time points after infection was expected, as viral antigen is still present. Interestingly, by day 8 after infection, P14-GFP CD8+ T cells in both the crypt and villi accelerated nearly 1.6 fold. This might suggest that the motility stop-signals induced through TCR engagement were no longer present at this time. Supporting this, the increase in P14-GFP CD8+ T cell velocity at day 8 coincided with what has been shown for LCMV clearance. The initial arrest during TCR engagement and increase in speed shortly after antigen clearance has been observed in solid tumor models. In a study by Boissonnas et al., evaluating thymoma specific CD8+ T cell responses in situ, tumor-specific CD8+ T cells initially arrested on the tumor periphery until tumor cells were eliminated. After tumor cell clearance, tumor-specific CD8+ T cells sped up and moved along collagen fibers and blood vessels to reach other areas with living tumor cells. These data suggest that CD8+ T cells may increase speed to seek out remaining antigen in other areas of the tissue, which could be happening in our model at day 8 after LCMV in the SI. Interestingly, the median turning angle and confinement ratio was not different between P14-GFP CD8+ T cells at day 5 and day 8 after infection in either the crypts or the villi. It might be
expected that if day 8 P14-GFP CD8$^+$ T cells were searching for remaining antigen that the median turning angle would decrease and the confinement ratio would increase, indicating a more exploratory search pattern. It is possible that the tissue architecture might supercede targeted motility, as perhaps the most direct route from one point to another may be through the intestinal lumen, and we did not observe any “space walkers”. Indeed CD8$^+$ T cells were confined to orbiting crypts and circling within villi.

Many groups have shown the impact of tissue composition on CD8$^+$ T cell motility$^{82,101,104,114}$. In addition to the mucosa, the SI also contains a complex muscle layer, which facilitates peristalsis$^{223}$. In the muscularis externa of the SI, effector P14-GFP CD8$^+$ T cells elongated, and were largely immobile, moving at nearly 2µm/minute. P14-GFP CD8$^+$ T cells also displayed a very wide median turning angle indicative of confinement. This behavior did not change after antigen clearance, which suggested that, unlike in the crypt and villi, this arrest seemed to be independent of TCR engagement. Visually, the cells appear to be held between muscle cells, which could be guiding the lethargic behavior of P14-GFP CD8$^+$ T cells in this part of the tissue. Researchers evaluating CD8$^+$ T cells in the skin observed similar phenomena, where motility of T cells within the epidermis was restricted to 2 dimensional movements. This behavior was consistent irrespective of whether T cells were effector or memory cells, indicating a role for tissue architecture in governing T cell mobility$^{120,121}$.

At a memory time point following LCMV infection, T$_{RM}$ were only detectable in the villi. In this compartment, memory T$_{RM}$ decelerated and
returned to a speed of 5 \( \mu \text{m/minute} \). These cells had a greater median turning angle and a lower confinement ratio than P14-GFP CD8\(^+\) T cells in the villi at day 5 and 8 after LCMV infection. It is somewhat confounding that CD8\(^+\) T cells in the SI were slower and more confined at a memory time point, given the large area of tissue to survey. In experiments using Kaede excitation to track CD8\(^+\) T cell location from a designated point of origin we did not observe migration outside the small area of photoconversion. This suggests that memory CD8\(^+\) T cells in this compartment may have a designated “area code” that they are responsible for carefully surveying.

Since antigen from LCMV was no longer present at this time point, TCR engagement was not responsible for the confined motility of memory P14-GFP CD8\(^+\) T cells. In both the skin and liver, CD8\(^+\) T cell interactions with integrins have been shown to be important for T cell behavior \(^{227–229}\). Since the \( \alpha_E \) integrin, CD103, gradually increased in expression on P14-GFP CD8\(^+\) T cells during infection with LCMV to nearly 100% of IELs and LPLs, we wondered if perhaps CD013 interacting with E-cadherin might be responsible for the movement of memory CD8\(^+\) T cells in the SI. Furthermore, studies have shown that CD103 is required for conventional CD8\(^+\) T cell maintenance in the epithelium of the SI \(^{209}\). At all time points after LCVM infection in both the crypts and the villi, P14-CFP CD103\(^{-/-}\) CD8\(^+\) T and P14-GFP CD8\(^+\) T cells displayed identical behavior. Similar results were observed when CD8\(^+\) T cells lacked \( \beta_1 \) integrin (data not shown). These results show that motility of memory CD8\(^+\) T cells in the SI is likely not
dependent on integrins. During infection, the ECM can be drastically reorganized. Perhaps there was some restructuring of connective tissue during LCMV infection that gave effector P14-GFP CD8$^+$ T cells more liberty to migrate within the SI. This may have subsided after pathogen clearance, leading to the restricted locomotion of memory P14-GFP CD8$^+$ T cells. Further investigation into any change in ECM composition after LCMV infection will be helpful to define what instructs memory CD8$^+$ T cell behavior in the SI.

As CD8$^+$ T cells require migration to mount an effective immune response, it could be that alterations in T cell motility serve as a tolerance mechanism to suppress the ability of self-specific CD8$^+$ T cells to respond to cognate antigen. Supporting this claim, in a study evaluating self-specific T cell responses to islet protein, PD-1 expression inhibited T cell arrest\textsuperscript{128}. As the SI actively maintains tolerance of inappropriate immune responses, we sought to investigate motility of self-specific CD8$^+$ T cells to intestinal protein. Here we adoptively transferred OT-I-GFP CD8$^+$ T cells to mice expressing OVA in the enterocytes of the SI. While the majority of self-specific OT-I-GFP CD8$^+$ T cells were unable to respond to self-antigen \textit{ex vivo} and failed to make cytokine in response to cognate peptide stimulation, the cells were found to be actively scanning the tissue. This behavior was dependent on self-antigen, as blocking interactions using an anti-K$^b$-SIINFEKL antibody prevented OT-I-GFP CD8$^+$ T cell arrest. These results unveiled drastic differences between self-specific CD8$^+$ T cell responses to antigen \textit{ex vivo} and \textit{in vivo} that would not have been observed without the use of TPLSM. Interestingly, by day 10 after OT-I-GFP CD8$^+$ T cells
were transferred to I-FABP OVA mice, the cells were no longer arrested and had accelerated nearly two-fold. It might be that these cells had more fully differentiated into a tolerogenic phenotype and were less sensitive to endogenous antigen by day 10. At both 5 and 10 days after OT-I-GFP CD8+ T cell transfer to I-FABP OVA mice, no differences in median turning angle or confinement ratio were observed. Interestingly, these measurements of motility were comparable to those of P14-GFP CD8+ T cells at a memory time point. This enforces the notion that the less hindered mobility of P14-GFP CD8+ T cells at days 5 and 8 is due to microbial induced inflammation and tissue restructuring.

Our studies show that CD8+ T cell mobility in the SI is disparate throughout the course of viral infection. While this motility is not dependent on αE and β1 integrins, it is governed by the complex tissue architecture within the SI, and CD8+ T cell locomotion varies between the muscle layers and the mucosa. Our findings also demonstrate the potential of self-specific CD8+ T cells to survey and respond to self-protein at early time points during tolerance induction. Knowledge from this work furthers our understanding of the requirements for effective CD8+ T cell immunosurveillance of SI.

3.4 Materials and Methods

3.4.1 Mice and infections.

C57BL/6J (B6), C57BL/6-Tg(UBC-GFP)30Scha/J (ubiquitin-GFP), Tg(CAG-ECFP)CK6Nagy/J (actin-ECFP) and B6.129S2(C)-Itgae<sup>tm1Cmp</sup>/J
(CD103KO) mice were purchased from The Jackson Laboratory and were maintained in specific pathogen free conditions at the University of Minnesota. I-FABP OVA, CD45.1 P14, and CD45.1 OT-I mice were maintained in house. Kaede mice were a generous gift from the Jameson/Hogquist labs. P14 and OT-I reporter mice were generated by crossing the mice with ubiquitin-GFP or actin-ECFP mice. P14-CFP+ CD013-/- mice were generated by crossing P14-CFP+ mice to P14+ CD103-/- mice. Kaede B6 231 mice were crossed to OT-I mice to generate OT-I Kaede reporter mice. All mice were used in accordance with the Institutional Animal Care and Use Committees guidelines at the University of Minnesota. P14 immune chimeras were generated by transferring 5 X 10^4 P14-GFP CD8+ T cells into naïve B6 mice followed by infection with 2 X 10^5 plaque-forming units (PFU) LCMV Armstrong via intraperitoneal (i.p.) injection one day later. For CD103-/- experiments, 2.5 X 10^4 P14-GFP CD8+ T cells and 2.5 X 10^4 P14-CFP CD8+ CD103-/- T cells were transferred to naïve B6 mice and infected with LCMV-Armstrong via i.p. injection one day later. OT-I immune chimeras were generated by transferring 5 X 10^4 naïve OT-I-GFP CD8+ T cells to I-FABP OVA mice. For OT-I Kaede immune chimeras, 5 X 10^4 naïve OT-I Kaede cells were adoptively transferred to B6 mice. One day later, mice were infected with 1 X 10^6 PFU of VSV-OVA.

### 3.4.2 Tissue harvesting and processing

Organs were harvested and digested as previously described 138. For isolation of SI IEL, Peyer's patches were removed, the SI was cut longitudinally
and then laterally into small pieces. Pieces were incubated for 30 minutes with stirring at 37°C with 0.154mg/mL dithioerythritol (Sigma-Aldrich, St. Louis, MO) in 10% HBSS/HEPES. The pieces were then vortexed on high speed to dislodge intraepithelial lymphocytes and the supernatant was collected. The SI pieces were washed in RPMI containing 5% FBS and put in RPMI 1640 containing 5% FBS, 2 mM MgCl2, 2 mM CaCl2 and 0.5mg/mL 100 U/mL type I collagenase (Worthington, Lakewood, NJ) and incubated for 45 minutes at 37°C with stirring to obtain LPLs. After enzymatic digestion, the remaining tissue pieces were mechanically disrupted using a gentleMACs dissociator (Miltenyi Biotec, San Diego, CA). Single cell suspensions from supernatant of the digestions were further separated using a 44/67% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient. Spleen and lymph nodes (LN) were dissociated mechanically. Splenocytes and blood were treated with ACK lysis buffer to lyse red blood cells.

**3.4.3 Flow cytometry and peptide stimulation**

The following antibodies were used for flow cytometry: anti-CD44 (IM7) and anti-Thy1.1 (HIS15)( eBioscience, San Diego, CA), anti-CD8α (53-6.7, eBioscience, Biolegend, San Diego, CA) anti-CD69 (H1.2f3, BD biosciences, San Jose, CA, eBiosciences, San Diego, CA), and anti-CD103 (m290, BD Horizon, BD Pharmingen, San Jose, CA 2E7, Biolegend, San Diego, CA), anti-CD45.1 (A29, Tonbo Biosciences, San Diego, CA). Cell viability was determined using Ghost Dye™ Red 780 (Tonbo Biosciences, San Diego, CA).
Peptide stimulation was performed as previously described \textsuperscript{217}. Briefly, splenocytes were plated in RPMI 1640 containing 10% FBS, 1x NEAA, 2mM L-glutamine, 1mM sodium pyruvate, 1x penicillin/streptomycin and 0.05mM \(\beta\)-mercaptoethanol and incubated with 1\(\mu\)g/mL SIINFEKL peptide for four hours at 37\(^\circ\)C. All samples received 1\(\mu\)g/mL GolgiPlug (BD Biosciences, San Jose, CA). Cells were washed and stained for surface and intracellular staining. Ghost Dye\textsuperscript{TM} Red 780 (Tonbo Biosciences, San Diego, CA) was used during surface staining to evaluate cell viability. Samples were acquired on an LSRII or LSR fortessa flow cytometer (BD Biosciences, San Diego, CA). For intracellular stains, the BD Biosciences intracellular kit for cytokine staining was used in accordance with manufacturer’s instructions.

3.4.4 Tissue freezing, immunofluorescence and microscopy

Harvested murine tissues were fixed in 2% paraformaldehyde for 2hrs before being treated with 30% sucrose overnight for cryoprotection. The sucrose treated tissue was embedded in tissue freezing medium OCT and frozen in an isopentane liquid bath. Frozen blocks were processed, stained, and imaged. Stains included antibodies against the following markers- E-cadherin (DECMA-1, Abcam), Collagen IV (Millipore), CD31 (MEC13.3, BD Horizon, San Jose, CA). Bovine \(\alpha\)-goat (polyclonal) was used as a secondary antibody (Jackson Immunoresearch).
3.4.5 Photoconversion of OT-I Kaede CD8+ T cells

Prior to surgery, mice were anesthetized with i.p. injection of Avertin (2,2,2-Tribromalethanol) (200mg/Kg). The upper abdominal area of the anesthetized mice was shaved and cleaned with Providone iodine solution followed by 70% alcohol. A 1-2cm long, horizontal midline incision was made in the belly skin to expose the peritoneum. The intestine was visualized by gently retracting the skin and then a second 1-1.5cm incision was made to open the peritoneal cavity at this point. A small loop (1cm²) of the ileum was carefully pulled through the opening in the peritoneal cavity. This piece of tissue was exposed to a 405nm UV laser at 100mW/cm² for periods of 2.5 to 10 minutes. Then the loop of intestine was returned to the peritoneal cavity and the peritoneal wall was closed with monofilament synthetic absorbable surgical suture. The skin was closed with 3-4 wound clips. Extreme care was taken to avoid Peyer’s patches and tears in the mesentery.

3.4.6 Two-photon laser scanning microscopy and analysis

A piece of apical jejunum was harvested, cut longitudinally, and mounted on a cover slip. For experiments with Hoescht (Invitrogen), mice were injected with 1mg of Hoescht 30 minutes- 24 hours before imaging. In Kb-SIINFEKL blocking experiments, mice were i.v. injected with 100µg of anti-Kb-SIINFEKL
antibody 1 hr before imaging (25-D1.15, BioXcell) \textsuperscript{111}. Movies were acquired using a MP SP5 two-photon microscope TCS (Leica) equipped with a Mai Tai HP DeepSee lasers (SpectraPhysics), an 8,000-Hz resonant scanner, a 25×/0.95 NA objective, and two-NDD and two HyD photo multiplier tubes. Samples were excited at 860 or 890nm and multiple fluorophores were imaged using the custom dichroic mirrors with the following collections: SHG <440nm, CFP 435-485nm, GFP 500-520nm, Hoescht 461nm. Data were processed with Imaris software (version 7.6.4 and version 8.0.1). Migrational analysis were performed using motility lab (created by the MotilityLab team, 2015: Johannes Textor, Jeffrey Berry, Mark J. Miller) or a macro \textsuperscript{128}. The drift correct function in Imaris and motility lab was used to correct SI contraction during imaging.

3.4.7 Statistical analysis For experiments comparing a single variable between two unpaired groups, a Mann-Whitney test was performed. When a single variable between multiple groups was compared, a one-way ANOVA with Dunn’s multiple comparison was employed. All statistics were performed using Prism (GraphPad Software, V7) and for all analysis $p$ values of less than 0.05 were considered significant an indicated by one or more asterisks (*).
Chapter 4

Repeated antigen stimulation generates functional self-specific CD8+ T cells
4.1 Introduction

A major function of CD8$^+$ T cells is to eliminate target cells through the directed release of perforin and granzyme B. This is a highly controlled process, as CD8$^+$ T cells express a T cell receptor (TCR) that recognizes a particular peptide fragment presented in the context of major histocompatibility complex I (MHCI)$^{2,151}$. All nucleated cells express MHCI, which serves as a means for CD8$^+$ T cells to survey all cells for infection and malignancy. As TCR development is a random process, and uninfected cells express self-protein in the context of MHCI, it is important for the immune system to eliminate CD8$^+$ T cells with TCRs that are specific for self-epitopes to prevent a potential autoreactive response$^{141}$. Central tolerance mechanisms aim to eliminate CD8$^+$ T cells that bind with high affinity to self-peptide:MHCI through autoimmune regulator (AIRE) mediated expression of tissue proteins on medullary thymic epithelial cells$^{142,143,145}$. While central tolerance negatively selects many self-reactive CD8$^+$ T cells, the process is leaky, and low affinity self-specific cells occasionally escape$^{141}$. Similarly, it is not feasible for every tissue antigen to be presented in the thymus. Mechanisms in the periphery serve as a failsafe to ensure that self-specific T cells in circulation undergo activation induced cell death, remain ignorant of antigen, are suppressed by T regulatory cells, or rendered nonfunctional through lack of co-stimulation$^{147}$.

These intrinsic mechanisms to maintain homeostasis and prevent attack of self-tissue can be very effective; however, there are instances where these
mechanisms are breached and lapses in self-tolerance result in the generation of a self-specific CD8+ T cell response. While these T cells present a possible threat to healthy tissues and have been shown to cause disease, they also serve as a CD8+ T cell pool that could be exploited for use in cancer immunotherapy, as many tumor cells express self-antigen:MHCI. Characterizing the generation and expansion of self-specific T cells and understanding their potential in mounting an effector response may not only aid in dissecting the mechanisms behind tissue homeostasis and autoimmune disease development, but can also inform strategies to manipulate self-specific CD8+ T cells for the treatment of cancer.

The small intestine (SI) is a unique tissue to study self-specific T cell responses, as it has the responsibility of maintaining balance between immune activation and tolerance to normal flora and food antigen. Inappropriate immune responses in this compartment can lead to autoimmune disorders, such as Crohn’s disease, whereby damage to intestinal tissue can cause extreme discomfort and digestive problems. Historically, self-specific CD8+ T cell responses to gut tissues have been difficult to assess because an exact self-antigen responsible for disease development has yet to be identified. Therefore, the development of a mouse expressing the model self-antigen ovalbumin (OVA) in the SI under the control of the intestinal fatty acid binding protein (I-FABP) promoter has allowed for study of self-specific CD8+ T cell responses to intestinal protein. Many groups have used heterologous-prime boost approaches to expand self-specific CD8+ T cells, however, to our knowledge, this has not been
used in generating an intestinal protein specific immune response, which might be under higher peripheral tolerance pressure than other tissues \cite{166,167,174,177}.

Here, we used three serologically distinct vectors delivering OVA antigen, and were able to generate a large population of self-specific CD8\(^+\) T cells. These T cells were functional and displayed a memory phenotype similar to CD8\(^+\) T cells generated against a foreign antigen. Interestingly, while an appreciable population of self-specific T cells as not observed with MHCI OVA tetramer binding after primary and secondary infection, we were able to detect large populations of self-specific CD8\(^+\) T cells at these time points using peptide to stimulate interferon-\(\gamma\) (IFN\(\gamma\)) production. T Cells after the tertiary infection were able to bind tetramer readily, were more sensitive to cognate antigen, and able to produce higher IFN\(\gamma\) on a per cell basis than T cells after secondary infection. This suggested that T cells at earlier time points during heterologous prime-boost-boost (HPBB) were of lower affinity and the response matured over time. The self-specific response at tertiary was not due to the outgrowth of a single, high avidity T cell clone specific for self-antigen, as the response was polyclonal and unique among individual mice in comparison to nonself-specific CD8\(^+\) T cell responses. Our studies show the great potential of self-specific CD8\(^+\) T cells. While self-specific T cells have a low affinity TCR that allowed them to go undetected by peripheral tolerance mechanisms, they could still be expanded with robust stimulation and produce cytokine in response to peptide. Thus, this self-specific CD8\(^+\) T cell pool in the periphery represents a kind of stealth
population of T cells that under the appropriate conditions, could safely be
harnessed for use in cancer immunotherapy.

4.2 Results

4.2.1 Repeated antigenic stimulation generates a large, functional
population of self-specific CD8\(^+\) T cells.

Self-specific CD8\(^+\) T cells that have escaped central and peripheral
tolerance mechanisms are likely low affinity and robust antigenic stimulation
may be required for their expansion. Heterologous prime-boost-boost (HPBB)
approaches, whereby three serologically distinct vectors carrying the same
epitope are used to generate antigen-specific CD8\(^+\) T cells, have been shown to
induce large numbers of CD8\(^+\) T cells \(^{71,72,74}\). Several groups have also used this
approach to expand self-specific CD8\(^+\) T cells \(^{166,169,170,174-177}\). To evaluate if
HPBB can break tolerance to an intestinal protein, we used I-FABP OVA mice,
where OVA is expressed as a self-protein in mature enterocytes of the SI.

To adapt HPBB to I-FABP OVA mice, mice were sequentially infected with
vesicular stomatitis virus (VSV)-OVA, *Listeria monocytogenes* (Lm)-OVA and
vaccinia virus (VV)-OVA with at least 30 days between each boost (Figure 4.1A).
K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells were evaluated from splenocytes at a memory
time point after each infection. Even after 2 rounds of infection (VSV-OVA+Lm-
OVA, 2°), there is minimal expansion of K\(^b\)-SIINFEKL-specific cells in I-FABP
OVA mice, resulting in less than 1% of CD8\(^+\) T cells (Figure 4.1B). In contrast,
non-OVA-antigen bearing B6 control mice mount a large response to 2°, resulting in 16% K\(^{b}\)-SIINFEKL-specific CD8\(^{+}\) T cells (Figure 1B). As expected, after 3° infection in B6 mice, a large percent of K\(^{b}\)-SIINFEKL-specific CD8\(^{+}\) T cells was observed and maintained at memory, reaching \(\sim 53\%\) of total CD8\(^{+}\) T cells \(^{71,74}\). Interestingly, while K\(^{b}\)-SIINFEKL cells in I-FABP OVA mice following 2° infection were rare, they were readily detectable following the third infection (Figure 4.1B). While there was a significantly lower number of K\(^{b}\)-SIINFEKL-specific CD8\(^{+}\) T cells in I-FABP OVA mice as compared to B6, they increased nearly 14-fold in percentage from 2°, and accounted for \(\sim 500,000\) K\(^{b}\)-SIINFEKL-specific CD8\(^{+}\) T cells (Figure 4.1B-C). The expansion of self-specific T cells was not the result dual TCR expression, where another TCR would be vector specific. T cells singly bound to K\(^{b}\)-SIINFEKL tetramer or MHCI tetramers with peptides from the nucleoprotein of VSV (K\(^{b}\)-N) or the B8R epitope from VV (K\(^{b}\)-B8R) (Figure 4.1D).

It might be expected that self-specific T cells would be exhausted or anergic given the constant presence of self-antigen in I-FABP OVA mice \(^{235}\). We measured inhibitory receptor expression on self-specific T cells and assessed their ability to receive survival signals at day 30 following 3° infection. Self-specific CD8\(^{+}\) T cells in I-FABP OVA mice remained low for programmed cell death protein-1 (PD-1) and lymphocyte activation gene-3 (Lag-3) and expressed higher levels of the anti-apoptotic marker B cell lymphoma-2 (Bcl-2) in comparison to B6 control mice (Figure 4.1E). Self-specific CD8\(^{+}\) T cells were responsive to antigen and produced IFN\(\gamma\) and tumor necrosis factor \(\alpha\) (TNF\(\alpha\)).
after SIINFEKL peptide stimulation (Figure 4.1F). Thus, self-specific CD8+ T cells generated through HPBB were functional, did not exhibit a phenotype associated with exhaustion, and expressed high levels of Bcl-2.

**Figure 4.1 Heterologous prime-boost-boost generates functional self-specific CD8+ T cells.** A) I-FABP OVA or B6 mice were boosted with the indicated vectors i.v. at least 30 days apart. B) At least 30 days after 1°, 2°, 3° infections, splenocytes from B6 (top) or I-FABP OVA (bottom) mice were evaluated for Kb-SIINFEKL+ CD8+ T cells. Plots are gated on CD8+ cells. C) Frequency of cells in (B). D) 30 days after 3° infection, splenocytes from I-FABP OVA mice were evaluated for dual binding of Kb-SIINFEKL cells to Kb-N (left) or Kb-B8R (right). E) Phenotypic markers on Kb-SIINFEKL+ CD8+ T cells on >day 30 splenocytes from B6 (black) or I-FABP OVA (red) mice in comparison to CD44lo naïve control CD8+ T cells. GMFI is quantified below. F) Cytokine expression after SIINFEKL peptide stimulation of splenocytes >day 30 after 3° infection. Plots are gated on CD8+ T cells. G) I-FABP OVA and B6 mice were boosted with the 3 sequential vectors as in (A) but lacking OVA. Kb-SIINFEKL responses were measured in splenocytes at least 30 days after 3° infection. The control plot is from a VSV-OVA infected B6 mouse. Plots are gated on CD8+ T cells. Data are representative of at least 2 experiments totaling ≥6 mice per group. All error bars are SEM. A Mann-
Whitney test was performed to evaluate significance. **** indicates a $p$ value of <0.0001, and *$p$ <0.03.

In experiments transferring transgenic T cells with a TCR specific for SIINFEKL (OT-I CD8$^+$ T cells) to I-FABP OVA mice, inflammation alone is enough to drive a destructive response against self-protein $^{181}$. We wondered whether vector delivered antigen was required to expand endogenous K$^b$-SIINFEKL-specific CD8$^+$ T cells. To test this, we administered the same three vectors used for HPBB but lacking OVA. Despite the presence of OVA in the SI of I-FABP OVA mice, there was no measurable K$^b$-SIINFEKL CD8$^+$ T cell response, indicating that OVA expressing vectors are necessary to induce self-specific CD8$^+$ T cell expansion (Figure 4.1G).

### 4.2.2 Self-specific CD8$^+$ T cells exhibit a canonical memory phenotype.

In B6 mice, HPBB generates a large memory population of CD8$^+$ T cells that is sustained long-term $^{71,72,74}$. We therefore wanted to understand if the self-specific T cells population generated through HPBB was able to establish memory. At least 30 days after the last infection, splenocytes from both B6 and I-FABP OVA mice were evaluated for a memory phenotype. Long-lived memory precursor cells express the interleukin-7 receptor α-chain, CD127, while short-lived effector cells lack this molecule and have high levels of killer cell lectin-like receptor subfamily G1 (KLRG1) $^{56,57}$. All K$^b$-SIINFEKL-specific CD8$^+$ T cells in both I-FABP OVA and B6 mice expressed CD127 at a memory time point,
however, self-specific T cells were nearly 100% double positive for KLRG1 and CD127 (Figure 4.2A-B). In memory CD8+ T cells, the transcription factors Tbet and eomesodermin (Eomes) work together to sustain memory homeostasis by regulating expression of cytokine receptors important for survival. While sustained high levels of Tbet has been associated with effector cell terminal differentiation, Eomes expression has also been shown to be important for memory generation and maintenance. Interestingly, both cell populations in our system had equal expression of the transcription factors Tbet and Eomes (Figure 2C) and also similarly expressed many other cell markers associated with memory formation and longevity such as CD27, CD25, lymphocyte antigen 6 complex (Ly6c), and CXCR3 (Figure 4.2D).

4.2.3 Self-specific CD8+ T cells are undetectable in the small intestine.

Even though large numbers of memory Kb-SIINFEKL CD8+ T cells in I-FABP OVA mice were generated following HPBB, mice did not exhibit any overt pathology in the SI. We questioned whether Kb-SIINFEKL-specific CD8+ T cells were located in tissues where self-antigen was present. While a robust population of Kb-SIINFEKL-specific CD8+ T cells was detected in the intestinal epithelial layer and lamina propria (LP) of B6 mice, there was little to no definable population of cells found in these compartments in mice expressing OVA in the SI (Figure 4.2E). Interestingly, in both control mice and I-FABP OVA mice, Kb-SIINFEKL-specific CD8+ T cells were present in the mesenteric lymph node (MLN). This was interesting as OVA is presented by dendritic cells (DCs) in
the MLN, which drains the SI \(^{181,236,237}\). We wondered why cells were able to survive in the MLN but were undetectable in the epithelium and LP. We investigated trafficking to those areas by evaluating the integrin \(\alpha_4\beta_7\), which is expressed at early time points after activation and important for entry into the small intestinal tissue\(^ {238–240}\). At day 4.5 in the blood of 3° mice, 30% of K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells in B6 mice expressed \(\alpha_4\beta_7\), and only 15% of K\(^b\)-SIINFEKL-specific CD8\(^+\) T cells in I-FABP OVA mice expressed this integrin (Figure 4.2F).

**Figure 4.2 Self-specific CD8\(^+\) T cells exhibit a memory phenotype.** A) At least 30 days after 3° infection, splenocytes were evaluated for CD127 and KLRG1 expression. Plots are gated on K\(^b\)-SIINFEKL specific CD8\(^+\) T cells. B) Frequency of CD127\(^+\) KLRG1\(^-\) (left) and CD127\(^+\) KLRG1\(^+\) cells (right) from (A). C) Transcription factor and D) memory marker expression were measured in memory splenocytes in I-FABP OVA (red) or B6 (black) mice. CD44\(^lo\) CD8\(^+\) T cells were used as a control. Plots were gated on K\(^b\)-SIINFEKL specific CD8\(^+\) T cells or CD44\(^lo\) CD8\(^+\) T cells. E) K\(^b\)-SIINFEKL specific CD8\(^+\) intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL), and mesenteric lymph node (MLN) were assessed >30 days after 3° infection. Plots are gated on CD8\(^+\) T
cells. F) Expression of α4β7 on K<sup>b</sup>-SIINFEKL specific CD8<sup>+</sup> T cells in B6 (black) or I-FABP OVA (red) d7 peripheral blood lymphocytes in histograms (left) and frequency (right). Plots are gated on K<sup>b</sup>-SIINFEKL specific CD8<sup>+</sup> T cells. Data are representative of at least 2 experiments totaling ≥6 mice per group. All error bars are SEM. A Mann-Whitney test was performed to evaluate significance. ** indicates a p value of <0.008.

4.2.4 Self-specific CD8<sup>+</sup> T cells bear TCR of low affinity compared to foreign antigen-specific T cells.

As K<sup>b</sup>-SIINFEKL-specific CD8<sup>+</sup> T cells from I-FABP OVA mice were not exhausted and able to produce cytokines in response to antigenic stimulation, we wanted to further dissect the functionality of K<sup>b</sup>-SIINFEKL-specific CD8<sup>+</sup> T cells from I-FABP OVA mice in comparison to B6 mice. Notably, K<sup>b</sup>-SIINFEKL-specific CD8<sup>+</sup> T cells from OVA bearing mice had significantly lower tetramer geometric mean fluorescent intensity (GMFI) than B6 control mice, although there were equal levels of TCRβ expression (Figure 4.3 A,C). This difference was not observed for vector-specific CD8<sup>+</sup> T cells, as the GMFI of tetramer staining detecting VSV-N specific or VV specific CD8<sup>+</sup> T cells was equal in T cells from B6 and I-FABP OVA mice (Figure 4.3 B-C). This suggested that K<sup>b</sup>-SIINFEKL self-specific CD8<sup>+</sup> T cells had TCR of lower avidity than TCR on B6 cells.
Figure 4.3 Kb-SIINFEKL-specific CD8+ T cells in I-FABP OVA mice have TCR of lower affinity compared to B6.  

A) Kb-SIINFEKL, TCRβ or B) Kb-N expression was evaluated in B6 (black) and I-FABP OVA (red) splenocytes at day 30 after 3° infection. C) Tetramer GMFI of samples in (A) (B) and Kb-B8R binding CD8+ T cells (not pictured in histograms). D) B6 and I-FABP OVA splenocytes were stimulated with SIINFEKL peptide (µg/mL) as in (E) GMFI of BrdU in K b SIINFEKL+ CD8+ T cells was evaluated in PBL at day 4 (top plots) or day 7 (bottom plots) and quantified as a frequency BrdU+ cells out of K b SIINFEKL specific CD8+ T cells. Plots are gated on CD8+ cells. F) As in (E) GMFI of BrdU in Kb-SIINFEKL-specific CD8+ T cells from I-FABP OVA mice (red) or B6 mice (black) on day 4 (left) or day 7 (right) in PBL. G) PD-1 expression at day 4 (left) and day 7 (right) after 3° infection on I-FABP (red) OVA or B6 (black) K b SIINFEKL-specific cells in PBL. H) CD62L expression on K b SIINFEKL specific CD8+ T cells in PBL in I-FABP OVA mice (red) or B6 mice (black) on days 4, 7, 10 after 3° infection. In all, CD44lo cells were used as a control. Data are representative of at least 2 experiments totaling >8 mice per group. All error bars are SEM. A Mann-Whitney test was performed to evaluate significance. * indicates a p value of <0.02, **<0.007, ***<0.0001.
To understand if this affected the sensitivity of self-specific T cells to SIINFEKL antigen, we stimulated memory splenocytes with differing concentrations of SIINFEKL peptide. IFNγ production was evaluated as a percentage of the highest level of IFNγ expressing population, which was set at 100%. Kb-SIINFEKL-specific CD8+ T cells from B6 mice never went below 65% maximum IFNγ production, even at very low concentrations of antigen stimulation. In contrast, Kb-SIINFEKL-specific CD8+ T cells from I-FABP OVA mice were less sensitive to lower amounts of antigen, and, at the lowest concentration of SIINFEKL peptide, were producing approximately 16% of what they were at maximum (Figure 4.3D). We next sought to determine if self-specific T cells with lower affinity TCR were as competent in their proliferative capacity as foreign antigen-specific CD8+ T cells. For this, we pulsed drinking water with bromodeoxyuridine (BrdU) starting immediately after 3° infection, and looked at incorporation into Kb-SIINFEKL-specific CD8+ T cells as a marker for proliferation at day 4 and 7. At day 4, fewer self-specific CD8+ T cells had incorporated BrdU as compared to B6 CD8+ T cells, and this percentage did not increase by day 7. This indicated that approximately 30% of Kb-SIINFEKL-specific CD8+ T cells had not undergone proliferation, while nearly 88% of B6 Kb-SIINFEKL-specific CD8+ T cells had (Figure 4.3E). Given the high level of expansion observed in frequency of self-specific CD8+ T cells from 2° to 3° this was somewhat surprising. Furthermore, of the T cells incorporating BrdU in I-FABP OVA mice, less BrdU was taken up on a per cell basis at day 4, indicating
that dividing self-specific CD8⁺ T cells were proliferating less than nonself-specific CD8⁺ T cells in B6 mice (Figure 4.3F). This difference was not seen at day 7, where cells had similar BrdU GMFI to B6 (Figure 4.3F). Even while 70% of self-specific CD8⁺ T cells were proliferating, they still remained low for PD-1 expression at early time points following 3° boost (Figure 4.3G). Upon activation, T cells may down-regulate CD62L. While Kᵇ-SIINFEKL-specific CD8⁺ T cells from B6 mice had low expression of CD62L, self-specific CD8⁺ T cells from I-FABP OVA mice displayed delayed down-regulation of CD62L in comparison to B6 cells over a ten-day period (Figure 4.3H). In both BrdU and CD62L experiments, GMFI cannot be compared between the different time points as the experiments were performed on different days.

### 4.2.5 Kᵇ-SIINFEKL-specific CD8⁺ T cells from OVA bearing mice can be detected at earlier time points with SIINFEKL peptide stimulation.

Nearly half a million self-specific CD8⁺ T cells were detected after 3° infection in I-FABP OVA mice with MHCI tetramer, yet, far fewer were found after 1° and 2° vaccination events. This was surprising, given the large population of Kᵇ-SIINFEKL-specific CD8⁺ T cells at 3°, and we wondered if we were neglecting to detect self-specific cells with tetramer at earlier time points during HPBB. Several groups have used peptide stimulation to detect self-specific T cells that are low affinity. For this reason, we stimulated splenocytes with high concentrations of SIINFEKL peptide at memory time points after 1°, 2°, and 3° infections and measured cytokine production.
Expectedly, a robust population of cytokine producing CD8+ T cells was observed at all time points in B6 mice and these levels were numerically similar to those observed with tetramer (Figure 4.4A-B). However, at 1° infection, very few cells were detected with peptide stimulation in I-FABP OVA mice and this was not above background (Figure 4A and data not shown). In contrast to tetramer, we were able to detect a 3 times more Kb-SIINFEKL-specific CD8+ T cells with peptide stimulation in 2° I-FABP OVA mice (Figure 4.4A-B). Furthermore, a 6 fold difference was observed between tetramer binding and cytokine producing cells in 3° mice, showing that while more CD8+ T cells were able to bind tetramer at this time point, we were still failing to detect all self-specific CD8+ T cells. In fact, the cytokine producing population at 3° in I-FABP OVA mice is 2 fold less than B6 cells, while the number of CD8+ T cells binding tetramer was 12 fold less than B6 tetramer binding CD8+ T cells (Figure 4.4A-B). Kb-SIINFEKL-specific CD8+ T cells from 2° I-FABP OVA mice that were producing cytokine were less sensitive to antigen than cells from B6 mice at this time point. By tertiary, self-specific CD8+ T cells were able to produce IFNγ on a per cell basis similar to B6 CD8+ T cells at this concentration of peptide, indicating a maturation of the self-specific response over the course of boosting (Figure 4.4C).
Figure 4.4 Self-specific CD8+ T cells which are undetectable by tetramer can be visualized with peptide stimulation. A) Splenocytes from B6 (top) or I-FABP OVA mice (bottom) were harvested at least 30 days after 1°, 2°, 3° infection and were stimulated with 100µg/mL of SIINFEKL peptide. Plots are gated on CD8+ cells. Gates were set on no peptide controls. B) Quantification of B6 (black) and I-FABP OVA (red) IFNγ producing cells (open bars) from (A) in comparison to Kb-SIINFEKL tetramer binding (filled bars) from the same time points. C) Splenocytes from B6 (black) and I-FABP OVA (red) mice were stimulated with 10ug of SIINFEKL peptide and evaluated for IFNγ GMFI at least 30 days after 2° (top) or 3° (bottom) infections. Bar graphs are calculated as a percentage of maximum IFNγ GMFI expression (highest expression was set at 100%). Data are representative of 2 experiments totaling ≥6 mice per group. All error bars are SEM. A 2-way ANOVA with Tukey’s multiple comparisons test was used to evaluate significance. ** indicates a p value of <0.009, *** >0.006.

4.2.6 Kb-SIINFEKL-specific CD8 T cells from I-FABP OVA mice are polyclonal.

To gain insight into whether tolerance to self-protein was ablated due to the boosting and expansion of a single T cell clone specific for Kb-SIINFEKL in
HPBB or if the self-specific CD8+ T cell response was polyclonal, splenic cells were isolated from 3° B6 or I-FABP OVA mice and evaluated for Vβ usage. Both B6 and I-FABP OVA mice displayed similar polyclonality of CD8+ T cells not binding to Kb-SIINFEKL tetramer (Figure 4.5B). There was considerably more variation in Vβ of usage of Kb-SIINFEKL-specific CD8+ T cells between I-FABP OVA mice than compared to B6 Kb-SIINFEKL-specific CD8+ T cells (Figure 4.5C). These data indicate that the Vβ chain usage in I-FABP OVA mice is unique across mice and not composed solely by the outgrowth of a single clone.
Figure 4.5 Self-specific CD8$^+$ T cell V$\beta$ clonality is distinct among mice. A) Splenocytes were harvested at least 30 days after 3$^\circ$ infection from B6 and I-FABP OVA mice and evaluated for V$\beta$ clonality. Representative flow plots of V$\beta$ chain staining from bulk (top, gated on CD8$^+$ K$^b$-SIINFEKL negative cells) or K$^b$-SIINFEKL specific (bottom, gated on K$^b$-SIINFEKL specific CD8$^+$ T cells) CD8$^+$ T cells. B) Pie graphs of the indicated V$\beta$ chains of bulk CD8$^+$ K$^b$-SIINFEKL negative CD8$^+$ T cells in B6 (top) and I-FABP OVA mice (bottom). C) Pie graphs of the indicated V$\beta$ chains of K$^b$-SIINFEKL specific CD8$^+$ T cells in B6 (top) and I-FABP OVA mice (bottom). Each pie graph displays results from individual mice. Data are representative of 2 experiments with totaling $\geq 6$ mice per group.
4.2.7 **Kb-SIINFEKL-specific CD8\(^+\) T cells can be detected in OVA bearing mice using tetramer stabilization and enrichment techniques.**

Tetramer enrichment techniques have long been used to identify rare populations of antigen-specific cells in the naïve T cell pool \(^{12,242,243}\). We suspected that the stabilization and cross-linking of tetramer with antibodies used in this processes would help us to also identify low affinity T cells \(^{244}\). We tested this in our studies using a dual staining technique, where Kb-SIINFEKL tetramers conjugated to either PE or APC were used together to enrich for self-specific cells in the naïve T cell pool in B6 and I-FABP OVA mice. Using this technique, we were able to identify a naïve precursor pool in both B6 and I-FABP OVA mice (Figure 4.6A). Kb-SIINFEKL cell frequency in B6 mice was comparable to that previously published at approximately 231 cells per mouse \(^{242,245}\). These antigen-specific CD8\(^+\) T cells were present at a significantly lower number in I-FABP OVA mice, resulting in roughly 176 cells per mouse, which was a 25% reduction in comparison to B6 cells (Figure 4.6B). Even though cells in the I-FABP OVA mice had access to antigen, they maintained a naïve phenotype similar to B6 mice, and were low for CD11a, CD44, PD-1, and high for CD62L (Figure 4.6C).
Figure 4.6 Naïve K\textsuperscript{b}-SIINFEKL specific CD8\textsuperscript{+} T cells in I-FABP OVA mice. 
A) Spleen and macroscopic lymph nodes were harvested from naïve B6 (top) or I-FABP OVA mice (bottom) and enriched for K\textsuperscript{b}-SIINFEKL specific cells. Gates were set on flow through (left) and applied to the bound fraction (right). Plots are gated on CD8\textsuperscript{+} cells. 
B) Quantification of cells in (A). 
C) Phenotype of B6 (black) and I-FABP OVA (red) K\textsuperscript{b}-SIINFEKL CD8\textsuperscript{+} T cells isolated from naïve mice in A. Data are representative of 4 experiments totaling at \( \geq 11 \) mice in each group. All error bars are SEM. A Mann-Whitney test was performed to evaluate significance. * indicates a \( p \) value of < 0.02.

4.3 Discussion

It has become increasingly more appreciated that central and peripheral tolerance mechanisms are not complete, and CD8\textsuperscript{+} T cells specific for a wide array of self-antigens exist in the periphery of healthy human adults \( ^{160-162} \). This is substantiated by the common autoimmune side effects that occur when the brakes on the immune system are removed with checkpoint blockade \( ^{165,246} \).
Richards et al., showed that in mice nearly 4% of CD4+ and CD8+ T cells are suppressed by peripheral tolerance mechanisms and several groups have demonstrated the ability of low affinity cells to facilitate an immune response to self-protein. It is widely accepted that autoimmune disease stems from the inappropriate response of self-reactive T cells, so characterizing the potential of the self-specific CD8+ T cell pool is of paramount importance to understanding their threat to healthy tissues. Additionally, many tumor associated antigens are nonmutated self proteins, so harnessing mechanisms of tolerance reversal for an anti-tumor response is an attractive option for cancer immunotherapy.

To explore this, many groups have used repeat antigen stimulation to generate a self-specific T cell response to tissue antigens. In a model evaluating self-responses to OVA expressed in the pancreas (Rat insulin promoter expressing OVA, RIP-mOVA), Enouz et al., showed that Lm-OVA prime followed with a VSV-OVA boost resulted in expansion of low affinity self-specific T cells that were able to contribute to pancreatic islet destruction. Additional studies have shown that even T cells specific for a ubiquitously expressed self-protein can respond to antigenic stimulation and that these T cells were able to reduce tumor burden. These studies and others provide evidence for targeted expansion of self-specific CD8+ T cells.

The SI is unique from many other tissues where self-specificity has been studied in that it is tasked with maintaining a balance between immune activation to invading pathogens and enforcing tolerance to self, normal flora,
and food antigen. Using a mouse expressing the model antigen OVA in mature enterocytes of the SI, we show that CD8+ T cells specific for an intestinal antigen can shirk central and peripheral tolerance mechanisms and can be expanded to large numbers using a HPBB approach. While self-antigen is readily accessible to CD8+ T cells, self-specific T cells are not exhausted and remained low for inhibitory receptors, such as PD-1 and Lag-3. Even while self-specific CD8+ T cells are actively proliferating at early time points after 3° infection, these cells maintain a PD-1 low phenotype, which perhaps speaks to the low affinity TCR and strength of signal needed to induce or maintain PD-1 expression. Indeed OT-I CD8+ T cells upregulate high levels of PD-1 in response to self-protein in I-FABP OVA mice, which is important for tolerance induction in this system. This may indicate different TCR signaling thresholds for specific CD8+ T cell functions, whereby low affinity cells are able to secrete cytokines and proliferate in response to high concentrations of antigen, but inhibitory molecule expression is not induced. Recent work has shown that PD-1 expression on CD8+ T cells boosted with a high affinity epitope led to less tumor immunogenicity, perhaps suggesting that targeting expansion of low affinity cells might be beneficial, as low affinity cells seem to be refractory to peripheral tolerance induction through inhibitory receptor signaling. Importantly, after HPBB in OVA bearing mice, 3° Kb-SIINFEKL CD8+ T cells display a similar memory phenotype to B6 control mice, suggesting the long-term survival of these cells, which is vital to a successful preventative cancer vaccine. Self-specific CD8+ T cells also expressed Bcl-2, indicative of their ability to receive survival signals.
It was possible that self-specific CD8$^+$ T cells escaped central tolerance due to incomplete allelic exclusion, which can lead to dual TCR expression that allows autoreactive T cell survival during negative selection \(^{249}\). These cells have been shown to contribute to autoimmunity \(^{250,251}\). Perhaps K$^b$-SIINFEKL-specific CD8$^+$ T cells in I-FABP OVA mice were escaping selection through use of a dual TCR that was specific for a vector epitope and were subsequently boosted through their vector specific TCR. To evaluate this, we looked at the potential of K$^b$-SIINFEKL-specific CD8$^+$ T cells to bear VSV or VV specific TCRs. We did not observe any dual tetramer binding cells, indicating that the self-specific CD8$^+$ T cell population generated with HPBB did not possess dual TCRs. Additionally, non-OVA containing viral and bacterial vectors were unable to boost K$^b$-SIINFEKL-specific CD8$^+$ T cells in I-FABP OVA mice, corroborating these results.

HPBB generated cells with increasing functional avidity throughout the boosting schema. While self-specific CD8$^+$ T cells were readily detectable with tetramer following the third boost, at earlier time points there was not an appreciable population of tetramer binding CD8$^+$ T cells. Interestingly, many more antigen-specific CD8$^+$ T cells were observed after 2$^\circ$ infection using peptide stimulation, uncovering a lower affinity population of cells presumably unable to stably bind to tetramer. Failure to detect K$^b$-SIINFEKL tetramer binding cells at 2$^\circ$ in I-FABP OVA mice was not due to a limit of detection of the reagent as the number of cells detected by K$^b$-SIINFEKL tetramer in 1$^\circ$ B6 mice was similar to K$^b$-SIINFEKL-specific CD8$^+$ T cells in I-FABP OVA mice observed after peptide
stimulation. Secondary self-specific CD8+ T cells were also less sensitive to antigen than 3° cells, and produced less IFNγ on a per cell basis. The increase in antigen sensitivity between boosts and the ability of cells to bind tetramer indicated a maturation of the response over time. This enhancement of functional avidity was not due to domination by a single high affinity clone for self-antigen, as Vβ staining of Kb-SIINFEKL tetramer binding CD8+ T cells in I-FABP OVA mice were polyclonal and distinct between individual mice. This perhaps speaks to the complex nature of central tolerance and differences in thymic selection across mice due to differing antigenic pressure. Single cell transfers of OT-I CD8+ T cells are readily detected after Lm-OVA induced expansion, showing that even low numbers of high affinity cells are readily detectable. This further supports our claims that a single high affinity self-specific CD8+ T cell clone is not expanding, as we would likely detect it after boosting even if numerically rare before, and that functional avidity maturation is occurring on the cell population level. Indeed, studies have shown that functional avidity maturation does not always depend on high affinity TCR and can occur within a single clonal transgenic TCR population. Future studies evaluating Vβ chain usage of cytokine producing cells at 2° and 3° time points will determine if and how clonality evolves throughout the boosting regimen.

These data demonstrate the remarkable breadth of self-specific CD8+ T cells whereby they are able to evade deletion and suppression but are responsive, can proliferate, and perform effector functions. Indeed, it has been shown that T
cells under the avidity threshold for negative selection are still able to proliferate in response to stimulation. It could be that OVA specific CD8+ T cells in naïve I-FABP OVA mice remain ignorant of self-antigen due to their low affinity, but are able to respond to vector delivered antigen, which has an exponentially higher antigen load than what is endogenously expressed. This suggests a need for more interactions with cognate antigen, as heterologous boosting with vectors lacking OVA were unable to mount a self-antigen specific response. This was similarly observed in RIP-mOVA x Vβ5 mice, were Lm lacking OVA was insufficient to generate an autoimmune response. Further supporting this claim, Kb-SIINFEKL-specific CD8+ T cells in naïve I-FABP OVA mice were not antigen experienced, and remained CD44, CD11a, and PD-1 low. This was not due to inability to access OVA antigen, as adoptively transferred naïve OT-I CD8+ T cells readily divide in these I-FABP OVA mice and enter the SI.

Interestingly, in OT-I CD8+ T cell transfers to I-FABP OVA mice, inflammation alone is enough to drive a destructive autoimmune response to small intestinal tissue, also citing a role for TCR affinity in the varying responses to self-antigen.

Even though self-specific CD8+ T cells had undergone maturation and had higher functional avidity following HPBB, they were still lower affinity than antigen-specific CD8+ T cells from B6 controls. A study evaluating low affinity ligands showed that weaker interactions with antigen lead to reduced proliferation. While more than 60% of Kb-SIINFEKL self-specific CD8+ T cells in I-FABP OVA mice were able to proliferate, they incorporated less BrdU on a
per cell basis than compared to B6 T cells. Interestingly, even at this time point, 6x more K\^b-SIINFEKL-specific CD8\(^+\) T cells were present in I-FABP OVA mice as detected through peptide stimulation than what was able to bind tetramer. This vast population of self-specific cells remains PD-1 low and is remarkably not rendered anergic. While we expected less proliferation than B6 controls, it was surprising given the expansion of K\^b-SIINFEKL cells in I-FABP OVA mice at 3\(^\circ\) that not all cells were incorporating BRDU. It might be that the substantial increase of tetramer binding cells at 3\(^\circ\) is not entirely due to proliferation.

Researchers have shown in other model systems that altering the cellular membrane composition of tolerant cells in regards to TCR and CD8\(^\alpha\) proximity can rescue tetramer binding and interactions with antigen presenting cells \(^{253,254}\). Restoration of TCR and CD8\(^\alpha\) interactions may be contributing to tetramer binding our model, but further investigation is needed to definitively conclude this.

Interestingly, even with nearly 4 million functional self-specific CD8\(^+\) T cells present in the circulation of I-FABP OVA mice, we do not detect any overt intestinal pathology. This might be explained by our inability to detect SI intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), which could be due to several reasons. It could be that K\^b-SIINFEKL-specific CD8\(^+\) T cells cannot be detected and remain “hidden” in this compartment because of TCR down-regulation due to antigen-encounter in the intestine. Supporting this possibility, OT-I CD8\(^+\) T cells transferred to I-FABP OVA mice are only recovered
from the SI with congenic markers, as the TCR is downregulated and cells no longer bind to K<sup>b</sup>-SIIFEKL tetramer (Nelson, unpublished data). Another possibility is that K<sup>b</sup>-SIINFEKL-specific CD8<sup>+</sup> T cells are deleted through peripheral tolerance mechanisms after entry into the small intestine, but the long-term maintenance of high affinity cells in this compartment indicates that this is likely not the case<sup>181–183</sup>. A more plausible explanation may be that cells are simply poor at entering the SI, because of the reduced expression of α4β7. In any case, it is tempting to consider HPBB as a means to generating low affinity self-specific cells that are “at the ready” to potentially fight a potential cancerous threat while maintaining normal tissue homeostasis.

Our studies provide important insight into what characterizes and contributes to an endogenous, self-specific CD8<sup>+</sup> T cell response. HPBB targeting intestinal protein is able to expand self-specific CD8<sup>+</sup> T cells to very high levels but these T cells can remain in the host long-term without undergoing pruning by peripheral tolerance mechanisms. These cells represent a possible reserve that can be called upon for use in cancer therapy.

### 4.4 Materials and Methods

#### 4.4.1 Mice and infections

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or The National Cancer Institute (Frederick, MD) and I-FABP OVA mice were maintained in house. To generate heterologous prime-boost-boost
mice, animals were primed with $1 \times 10^6$ PFU VSV-OVA i.v. (1°), rested for at least 30 days, boosted with $1 \times 10^4$ CFU Lm-OVA i.v. (2°), rested for at least an additional 30 days, then given a final boost with $2 \times 10^6$ PFU VV-OVA i.v. (3°).

When evaluating the need for OVA antigen in generating self-specific responses, both B6 and I-FABP OVA mice were primed with $1 \times 10^6$ PFU VSV (1°), rested for at least 30 days, boosted with $1 \times 10^4$ CFU Lm-gp33 i.v. (2°), rested for at least an additional 30 days, then given a third boost with $2 \times 10^6$ PFU VV-Simian immunodeficiency virus-Gag i.v. (3°). All mice were used in accordance with the National Institutes of Health and the University of Minnesota Institutional Animal Care and Use Committee guidelines.

### 4.4.2 Tissue harvesting and processing

Organs were harvested and digested as previously described. For isolation of SI IELs, Peyer’s patches were removed, the SI was cut longitudinally and then laterally into small pieces. Pieces were incubated for 30 minutes with stirring at 37°C with 0.154mg/mL dithioerythritol (Sigma-Aldrich, St. Louis, MO) in 10% HBSS/HEPES. The pieces were then vortexed on high speed to dislodge IELs and the supernatant was collected. The small intestinal pieces were washed in RPMI containing 5% FBS and put in RPMI 1640 containing 5% FBS, 2 mM MgCl$_2$, 2 mM CaCl$_2$ and 0.5mg/mL 100 U/mL type I collagenase (Worthington, Lakewood, NJ) and incubated for 45 minutes at 37°C with stirring to obtain LPLs. After enzymatic digestion, the remaining tissue pieces were mechanically disrupted using a gentleMACs dissociator (Miltenyi Biotec, San Diego, CA).
Single cell suspensions from supernatant of the digestions were further separated using a 44/67% Percoll (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient. Spleen and lymph nodes (LN$s$) were dissociated mechanically. Splenocytes and blood were treated with ACK lysis buffer to lyse red blood cells.

### 4.4.3 Flow cytometry and peptide stimulation

The following antibodies were used for flow cytometry: anti-KLRG1 (2F1), anti-Eomes (Dan11ma), anti-T-bet (4B10), anti-CD44 (IM7), anti-CD27 (LG.7F9) (all from eBioscience, San Diego, CA), anti-CD8α (53-6.7, eBioscience, Biolegend, San Diego, CA), anti-CD25 (PC61), anti-Bcl-2 (Bcl-2/100) and anti-CD127 (SB/199) (BD Biosciences, San Jose, CA). Cell viability was determined using Ghost Dye™ Red 780 (Tonbo Biosciences, San Diego, CA). K$^b$-SIINFEKL-specific CD8$^+$ T cells were identified using H-2K$^b$ tetramers made in house containing the SIINFEKL peptide (New England Peptide, Gardener, MA).

Peptide stimulation was performed as previously described. Briefly, splenocytes were plated in RPMI 1640 containing 10% FBS, 1x NEAA, 2mM L-glutamine, 1mM sodium pyruvate, 1x penicillin/streptomycin and 0.05mM β-mercaptoethanol and incubated with 10 fold dilutions of SIINFEKL peptide ranging from 100µg/mL-0.0001µg/mL for four hours at 37°C. All samples received 1µg/mL GolgiPlug (BD Biosciences, San Jose, CA). Cells were washed and stained with fixable LIVE/DEAD aqua dead cell stain (Life Technologies, San Diego, CA) before surface and intracellular staining or with Ghost Dye™ Red 780.
(Tonbo Biosciences, San Diego, CA) during surface staining. Samples were acquired on an LSRII or LSR fortessa flow cytometer (BD Biosciences, San Diego, CA). For intracellular stains, the BD Biosciences intracellular kit for cytokine staining and the eBioscience FoxP3 kit for transcription factor staining were used in accordance with manufacturer’s directions.

4.4.4 Naïve CD8$^+$ T cell tetramer enrichment

The spleen and all macroscopic LNs were isolated from naïve mice, mechanically dissociated, and filtered through nylon mesh to achieve single cells suspensions. Red blood cells were removed using ACK lysis buffer. Cells were pelleted and resuspended in 100uL of EasySep buffer (Stem cell Technologies) with 1µg of both K$^b$-SIINFEKL-PE and K$^b$-SIINFEKL-APC and incubated at room temperature in the dark for 1 hour. Following incubation, cells were washed and resuspended in 500uL EasySep buffer containing 25uL of each anti-PE and anti-APC selection cocktails and incubated for 15 minutes at 4°C. Following the incubation, 25uL of EasySep Magnetic particles for positive selection were added to each sample and they were incubated at room temperature for 10 minutes in the dark. Cells were then brought up to 2.5mL in EasySep buffer and placed in the EasySep magnet for 5 minutes. The unbound sample was removed by decanting the tubes while they were still in the magnet. The tubes were then removed from the magnet, raked to resuspend the bound fraction and washed with an additional 2.5mL of EasySep buffer. Samples were put back into the magnet and incubated for an additional 5 minutes before repeating the decanting
Step. Steps to wash the bound faction were repeated twice more (for a total of three washes) before the samples were stained for flow cytometry analysis.

4.4.5 BrdU experiments

BrdU was added to drinking water of mice at day 0 of VV-OVA infection at a concentration of 0.8mg/mL with 2% sucrose. Water was refreshed daily for 7 days. BrdU staining with anti-BrdU (eBioscience) was performed on PBL on days 4 and 7 after infection using the BrdU flow kit (BD biosciences, San Jose, CA) as per manufacturer's instructions. BrdU staining was analyzed by flow cytometry.

4.4.6 Statistical analysis

For experiments comparing a single variable between two unpaired groups, a Mann-Whitney test was performed. When a single variable between multiple groups was compared, a one-way ANOVA with Dunn's multiple comparisons was employed. A two-way ANOVA with Tukey's multiple comparison analysis was done to evaluate multiple comparisons of two or more variables between two or more groups. All statistics were performed using Prism (GraphPad Software, V7) and for all analysis p values of less than 0.05 were considered significant as indicated by one or more asterisks (*).
Chapter 5

Conclusion
5.1 The potential of self-reactive CD8$^+$ T cells

CD8$^+$ T cells have the capacity to recognize any infected cell in the context of peptide:MHCI. This makes CD8$^+$ T cells indispensable for the immune response against intracellular pathogens; however, the pan expression of MHCI on all nucleated cells can be problematic when CD8$^+$ T cells have TCR that recognize self-protein. To counteract the threat of an autoreactive CD8$^+$ T cell response, the immune system imposes mechanisms to delete self-specific T cells, or render them nonresponsive to antigen. While effective, self-specific T cells can sometimes escape these processes. Autoreactive CD8$^+$ T cells may go on to cause tissue destruction, but could also be important for eliminating transformed cells, which often express self-antigen. It is vital to understand the potential of self-specific CD8$^+$ T cells to mount a response to self-antigen not only as applied to autoimmune disease development but also for targeting these responses to tumors.

5.1.1 Self-reactivity: a blessing in disguise?

Part of my thesis work aimed to understand what characterizes and contributes to a self-reactive CD8$^+$ T cell response. We found that by using a heterologous-prime-boost-boost (HPBB) approach with three serologically distinct vectors delivering the same self-antigen that tolerance to a self-protein in the small intestine (SI) could be thwarted. My work has shown that self-specific CD8$^+$ T cells generated with HPBB were functional, not exhausted, and
underwent avidity maturation during the course of boosting. Remarkably, self-specific CD8+ T cells generated through HPBB were phenotypically similar to foreign-antigen specific CD8+ T cells and exhibited a canonical memory T cell signature. These studies show the ability of self-specific CD8+ T cells to escape central tolerance mechanisms and respond to cognate antigen in the periphery, expanding to large numbers. How were self-specific CD8+ T cells able to evade peripheral tolerance mechanisms during boosting? In fact, how could self-specific CD8+ T cells make it past negative selection? It is likely that the answer concerns the affinity of the self-specific CD8+ T cell pool. Indeed many studies have shown that low affinity T cells are able to bypass negative selection and contribute to autoimmune responses in the periphery. My thesis work provides evidence for the incredible breadth of self-specific CD8+ T cells in that they can respond to antigen given the right context (inflammation plus high levels of antigen) but remain masked from, or ignored by, peripheral tolerance mechanisms. Simply put, self-specific CD8+ T cells are not “dead in the water” and have the ability to do a job. It is interesting to speculate why the immune system did not evolve to completely eliminate low affinity self-specific CD8+ T cells, considering the possible threat these cells pose to homeostasis, evidenced by the rampant prevalence of autoimmune disease.

It could be that low affinity self-specific CD8+ T cell survival is not a passive process, and is purposeful. The high affinity self-specific CD8+ T cells that would immediately cause damage are presumably efficiently deleted, and low affinity cells that have more requirements to react to self-antigen are
sometimes left unabated. Could this be a way to curtail cancerous cells? Many tumor cells express unaltered self-protein\textsuperscript{173}. Although it can be argued that the tumor microenvironment does not provide the necessary signals to generate an effective self-specific CD8\textsuperscript{+} T cell response, it might be that self-specific CD8\textsuperscript{+} T cells are beneficial for eliminating transformed cells that have not yet become cancerous.

Self-specific CD8\textsuperscript{+} T cells may also exist to contain pathogens with molecular mimicry to self-proteins. Many have proposed microbes take advantage of potential holes in the immune repertoire due to negative selection\textsuperscript{161,255}. Perhaps low affinity self-specific CD8\textsuperscript{+} T cells are a means for the immune system to protect against these pathogens without causing damage to tissue after clearance of infection. Our work has shown that several rounds of infection are required for functional avidity maturation of self-specific CD8\textsuperscript{+} T cells to occur, which may support this idea. While many bacteria and viruses do trigger autoimmune disease through mimicry to self-protein, this is likely the result of several confounding factors, such as genetic susceptibility, as not everyone who is infected with these pathogens acquires autoimmune disease\textsuperscript{256}.

While much still remains to be uncovered regarding the mechanisms behind self-specific CD8\textsuperscript{+} T cell expansion and maintenance, our studies have shown the capability of self-specific CD8\textsuperscript{+} T cells to mount a functional response against self-protein.
5.1.2 Heterologous prime-boost-boost in practice

We and others have shown that repeated antigenic stimulation against a self-protein generates self-specific CD8+ T cells. These expanded self-specific CD8+ T cells can be exploited for use in cancer immunotherapy, targeting a specific tumor. In humans, many tumor-associated antigens (TAA) have been identified. As a therapeutic approach, a known TAA or one isolated from a patient's tumor biopsy, could be cloned into three different vectors for use in HPBB to generate CD8+ T cells specific to the tumor. In this way, HPBB can be personalized or broadly applicable. Importantly, this approach is highly specific and may have less autoreactive side effects than with broadly targeted immunotherapies, such as checkpoint blockade. For HPBB to be an effective therapy, tumor-specific CD8+ T cells would need to overcome the suppressive tumor microenvironment and respond to presumably low levels of cognate antigen. This brings up many pressing questions about the use of HPBB in cancer therapy: At what stage of diagnosis would HPBB be feasible? Would tumor-specific CD8+ T cells already be exhausted at the time of HPBB therapy? Is the lengthy time needed to complete HPBB problematic for fast growing tumors? In regards to the last point, my research has shown that shortening the intervals between HPBB events can still generate functional CD8+ T cells and break tolerance to self-protein (data not shown), but that this comes at the cost of forming stable long term memory. Perhaps in the case of a tumor response, this may be beneficial to prevent cytotoxic T cell responses to healthy tissue after tumor antigen is cleared. It might be that HPBB would be more
useful as a prophylactic vaccine. In this instance, individuals with high risk for developing cancer might undergo HPBB to prevent disease.

5.2 **CD8^+ T cells: the shape shifters of the immune system**

Mobility is paramount for successful CD8^+ T cell mediated immune responses. As such, CD8^+ T cells must rapidly adapt cell shape and migratory machinery to the surrounding environment. This makes CD8^+ T cell locomotion context dependent, showing the need to evaluate CD8^+ T cell *in situ* behavior in a multitude of conditions.

5.2.1 **If T cells could talk**

By design of the dependency of CD8^+ T cells to directly contact target cells to perform cytotoxic lysis, CD8^+ T cells must be motile. The immune system also cannot predict what tissues CD8^+ T cells must traverse to fight infection, so by nature, CD8^+ T cells are remarkably adaptable to the environment. A major goal of my thesis work was to evaluate CD8^+ T cell motility within the SI to better understand how CD8^+ T cells can survey such an expansive tissue. My research showed that antigen-specific CD8^+ T cell locomotion varied during the course of infection. Surprisingly, at a memory time point, antigen-specific CD8^+ T cells had somewhat more narrowed migration in comparison to other time points evaluated. To this end, it appeared that there was a specific “area code” that memory CD8^+ T cells were tasked with surveying. What does this mean for
effective immunosurveillance in this compartment? It might suggest that for vaccines targeting quickly replicating pathogens, large numbers of CD8+ T cells positioned in the SI might be necessary to rapidly snuff out infection. Further mathematical analysis of the amount of 3D tissue scanned in a given period of time is necessary to predict the complete surveillance capacity of CD8+ T cells in the SI.

At earlier time points during infection, CD8+ T cells were not as confined, suggesting that the extracellular matrix (ECM) may have been altered during infection and returned to normal after pathogen clearance. Further supporting this idea in the SI, self-specific CD8+ T cells, which may not elicit the same level of tissue remodeling as a virus, exhibited similar behavior to memory foreign antigen-specific CD8+ T cells. CD8+ T cell behavior was also disparate between the muscle layers and the mucosa of the SI. While CD8+ T cells were tightly packed between muscle cells and were restricted to very little 2D movement, CD8+ T cells in the crypt and villi were motile in 3D and actively scanned the tissue. Each type of unique behavior requires significantly different actin remodeling. Migration through denser tissue requires actomyosin contractions at the trailing edge of the cell to overcome the resistance posed by the rigid nucleus. In less dense tissue, this myosin activity is dispensable.

With surmounting evidence that the tissue structure plays a role in CD8+ T cell motility, maybe it is not surprising that we did not see reliance on integrins for in situ behavior. Indeed, perhaps the adaptability of CD8+ T cells to varying
conditions masks what is preferred for their locomotion. As such, only two things are considered *sine qua non* for CD8⁺ T cell motile behavior: TCR signaling and actin remodeling.

While we have gained significant insight about CD8⁺ T cell motility from TPLSM, it is still just a small window into the lifespan of a T cell. In fact, it is likely that CD8⁺ T cells, as seasoned travelers, made many stops along the journey to the location being imaged. What if all that a T cell had “experienced” could be recorded? If every tissue visited, cell contacted, and infection thwarted by the CD8⁺ T cell was imprinted in some fashion, we could learn so much more about how CD8⁺ T cells integrate signals and adapt to the environment. This perhaps could be accomplished in the form of a T cell “GoPro” or GPS that recorded tracking information throughout the CD8 T cell lifespan. While we still have much to uncover regarding T cell mobility, our current understanding shows the incredible adaptability of CD8⁺ T cell locomotion. If we could discover the environmental signals governing these responses, we could harness this knowledge of plasticity for use in immunotherapy and other applications.
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