Derivation, Maintenance, and Functions of Virtual Memory cells

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Dedication

This dissertation is dedicated to my parents, Adote Amegnona Akue and Ayaba Minasseh. I would not have accomplished this without your hard work and example. Thank you for bringing me into this world. I am forever grateful for all you have done for me.

Abstract

Memory phenotype $CD8^+$ T cells are typically thought to have undergone an immune response to foreign antigen and to have differentiated from antigen-specific precursors in the naïve pool. However, using a peptide-MHC I tetramer enrichment technique, we identified foreign antigen-specific memory-phenotype $CD8^+$ T cells in unimmunized mice. These cells (termed "virtual memory" T or VM cells) were observed in mice maintained in both specific-pathogen- and germ-free (SPF and GF respectively) housing.

This thesis focuses on the relationship between VM cells and "conventional" memory cells: memory cells arising from homeostatic proliferation (HP), and innate-like memory $CD8^+$ T cells such as IL-4 bystander memory $CD8^+$ T cells.

Our data indicate physiological HP and IL-4-driven bystander processes are the main mechanisms that drive the generation of VM cells and not the exposure to foreign antigens. VM cells arise in the periphery during the neonatal period and are maintained long term. We also show that VM cells respond in vitro to innate cytokines (similar to conventional memory $CD8^+$ T cells) and they outcompete antigen-specific naive $CD8^+$ T cells in *in vivo* responses. Overall our observations suggest that VM cells arise out of normal homeostatic and IL-4-driven bystander processes in unimmunized SPF and GF mice, and express at least some memory-like capabilities.

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Chapter 1

Introduction

The Immune System

The survival of eukaryotic organisms depends on their ability to defend themselves against pathogens. This defense mechanism called, the immune system, is composed of specialized cells and soluble factors; components of this system exist in all multicellular organisms. The mammalian immune system is composed of two different arms: the innate immune system that encompasses non-specific defense mechanisms, and the adaptive immune system made of specific defense mechanisms (1). Although these two arms of the immune system are separated for simplicity and didactic purposes, in reality they are intricately connected systems whose components interact and communicate with each other through various means to assume the functions of the immune system.

In order for the immune system to fulfill its functions of protecting the host, it has to maintain certain features. First, the immune system has to sense pathogens through receptors on the surface of the specialized cells of the innate immune system (2, 3). Second, the immune system has to eliminate the pathogens it senses using both cellular and soluble components while limiting the collateral damage caused by these immune components to the organism itself (3). Finally, the immune system has to maintain antigen-specific and long-lived cells that are generated in response to infections in order to readily eliminate subsequent challenges with similar pathogens (1). This last feature is a hallmark of the adaptive immune system and the main objective of vaccination (4).

Innate Immune system

Response of the innate immune system is fast, non-specific, and represents the first line of defense against pathogens (5). The innate response is mediated through recognition of danger- and pathogen-associated molecular patterns (DAMPs and PAMPs respectively) by pattern recognition receptors (PRRs) on the surface of cells of the innate immune system such as dendritic cells (DCs) (2). These PRRs recognize DAMPs and PAMPs that are only present on the surface of pathogens; this way the innate immune system is able to tell self from non-self and avoid autoimmune attacks (2, 6, 7). Diversity of these receptors is restricted and memory cells are not associated with most cell types of the innate immune system. The exception is natural killer (NK) cells that display functional characteristics associated with memory T cells (8, 9), a hallmark of the adaptive immune system. Upon recognition of DAMPs and/or PAMPs, a signaling cascade is initiated within the cells of the innate immune system leading to the production of inflammatory cytokines that activate and recruit other cells of the innate immune system to the site of infection and increase their ability to activate the adaptive immune system (10-13). Some of the cells of the innate immune system are macrophages, neutropils, monocytes, DCs, NK cells. Because they are not the focus of the work described in this manuscript they will not be discussed in detail.

Adaptive Immune system

The adaptive immune system is composed of T and B cells. These cells express a vast array of receptors (T cell receptor/TCR and B cell receptor/BCR respectively) on their surface that serve to recognize antigens from various sources in a very specific

manner. B cells develop in the bone marrow and recognize whole antigens with their receptor while T cells are generated in the thymus and recognize fragments of antigens that are processed and presented in the context of major histocompatibility (MHC) molecules by antigen presenting cells (APCs) such as DCs, macrophages, and activated B cells.

The difference between the receptors on B and T cells and PRRs on cells of the innate immune system is that each BCR and TCR recognizes a unique feature of each antigen while PRRs recognize features that are common to microbes and that are not expressed on cells of the host in steady state (2, 6, 7). Due to that specificity the repertoire of the TCR has to be very diverse for naïve T cells to recognize the vast array of antigens expressed by microbes. The potential diversity of the TCR repertoire was originally estimated to be over $1x10^{15}$ (14), but other calculations later estimated the actual diversity of the peripheral TCR repertoire at $2.5x10^{7}$ in humans and $2x10^{6}$ in mice, presumably because there could not be more TCR specificities than T cells in the body at any given time (15, 16).

T cells can be subdivided based on the type of co-receptor they express. $CD4^{+}$ T cells express CD4 co-receptor and their TCR recognize their specific peptide antigen in the context of MHC class II molecules. When activated, naïve $CD4^{+}$ T cells differentiate into several types of effector cells such as Th1, Th2, Th17, T follicular helper (TFH), and regulatory T cells (Tregs). Th1, Th2, and Th17 cells are important in the defense against extracellular pathogens and parasites, while Tregs suppress T cell responses and thus control the breath of the immune response and prevent autoimmune diseases. TFH cells,

as their name suggests can help B lymphocytes, but whether they represent a separate lineage of cells or whether they derive from activated CD4$^+$ T cells is still debated. CD8$^+$ T cells on the other hand express CD8 co-receptor and bind peptide antigens presented in the context of MHC class I molecules. They differentiate into cytotoxic CD8$^+$ T cells when activated and are important for immunity against intracellular pathogens (17).

CD8$^+$ T cell response to pathogens

In steady state conditions, the CD8$^+$ T cell compartment is mainly composed of naïve T cells. These cells are maintained as quiescent, non-dividing cells through interaction of their TCR with self peptide-MHC class I molecules and interaction with IL-7 (18-21). Upon disturbance of this homeostasis, the dynamics of the CD8$^+$ T cell compartment changes depending on the nature, severity, and duration of the disturbance.

An acute viral infection for instance triggers responses from the innate immune system first, leading to inflammation. This innate defense mechanism activates DCs to mature and upregulate MHC and costimulatory molecules such as B7 and B7.1 (CD80 and CD86 respectively). This upregulation of MHC and B7 molecules on activated DCs increases their ability to effectively process protein antigens and present antigenic peptides to T cells. Activated DCs then migrate from the site of infection to secondary lymphoid organs (lymph nodes and spleen) where they present antigens to naïve CD4$^+$ T cells in the context of MHC II molecules. In turn DCs receive help from activated CD4$^+$ T cells through interaction between CD40 molecules on DCs and CD40ligand (CD40L) on CD4$^+$ T cells. This CD4 help enable DCs to effectively prime good cytotoxic

functions in $CD8^+$ T cells. With the right costimulation (B7 and B7.1 interaction with CD28) and inflammatory cytokines such as IFN-I and IL-12, naïve $CD8^+$ T cells that recognize their nominal antigen differentiate and undergo massive clonal expansion leading to a dramatic increase in the number of effector $CD8^+$ T cells specific for that antigen. Activated $CD8^+$ T cells then migrate from secondary lymphoid organs into the site of infection and other peripheral sites where they can eliminate pathogens by killing infected cells and/or producing factors such as IFN-γ. Following clearance of the pathogen by effector $CD8^+$ T cells, a contraction period ensues where the number of effector T cells is reduced to a minimum such that further tissue damage is limited. The remaining effector cells become memory $CD8^+$ T cells (22).

Immunologic memory: a hallmark of the adaptive immune system

As mentioned above, naïve T cells clonally expand in response to foreign antigens and generate larger numbers of memory cells. Naïve and memory T cells can be distinguished based on surface markers, but there are also several qualitative differences between the two populations. Naïve T cells have a defined lifespan and die within weeks to months unless they become activated, while memory cells can persist a lifetime (20, 23, 24). Memory T cells very rapidly acquire effector functions upon restimulation, while naïve T cells require at least several divisions before acquiring them (25). Memory T cells express higher levels of many adhesion markers; this allows them to access peripheral non-lymphoid sites more efficiently. In contrast naïve T cells preferentially recirculate through secondary lymphoid organs (26-29). This positioning of memory T

cells at the major ports of entry of many pathogens may contribute to their readiness in clearing infections, and recent reports even suggest the existence of tissue resident memory cells that do not recirculate between peripheral sites and blood (29, 30). The focus of the following sections will be on memory $CD8^+$ T cells.

Conventional memory $CD8^+$ T cells

Conventional memory $CD8^+$ T cells are memory cells generated in response to foreign antigens as described above. Phenotypically they are characterized by higher expression of CD44 (adhesion molecule), CD122 (IL-2 and IL-15 receptor β), CD127 (IL-7 receptor α), and CXCR3 (chemokine receptor that binds CXCL9/10/11). This is by no mean an exhaustive list of markers since there is heterogeneity within the memory pool. It is broadly accepted that there are two main memory populations within the memory pool: effector memory T cells (T_{em}) and central memory T cells (T_{cm}) based on the expression of CCR7 (chemokine receptor for CCL19 and 21) and CD62L (receptor for lymph node homing molecules). T_{em} express low levels of CCR7 and CD62L while T_{cm} express high levels of CCR7 and CD62L. The differential expression of these two trafficking molecules on memory T cells suggests localization and functional differences between these two memory subsets (31). Due to their lack of CCR7 and CD62L expression, T_{em} can rapidly access non-lymphoid peripheral organs, major port of entry of many pathogens, and can rapidly differentiate into effector cells and produce effector cytokines. T_{cm} on the other hand have the potential to recirculate through secondary lymphoid organs due to the expression of CCR7 and CD62L, and take longer to

differentiate into effector cells (31). This grouping of memory cells into T_{em} and T_{cm} does not necessary exclude further diversity. Regardless of the subsetting of memory T cells, it is widely accepted that effector cells that live beyond the contraction phase during the immune response to antigens become memory cells. This brings up a very fundamental question regarding which factor(s) determine(s) which cell becomes a memory cell within the effector pool and when that decision is made. Studies using killer cell lectin-like receptor G1 (KLRG1) and CD127 (IL-7Rα) expression showed that $KLRG1^{lo}/CD127^{hi}$ cells represent memory precursor effector cells (MPECs), while $KLRG1^{hi}/CD127^{lo}$ cells are short-lived effector cells (SLECs) (32, 33). Other reports suggest a cell can either become a SLEC or an MPEC depending on the duration and the strength of the stimulus it receives (34-37) and the exposure to inflammatory cytokines such as IL-12 and IFN-I (32, 38-40). On the other hand, reports show that one naïve T cell can generate both effector and memory cells (41-43). Regardless of the path that leads to memory generation and despite the diversity within the memory pool, the phenotype of memory is associated with an increase in the number of cells and the quality of the immune response compared to naïve cells. That increase in quality translates into a heightened readiness of memory cells to proliferate and eliminate infectious agents they have encountered in the past.

Memory cells have to be maintained long term in a state of readiness to protect the host against subsequent infections. We will next discuss three common-γ chain (ϒc) cytokines involved in memory $CD8^+$ T cell homeostasis and function.

IL-2

IL-2 is produced by T cells and it functions in autocrine and paracrine fashion. The IL-2 receptor is a trimeric molecule composed of the common γ-chain receptor (CD132), IL-2Rα (CD25), and IL-2 Rβ/15-Rβ (CD122). Upon activation of naïve T cells, a signaling cascade ensues leading to the production of IL-2, which in turn drives the proliferation of T cells. CD25 increases the affinity of the IL-2 receptor and its expression is increased on activated T cells. IL-2 induces potent T cell proliferation and is used in therapies against cancer and viruses (44, 45). Although IL-2 is not required for the generation of memory T cells, it is required for the recall function of memory cells (46). Also IL-2 expands memory cells when complexed with certain anti-IL-2 antibodies (47-49).

IL7

Produced by stromal cells in the bone marrow, thymus, and lymph nodes. IL-7 is required for lymphocyte development, and for memory $CD8^+$ T cell survival as indicated by the failure of memory $CD8^+$ T cells to persist in the absence of IL-7Rα (50, 51).

The receptor for IL-7 is a dimer composed of the IL-7 receptor α (IL-7Rα or CD127), and CD132. IL-7 is unique among all ϒc cytokines in that its receptor is downregulated when it binds IL-7 (52). The expression of IL-7Rα changes over the course of an immune response. When activated, naïve T cells differentiate into effector cells and downregulate IL-7Rα. IL-7Rα is re-expressed by effector cells that go on to survive the contraction and reach the memory phase, suggesting a role for IL-7 signaling

in the progression of effectors into the memory pool during the contraction phase. However, experimental designs forcing IL-7Rα expression on T cells did not lead to the generation of more memory cells (53, 54), suggesting the expression of IL-7Rα alone during the contraction phase is not sufficient for the generation of memory $CD8^+$ T cells.

IL-15

IL-15 is produced by DCs, monocytes, and macrophages during inflammation. Its receptor is also trimeric, composed of CD132, IL-15-Rα, and IL-2/15-Rβ (CD122). Specificity of the receptor is conferred by the IL-15-Rα since it shares the other two chains with other ϒc cytokines. IL-15 is required for the long-term survival and basal proliferation of memory $CD8^+$ T cells (55-58).

"Homeostatic memory" cells

As mentioned in the previous section memory cells are typically generated as a result of foreign antigen exposure, but it has become clear that there are alternative ways memory cells can be generated. Evidence came from studies where naïve cells slowly proliferate and acquire the phenotype of memory cells in lymphopenic environments. This process is termed homeostatic proliferation (HP) or lymphopenia-induced proliferation (LIP). TCR engagement with self peptide-MHC molecules, and cytokines such as IL-7 and IL-15 are required during HP (51, 59-67). Cells undergoing HP do not require costimulation and do not transition through the effector stage prior to acquiring the phenotype of memory, contrary to foreign antigen-driven cells (65, 68). All these

findings suggest a different pathway of differentiation that could result in different functional properties, but HP memory cells not only carry the phenotype but are functionally similar to conventional memory cells as well (60, 61, 69, 70). The T cell lymphopenia required for HP can be artificially induced with radio- and chemo-therapy or gene knockout techniques, but viral infections such as HIV also lead to the reduction of T cell numbers (71-73). Natural lymphopenia during the neonatal period and aging also contribute to HP (74-77).

"Bystander memory" cells

Yet another memory-phenotype population called "innate memory" T cells exists and is different from conventional memory and homeostatic memory cells. These innate memory cells include MHC class I^b-restricted cells (78-80). One main characteristic of "Innate memory" cells is that they are generated in the thymus as memory (CD44hi) cells but not in response to foreign antigens in the periphery. One example of such "innate memory" cells is iNKT cells. Many of the same signaling pathways involved in T cell selection are involved in the selection of "innate memory" cells, but they also require the interaction of signaling lymphocyte activation molecule (SLAM) receptors with their corresponding adaptor SLAM associated protein (SAP) for their selection (81-84). Similar innate $CD8^+$ memory cells were also described in Tec kinase deficient (ITK-KO) mice (85, 86) and it was not until recently that a report from our group and others showed that these "innate-like memory" cells in ITK-KO and other gene knockout mice represent a distinct population of memory $CD8^+$ T cells generated via exposure of naïve single

positive $CD8^+$ T cells to IL-4 (87-91). These IL-4-driven memory $CD8^+$ T cells were termed "bystander memory" cells and shown to exist in intact BALB/c mice. Whether IL-4 effect on naïve T cells is also seen in B6 mice is not totally clear.

IL-4

IL-4 is another member of the ϒc cytokine family. It is produced by iNKT cells, mast cells, and basophils upon activation (92-94). IL-4 has a dimeric receptor composed of CD132 and IL-4Rα (CD124). It is a Th2 cytokine that is also necessary for an effective $CD8^+$ T cell response to liver stage malaria parasites (95, 96). Being a ϒc cytokine, it was thought that IL-4 would have a role in homeostatic proliferation. Although it can enhance HP, experiments where naïve T cells were transferred in IL-4 deficient mice suggest that it is dispensable for homeostatic proliferation (67). Our group and others recently reported its involvement in the generation of thymic memory $CD8^+$ T cells or bystander memory cells.

Purpose

Overall we discussed several mechanisms that lead to the generation of memory $CD8^+$ T cells (immune responses to pathogens, homeostatic proliferation, and IL-4 exposure in the thymus), but normal SPF and germ-free mice are known to contain memory-phenotype $CD8^+$ T cells and our study sought to determine what type(s) of memory cells are represented in this pool and whether they are functionally relevant.

Chapter 2

The antigen-specific $CD8^+$ T cell repertoire in unimmunized mice includes memory-phenotype cells bearing markers of homeostatic expansion

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Memory T cells exhibit superior responses to pathogens and tumors compared to their naïve counterparts. Memory is typically generated via an immune response to a foreign antigen, but functional memory T cells can also be produced from naïve cells by homeostatic mechanisms. Using a recently developed method, we studied CD8 T cells, specific for nominal model (ovalbumin) and viral (HSV, vaccinia) antigens in unimmunized mice, and found a sub-population bearing markers of memory cells. Based on their phenotypic markers, and by their presence in germ free mice, these pre-existing memory-like CD8 T cells are likely to arise via physiological homeostatic proliferation rather than a response to environmental microbes. These antigen-inexperienced memory-phenotype CD8 T cells display a number of functions which distinguish them from their $CD44^{lo}$ counterparts, including a rapid initiation of proliferation after T cell stimulation. Collectively, these data indicate that the unprimed antigen-specific CD8 T cell repertoire contains antigen inexperienced cells that display phenotypic and functional traits of memory cells.

Introduction

During normal primary T cell responses, naïve T cells are induced to proliferate and differentiate, giving rise first to an effector pool and then to a long lived memory population (97, 98). The size of the antigen-specific memory T cell pool is typically larger than the naïve population from which they derived, but in addition to their elevated frequency, memory T cells exhibit multiple qualitative advantages over their naïve counterparts with respect to their functional versatility, speed of response and capacity to migrate to multiple sites besides lymphoid tissues (97, 98).

Aside from this standard pathway, however, there is accumulating data that memory-phenotype T cells can arise from naïve T cells via homeostatic mechanisms, without activation of the T cell by foreign antigen. This has been well studied in situations of extreme lymphopenia, as induced by irradiation or genetic T cell deficiency, which induces "homeostatic proliferation" (HP) of naïve T cells (99-101). In addition, there is also evidence that HP can occur within unprimed neonatal mice (74, 76, 77, 99-102). T cell HP in the lymphopenic environment is thought to be driven by reduced competition for limiting resources, including IL-7 and low affinity TCR ligands (51, 67, 99-101), and can be further influenced by other cytokines (103-106).

HP memory cells resemble conventional memory cells in many of their phenotypic and functional traits. Following HP, T cells display numerous phenotypic

markers similar to true antigen-driven memory cells, such as increased CD44, LFA1, Ly6C and CD122 expression (70, 99-101, 107). Antigen-driven memory cells display increased sensitivity to antigen stimulation leading to a more rapid proliferative response and enhanced cytotoxic and cytokine-producing effector functions (108, 109). Similarly, HP memory cells display proliferative responses, increased effector cytokine production, and an acquisition of cytotoxic functions that are significantly elevated compared to naïve T cells (70, 99-101, 107). This increased functionality of HP memory cells is significant as demonstrated by the fact that HP memory cells can provide a substantial degree of protective immunity against infectious challenge (70). Besides their enhanced functional responses, antigen-driven memory cells can traffic outside of traditional secondary lymphoid tissues (110-112) and indeed may even show a preference for circulating within peripheral tissues, particularly under inflammatory conditions (111, 113), though whether this also applies to HP memory cells has not been extensively studied.

The representation of HP memory T cells in the bulk memory T cell pool within a normal, un-manipulated host is unclear. Analysis of "naïve" mice (i.e. animals which have not been deliberately immunized) consistently shows an abundant population of memory-phenotype T cells, which can amount to 15-20% of total CD8 T cells. This population is typically assumed to be the result of T cell activation and formation of memory in response to environmental antigens. However, a memory CD8 T cell population is also present in unprimed animals housed under gnotobiotic ("germ-free"

GF) conditions (114), which are free of what would be considered a dominant source of environmental antigens. This suggests that at least some memory-phenotype cells in unprimed mice might be HP memory cells, generated during physiological lymphopenia. Importantly, little is known about the antigen specificity of endogenous memory-phenotype T cells, nor their ability to engage in "primary" immune responses.

Using a novel technique recently developed by ourselves (115) and others (116), we have isolated the pool of antigen specific $CD8^+$ T cells from unimmunized hosts and found evidence not only for naïve antigen specific cells, but also for memory-phenotype T cells within this pool. These cells bear cell surface markers typical of HP memory cells, and are detected in GF as well as Specific Pathogen Free (SPF) animals. We present evidence suggesting that these pre-existing memory-like CD8 T cells represent a novel, additional arm of the immune repertoire capable of participating in the "primary" immune response.

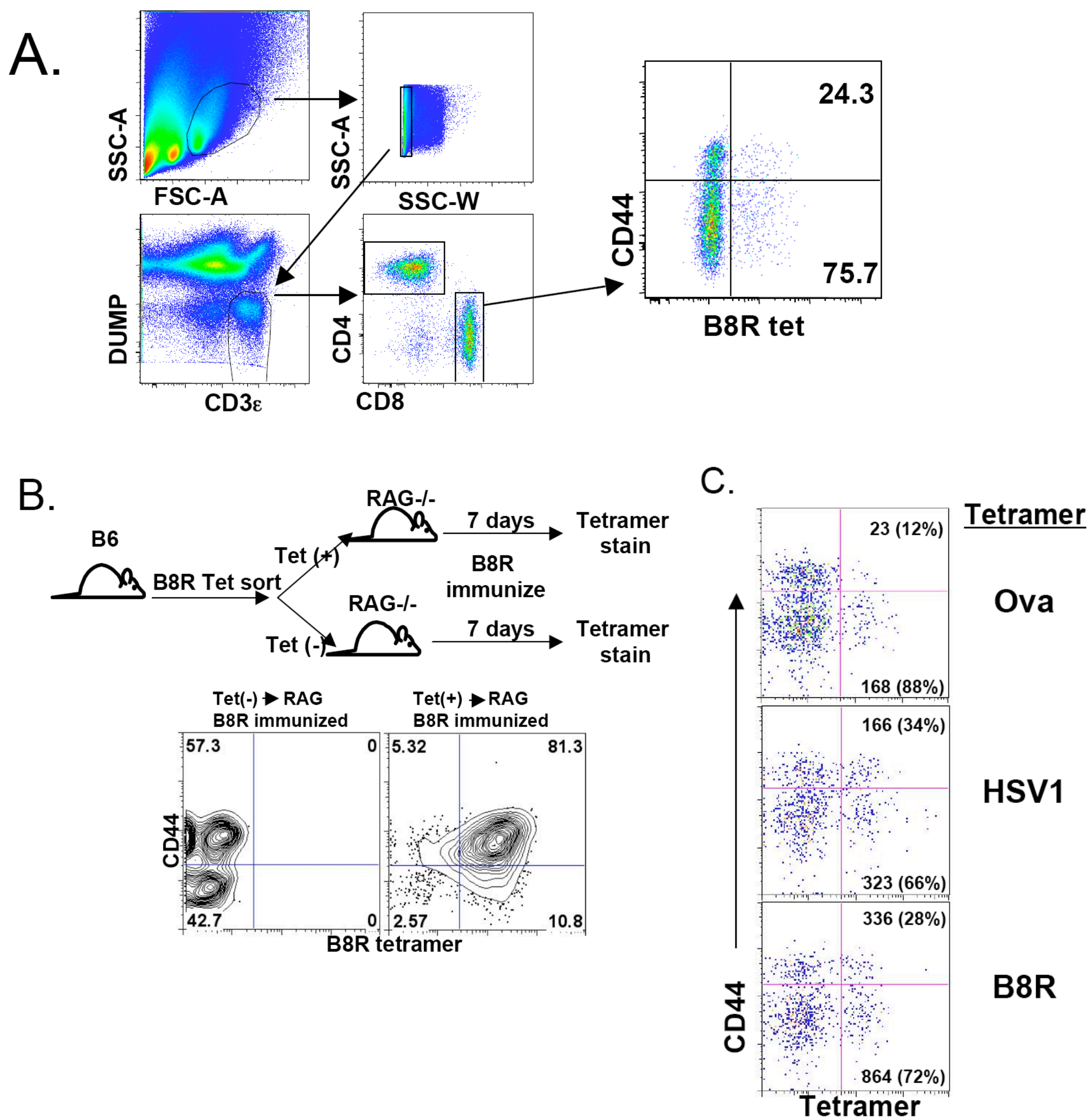
Results

Isolation of naïve antigen specific T cells from an unprimed host

We (115) and others (116) recently devised a method for isolating antigen specific $CD4^+$ T cells from the normal T cell repertoire in an unprimed animal. Using a modified approach to this method, we used class I MHC tetramers loaded with various peptide antigens (OVA)- K^b/SIINFEKL (117, 118), vaccinia virus B8R-K^b/TSYKFESV (119), HSV-1gB-K^b/SSIEFARL (120) to isolate antigen-specific $CD8^+$ T cells, from unimmunized normal B6 mice. Spleen and/or LN cells were isolated as previously described (121) and stained with PE-labeled tetramer in the presence of azide to block internalization of the TCR. After tetramer staining, the cells were incubated with anti-PE magnetic beads. Following separation on a magnetic column, the cells were stained and gated so as to most effectively capture all tetramer stained $CD8^+$ T cells and eliminate noise (Fig. 2-1A). We confirmed that this tetramer enrichment approach was reliable at capturing antigen-specific cells by adoptively transferring either positive (column bound) or negative (flow through) fractions of B8R/K^b stained spleen cells into $RAG^{-/-}$ hosts (Fig. 2-1B). Following immunization of these transfer recipients, B8R specific T cells were only detectable in the $RAG^{-/-}$ mice transferred with the positive column fraction (Fig. 2-1B), confirming that all antigen specific T cells were contained within this fraction.

Analysis of the population of tetramer-bound T cells from unprimed animals showed a number of interesting properties. First, depending on the epitope, the total

number of antigen-specific CD8$^+$ T cells detected in these unprimed animals averaged ~ 170 (OVA-specific T cells) to 1,070 (B8R-specific T cells) cells per mouse spleen (Fig. 2-1C, D), a frequency which is significantly higher than that observed for antigen specific CD4$^+$ T cells using a similar method (115), but is in line with recent studies of CD8 T cells using a similar method of naïve T cell isolation (116).



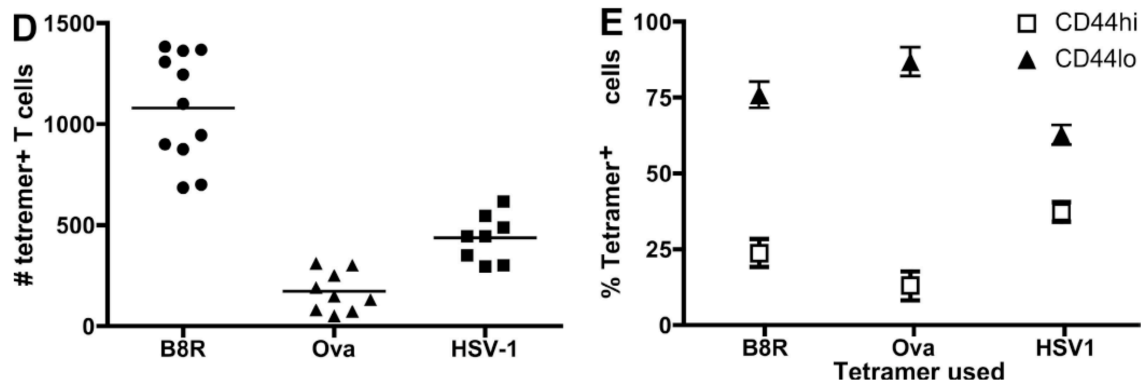


Figure 2-1. Quantitation and characterization of antigen specific $CD8^+$ T cells from unprimed mice. (A) Gating scheme for FACS analysis of peptide/MHC tetramer-binding cells isolated by magnetic bead sorting. Left dot plots show a representative gating strategy used to identify $CD8^+$ T cells isolated by magnetic bead column after tetramer staining. Following this gating strategy, the cells were then analyzed as displayed in the right dot plots and show the general phenotype of the tetramer and CD44 staining profile. Numbers in upper and lower right quadrants indicate the percent of $tetramer^+$ cells that are either $CD44^{hi}$ or $CD44^{lo}$, respectively. (B) As shown in the flow diagram, B6 spleen cells stained with the B8R tetramer were subjected to magnetic bead separation. The flow through and bound fraction were then injected into separate $RAG^{-/-}$ hosts which were subsequently immunized with the B8R peptide plus polyI:C and anti-CD40. Seven days after immunization, peripheral blood (depicted) and spleen (not depicted) were analyzed by B8R tetramer staining. The results shown are representative of 6 mice from 3 independent experiments. (C) Splenocytes from unprimed B6 mice were isolated using MHC tetramers loaded with the indicated peptides. Numbers outside the parentheses indicate the total number of cells in the mouse with the phenotype in that quadrant. Numbers inside the parentheses indicate the percent of cells, out of all $tetramer^+$ cells, that are either $CD44^{hi}$ or $CD44^{lo}$. (D) Scatter plot shows the distribution of total $tetramer^+$ cell numbers per spleen as isolated by the indicated MHC tetramer, one mouse spleen per data point. (E) The frequency of $CD44^{lo}$ and $CD44^{hi}$ cells within tetramer-binding CD8 T cells from unprimed mice is shown for the indicated tetramers. The results shown are representative of at least 10 independent experiments, with the staining results pooled from 4 independent experiments showing 11 mice for the B8R tetramer, 9 mice for the OVA tetramer, and 6 mice for the HSV-1 tetramer. Error bars show SD.

Second, in contrast to our expectations that antigen-specific cells in unimmunized animals would all be of naïve phenotype, a significant percentage of these $CD8^+$ T cells expressed high levels of CD44 (Fig. 2-1A and C), a phenotype typically associated with

antigen-experienced effector or memory CD8 T cells. The percent of $CD44^{hi}$ cells varied from as little as 10% of OVA-specific $CD8^+$ T cells, to 30-40% of all HSV-1 specific $CD8^+$ T cells (Fig. 2-1C and E). Changes in cell isolation approaches, tetramer staining conditions and gating schemes were evaluated to determine whether such $CD44^{hi}$ tetramer-binding cells were artifacts, but the population was reliably observed under various protocols (Fig. S2-1; and see Materials and methods). Therefore, a significant percentage of antigen inexperienced T cells in an unprimed host expressed a phenotypic marker usually associated with activation or memory.

Antigen specific T cells from an unprimed host are both $CD44^{hi}$ and $CD44^{lo}$

It was plausible that a population of $CD44^{hi}$ CD8 T cells are simply prone to non-specific tetramer binding. As an initial test of this premise, we isolated T cells from a $RAG^{-/-}$gBT-1 TCR transgenic mouse, specific for an HSV-1 glycoporotein B (gB) peptide (120), using K^b MHC tetramers loaded with the OVA, B8R or HSV-1 peptides. Two observations are noteworthy from these experiments. First, the B8R- or OVA-loaded tetramers failed to isolate any cells at all from the $RAG^{-/-}$gBT-1 mouse (Fig. 2-2A). Second, staining with the HSVgB tetramer detected both $CD44^{lo}$ and ^{hi} gB specific $CD8^+$ T cells within the $RAG^{-/-}$gBT-1 host. These $CD44^{hi}$, TCR^+ cells are typically seen in TCR transgenic mice on a RAG-expressing background, their presence usually attributed to the expression of endogenous alpha chains, whereas our data indicate they are also detected in situations where a monoclonal TCR is utilized. More importantly, this $CD44^{hi}$ population does not bind non-specifically to MHC tetramers, as evidenced by

the lack of binding to OVA or B8R tetramers. We therefore concluded that the interaction of our tetramers with either CD44^{lo} or CD44^{hi} T cells is antigen specific, and that CD44^{hi} T cells do not have an increased propensity to non-specifically bind to class I MHC tetramers.

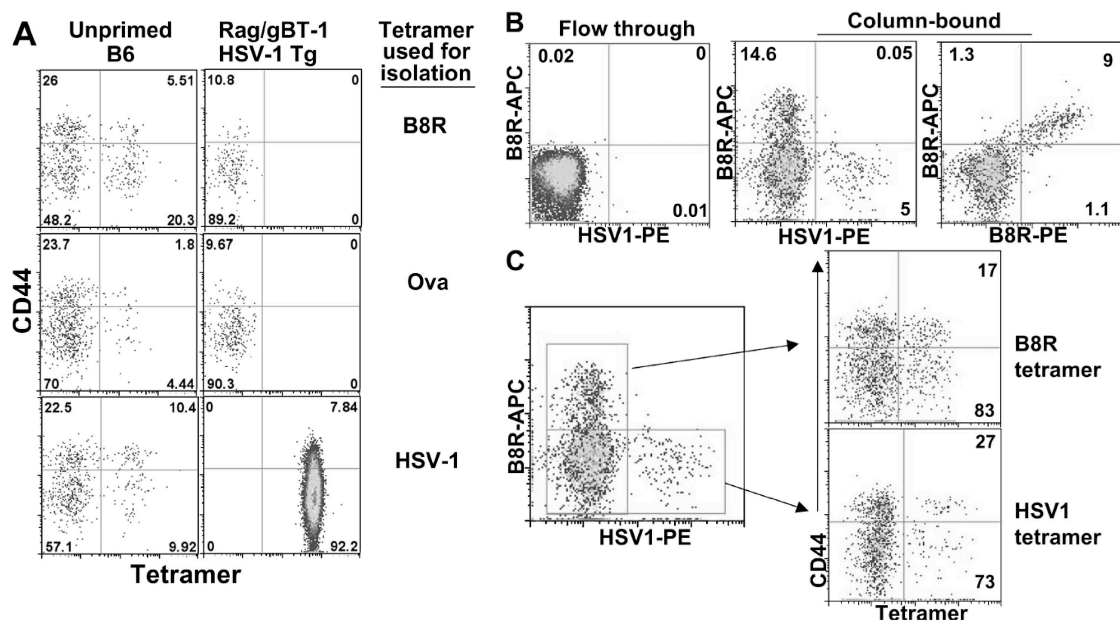


Figure 2-2. Specific tetramer staining of endogenous CD44^{hi} CD8 T cells. (A) Tetramer staining and magnetic bead sorting was performed on spleen cells from B6 and RAG$^{-/-}$ gBT-1 mice using the indicated tetramers. Numbers in the plots indicate percent of cells in that quadrant after having been gated as shown in Figure 1A. The results shown are representative of at least 3 independent experiments using a total of 6 mice from each strain. (B) Splenocytes from unprimed B6 mice were isolated using both PE- and APC-labeled MHC tetramers, loaded with the indicated peptides, as described in the Materials and Methods. Dot plots show all B220^{-}CD3^{+}CD8^{+} events from either the flow through or column bound fractions as indicated. Numbers in each quadrant indicate the percent of total CD8^{+} cells in that quadrant. (C) Cells analyzed in (B) were further gated as indicated in the left dot plot and analyzed as shown in the right dot plots. Numbers in the upper and lower quadrants indicate the percent of all tetramer^{+} events that are either CD44^{hi} or CD44^{lo}, respectively. The results for B and C are representative of 15 mice from at least 5 independent experiments.

As a further confirmation of this conclusion, we determined whether the tetramer positive cells were staining in a peptide selective manner by simultaneously staining the cells with two different tetramers (PE and APC labeled) loaded with either the same or different peptides. Consistent with our prediction, antigen specific cells isolated using both B8R-PE and B8R-APC labeled tetramers were found to bind both tetramers, while cells enriched using both HSV-1-PE and B8R-APC tetramers did not demonstrate any degree of double tetramer staining (Fig. 2-2B). Additionally, both $CD44^{hi}$ and ^{lo} cells were present within the $tetramer^{+}$ fraction (Fig. 2-2C). These data reinforce the conclusion that the interaction of our tetramers with either $CD44^{lo}$ or $CD44^{hi}$ T cells is indeed antigen specific.

T cells isolated by tetramer binding respond to specific antigen stimulation in vivo

Confident in the specificity of our tetramer staining method, we next determined whether both $CD44^{lo}$ and $CD44^{hi}$ $tetramer^{+}$ cells were capable of responding to peptide stimulation. B6 mice were injected IV with either the B8R or HSV-1 peptide. Two hours later, the mice were sacrificed and half of the spleen cells from each peptide-injected mouse were stained with the K^{b}/B8R tetramer and the other half stained with the K^{b}/HSVgB tetramer. The B8R- and HSV-1-specific cells were then isolated as before on the magnetic columns and the resulting cells were analyzed for CD69 expression. Both $CD44^{hi}$ and $CD44^{lo}$ B8R-specific T cells isolated from the B8R peptide-injected mice showed an increase in CD69 expression (Fig. 2-3A). In contrast, cells isolated from the same B8R peptide injected mice using the HSV-1 tetramer did not show increased CD69

expression (Fig. 2-3A). The reciprocal results were true for HSV-1 peptide injected mice. Not all tetramer staining T cells increased expression of CD69 in response to peptide challenge (the frequency of $CD69^+$ cells was ~30-40% of the tetramer-binding pool). This may be due to incomplete peptide distribution and/or specific T cell activation within the 2-hour stimulation, or may reflect a true inability of some tetramer binding T cells to respond to the dose of antigen used.

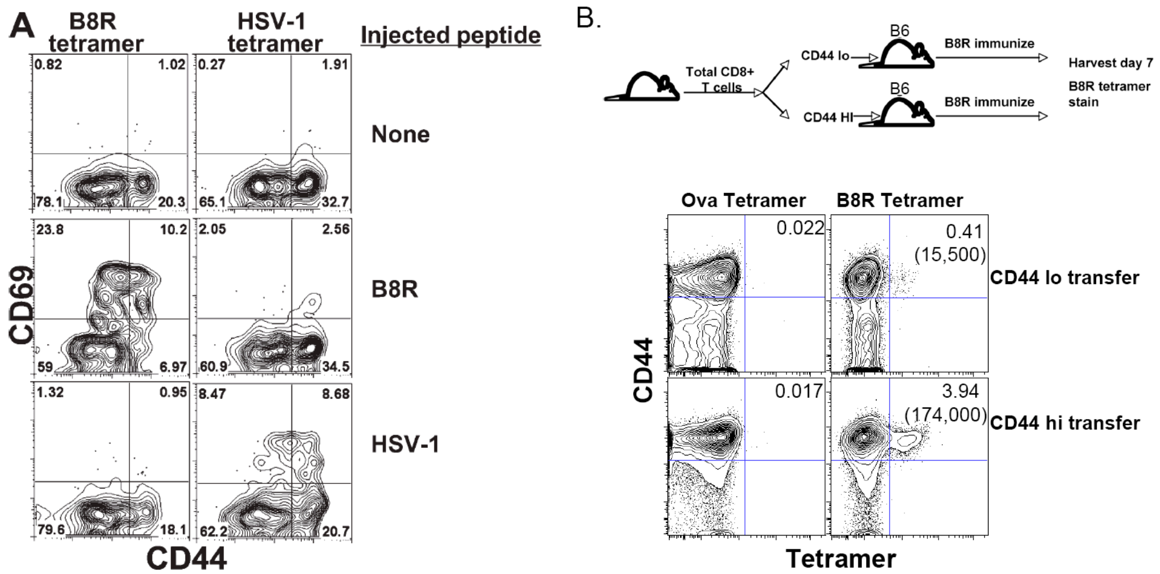


Figure 2-3. Foreign antigen-reactive CD44hi CD8 T cells found in unprimed SPF mice. (A) B6 mice were injected IV with 400mg of either B8R or HSV-1 peptide. Two hours later, the mice were sacrificed and half of the spleen cells from each peptide injected mouse were stained with the B8R/K^b tetramer and the other half stained with the HSVgB/K^b tetramer. B8R and HSV specific T cells from either peptide challenged mice, as well as control mice, were then isolated by magnetic column and analyzed for CD44 and CD69 expression on all tetramer-staining cells. Numbers in the upper quadrants represents the percent $CD69^+$ cells of all $B220^-CD8^+CD3^+tetramer^+$ cells shown. The results shown are representative of 6 mice for each treatment group from 3 independent experiments. (B) As shown in the schematic, $CD8^+$ T cells from unimmunized B6/SJL mice were sorted based on their expression of CD44. $CD44^{hi}$ and $CD44^{lo}$ cells were transferred into separate B6 mice which were subsequently immunized with the B8R peptide in conjunction with polyI:C and anti-CD40. Seven days after immunization, the spleen cells from each transferred and immunized host were stained with the indicated

tetramers. The K^b/OVA tetramer was used as a staining control as shown. Numbers above each gate represent the percent of tetramer-staining T cells out of total $CD8^+$ T cells. The numbers in parentheses indicate the total number of tetramer-staining cells per mouse after immunization. The results shown are representative of 8 total recipient mice from 2 independent experiments.

Importantly, however, $CD44^{hi}$ T cells were activated at least as efficiently as $CD44^{lo}$ cells within the appropriate tetramer-binding population, suggesting that the pre-existing $tetramer^+$ $CD44^{hi}$ pool include bona fide antigen reactive cells. The isolation of OVA-, HSV-1-, or B8R-specific T cells from vaccinia virus-challenged mice demonstrated a similar finding; namely that, while cells isolated with the B8R tetramer show signs of antigenic stimulation, T cells isolated from the virus challenged host with the HSV-1 or OVA tetramers show no signs of overt antigen stimulation (Fig. S2-2). Collectively, these results further support the conclusion that both $CD44^{hi}$ and $CD44^{lo}$ cells isolated with a given peptide MHC tetramer demonstrate specificity for that peptide/MHC complex.

Antigen responsive cells are present in both the $CD44^{hi}$ and $CD44^{lo}$ $CD8^+$ T cell pools of unprimed mice

Standard models of adaptive immunity would predict that foreign antigen-specific T cells in an unprimed animal would be exclusively of naïve phenotype ($CD44^{lo}$). In contrast, the data discussed above indicates that antigen specific precursors should be present in both the $CD44^{lo}$ and $CD44^{hi}$ (memory-phenotype) populations of $CD8^+$ T cells in an unprimed animal. To test this hypothesis using a method not influenced by the use of tetramer sorting, we sorted total $CD8^+$ T cells from unprimed animals based only on their CD44 expression (Fig. 2-3B). $CD44^{lo}$ and $CD44^{hi}$ populations were then transferred

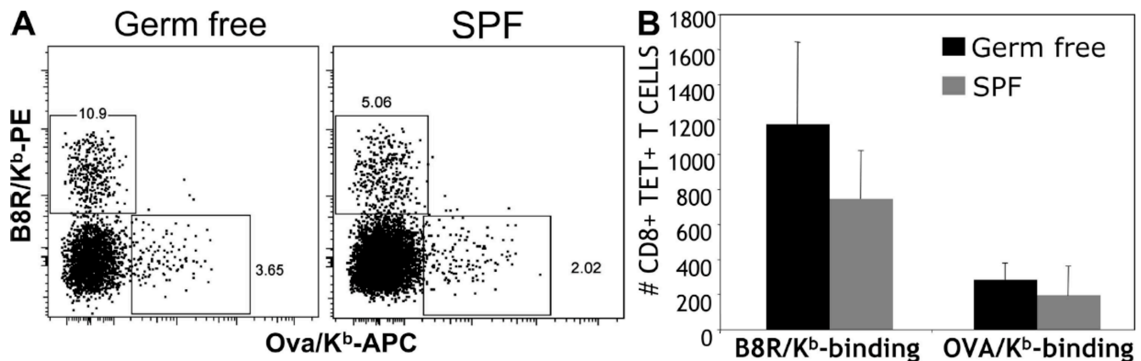
into separate B6 hosts and the recipients immunized with B8R peptide, together with adjuvants. Following immunization, B8R specific T cells were readily identified in mice transferred with either $CD44^{lo}$ or $CD44^{hi}$ precursors (Fig. 2-3B), suggesting that specific precursors are present in both pools. We therefore concluded that antigen specific precursors are present in both the $CD44^{lo}$ and $CD44^{hi}$ fractions of $CD8^{+}$ T cell in unprimed mice.

Antigen specific $CD44^{hi}$ CD8 T cells are found in unprimed germ-free mice

We postulated two possible explanations for the existence of antigen specific $CD44^{hi}$ T cells in unimmunized animals. First, these cells may be authentic antigen-experienced memory T cells, which fortuitously cross-react on the peptide/MHC complex used for tetramer based isolation. Previous studies have shown that the memory CD8 T cell pool specific for one pathogen may contain cells reactive to an unrelated pathogen (a response called heterologous memory) (122, 123). Alternatively, the memory-like cells we detected in unimmunized animals may be antigen-inexperienced, having been generated from the naïve T cell pool through homeostatic mechanisms. Conversion from naïve to functional memory-phenotype cells occurs in response to induced lymphopenia (64, 70, 107), but has also been reported to occur in physiological circumstances of lymphopenia, such as in neonatal mice (76, 77). To begin distinguishing these possibilities, we analyzed the antigen-specific T cell pool in germ-free mice. These animals are raised in sterile conditions and lack culturable gut flora. In contrast to the

SPF animals studied above, which are colonized by a diverse gut flora, the T cell pool of GF mice should be unbiased by exposure to commensal or environmental microbes.

Use of the tetramer enrichment protocol revealed a notable population of antigen-specific CD8 T cells binding B8R/K^b or OVA/K^b tetramers in unimmunized GF B6 splenocytes (Fig. 2-4A), which in absolute numbers were similar to SPF B6 animals analyzed in the same experiments (Fig. 2-4B). Critically, the tetramer-bound populations from GF animals also contained a population of $CD44^{hi}$ cells, which were present at similar frequencies as found in SPF mice (Fig. 2-4C, D; Fig. S2-4A). These studies cannot rule out the contribution of non-microbial antigens in appearance of these memory-phenotype CD8 T cells. However, our data do suggest that the presence of $CD44^{hi}$ antigen-specific pool in unprimed animals does not require exposure to environmental microbes and hence is unlikely to arise from the cross-reactive response to pathogens characteristic of heterologous memory (122, 123).



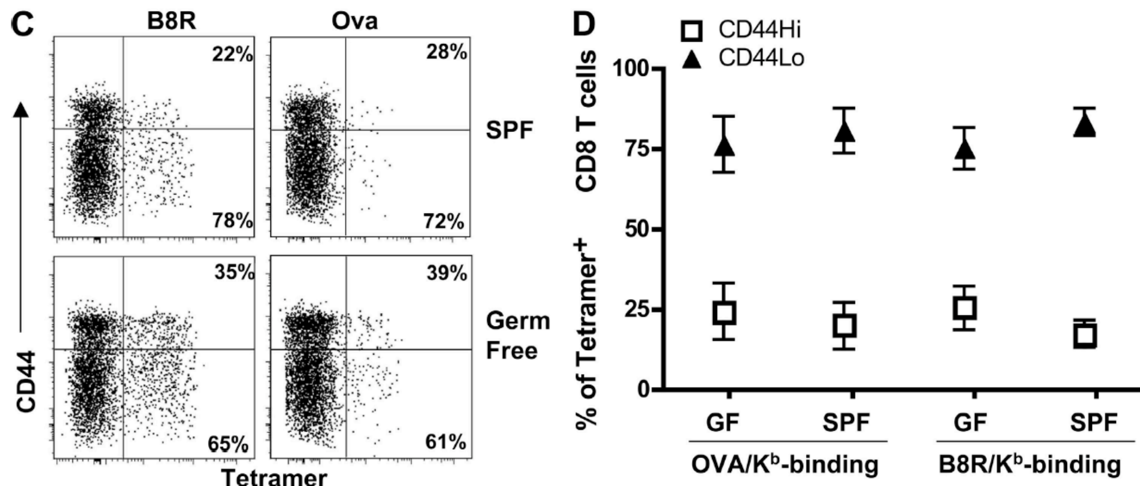


Figure 2-4. Foreign antigen-specific CD44hi CD8 T cells are found in germ-free mice. Spleen and lymph nodes were collected from B6 mice maintained in germ free versus SPF conditions, and subjected to tetramer enrichment using B8R/K^b and OVA/K^b tetramers. In (A) the dot plots show representative tetramer staining of the column bound pool, gated on $CD3^+$, $CD8^+$ B220/CD4- cells. (B) Shows ther total numbers of tetramer-bound CD8 T cells detected in unprimed SPF and germ-free mice, compiled from three independent experiments (n=9 for both SPF and germ-free mice). The graph shows mean and SD. (C) Representative data showing phenotype of column bound cells. The data is gated on dump-ve. CD3+$CD8^+$ T cells, and shows CD44 versus tetramer staining for cells from SPF and germ-free animals. Percentages are of tetramer-bound cells. (D) Shows average percentage of $CD44^{hi}$ and $CD44^{lo}$ phenotype cells, detected in unprimed SPF and GF mice, within the indicated $tetramer^+$ population. Numbers reflect mean and SD, with the data compiled from 9 mice in each group.

$CD44^{hi}$ T cells in unprimed mice bear the phenotypic signature of HP

These data lead us to speculate that the presence of the $CD44^{hi}$ $tetramer^+$ T cells might be the result of HP rather than a foreign antigen-driven response. We therefore sought to more comprehensively determine whether the phenotype and function of the $CD44^{hi}$ $tetramer^+$ cells was more consistent with foreign antigen experience or with HP. B8R specific T cells were isolated from unprimed B6 mice and stained for a variety of markers known to be differentially expressed on naïve and activated/memory T cells. As

a control for a phenotype representative of antigen-driven activation, B8R-specific cells were simultaneously isolated from mice challenged 4 days previously with vaccinia virus. B8R-specific cells from vaccinia-challenged mice displayed a classic activation phenotype, uniformly expressing high levels of CD44, LFA1, VLA4, Ly6C, and CD122 (Fig. 2-5A). A significant population of cells also expressed CD69 and CD25, indicative of ongoing antigenic stimulation as would be expected in mice only 4 days after vaccinia challenge. This phenotype was similar to peptide-immunized mice as well, though the peptide challenged mice displayed a slightly advanced time course of activation compared to viral challenge (Fig. S2-3). The specific $CD44^{hi}$ CD8 T cells isolated from unprimed mice expressed noticeably increased levels of CD122, LFA1 and Ly6C, but were low for CD69 and CD25, consistent with a memory (rather than effector) phenotype (64, 70, 107). The $CD44^{hi}$ population in GF mice also expressed elevated levels of CD122, which is similar to cells from SPF mice (Fig. S2-4A/B). Together these data reinforce the designation of the pre-existing $tetramer^{+}$ $CD44^{hi}$ pool as memory-like.

Unexpectedly, we found that tetramer-binding $CD44^{hi}$ cells in unimmunized animals expressed low levels of α4-integrin (Fig. 2-5A). This protein (also called CD49d) is a component of the integrins VLA-4 and LPAM, and is typically expressed at high levels on antigen stimulated effector and memory CD8 T cells (28, 113, 124-127) (Fig. 2-5A). Interestingly, we saw similarly low expression of α4-integrin on memory CD8 T cells produced via HP. Conventional- and HP- memory CD8 T cells were generated from naïve OT-I T cells by adoptive transfer into LM-OVA infected or lymphopenic mice, respectively as previously described (70). While antigen driven

memory OT-I T cells display elevated levels of α4-integrin (compared to bulk, polyclonal CD8 T cells), HP-memory OT-I cells show markedly reduced α4-integrin expression (Fig. 2-5B).

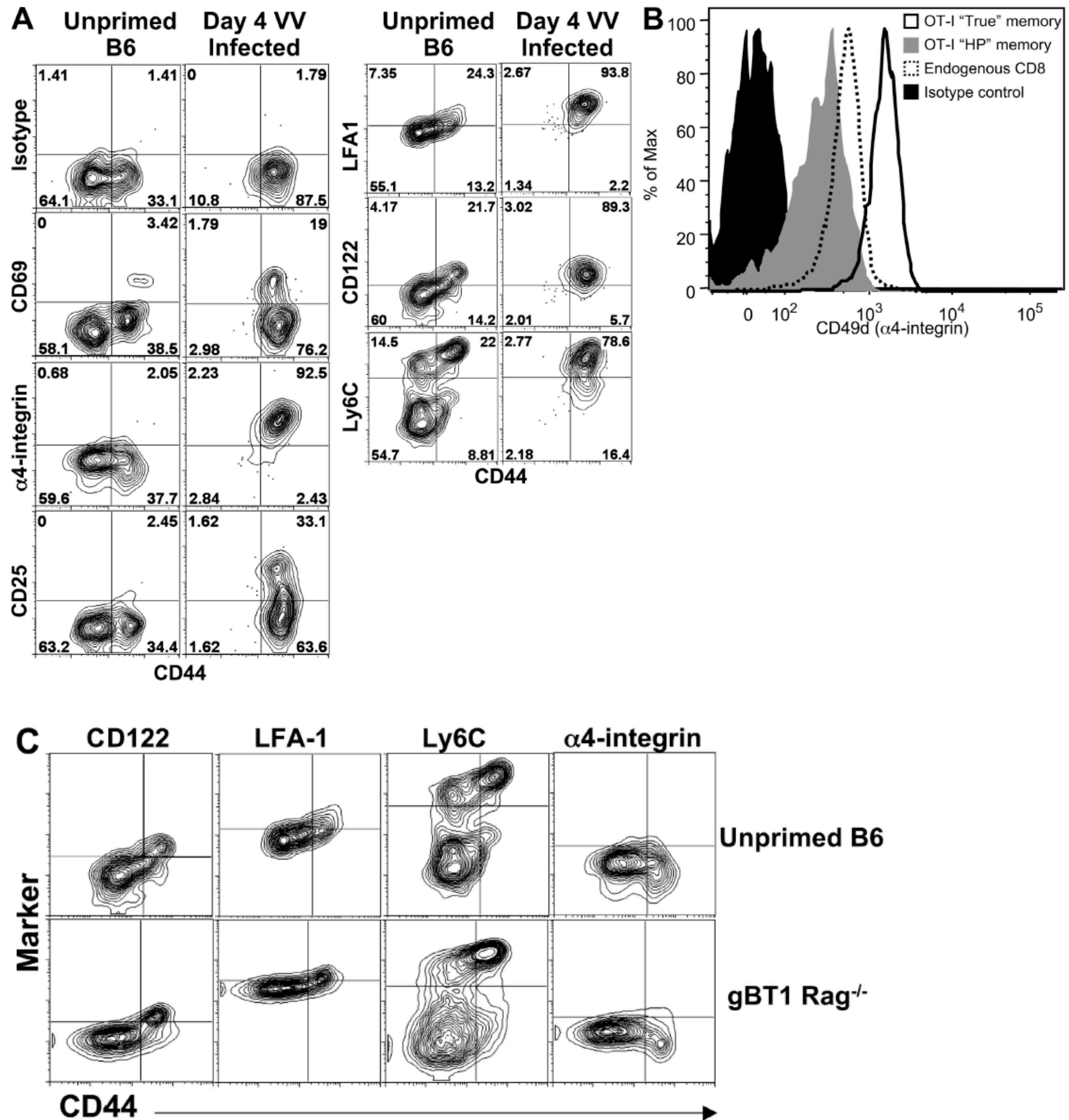


Figure 2-5. Phenotypic analysis of CD44hi and CD44lo tetramer-bound T cells from unprimed mice. (A) B8R specific T cells were isolated by tetramer staining and magnetic bead separation from the spleens of both unprimed and day 4 vaccinia virus-challenged mice. The cells were stained with CD44 and the indicated activation markers.

The data shown is gated as in Figure 1A and shows all B220$^-$ /CD8$^+$ /CD3$^+$ /tetramer$^+$ events. Numbers in each quadrant indicate the percent of B8R tetramer$^+$ cells within that quadrant. The results shown are representative of 8 mice from 4 independent experiments. (B) Naive OT-I CD8 T cells were transferred into either irradiated B6 mice (to generate HP memory cells) or LM-OVA infected mice (to generate "true" memory cells). At least 30 days later, the donor OT-I cells (identified using OVA/K^b tetramer) were assessed for their expression on α4-integrin. For comparison, α4-integrin on bulk endogenous CD8 T cells is shown, as is isotype control staining. These data are representative of at least 3 experiments with 2-3 mice per group. (C) B8R specific CD8$^+$ T cells isolated by magnetic bead separation as in A from an unprimed B6 (top contour plots) were compared to the CD8$^+$ spleen cells from a gBT-1RAG$^{-/-}$ mouse (lower contour plots) with respect to the activation markers shown. Cells were gated on all CD8$^+$ /B220$^-$ /CD3$^+$ /tetramer$^+$ events.

Hence, by this marker, the tetramer-binding CD44^{hi} cells in unprimed animals resembled HP memory CD8 T cells (Fig. 2-5A/B; Fig. S2-4B and S2-5). This pattern of α4-integrin expression was observed on specific T cells isolated from either LN or spleen (Fig. S2-4B, S2-5). In addition, low expression of α4-integrin was also observed on the CD44^{hi} pool of CD8$^+$ T cells isolated from unmanipulated RAG$^{-/-}$TCR transgenic gBT mice (Fig. 2-5C). Again, the T cells in these mice are all specific for the HSV glycoprotein B and cannot express endogenous receptors due to the RAG deficiency, and therefore cannot respond to environmental antigens. Therefore the phenotype of these cells must arise via a mechanism independent of antigen/TCR stimulation. In summary, the antigen-specific pool of CD44^{hi} T cells in unprimed WT mice, which is similar to those in TCR transgenic RAG$^{-/-}$ hosts, display a phenotype (CD122^{hi}, LFA-1^{hi}, and Ly6C^{hi}, α4-integrin^{low}) consistent with their being derived from homeostatic, rather than foreign antigen mediated, expansion.

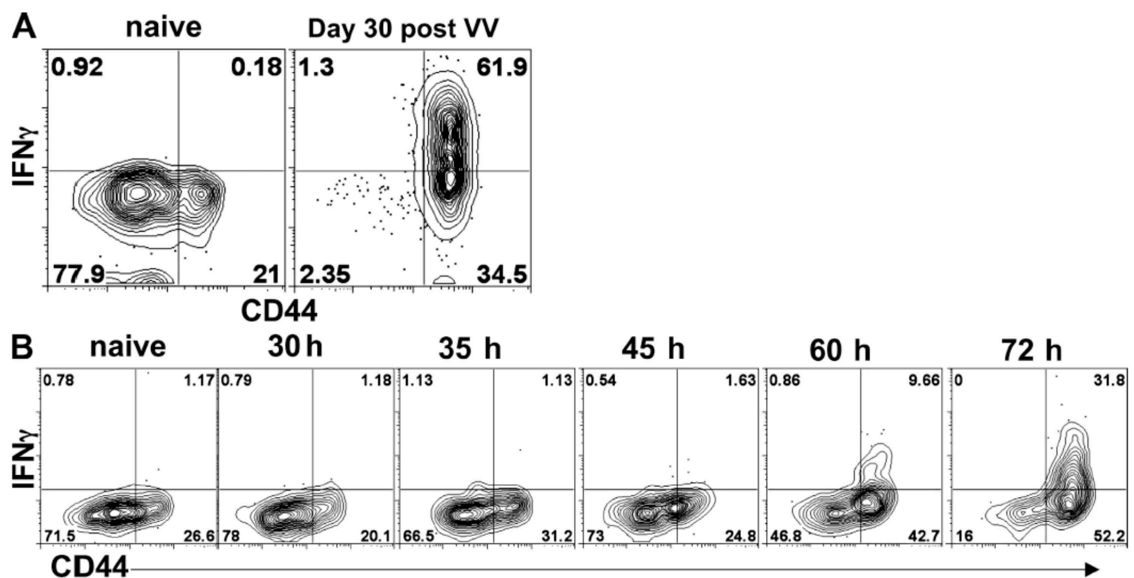
Enhanced functional reactivity of CD44^{hi} T cells isolated from unprimed animals

As discussed earlier, memory and naïve CD8 T cells differ not only in their phenotype but also in their functional properties. Enhanced functional reactivity is a hallmark of both antigen-experienced and HP memory CD8 T cells (64, 70, 107). Hence we sought to test the functional capacity of antigen specific CD44^{hi} cells from unprimed mice.

In initial experiments, we studied the response of endogenous CD44^{hi} and CD44^{lo} CD8 T cells following *in vivo* antigen stimulation. Unprimed mice were injected IV with the B8R peptide to provide an acute TCR stimulus for all B8R specific T cells, analogous to the addition of peptide in an *in vitro* intracellular cytokine staining assay. This was followed 30 minutes later by iv injection of Brefeldin A (BFA), to facilitate retention of intracellular cytokines (128, 129). As a positive control, this procedure was also performed on mice possessing a functional pool of memory B8R specific T cells, having been challenged 30 days prior with vaccinia virus. Two hours after initial peptide injection, the B8R specific T cells were isolated by magnetic bead sorting, in the continued presence of BFA, and then were stained and analyzed to detect intracellular IFN-γ in the CD44^{hi} and ^{lo} B8R specific T cells. In keeping with published data on conventional and HP memory cells (64, 70, 107), the majority of memory B8R-specific T cells from the vaccinia immune mice readily stained positive for both tetramer and IFN-γ (Fig. 2-6A). In contrast however, the CD44^{hi} antigen specific T cells from unprimed mice did not. These data indicate that the lack of IFN-γ production from the B8R-specific T cells in the unprimed host was due neither to a failure of the peptide to provide an adequate TCR stimulus *in vivo,* nor to a failure to isolate the unprimed T cells due to

downregulation of the TCR (130). These data suggested that antigen specific CD44^{hi} T cells differ from other memory CD8 T cell populations in that they do not make effector cytokines, such as IFN-γ, immediately upon TCR stimulus.

We next reasoned that perhaps the antigen-specific CD44^{hi} T cells would convert to IFN-γ-producing effectors, following antigen priming, more rapidly that their antigen-specific CD44^{lo} counterparts. We therefore immunized mice with B8R peptide and anti-CD40 antibody (as an adjuvant) at various time points prior to isolation of the specific cells by B8R/K^{b}-tetramer sorting. Again, analogous to the addition of peptide in an *in vitro* intracellular cytokine staining assay, mice were injected IV with peptide two hours prior to harvest, followed 30 minutes later by injection of BFA. The B8R specific T cells were isolated and were again stained and analyzed to detect intracellular IFN-γ in the CD44^{hi} and ^{lo} B8R specific T cells. By 60 hours after immunization, IFN-γ production could be induced selectively in the CD44^{hi} pool (Fig. 2-6B).



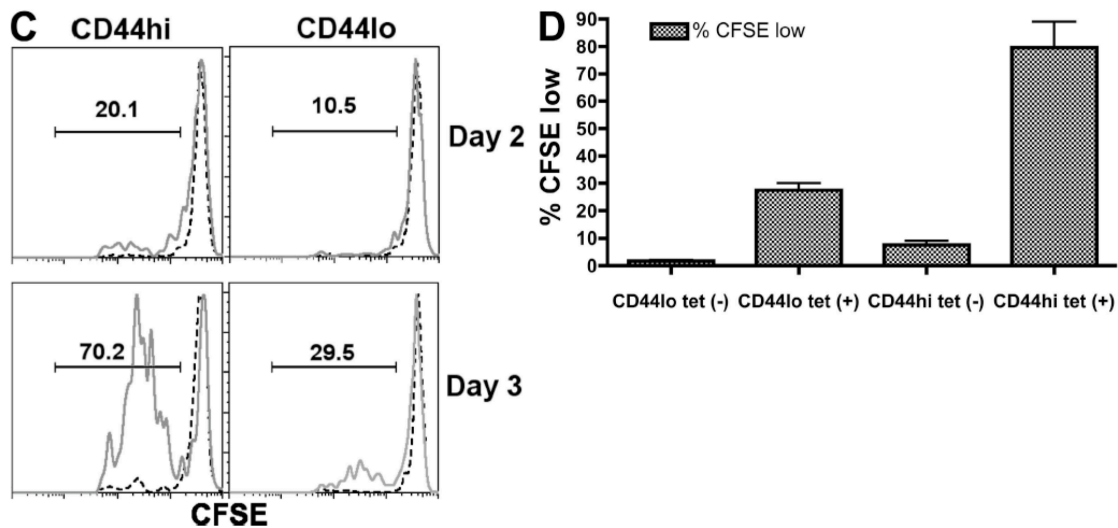


Figure 2-6: During primary stimulation, antigen specific $CD44^{hi}$ $CD8^{+}$ T cells respond more quickly than $CD44^{lo}$ $CD8^{+}$ T cells. (A) Unprimed B6 mice, or mice challenged with vaccinia virus 30 days prior, were injected IV with 300ug B8R peptide and BFA 2 hours before spleen harvest as described in the Materials and Methods. The B8R specific T cells were isolated by tetramer staining and magnetic bead sorting as described in Figure 1, with the exception that BFA was included in all buffers. The cells were stained for surface markers and intracellular IFN-γ as previously described (131). (B) B6 mice were immunized with 100mg B8R peptide and 50mg antiCD40 antibody at the indicated times prior to the harvest of their spleen cells. As in (A), the mice were injected with B8R peptide and BFA 2 hours prior to their sacrifice, and their B8R specific T cells isolated by tetramer staining and magnetic bead sorting. The cells were stained for surface markers and intracellular IFN-γ as previously described (131). The contour plots shown were gated on all $CD8^{+}$ $B220^{-}$ $tetramer^{+}$ T cells. (C) B6 splenocytes were harvested from unprimed animals and tetramer enrichment performed (using both $B8R/K^{b}$ and $HSV\text{-}1/K^{b}$ tetramers). $Tetramer^{+}$ and tetramer- CD8 T cells were both sorted into $CD44^{hi}$ and $CD44^{lo}$ populations, each was CFSE labeled and the cells stimulated in vitro on antigen (B8R and HSV peptide) pulsed splenocytes. Two or 3 days later (as indicated) the proliferation of the $tetramer^{+}$ (solid lines) and tetramer- (dashed lines) pools were assessed by CFSE dye dilution. (D) Compiled data from 3 experiments performed as in (C), showing the frequency of CFSE-diluted responder cells on day 3 after stimulation. Error bars show SD.

This response was specific since IFN-γ was not produced by T cells from the same animals isolated by MHC tetramers of an unrelated antigen (unpublished data). However, by this 60 h time point, there was a substantial increase in the fraction of

B8R/K^b tetramer binding $CD44^{hi}$ T cells, making it difficult to determine whether the responding cells were derived from the original $CD44^{hi}$ or $CD44^{lo}$ pool. In addition, although we did not observe this problem in the analysis of B8R specific T cells from the vaccinia-immune hosts, it was formally possible that the increased degree of TCR stimulation provided by the peptide+adjuvant immunization facilitated a greater loss of antigen responsive cells due to TCR downregulation (130).

To avoid these concerns, we first isolated tetramer-binding $CD44^{lo}$ and $CD44^{hi}$ CD8 T cells (from unprimed animals), and then tested their response to antigen stimulation in vitro. Specific CD8 T cells were enriched by tetramer-based isolation, and these cells were FACS sorted into $tetramer^{+}$ (and $tetramer^{-}$) $CD44^{lo}$ and $CD44^{hi}$ populations. These were labeled with CFSE and stimulated in vitro with peptide pulsed APC. Both $CD44^{lo}$ and $CD44^{hi}$ populations responded to stimulation, but we consistently observed a greater fraction of $CD44^{hi}$ cells undergoing proliferation in these assays (Fig. 2-6C, D). These data confirm that tetramer-binding cells are competent to functionally respond to the specific peptide/MHC molecule, and suggest that tetramer-binding $CD44^{hi}$ cells can proliferate more efficiently than their $CD44^{lo}$ counterparts.

Discussion

Collectively our studies indicate that the peptide/MHC specific CD8 T cell repertoire within an unprimed host contains both the predicted naïve phenotype pool, but also a memory-phenotype population. Furthermore, our data indicate that these cells are distinct from their naïve-phenotype counterparts in their proliferative.

The existence of memory-like T cells in non-immunized animals has long been known. However, this is the first demonstration, to our knowledge, that the pool contains cells specific for (and reactive to) nominal foreign antigens. We propose the term "Virtual Memory" (VM) to describe this novel population of antigen specific T cells within the unprimed T cell repertoire (in computing, virtual memory describes a form of working memory, based on alternative utilization of existing space). Our findings differ from a recent publication by Obar et al. (116), who used very similar approaches to study antigen specific CD8 T cells in the unprimed repertoire, yet concluded that these cells were all of naïve phenotype. Those authors did observe a range in CD44-expression among tetramer binding cells specific for VSV-N/K^b or M45/D^b (the main specificities studied in that report; Fig. 1) (116), but other phenotypic markers (such as LFA-1 expression levels) did not suggest a memory-phenotype. However, it is clear from our data that the frequency of VM cells varies greatly within any given specificity (Fig. 2-1,C-E). That being said, our preliminary studies on cells isolated from unprimed mice using VSV-N/K^b and M45/D^b tetramers indicates the presence of VM CD8 T cells, similar to the other epitopes studied here (unpublished data). The discrepancy between our findings and those of Obar et al. (116) may either reflect the natural variation in VM

frequency within the M45 and VSV-N specific T cells or reflect differences in our methods and/or analysis of tetramer-binding T cell isolation.

An important issue is how this memory-phenotype pool arises in unprimed animals. By standard models, the expectation would be that this pool represents cells primed by foreign antigens encountered in the environment, such as microbes or food antigens. Given such a scenario, one might anticipate that the endogenous memory CD8 T cells would be unlikely to show specificity for the nominal antigens studied here (including OVA, B8R and HSV epitopes), since their repertoire would be expected to be focused on the priming antigens. In contrast, we reliably found memory-phenotype CD8 T cells, in unprimed animals, for all of the foreign peptide/MHC ligands studied, and such cells were present at ~10-30% of the total tetramer-bound pool. These data suggest that the antigen specificity of VM cells has similar diversity as the naïve pool, arguing against the bias expected by reactivity to an environmental antigen.

Along the same lines, we consider it unlikely that VM cells are products of heterologous immunity, at least in the context in which this term is typically applied (122, 123). Heterologous immunity has been used to refer to the overlap in the reactivity of antigen specific T cells responding to distinct pathogens. This response arises because the pool of T cells responding to epitopes from one pathogen will sometimes include T cells cross-reactive with epitopes produced by a different pathogen. Such responses can lead to improved responses to a second microbe as a result of pre-existing memory against the first. However, since these responses involve fortuitous cross-reactivity between distinct epitopes, they often lead to dramatic changes in immunodominance of the immune

response (122, 123). In contrast, in our studies, the precursor frequency of VM cells responding to nominal foreign antigens was in proportion to the frequency of naïve-phenotype precursors, suggesting the absence of a severe bias in the repertoire of this pool. Secondly, VM cells isolated by tetramer sorting from unprimed animals displayed low expression of α4-integrin, this marker being expressed at levels lower than that of bulk CD8 T cells (Figure 2-5). In contrast, this marker is induced upon antigen stimulation, and typically remains high on the ensuing memory population (113, 126, 127, 132) (Fig. 2-5). Thus, the α4-integrin^{lo} phenotype of VM cells is inconsistent with their production by heterologous immunity, as classically defined (122, 123). It is worth noting that a substantial proportion (50-70%) of bulk CD44^{hi} cells in unprimed mice express low levels of α4-integrin (unpublished data). This suggests that the majority of the memory phenotype cells in unprimed mice may also be generated as a result of homeostatic proliferation. Finally, our studies using germ-free mice further argue against the model that the VM pool arises as a consequence of exposure to environmental microbes, although we cannot rule out the contribution of non-self antigens (e.g., components of food) in driving the generation of these cells.

The markers expressed by VM cells (high expression of CD122, LFA-1 and Ly6C, but low expression of α4-integrin) are the same as those expressed by HP memory CD8^{+} T cells produced in a lymphopenic host (64, 70, 103, 107) (Fig. 2-5). Together with the breadth of the antigen-specific repertoire discussed above, these data are most consistent with VM cells being produced by homeostatic mechanisms rather than conventional priming. On the other hand, both antigen-experienced and HP memory CD8

T cells rapidly produce IFN-γ upon TCR stimulation (64, 70, 103, 107), while antigen specific VM CD8 T cells appear unable to respond in this way (Fig. 2-6). Hence, the functional properties of VM CD8 T cells may be distinct from either conventional or HP memory CD8 T cells.

This report focuses exclusively on CD8 T cells. A memory-like pool of CD4 T cells in unprimed animals is also found, and it will be interesting to determine their specificity and function. Previous studies using the tetramer pull-down approach (115) showed that the majority of antigen-specific CD4 T cells in the unprimed pool were of naïve phenotype ($CD44^{lo}$). Whether this represents a true distinction between CD4 and CD8 T cells will require more extensive study. However, it is interesting to note that Class II-MHC specific TCR transgenic $RAG^{-/-}$ animals typically have very few $CD44^{hi}$ T cells (115), while several Class I-MHC specific TCR transgenic $RAG^{-/-}$ mice (Fig. 2-2 and 2-5), contain a clearly identifiable population of $CD44^{hi}$ T cells. In the absence of secondary TCRs, it seems likely that the appearance of $CD44^{hi}$ cells in such mice arises from HP. Indeed, the fact that Class I MHC-specific H-Y TCR transgenic $RAG^{-/-}$ T cells do not contain a $CD44^{hi}$ pool (133), and that these T cells also fail to undergo HP in a lymphopenic environment (60, 134) supports that model. Although the basis for the different incidence of memory-like cells in Class I-MHC versus Class II-MHC restricted TCR transgenics is unclear, it correlates with the greater propensity of CD8 T cells to undergo HP compared to the CD4 pool (60, 99-101, 103).

Regardless of how VM cells are generated, it is important to consider their potential significance for immune competence. Within the unprimed antigen specific T

cell pool, the majority of cells are naïve phenotype, with VM cells accounting for only 10-30% of the population. However, upon in vitro stimulation with antigen, VM CD8 T cells exhibit more robust proliferation compared to their naïve counterparts (Fig. 2-6). This property suggests that VM cells may participate in adaptive immune responses during a "primary" immune challenge. In addition, the proliferative advantage of VM cells may give them a competitive advantage during priming. Competition among T cells has been well described (131, 135, 136), although the molecular basis for this competition is yet to be fully elucidated (131), and it is interesting to speculate that VM cells may increase their proportional representation during an immune response. In addition, the fact that we observed variability in the frequency of VM cells depending on the peptide/MHC ligand studied is noteworthy, since the relative frequency of VM cells within an antigen specific pool may influence aspects of the response (such as immuno-dominance). Finally, if VM cells are indeed generated by homeostatic mechanisms, their functional relevance may be especially tailored to situations where the size of the naïve T cell pool is limited. It is interesting to note that young mice, although lymphopenic, contain a high proportion of memory phenotype CD8 T cells compared to adult animals (74, 75). Neither the specificity of these cells, nor their similarity to VM cells has been assessed, but it is tempting to propose that such cells may contribute to CD8 T cell-mediated immune responses in the neonate. Further studies will be required to determine the contribution of VM cells to the effector and memory populations following priming in young and adult animals.

In summary, our data suggest the unprimed T cell pool contains cells which bear phenotypic and functional traits of memory CD8 T cells, and which appear to arise via homeostatic mechanisms. Participation of these cells will need to be considered in understanding the nature of the "primary" immune responses and protective immunity against pathogens.

Materials and Methods

Mice and Reagents.

6-12 week old female C57BL/6J mice were purchased from NCI, and 6-12 week old female C57BL6/SJL mice were purchased from the Jackson laboratory. $RAG^{-/-}$, $RAG^{-/-}$ gBT1 TCR transgenic mice, and $RAG^{-/-}$OT-1 TCR transgenic mice were bred in the National Jewish Biological Resource Center or at the University of Minnesota Medical School. Spleen and lymph node cells from germ-free C57BL/6J mice were obtained from animals at the Gnotobiotic Core of the Center for Gastrointestinal Biology and Disease at the College of Veterinary Medicine, North Carolina State University, using conditions described previously (137). Germ-free mice were maintained in flexible film isolators under positive pressure and received autoclaved food and water. Aerobic and anaerobic cultures of freshly collected fecal samples from each isolator were performed biweekly, and were found to be negative. Peptides were ordered from Global Peptide (Ft. Collins, CO), New England Peptide (Gardner, MA) or Invitrogen (Carlsbad, CA). Class I MHC tetramers were produced as previously described ((135) or (70)) or purchased from Beckman Coulter (Immunomics, San Diego, CA). Fluorochrome conjugated antibodies against CD8, CD44, B220, CD4, CD11b, CD69, CD25, CD45.1, CD45.2, CD49d (α4-integrin), CD122, LFA1 and Ly6C were purchased from Beckton Dickinson (San Diego, CA) or eBioscience (San Diego, CA). Vaccinia Virus (WR strain) was produced by infection of Vero cells as previously described (135). All mouse protocols were approved

by the Institutional Animal Use and Care Committees at National Jewish Health or the University of Minnesota.

Tetramer staining and magnetic bead sorting

Tissues from mice (Spleen, LN, ovary) were removed and collagenase digested for 45 minutes as previously described (135). In the absence of collagenase digestion, antigen specific T cells are poorly isolated from antigen-challenged hosts (138). Therefore, collagenase treatment was typically used for isolating cells from both naïve and antigen stimulated mice. However, qualitatively similar recovery of naïve and VM populations were observed with or without inclusion of collagenase in the isolation procedure. Cells were resuspended in 500ul sorting buffer (consisted of 250 μl 24G2 supernatant and 250μl HBSS containing 2% FCS and 0.2% azide, to prevent internalization of the tetramer) per spleen. Cells were stained with tetramer and anti-CD8 for 60 minutes at 37°C. Cells were then washed, resuspended in HBSS plus azide, and stained with anti-PE coupled MACS MicroBeads (Miltenyi Biotec, Auburn, CA) for 30 minutes rotating at 4°. Cells were then washed, resuspended in HBSS and the tetramer^{+} cells isolated on a magnetized MACS column (Miltenyi Biotec, Auburn, CA) according to manufacturers instructions. Following elution, cells were centrifuged and resuspended in FACS buffer and stained for markers used to enhance gating of tetramer^{+} CD8^{+} T cells (CD8 and CD3 as a positive gate; B220 and, in some experiments, CD4 or CD11b as a dump gate), as well as with antibodies to determine activation status (CD44, α4-integrin, CD122, LFA-1, Ly6C, CD69, CD25).

As an alternative isolation and tetramer binding protocol (used for Figs. 2-1, 2-3, 2-4, S2-1, S2-4A, S2-5), tetramer binding was performed at 4°C (rather than 37°C) and the cells stained with fluorochrome (PE or APC) labeled tetramers at 4°C for 60 min in the absence of anti-CD8 antibodies. Cells were incubated with MACS MicroBeads coupled to anti-PE and/or anti-APC and isolated as above. In these experiments, fluorescent anti-CD8 antibodies were included with other stains after enrichment of tetramer-associated cells. For dual tetramer staining experiments, spleen cells were stained for an hour with both PE- and APC-labeled tetramers. Cells were then incubated with both anti-PE and anti-APC MicroBeads, isolated on MACS columns as above, and then stained with fluorescent antibodies to CD8, CD3, and CD44 in various fluorchrome combinations. In addition cells were staining with a cocktail of antibodies (all conjugated to Pacific Blue) specific for B220, CD11b, CD11c, F4/80 and (in some experiments) CD4. Cells positive for this "dump" stain were excluded from further analysis.

Cells were analyzed on an LSRII (Beckton Dickinson), Cyan ADP (Dako Cytomation) or a FACSan (Beckton Dickinson) retrofitted with a second laser (CyTek) to allow 5-color analysis. Data were analyzed using FlowJo software, following the gating strategy described in Fig. 2-1.

Immunizations

Where indicated mice were injected IV with 50-400ug peptide (B8R- TSYKFESV, OVA-SIINFEKL, or HSV-1-SSIEFARL). For the experiments shown in Figure 3, mice were challenged with peptide in the absence of any adjuvant in order to avoid any non-

antigen specific influence of the adjuvant on CD69 expression. In other experiments, mice were injected with peptide together with 25-50ug of the anti-CD40 antibody FGK45 (121, 139, 140) or a combination of FGK45 and 50 μg of poly-I:C (GE Life Sciences). This approach uses the capacity of CD40 stimulation, paired with poly-I:C, to act as a highly potent adjuvant for T cell responses (121, 139, 141, 142). For the experiments in which we transferred enriched cells into $RAG^{-/-}$ and B6 hosts, we used CD40/poly-I:C as an adjuvant to give us the best opportunity at expanding the small numbers of antigen specific cells transferred. For immunizations in which we required an adjuvant but wished to limit non-antigen specific adjuvant effects, we used peptide in combination with CD40 alone. For viral challenge, mice were injected i.p with $1x10^7$pfu of vaccinina virus strain WR.

In vivo and in vitro T cell activation assays

Mice were either left as unimmunized controls or were immunized with B8R peptide and anti-CD40 at various times to prior to sacrifice. Two hours before sacrifice, the mice were injected with 300ug of either B8R peptide or HSV peptide. Thirty minutes later, they were injected IV with 250ug brefeldin A in 500ul PBS as previously described (128, 129). 1.5 hours after BFA injection, the mice were sacrificed and tetramer-based enrichment performed as described previously (with the exception that BFA was in included in all buffers during the course of T cell staining and isolation). Following magnetic column isolation, the cells were stained with the remaining surface markers as

described above, then fixed, permeabilized, and stained for intracellular IFN-γ as previously described (131).

For in vitro T cell activation assays, B6 splenocytes were first subjected to tetramer-based enrichment, using both B8R/K^b and HSV-1/K^b tetramers in order to maximize the isolation of antigen specific T cells from the spleens. Bound cells were then sorted by flow cytometry to generate four populations: tetramer$^+$ CD44^{lo}, tetramer$^+$CD44^{hi}, tetramer$^-$ CD44^{lo} and tetramer$^-$ CD44^{hi}. The cells were labeled with CFSE and included with irradiated splenocytes (from B6.SJL animals) pre-coated with 1μM HSV and B8R peptides and with polyIC and anti-CD40 for one hour at 37 degrees. Flow cytometric analysis was performed on days 2 and 3 of the culture, gating on live, CD45.2$^+$ cells, representing the B6 responder cells.

Preparation of HP and "True" memory OT-I cells

CD44^{lo} (naïve) CD8$^+$ OT-I T cells were isolated from OT-I mice and adoptively transferred into B6 animals to generate memory populations. For "True memory" cells, naïve OT-I T cells were transferred into normal B6 mice which were then infected with LM-OVA and left for >30 days to allow memory generation. To generate "Homeostatic Proliferation" memory cells (HP), naïve OT-I cells were transferred into sublethally irradiated (~450 rads) B6 mice and left for >30 days to allow for expansion and differentiation in response to lymphopenia. Expression of α4-integrin on these populations is indicated, as is an isotype control staining profile.

Chapter 2 supplemental figures

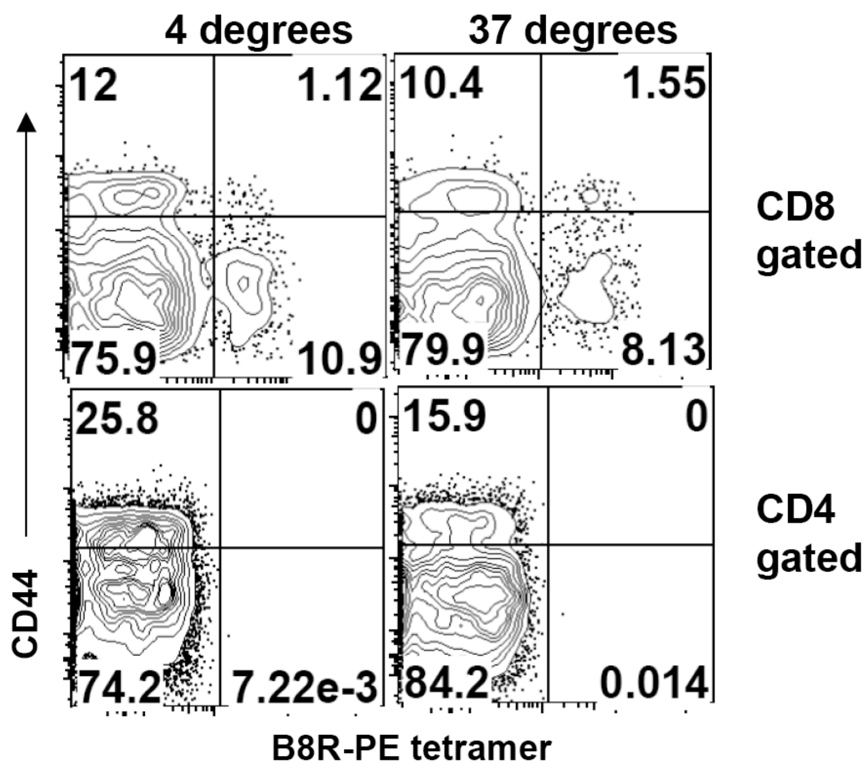
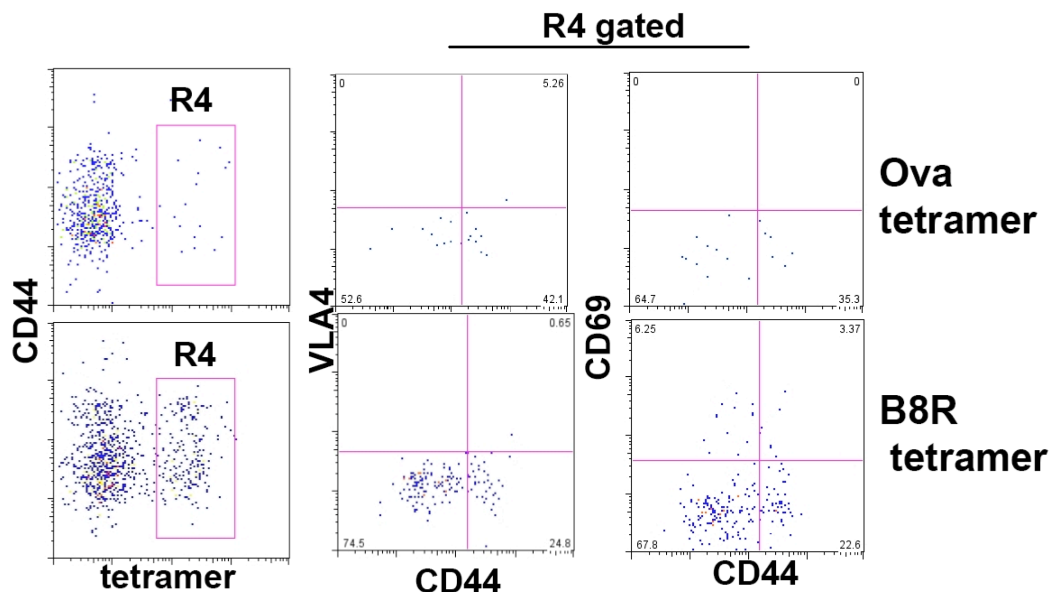


Figure S2-1. Minimal impact of tetramer staining conditions on recovery of tetramer-binding CD44hi cells from unprimed mice. Spleen and lymph node cells were isolated from unimmunized B6 mice and subjected to tetramer enrichment. In these experiments, tetramer binding was performed at either 4°C or 37°C as indicated, in the absence of anti-CD8 antibody. Cells were enriched using anti-PE coupled magnetic particles and the data shown are gated on CD3 positive events which were negative for the “dump” cocktail (CD11b, CD11c, F4/80, B220). In this case, CD4 and CD8 were stained individually (as indicated) on the column-bound pool. Similar data were observed in a separate experiment.



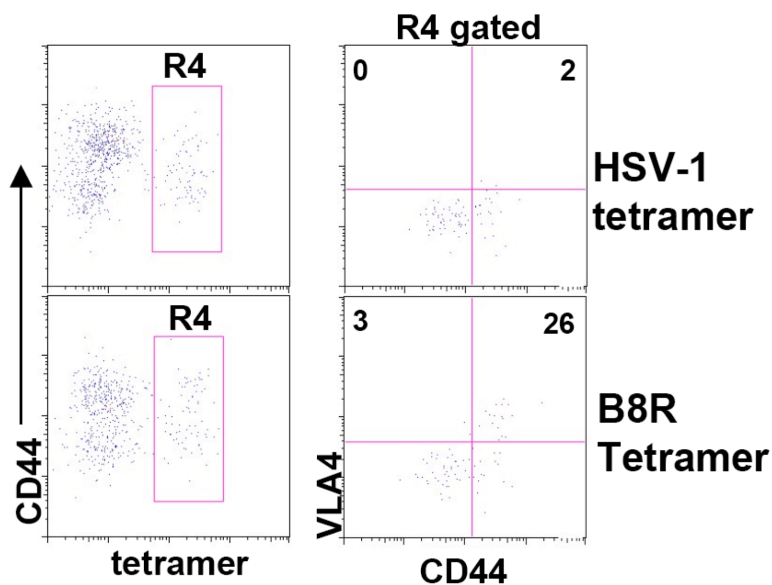


Figure S2-2. T cells identified by tetramer staining and magnetic bead isolation are antigen specific. B6 mice were challenged with VV-WR and at the indicated time points, either HSV-, Ova-, or B8R-specific T cells were isolated by tetramer staining and magnetic bead separation as described in Fig. 2-1. Far left dot plots in A and B show gating on all tetramer^{+} events and the right dot plots indicate the phenotype of either HSV or B8R tetramer $^{+}$ events with respect to the indicated markers. Only cells with the B8R tetramer from VV challenged mice show signs of phenotypic activation. These data are representative of 3 independent experiments.

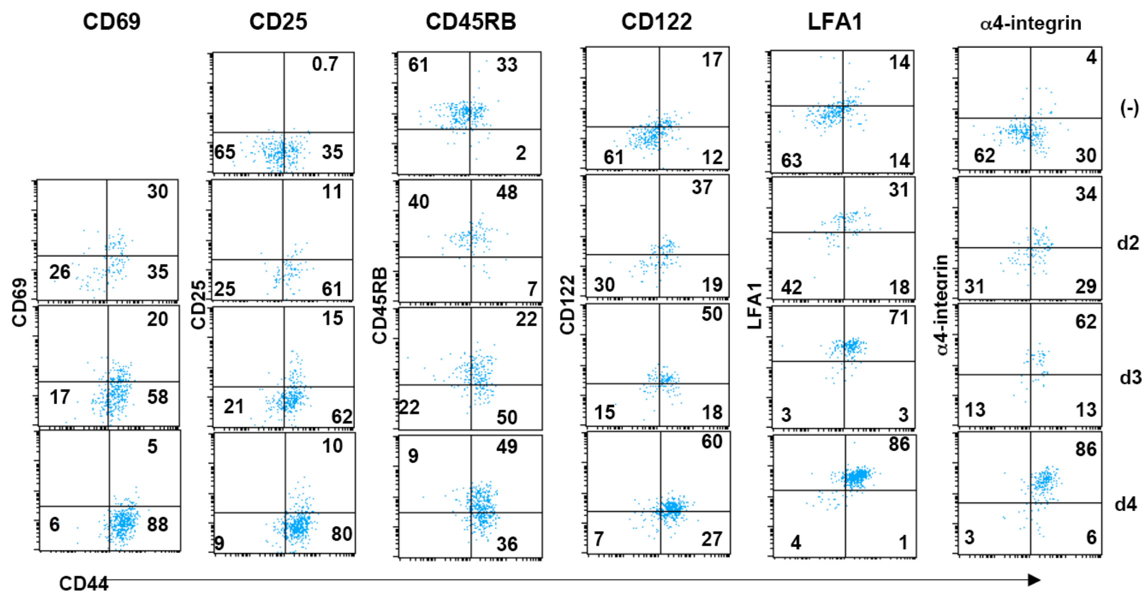
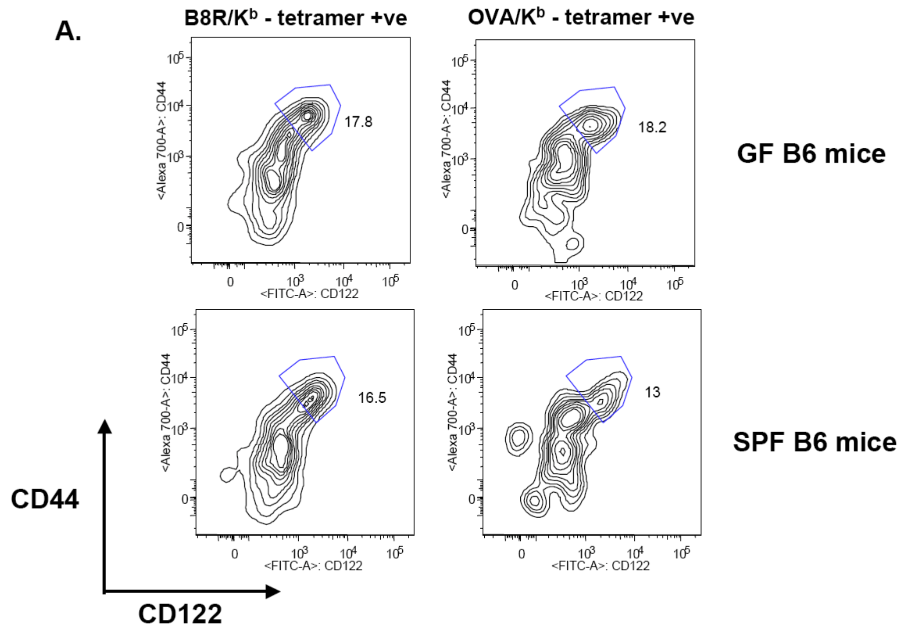


Figure S2-3. Peptide immunization results in an activation phenotype of B8R specific T cells similar to that observed following VV-WR challenge. B6 mice were immunized i.v. with 100μg of B8R peptide in combination with 50μg of the antiCD40 antibody FGK45. On the indicated time points, B8R specific T cells were isolated from the spleens as described in Figure 1 and analyzed for their expression of the indicated activation markers. Data shown was gated on all $CD8^{+}B220\text{-}CD3^{+}$ events. Data is representative of 3-4 mice per time point from two independent experiments.



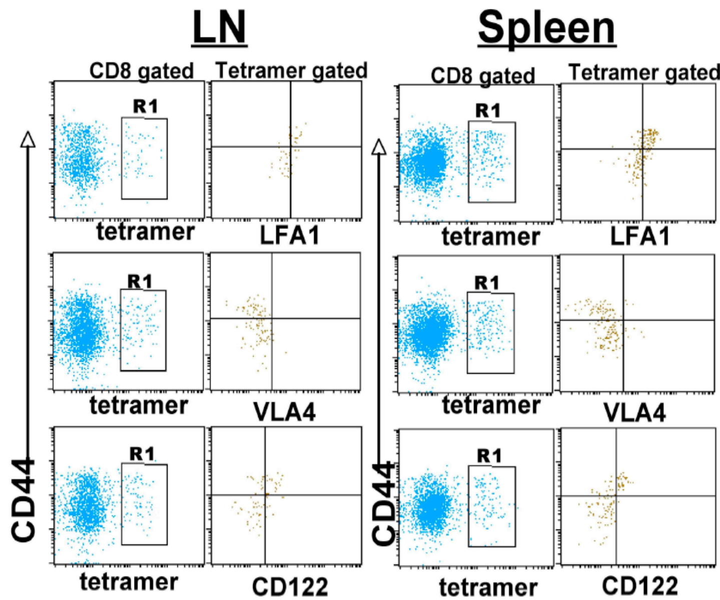


Figure S2-4. Phenotype of CD44hi tetramer$^+$ T cells, from unprimed animals, isolated from spleen and lymph node. (A) Spleen and lymph node cells were isolated from SPF or GF mice and isolated for binding to B8R/K^b or OVA/K^b tetramer, as in Figure 4. Cells were gated on dump-ve, CD3+, CD8+ tetramer+ cells (for the tetramer indicated) and staining for CD44 and CD122 is shown. (B) Lymph node (LN) and spleen cells were isolated, stained with B8R tetramer, and isolated by magnetic column separation as described in the Materials and Methods and Figure 5. The phenotype of all CD8$^+$B220-CD3$^+$tetramer$^+$ events are shown with respect to the indicated markers. (VLA-4 indicates staining for α4-integrin). Data is representative of 5 experiments.

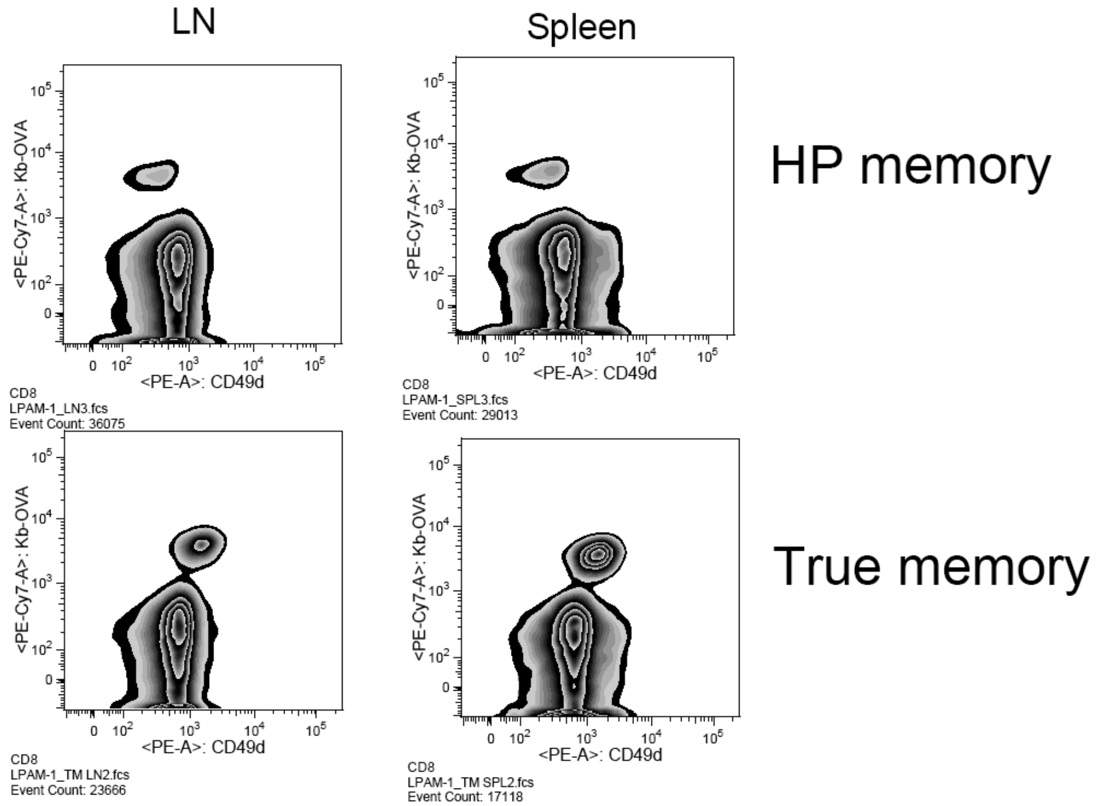


Figure S2-5. Expression of α4-integrin by antigen-driven versus HP memory OT-I T cells. HP and antigen-driven ("True" memory) OT-I CD8 T cells were generated following adoptive transfer of naïve OT-I cells, as described for Fig. 5B and the Materials and Methods. At least 30 days following OT-I adoptive transfer, spleen and LN cells were isolated and stained for flow cytometry. Data are gated on total CD8 T cells, with donor OT-I cells identified using Ova/K^b tetramer. Staining for α4-integrin is indicated. These data are representative of at least 3 experiments, with 2-3 mice per group.

Chapter 3

Derivation and maintenance of virtual memory cells

Memory $CD8^+$ T cells are an important component of the adaptive immune response against many infections, and understanding how antigen-specific memory $CD8^+$ T cells are generated and maintained is crucial for the development of vaccines. We recently reported the existence of memory-phenotype, antigen-specific $CD8^+$ T cells in unimmunized mice ("virtual memory" or VM cells). However, it was not clear when and where these cells were generated during normal development, nor the factors required for their production and maintenance. This issue is especially pertinent given recent data showing that memory-like $CD8^+$ T cells can be generated in the thymus, in a "bystander" response to IL-4.

Here we show that the size of the VM population is reduced in IL-4R-deficient animals. However, the VM population appears first in the periphery and not the thymus of normal animals, suggesting this role of IL-4 is manifest following thymic egress. We also show that the VM pool is durable, being maintained for many months in normal animals, and also being retained during responses to unrelated infection.

Introduction

Conventional memory $CD8^+$ T cells are generated in response to foreign antigens, following the production and contraction of the effector response (143-146). However, memory-like CD8 T cells can also be generated by homeostatic mechanisms, without TCR engagement with foreign peptide/MHC ligands. In response to T cell deficiency, naïve T cells proliferate and differentiate into memory-phenotype cells, in the process of lymphopenia-induced proliferation (60-65, 147). Such "homeostatic memory" $CD8^+$ T cells express numerous phenotypic and functional traits of conventional memory $CD8^+$ T cells, including their ability to effectively control infection (70, 143, 148). While initially studied in the context of induced lymphopenia (21, 100, 143, 149), additional studies suggested that physiological lymphopenia occurs during the neonatal period in mice, and induces production of homeostatic memory T cells (74-77, 100, 149). Finally, recent studies from our group and others have identified a third mechanism for generating memory-like $CD8^+$ T cells, which involves the response of thymocytes to the cytokine IL-4. This pathway was identified in mouse models in which the thymic NK-T (or NK-T like) pool was enlarged, resulting in elevated frequencies of IL-4-producing cells. Reactivity to IL-4 leads to upregulation of the transcription factor Eomes and acquisition of memory-phenotype by "bystander" $CD8^+$ mature thymocytes (87-91). Although the mechanism for generation of "bystander memory" $CD8^+$ T cells was initially identified in genetically manipulated mice, a similar population was observed in some conventional

mouse strains (e.g. BALB/c), suggesting such cells may also contribute to the $CD8^+$ memory pool.

Using peptide/MHC tetramers in combination with magnetic enrichment protocol (115, 116, 150) we showed that unimmunized mice contained a population of antigen-specific memory-phenotype $CD8^+$ T cells (150). Similar populations have subsequently been reported by others (151, 152). These cells, which we termed "Virtual memory" (VM) T cells, express multiple phenotypic and functional characteristics of memory $CD8^+$ T cells, and were found both in unimmunized conventional mice and also in germ-free animals. These and other data suggested that the VM population was not generated via conventional priming. However, it was unclear whether these cells arose from lymphopenia-induced proliferation or as bystander memory cells produced in the thymus. In addition, previous studies did not address whether the VM pool was maintained during normal T cell homeostasis and during conventional immune responses.

In this report we demonstrate that both bulk memory $CD8^+$ T cells and the VM population are reduced in IL-4R-deficient C57BL/6 mice. However, we also show that production of VM cells initiates in the periphery and not the thymus arguing that VM cells are not generated exclusively as IL-4-induced thymic memory $CD8^+$ T cells. We also show that the VM population appears during a period of neonatal lymphopenia, and is sustained throughout adulthood. Furthermore, the VM pool is maintained during a $CD8^+$ T cell response to unrelated antigens, suggesting these memory-like cells are not a consequence of immunological naiveté.

Materials and methods

Mice.

C57BL/6 (B6), T cell receptor alpha-deficient ($TCR\alpha^{-/-}$), and Interleukin-4 receptor alpha (CD124) deficient ($IL\text{-}4R\alpha^{-/-}$) B6 mice were purchased from the Jackson laboratory. Female B6 mice were bred with male $TCR\alpha^{-/-}$ B6 to generate $TCR\alpha^{+/-}$ B6 mice. All mice were maintained in specific pathogen-free conditions at the University of Minnesota (Twin Cities). All animal protocols were approved by the Institutional Animal Use and Care Committees at the University of Minnesota.

Magnetic bead enrichment and flow cytometry

Spleen, major lymph nodes and thymus were harvested from 1 to 25-week-old mice as indicated. Tissues were injected with 1X collagenase D (Roche, Germany) and minced to generate single cell suspension. Tetramer-binding cells (from thymus or pooled spleen and lymph nodes) were isolated by magnetic bead enrichment as previously described in detail (150). Tetramers generated with K^b contained epitopes from vaccinia B8R (TSYKFESV), OVA (SIINFEKL), HSVgB (SSIEFARL) or the following MCMV-derived peptides: M38 (SSPPMFRV), M57 (SCLEFWQRV), m-139 (TVYGFCLL) or IE3 (RALEYKNL). D^b based tetramers contained epitopes from LCMV GP_{33} or MCMV M45 (HGIRNASFI). Monomers and tetramers were generated as previously described (153). The MCMV epitope based tetramers were a generous donation of Maire Quigly (University of Massachusetts). Phenotypic analysis was done by staining with antibodies

to CD19 (clone 6D5 Biolegend), CD11b (clone M I/70 eBiosciences), CD11c (clone), F4/80 (Invitrogen), CD3ε (clone 145-2C11, eBiosciences), CD4 (clone RM 4-5, BD Biosciences), CD8 (Invitrogen), CD44 (clone IM7, eBiosciences), CD122 (BD Biosciences). Data was acquired using LSR II (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

Viral infection.

6- to 10-week-old B6 mice were infected via intraperitoneal injection with $2x10^{\wedge 5}$ PFU of LCMV Armstrong (kindly provided by Dr. David Masopust, University of Minnesota). Spleens were harvested 30 to 45 days later for magnetic bead enrichment using GP_{33}/D^b and $B8R/K^b$ tetramers.

Statistics. A two-tailed, unpaired student's t-test was performed on the indicated data samples. P values are displayed within figures. P values of <0.05 are considered significant.

Results

Virtual memory CD8$^+$ T cells are detected among CD8$^+$ T cells of diverse specificities, and are not a property of dual reactive T cells

Our previous studies using a peptide/MHC tetramer enrichment procedure revealed the presence of foreign antigen-specific memory-phenotype CD8$^+$ T cells (VM cells) in unimmunized conventional and germ-free B6 strain mice (150). In that report, VM cells were detected among the precursor pool specific for three well characterized peptide/MHC complexes (OVA/K^b, B8R/K^b and HSVgB /K^b). Because each of these antigens induce dominant immune responses in their respective systems, it is possible that the VM population is solely a feature of cells responding to immunodominant epitopes. Also, a previous study by Obar et al using a similar experimental approach concluded that foreign-antigen specific CD8$^+$ T cells in unimmunized mice were uniformly of a naïve phenotype (116). To explore this further we used peptide/MHC tetramer enrichment (115) to assess the VM frequencies for five epitopes recognized in the B6 response to MCMV. These represent the three most immunodominant specificities (M45/D^b being dominant, followed by M139/K^b and M57/K^b) and two epitopes (M38 and IE3, both K^b restricted) which are barely detectable in the acute MCMV response (although the response is higher during chronic infection) (154, 155). This analysis includes the M45/D^b specificity studied in the report by Obar et al. (116). The precursor frequencies in unimmunized animals for these antigens varied depending on the specificity studied, and were in rough correlation with their reported immunodominance

during acute MCMV infection (Fig. 3-1A). Significantly, VM cells ($CD44^{hi}$) could be detected for each specificity, and there was no notable correlation between the frequency of VM cells and the immunodominance characteristics of the specificity examined (Fig. 3-1B). Such data extend our previous observations and indicate VM cells are consistently found within $CD8^+$ T cells of varying specificities, precursor numbers and immunodominance characteristics.

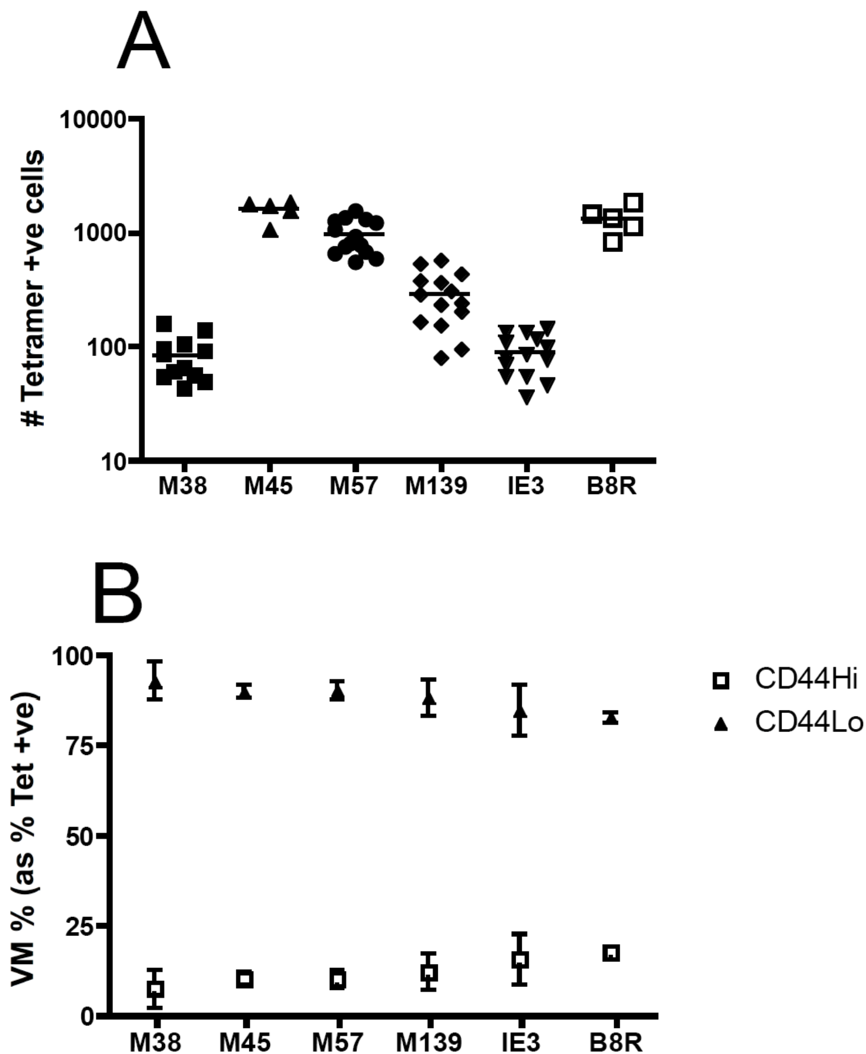


Figure 3-1. VM cells are detected among diverse foreign-antigen specific precursor pools. Peptide MHC-I tetramer-based enrichment was performed on spleen and lymph

node cells from unprimed B6 mice using PE and APC-coupled tetramers representing five epitopes from MCMV (M38/K^b, M45/D^b, M57/K^b, M139/K^b, and IE-3/K^b) and one from vaccinia virus (B8R/K^b). (A) Shows the number of tetramer positive CD8$^+$ T cells in the bound fraction. (B) Represents the percentage of each tetramer positive CD8$^+$ T pool that are either memory (CD44^{hi}/CD122$^+$) or naïve (CD44^{lo}/CD122$^-$). The results in A and B are compiled from at least 3 individual experiments.

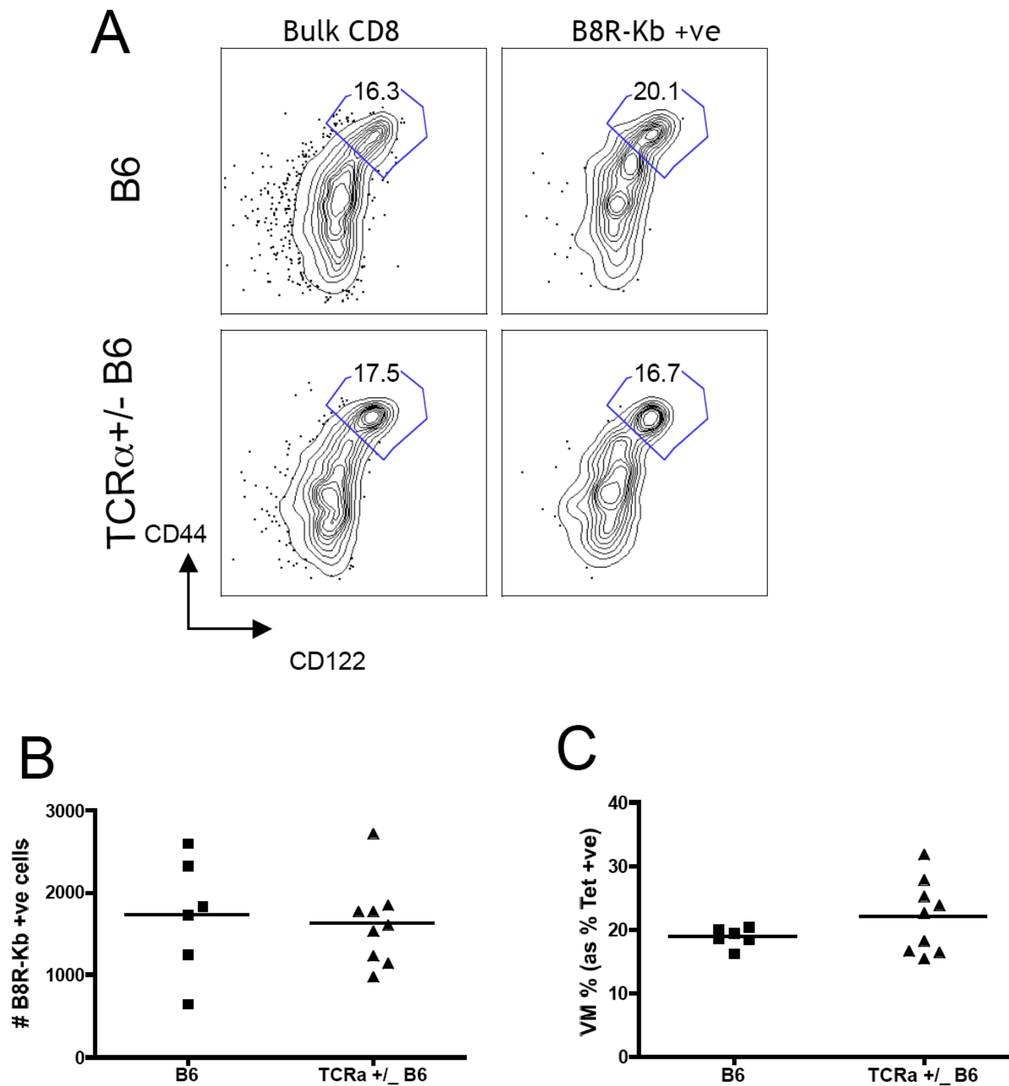


Figure 3-2. VM cells are not dual TCR reactive T cells. Cells from lymph nodes and spleen were obtained from TCRα$^{+/-}$ B6 and wild type B6 mice, and stained for CD8, CD44, and CD122. Magnetic enrichment was also performed using B8R/K^b tetramers. (A) Contour plots of both bulk CD8$^+$ T cells and B8R/K^b positive CD8$^+$ T cells. Numbers

represent the percentage of cells with the phenotype of memory ($CD44^{hi}/CD122^{+}$). (B) Depicts the number of $B8R/K^{b}$ positive $CD8^{+}$ T cells in the spleen and lymph nodes of $TCR\alpha^{+/-}$ and B6 mice. (C) Shows the percentage of VM cells among the $B8R/K^{b}$ positive $CD8^{+}$ T cells of $TCR\alpha^{+/-}$ and B6 mice. A total of 3 experiments were performed, each with 3 $TCR\alpha^{+/-}$ B6 mice and 2 wild type B6 controls.

The fact that VM cells are reliably seen for multiple specificities in unprimed animals makes it unlikely that VM cells were induced by prior encounter with the foreign antigens recognized by these cells. However, a significant fraction of T cells express dual TCRs, as consequence of incomplete TCRα-chain allelic exclusion – a phenomenon reported to occur for up to 30% of T cells (156, 157). It was possible that VM cells represent dual reactive T cells, primed against one foreign antigen but bearing a second TCR for an antigen not yet encountered. To test this hypothesis, we explored the frequency of VM cells in T cells from $TCR\alpha^{+/-}$ mice, which are limited to a single functional TCRα-chain. Frequencies of memory within the bulk (Fig. 3-2A) and $B8R/K^{b}$ tetramer-binding $CD8^{+}$ T cells (Fig. 3-2A-C) were similar for normal and $TCR\alpha^{+/-}$ animals, arguing against dual reactivity as a likely basis for the appearance of VM cells.

Together with earlier studies (150), these findings suggest that the VM population is a feature of diverse foreign-antigen specific precursor pools, and that these cells are unlikely to be generated by conventional priming or as a by-product of incomplete TCRα-chain allelic exclusion.

The size of the VM pool is dependent on IL-4 reactivity

Two distinct mechanisms have been shown to induce memory-phenotype $CD8^{+}$ T cells in the absence of foreign antigen priming. Homeostatic memory T cells are

generated during the response of naïve $CD8^+$ T cells to lymphopenic conditions in secondary lymphoid tissues, while bystander memory $CD8^+$ T cells are generated in the thymus in response to IL-4. Our studies and others (88, 158) suggested that the bystander memory mechanism was prominent in BALB/c but not B6 strain mice, which correlated with an expanded pool of PLZF-expressing NK-T cells, capable of IL-4 secretion, in BALB/c mice. Nevertheless, it was possible that such IL-4 dependent bystander memory cells were part of the VM population studied in B6 strain mice.

To address this hypothesis, we examined the VM population in IL-$4R^{-/-}$ B6 mice. The total numbers of B8R/K^b and HSVgB/K^b specific precursors was similar in WT and IL-$4R^{-/-}$ animals (Fig. 3-3A), yet frequency of VM cells within this pool was significantly reduced in IL-$4R^{-/-}$ animals (Fig. 3-3B). We also noted a reduction in the percentage of memory-phenotype cells in the bulk $CD8^+$ T cell pool (Fig. 3-3B/C). Together, these data suggest that IL-4Rα-deficiency leads to a reduction (but not complete loss) of the VM population in B6 mice. Previous work showed that IL-4 induced generation of bystander memory $CD8^+$ T cells in the thymus of some mouse strains (87-89) and, although this population is quite rare in B6 mice, we observed a significant reduction in the frequency of memory-phenotype thymic CD8 single positive T cells in IL-4R deficient animals (Fig. 3-3C).

VM cells arise in the peripheral pool during the neonatal period

The data above demonstrated a role for IL-4 in production of VM cells, and raised the possibility that these memory-phenotype cells are produced during thymic

development, rather than during physiological homeostatic proliferation in the periphery, as we had previously proposed (150). However, reports have indicated that homeostatic memory CD8$^+$ T cells produced in the periphery can home to the thymus (159), making it difficult to determine where the thymic memory-like CD8$^+$ T cell pool was generated.

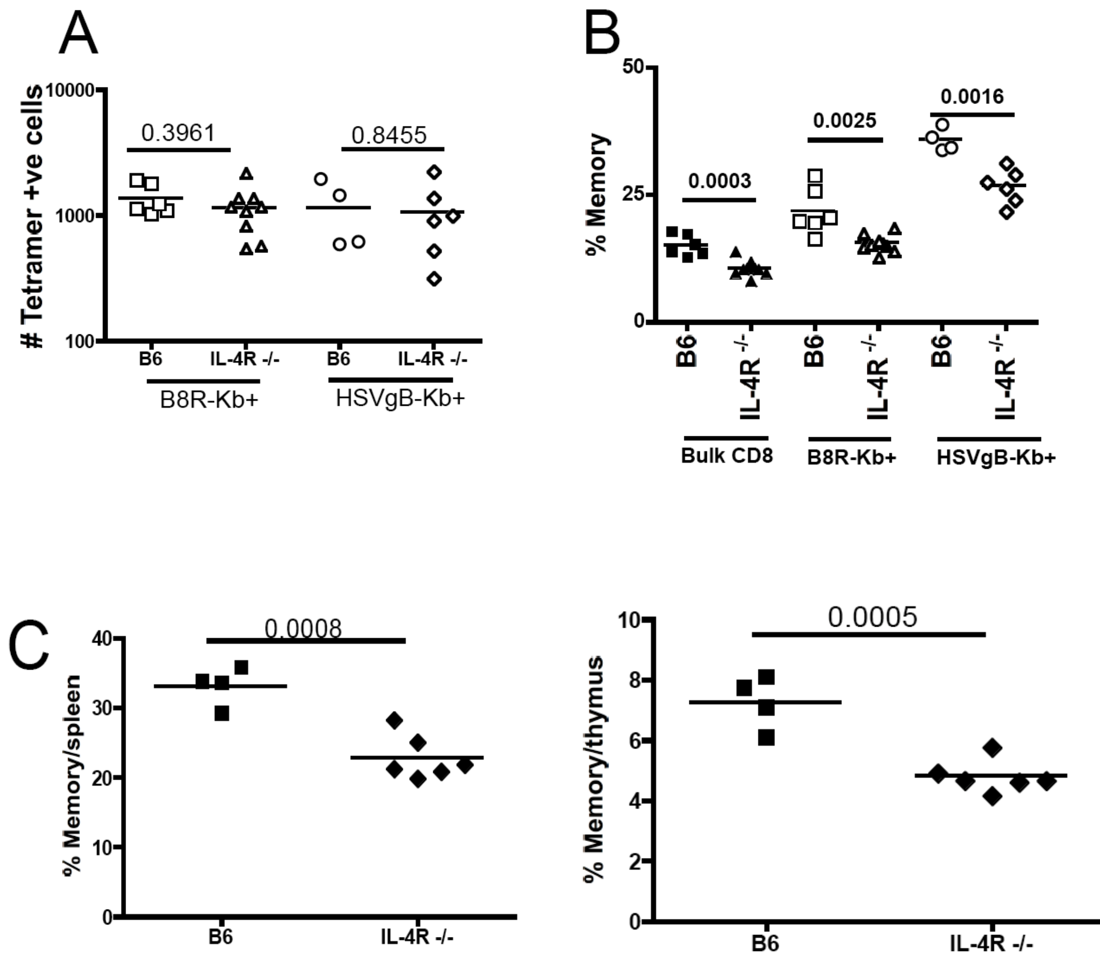


Figure 3-3. The size of the VM pool depends on IL-4 reactivity. (A/B) Cells from lymph nodes and spleen were obtained from 9-10-week-old IL-4R$^{-/-}$ B6 and wild-type B6 mice, and stained for CD8, CD44, and CD122. MHC-I tetramer enrichment was also performed using B8R/K^b and HSVgB/K^b tetramers. (A) Depicts the number of B8R/K^b and HSVgB/K^b tetramer positive CD8$^+$ T cells in the spleen and lymph nodes of IL-4R$^{-/-}$ and B6 mice. (B) Shows the percentage of memory phenotype cells among total, B8R/K^b and HSVgB/K^b tetramer positive CD8$^+$ T cells in adult IL-4R$^{-/-}$ and B6 mice. A total of 3

experiments were performed, each with 3 IL-4R$^{-/-}$ B6 and 2 wild-type B6 control mice. In (C), 4-5-week-old wild type or IL-4R$^{-/-}$ mice were analyzed for frequency of memory-phenotype cells among CD8 single positive T cells from spleen and thymus.

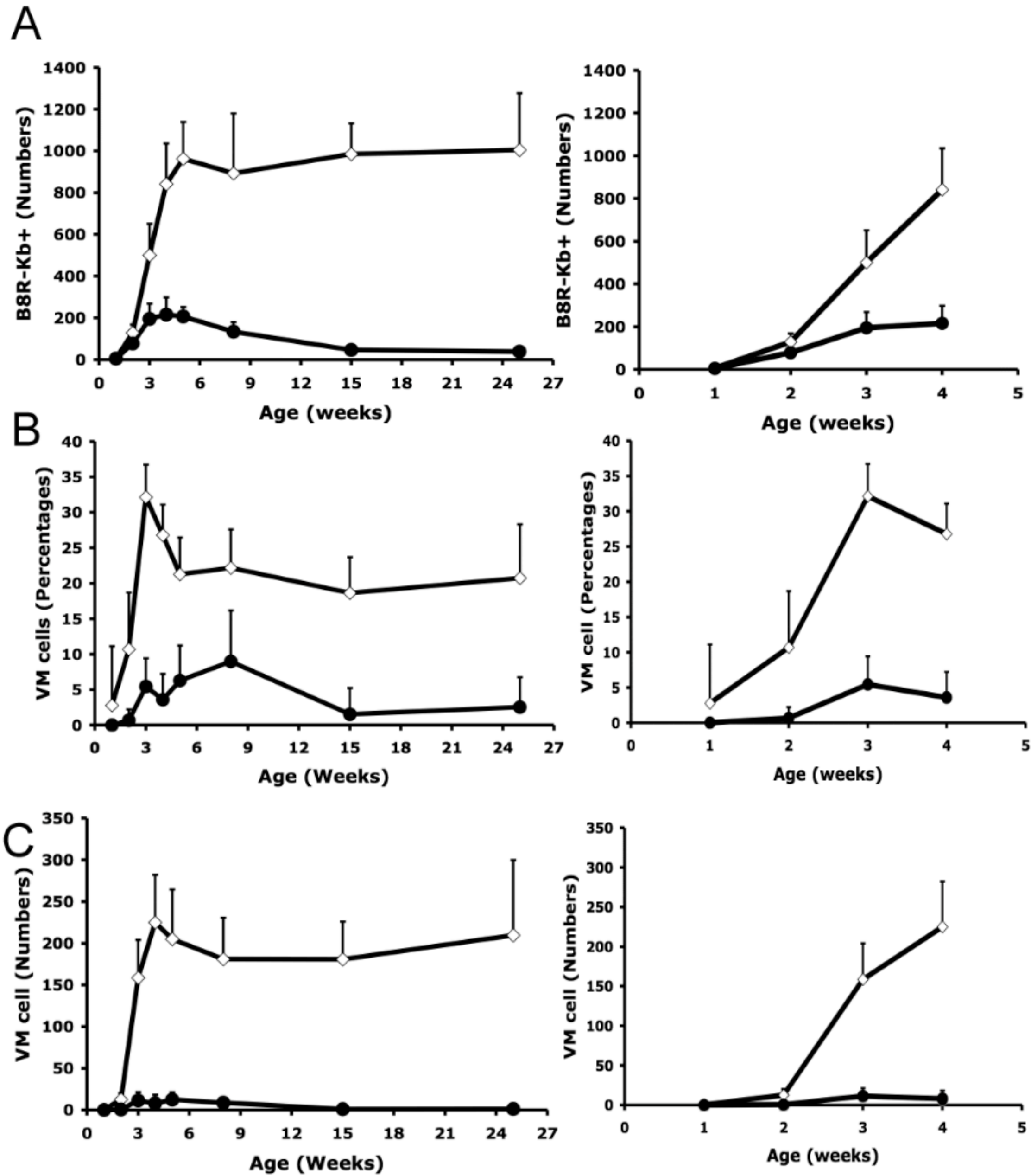


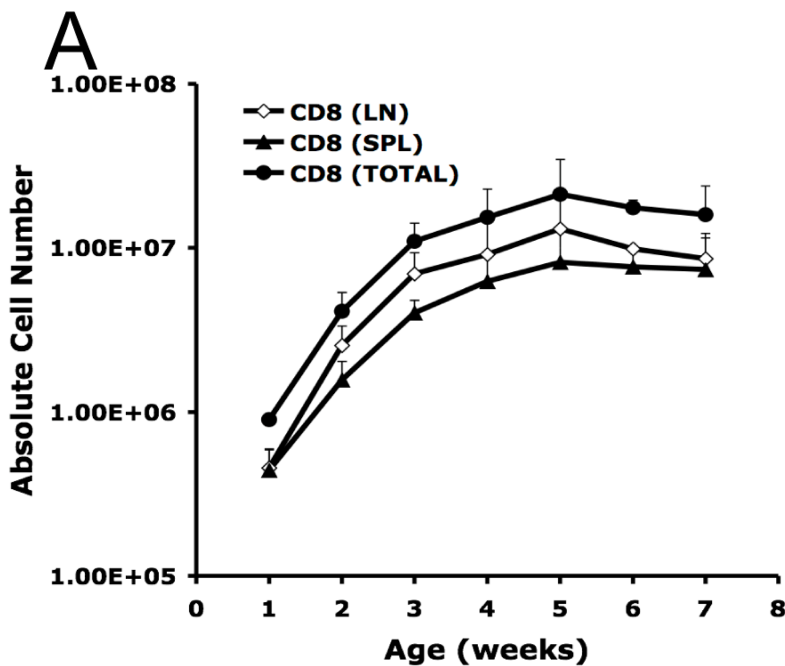
Figure 3-4. VM cells arise first in the periphery during the neonatal period. Thymus (filled circles) and pooled spleen/lymph nodes (open diamonds) were harvested from mice ranging from 1 to 25 weeks of age and tetramer enrichment was performed using B8R/K^{b} tetramers. CD8 single positive/CD3^{+} cells were gated on. (A) the number of

B8R/K^b tetramer binding $CD8^+$ T cells, (B) The percentage of memory-phenotype cells within this population and (C) shows the absolute number of memory-phenotype B8R/K^b tetramer binding $CD8^+$ T cells in the thymus and pooled spleen and lymph nodes. For each group, the right-hand panel highlights the data from animals of 1- to 4-week old. Each time point represents a minimum of 2 experiments with at least 4 mice per experiment.

To address this issue, we studied when and where the VM population first appears, by performing tetramer enrichment assays on thymic and peripheral lymphoid tissues from mice of 1-4 weeks after birth. Peripheral B8R/K^b specific $CD8^+$ T cells accumulate over this time, and while B8R/K^b specific pool of thymic CD8 SP cells is predictably small, these cells could be detected in mice from 2 weeks of age (Fig. 3-4A). Strikingly, by two weeks there was clear appearance of memory-phenotype B8R/K^b specific $CD8^+$ T cells in the peripheral but not the thymic pool (Fig. 3-4B/C). When we extended this analysis through the neonatal period, we observed a small population of VM cells that appeared in the thymus from 3 weeks, a timepoint when the peripheral pool of B8R/K^b specific VM cells had peaked (Fig. 3-4B). Both the low frequency of thymic VM cells, and their appearance after the VM cells have arisen in the periphery argue against thymic generation of VM cells as a major component of their derivation in B6 mice.

We also extended this time course to explore the maintenance of the VM pool in adult animals. The frequency of B8R/K^b specific VM cells reached a peak (>30%) at around 3 weeks of age, and then declined to the frequencies observed in adult animals (around 20%) by 5 weeks (Fig. 3-4B). However, the absolute number of B8R/K^b specific cells (both total and memory phenotype) was maintained at a plateau from around 4-5 weeks of age into adulthood. These findings are similar to the changes observed in the

bulk CD8$^+$ T cell pool, which gradually accumulates in the periphery from birth to 5 weeks of age (Fig. 3-5A). We found that the frequency of bulk memory-phenotype CD8$^+$ T cells peaks in younger animals (Fig. 3-5B) – similar to the findings reported by Ichii et al. earlier (75). Previous studies showed that adoptively transferred naïve T cells proliferate in neonatal mice and acquire the phenotype of memory cells (74-77), suggesting that the neonatal environment is functionally lymphopenic. Hence, taken together, our data are most consistent with VM cells arising in the peripheral pool, in response to neonatal lymphopenia, rather than generated in the thymus.



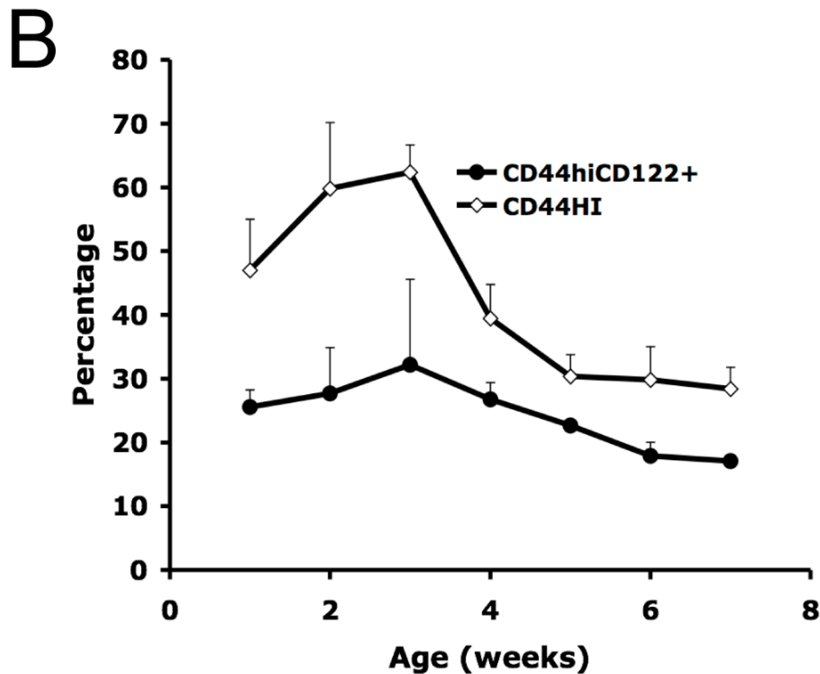


Figure 3-5. The frequency of memory-phenotype CD8 T cells is elevated during establishment of the peripheral T cell pool. Lymph node and spleen cells were obtained from B6 mice ranging from 1- to 7-week-old and stained with antibodies to CD4, CD8, CD44, and CD122. (A) Shows the absolute number of bulk $CD8^+$ T cells in the spleen, lymph nodes, and both tissues combined, while (B) shows the percentage of splenic $CD8^+$ T cells that are of memory-phenotype ($CD44^{hi}$ and $CD44^{hi}/CD122^+$). These data derive from at least 2 individual experiments for each time point.

VM cells are maintained long term under steady state conditions and during bystander immune responses

Our data show that the frequency and number of $B8R/K^b$ binding memory-phenotype $CD8^+$ T cells was constant in mice from 5 to 25 weeks of age (Fig. 3-4B,C), indicating that the VM pool was stable. However, such maintenance may be a consequence of the minimal exposure to immune challenge associated with SPF housing. To test whether the VM population is affected by a vigorous $CD8^+$ T cell response, we infected B6 mice with LCMV Armstrong and tracked $B8R/K^b$ specific precursors >30

days post-infection. Since B8R/K^b specific T cells are not expected to participate in the LCMV response, this approach allows us to test how a bystander infection influences the stability of a VM population of distinct specificity.

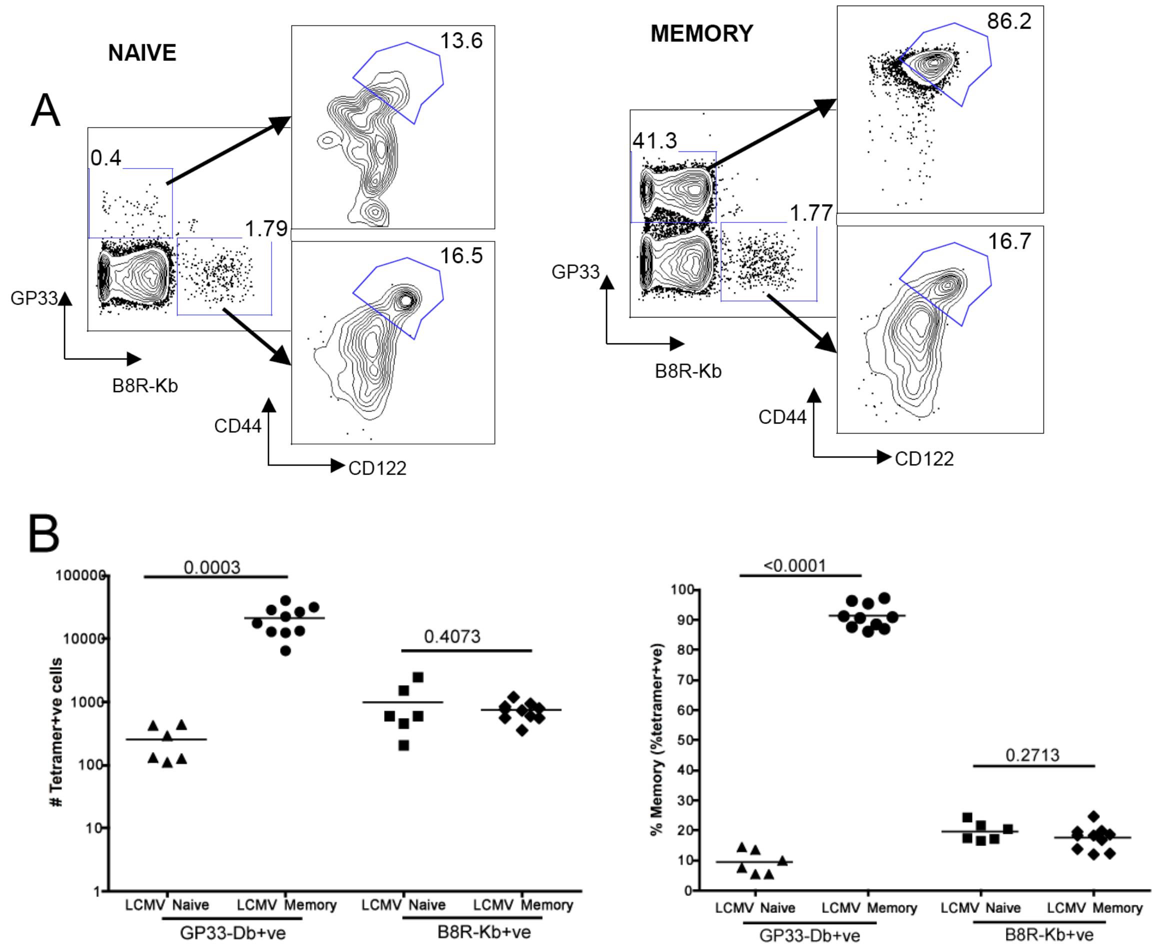


Figure 3-6. VM cells are maintained during bystander immunization. Wild type B6 mice were infected with LCMV. Splenocytes were harvested 30-45 days later from these animals ("memory") or uninfected controls ("naïve") and subjected to enrichment using both B8R/K^b and GP_{33}/D^b tetramers. (A) Numbers in the left-hand contour plots (of Naïve and Memory) represent the percent of $CD8^+$ T cells in the bound fraction that are GP_{33}/D^b and B8R/K^b positive, while numbers in the right-hand contour plots (of Naïve and Memory) represent the percent of VM cells for each specificity. These plots are representative of 3 experiments with a total of 10 LCMV memory mice and 6 unprimed B6 controls. (B) Shows the number of GP_{33}/D^b and B8R/K^b positive cells and the percentage of memory phenotype cells among each tetramer positive pool.

As expected, the LCMV GP_{33}/D^b specific T cell pool was greatly expanded and was uniformly of memory phenotype (Fig. 3-6A/B). However, the $B8R/K^b$ tetramer binding pool was of similar size (Fig. 3-6B) and, most importantly, contained a similar frequency of VM cells following LCMV infection (Fig. 3-6A/B). These data suggest that the persistence of the VM population is not a consequence of artificially low immune exposure, and that this population can be maintained in the presence of a greatly expanded antigen-driven memory $CD8^+$ T cell pool. Our finding suggests that preexisting memory (VM) cells are not displaced by new antigen-experienced memory cells with different specificities, but instead the memory pool expands in size with each immunological experience in order to accommodate newly generated memory cells (consistent with studies by Vezys et al (160)).

Discussion

It is now recognized that multiple pathways can lead to the production of $CD8^+$ memory-phenotype T cells in mice. In addition to conventional immune responses against foreign antigens, there is evidence for "homeostatic memory" cells produced by lymphopenia induced proliferation and "bystander memory" induced by IL-4 in the thymus (87-91). In this report, we sought to determine how VM cells, being memory phenotype $CD8^+$ T cells specific for unencountered foreign antigens, are generated and maintained.

Our data reinforce the concept that VM cells are not induced by conventional immune responses against foreign antigens: We find VM cells among $CD8^+$ T cells of multiple specificities, including immunodominant and subdominant cells responsive to MCMV epitopes, and these data are in agreement with studies finding VM cells among $CD8^+$ T cells specific for both dominant and sub-dominant epitopes in an influenza model (151). Between data presented here and elsewhere, VM cells have now been defined in more than a dozen specificities (150-152). Furthermore, our data using TCRα hemizygous mice make it unlikely that the VM pool derives from conventional memory cells bearing a second TCR specificity. These data indicate that VM cells are consistently found within $CD8^+$ T cells of various specificities from the unimmunized pool.

Of non-conventional mechanisms for memory-phenotype $CD8^+$ T cell generation, our recent findings that elevated levels of IL-4 can cause induction of thymic memory like cells prompted us to reassess this pathway in VM generation. Our published studies

demonstrated that IL-4 levels in normal BALB/c mice but not B6 mice were sufficient to drive production of a substantial "bystander memory" pool of mature $CD8^+$ thymocytes (88), and similar conclusions were reached by others (158). Hence the substantial decrease in the size of the bulk memory and VM pool in IL-4R$\alpha^{-/-}$ B6 mice was not anticipated. In any case, these data indicate the VM population is partially dependent on IL-4 sensitivity for induction and/or maintenance.

Since our previous work suggested IL-4 induces $CD8^+$ memory-like cell generation in the thymus (88, 89), these new findings prompted the question of whether VM cells were generated during or after thymic development. Also, although the conventional $CD8^+$ memory T cell pool is renown for its long-term maintenance, even in the face of subsequent immune responses (152, 160), the sustainability of the VM population has not been addressed. We explored these issues by tracking VM cells longitudinally across a range of mouse ages. These data showed that the VM population initially appears in peripheral tissues, while memory-phenotype cells are scarce in the thymus. At later time points, however, a small pool of VM cells was detected in the thymus: this might indicate a second "wave" of thymically-generated VM cells, but interpretation of such data is complicated, due to data suggesting peripheral homeostatic memory cells can recirculate to the thymus (159). These thymic VM cells appear at a stage (3 weeks of age) where thymic function is critical for generating the T cell pool, making alternative approaches - such as thymectomy - difficult to interpret. Hence, we cannot completely exclude a contribution of thymically differentiated VM cells at this stage. Our data also indicate that the VM pool is remarkably stable: For the tracked

B8R/K^b specificity, VM cell numbers peaked at around 4 weeks of age, and were sustained at similar numbers for the next ~5 months. Likewise, priming of a robust conventional immune response (against LCMV) did not reduce either the percentage or number of B8R/K^b specific VM cells. Together, these data suggest that the majority of the VM pool is generated in the peripheral compartment, and maintained long term, similar to conventional memory cells.

The studies in this report reinforce the concept that VM cells are a pool of unprimed, memory-like $CD8^+$ T cells, that this population is produced during the neonatal stage, in a process enhanced by IL-4, and VM cells are maintained long term in immunologically competent animals. Given the relatively high frequency of VM cells within multiple antigen specificities studied, a large fraction of the endogenous memory pool must be comprised of VM cells, in contrast to the typical view that such cells are the result of immune responses to environmental foreign antigens. Intriguingly, a similar population may be generated in humans during gestation (a stage typically aligned with mouse neonatal immune development): Studies on the fetal spleen revealed a prominent fraction of $CD8^+$ T cells with multiple memory traits (elevated CD122 and Eomes, competence to make IFN-γ rapidly) (161). Whether this pool includes bona fide memory cells will require further studies, but given the sterile environment of the fetus, it is unlikely that this population was primed by foreign antigen exposure. Such findings underscore the importance of future studies to define how the VM population may contribute to primary immune responses, perhaps especially during the neonatal period.

Chapter 4

Virtual memory (VM) cells outcompete naïve $CD8^+$ T cells during the immune response to foreign antigen

Figure 4-4 was reprinted from the Journal of Experimental Medicine. Catherine Haluszczak, Adovi D. Akue, Sara E. Hamilton, Lisa D.S. Johnson, Lindsey Pujanauski, Lenka Teodorovic, Stephen C. Jameson, and Ross M. Kedl. "The antigen-specific $CD8^+$ T cell repertoire in unimmunized mice includes memory-phenotype cells bearing markers of homeostatic expansion". **©2009 Rockefeller University Press. Originally published in *J. Exp. Med.* 206:435-448.**

C. Haluszczak, A.D. Akue, and S.E. Hamilton contributed equally to this paper.

The $CD8^+$ T cell compartment of unprimed mice contains antigen-specific memory-phenotype (VM) cells generated by homeostatic mechanisms and a response to IL-4. However we do not know how well VM cells respond to antigen and how they compete with their naïve counterparts.

Here we show that VM cells display rapid IFN-γ production following exposure to pro-inflammatory cytokines, expand faster during *in vivo* response to priming with both peptide and live bacteria, and also contribute more cells to the memory pool than naïve $CD8^+$ T cells. In competition with naïve T cells, VM cells have an early proliferative advantage that is maintained over the course of the immune response. These differences in responses within the $CD8^+$ T cell pool of unprimed mice confirm the functional heterogeneity within the pool and show that VM cells share certain functional characteristics of conventional memory $CD8^+$ T cells and could be a first line of defense against pathogens.

Introduction

Antigen-specific T cells expand during immune responses to pathogens and generate effector T cells. These effector cells participate in the clearance of the infection and in the process the majority of them die. The remaining effector cells become memory cells capable of faster recall responses and protection against subsequent encounters with the same pathogens (22, 143-146). Memory cells can also be generated in response to self-antigen in T cell lymphopenic environments through the process of homeostatic proliferation (HP) (60, 61, 63-65, 67). This homeostatic mechanism serves to replenish the T cell compartment when T cell numbers fall below a certain threshold during the neonatal period, acute viral infections, senescence, and radio- and chemotherapies (74-77, 162-164). Recent reports from our group and others suggest memory $CD8^+$ T cells generated via a response to IL-4 in the thymus further enhance diversity within the memory pool. These IL-4-driven thymic memory cells termed “bystander memory” cells were reported to exist in BALB/c and several strains of gene knockout mice (87, 89, 90).

We previously reported that unprimed SPF and GF mice contain memory-phenotype $CD8^+$ T cells with specificities to various foreign antigens (“virtual memory” or VM cells) (150 or chapter 2). In chapter 2 we further show that VM cells are generated in the lymphopenic environment of neonatal mice and via a response to IL-4, suggesting that VM cells may comprise both HP and “bystander memory” cells. Although HP memory cells display functions similar to conventional memory $CD8^+$ T cells, we do not know how VM cells compete for antigen against naïve antigen-specific $CD8^+$ T cells.

Here we show that both naïve and VM cells contribute to the immune response against foreign antigens. However, VM cells outcompete naïve $CD8^+$ T cells during the early phase of the immune response and this early advantage is maintained throughout the immune response to antigen. Finally, VM cells also display innate-like functions, including their capacity (similar to NK and memory $CD8^+$ T cells) for production of IFN-γ in response to stimulation by IL-12 and IL-18 (170, 171). Together the data presented here suggest VM cells share certain functional features of conventional memory $CD8^+$ T cells and may be a first line of defense against pathogens, especially during the neonatal period.

Materials and methods

Mice

C57BL/6 (B6) CD45.2/Thy1.2, B6/SJL CD45.1/Thy1.2, B6/PL CD45.2/Thy1.1, and RAG/$\Upsilon c^{-/-}$ B6 mice were purchased from the Jackson laboratory and the National Cancer Institute. Female B6/SJL CD45.1/Thy1.2 were bred with male B6/PL CD45.2/Thy1.1 to generate B6/PL/SJL CD45.1/CD45.2/Thy1.1/Thy1.2 mice. All mice were maintained in specific pathogen-free conditions at the University of Minnesota (Twin Cities). All animal protocols were approved by the Institutional Animal Use and Care Committees at the University of Minnesota.

Adoptive transfer

CD45.1/Thy1.2 $CD44^{hi}$ CD8 T cells and CD45.1/CD45.2/Thy1.1/Thy1.2 $CD44^{lo}$ CD8 T cells were either column-purified (Miltenyi Biotec) or sorted from CD8 enriched cells using FACS Aria (BD Biosciences). $2x10^{\wedge 6}$ of each population ($CD44^{hi}$ and $CD44^{lo}$ CD8 T cells) were then transferred i.v. into either RAG/$\Upsilon c^{-/-}$ B6 or wild type B6 CD45.2/Thy1.2 mice. $CD44^{hi}$ cells were at least 75% pure while $CD44^{lo}$ cells were over 99% pure.

Magnetic bead enrichment and flow cytometry

Spleen was harvested from primed and unprimed recipient mice and injected with 1X collagenase D (Roche, Germany) and minced to generate single cell suspension.

Tetramer binding cells were isolated by magnetic bead enrichment as previously described in detail (150). K^b-associated-B8R/TSYKFESV, -OVA/SIINFEKL, and -HSVgB/SSIEFARL tetramers were generated as previously described (153). Further analysis was done by staining with antibodies to CD19 (clone 6D5 Biolegend), F4/80 (Invitrogen), CD3ε (clone 145-2C11, eBiosciences), CD4 (clone RM 4-5, BD Biosciences), CD8 (Invitrogen), CD44 (clone IM7, eBiosciences), CD45.1, CD45.2 antibodies. Data was acquired using LSR II (BD Biosciences) and analysis was performed using FlowJo software (Tree Star).

Immunizations

6- to10-week-old B6 mice were injected i.v. with $2x10^{\wedge 6}$ PFU of Vaccinia virus (VV-WR). Spleen and ovaries were harvested 4 days later for processing. In other experiments, mice were injected either with a cocktail of B8R /TSYKFESV peptide, agonistic anti-CD40 antibody (1C10 or FGK45, Bioxcell) and Poly-I:C (Amersham Biosciences) (121, 139, 150) or a cocktail of recombinant *Listeria monocytogenes* (LM-OVA-B8R, LM-OVA-HSVgB). LM-OVA-B8R and LM-OVA-HSVgB are kind gifts from Dr Ross M. Kedl at the University of Colorado and Dr SingSing Way at the University of Minnesota/Twin cities respectively.

In vitro cytokine stimulation assay

Spleen cells from B6 mice were cultured for 18 hours in the presence of 10 U/ml recombinant human IL-2 (Biological Resources Branch, NCI-Frederick) alone or with 10

ng/ml IL-12 (a kind gift of Wyeth) and 10 ng/ml IL-18 (Peprotech Inc.). During the last 4 hours of culture, brefeldin A was added at 2 μl/ml. Following the culture period, cells were harvested and B8R/K^b tetramer enrichment was performed as described previously. Post-enrichment, surface staining was performed followed by fixation, permeabilization and intracellular staining for IFN-γ.

Statistics. A two-tailed, unpaired student's t-test was performed on the indicated data samples. P values are displayed within figures. P values of <0.05 are considered significant.

Results

Foreign antigen enhances the proliferation of VM cells in lymphopenic hosts

We set up a dual adoptive transfer system to compare VM and naïve $CD8^+$ T cell proliferation in response to foreign antigens. Because antigen-specific cells are present at low frequencies in unmanipulated animals (115, 116, 150, 165-168), we initially decided to use lymphopenic hosts as recipients in order to eliminate competition from host cells. Polyclonal memory-phenotype and naïve T cells were enriched by purifying $CD44^{hi}$ and $CD44^{lo}$ $CD8^+$ T cells from either CD45.1 and CD45.1/CD45.2 unprimed SPF mice. A 1:1 mix of naïve and memory-phenotype $CD8^+$ T cells was transferred into RAG/$\Upsilon c^{-/-}$ mice. Recipient mice were subsequently primed with a cocktail of B8R peptide; agonistic anti-CD40 antibody and Poly-I:C (121, 139, 150). Because the frequency of memory-phenotype cells is the same within the B8R-K^b positive and the polyclonal pools (about 20%), a 1:1 mix would give us similar numbers of antigen-specific precursors within each donor population. B8R/K^b tetramer staining was performed at day 7 post-priming. Both naïve and VM cells expanded in response to priming at day 7 but with significantly different fold expansions (Fig. 4-1). Although naïve $CD8^+$ T cells responded to immunization, VM cells significantly outnumbered them at day 7 post-priming (Fig. 4-1/ empty squares and circles). This preferential accumulation of VM cells at day 7 post-priming could be due to proliferation in response to T cell lymphopenia. However in the absence of priming, the numbers of recovered cells 7 days post-transfer into the severely lymphopenic RAG/$\Upsilon c^{-/-}$ mice were similar between naïve and VM cells (Fig. 4-1/filled

squares and circles), suggesting that VM cells outcompete naïve $CD8^+$ T cells for antigen in lymphopenic conditions.

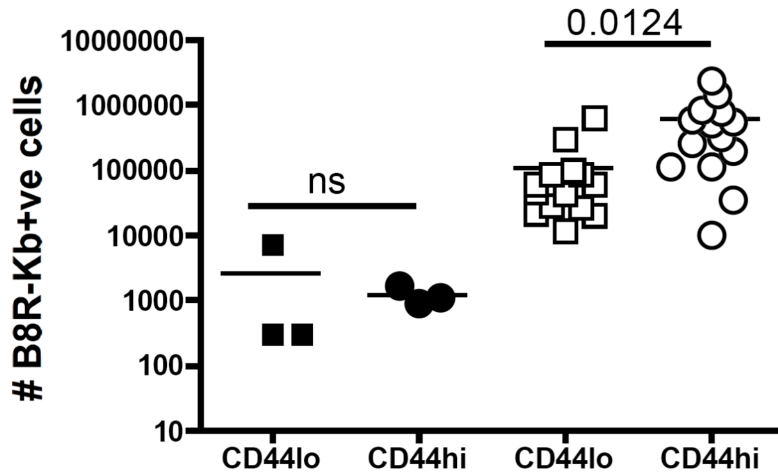
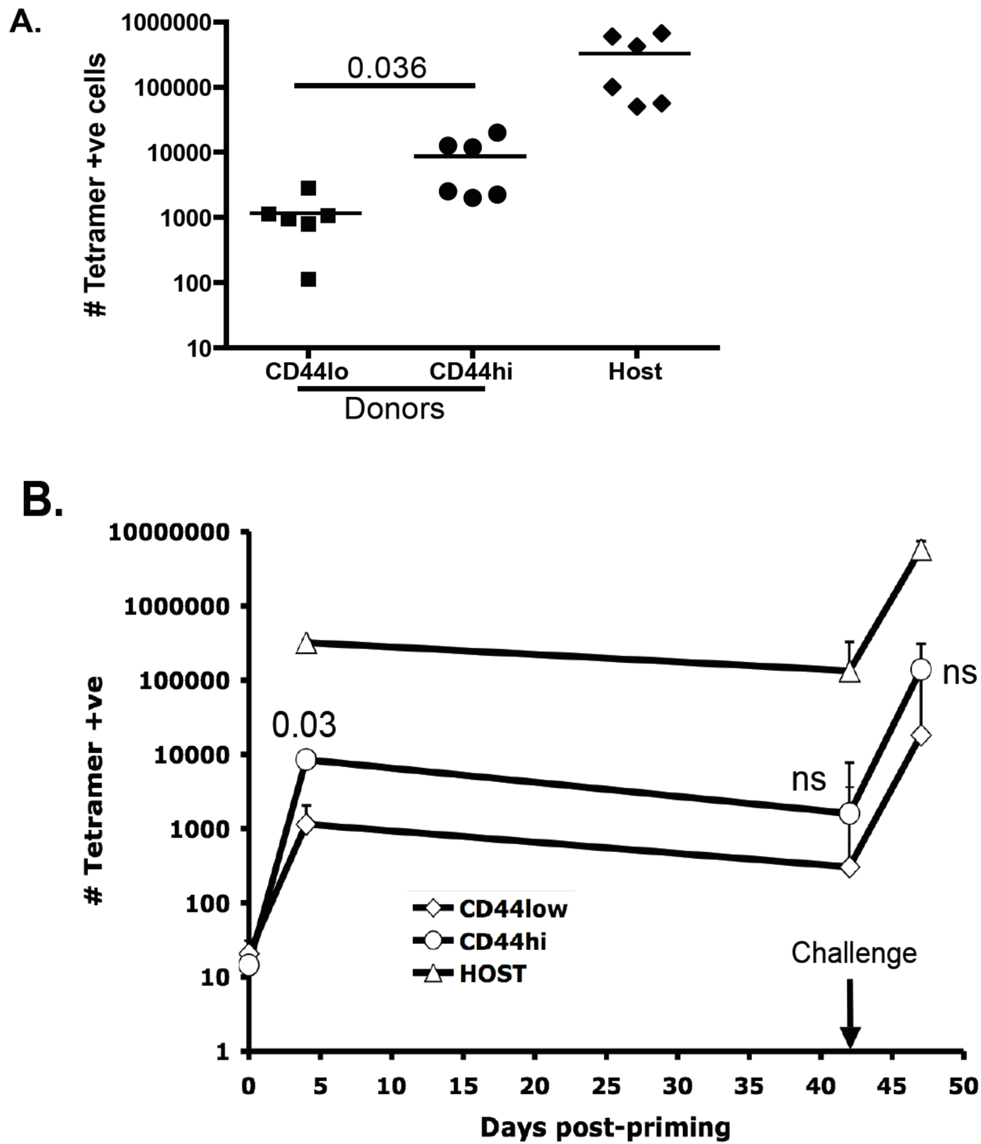


Figure 4-1. Foreign antigen enhances the proliferation of VM cells in lymphopenic hosts. Cells from lymph nodes and spleen were obtained from B6/SJL/CD45.1 and B6PL/SJL/CD45.1/45.2 mice, and $CD8^+$ T cells were enriched by negative selection. $CD44^{hi}$ and $CD44^{lo}$ $CD8^+$ T cells were column-purified or sorted and transferred in 1:1 ratio into RAG/$\Upsilon c^{-/-}$ B6/CD45.2 mice. Some recipient mice were then primed with B8R-K^b peptide, anti CD40 antibody and Poly-I:C and some remained unprimed. Figure shows the number of B8R/K^b positive $CD8^+$ T cells in primed and unprimed mice. Data is cumulative of 3 experiments with at least 4 primed and 1 unprimed mice per experiment.

VM cells outcompete naïve $CD8^+$ T cells early during the immune response and contribute more cells to subsequent memory pools

To eliminate any proliferative advantage lymphopenia would potentially give to memory-phenotype ($CD44^{hi}$) cells, we next used wild type B6 mice as recipients. We also used a cocktail of recombinant *Listeria monocytogenes* (LM) carrying different antigenic epitopes (LM-OVA-B8R, LM-OVA-HSVgB). This approach allows us to track

a larger number of antigen-specific cells at different stages of the immune response to live pathogens. 24 hours after transfer of 1:1 mix of naïve and VM cells, recipient mice were primed with the cocktail of LM.



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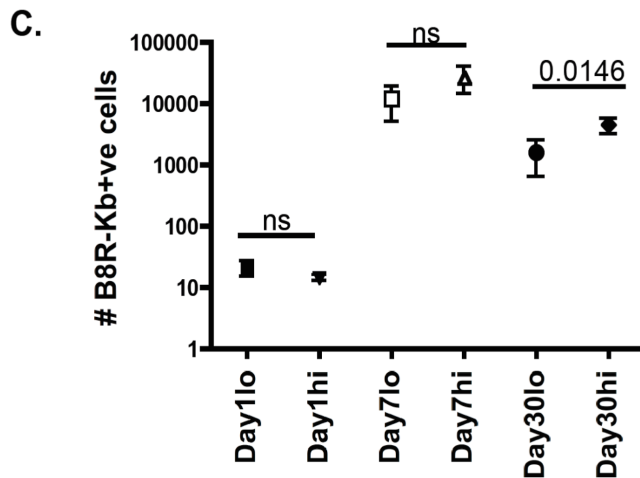


Figure 4-2. VM cells outcompete naïve $CD8^+$ T cells early during the immune response and contribute more cells to subsequent memory pools. Cells from lymph nodes and spleen were obtained from B6/SJL/CD45.1 and B6PL/SJL/CD45.1/45.2 mice, and $CD8^+$ T cells were enriched by negative selection. $CD44^{hi}$ and $CD44^{lo}$ $CD8^+$ T cells were column-purified or sorted and transferred in 1:1 ratio into wild type B6/CD45.2 mice. 1 day after transfer CD8 enrichment was performed and the number of B8R-K^b positive $CD8^+$ T cells within each donor population was estimated using the known precursor frequencies for OVA-K^b-, B8R-K^b-, HSVgB-K^b-positive $CD8^+$ T cells in unprimed mice (1 in $10^{\wedge 5}$, 1 in $10^{\wedge 4}$ to 1 in 2 x $10^{\wedge 4}$, and 1 in 4 x $10^{\wedge 4}$ $CD8^+$ T cells respectively) (150). The remaining recipients mice were then primed with a cocktail of LM-OVA-B8R and LM-OVA-HSVgB (A/B) or B8R peptide and adjuvants (C). (A) Number of OVA-K^b/B8R-K^b/HSVgB-K^b positive $CD8^+$ T cells within each donor population in the spleen of wild type B6 recipients 4 days post-priming. The number of tetramer positive $CD8^+$ T cells for the host is shown as reference for effective priming. (B) Shows the kinetics of the immune response to *Listeria monocytogenes* for each donor population and the host. (C) Depicts the park rate of B8R-K^b positive $CD8^+$ T cells within each donor population (day 1) and the number of B8R-K^b positive $CD8^+$ T cells at day 7 and 30 post-priming with B8R peptide. The data in A/B/C is cumulative of 2 experiments per time point with at least 4 mice per experiment.

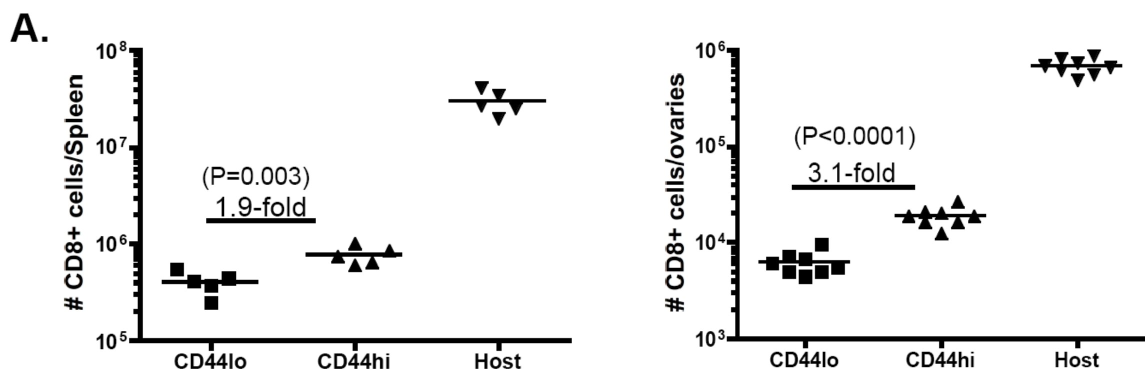
CD8 enrichment followed by staining with B8R-K^b/HSVgB-K^b/OVA-K^b tetramers were performed 4 days post-priming. Tetramer enrichment was done at day 42 post-priming to determine the number of memory cells derived from each population. At day 5 post-secondary challenge with the same LM cocktail (Day 47 post-priming), CD8 enrichment

and staining with B8R-K^b/HSVgB-K^b/OVA-K^b tetramers were done. Similar to our finding in figure 4-1, both subsets responded to infection (Fig. 4-2A/B). However, VM cells accumulated better than naïve donors at day 4 and also generated more primary memory cells than their naïve counterparts (Fig. 4-2A/B). We also saw that this advantage at day 4 and 30 was maintained during the recall response (Fig. 4-2B). The better accumulation of VM cells during the immune response to LM was not due to a better overall transfer efficiency relative to naïve cells since there were similar numbers of both donor cells at day 1 post-transfer (day 0 of priming) (Fig. 4-2B). We did not determine the size of the effector pool at day 7 post-priming during LM infection, but in separate experiments using B8R peptide priming, the day 4 advantage for VM cells carried over to the peak of the response (day 7), although not statistically significant (Fig. 4-2C). This day 7 result is in contradiction with what we saw in figure 4-1 for lymphopenic hosts, suggesting that indeed lymphopenia enhanced the antigenic response of VM cells compared to naïve donors. Overall our data suggest that in direct competition, VM cells expand faster than their naïve counterparts, and that this early advantage is maintained during the course of the immune response to antigens.

VM cells migrate faster and initiate immune response to infection in peripheral tissues

It is known that differential expression of adhesion molecules on effector and memory cells allows them to access peripheral sites of infections (110, 112). We showed in our previous report that VM cells share many of the phenotypic markers of memory

cells (150 or chapter 2) including high expression of adhesion markers such CD44 and LFA-1. Therefore we hypothesized VM cells will traffic to peripheral non-lymphoid tissues and initiate immune responses faster than naïve $CD8^+$ T cells. To test our hypothesis, we transferred purified and congenically marked polyclonal memory ($CD44^{hi}$) and naïve ($CD44^{lo}$) $CD8^+$ T cells into B6 mice and challenged with vaccinia virus one day after transfer. Phenotype and numbers of transferred cells were determined 4 days post-priming in the spleen and ovaries, the primary lymphoid and non-lymphoid reservoirs for vaccinia virus respectively. Analysis of the polyclonal donor cells revealed 2- to 3-fold higher accumulation of VM cells in the spleen and ovaries respectively (Fig. 4-3A). Analysis of B8R-K^b-specific cells shows even a more significant accumulation (4- and 6-fold) of VM cells in both the spleen and the ovaries (Fig. 4-3B). At this time point naïve donors contain activated cells ($CD44^{hi}$) in the ovaries, which makes it difficult to determine whether the cells have trafficked to the ovaries post-activation or whether they expanded in situ. Regardless of where the cells in the ovaries first encountered antigen, VM cells accumulated faster than naïve donors in these tissues suggesting that VM cells can traffic more efficiently to and/or initiate immune response faster in non-lymphoid sites of infections than naïve $CD8^+$ T cells.



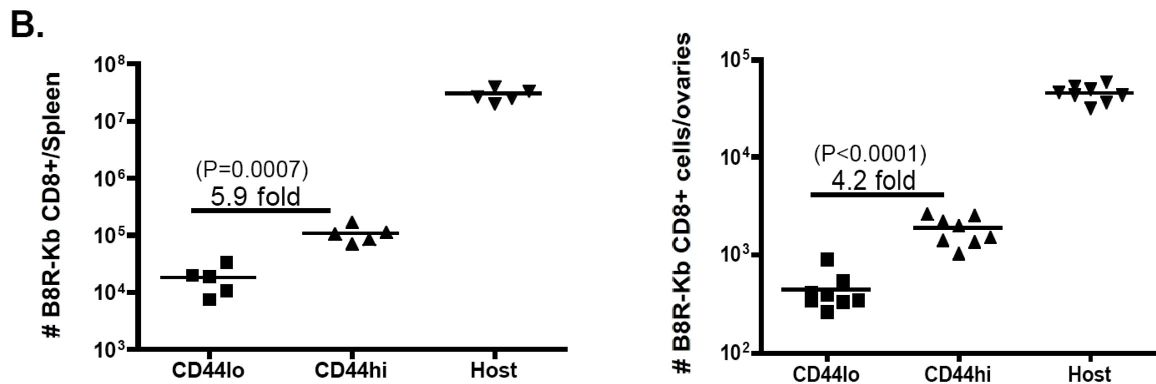
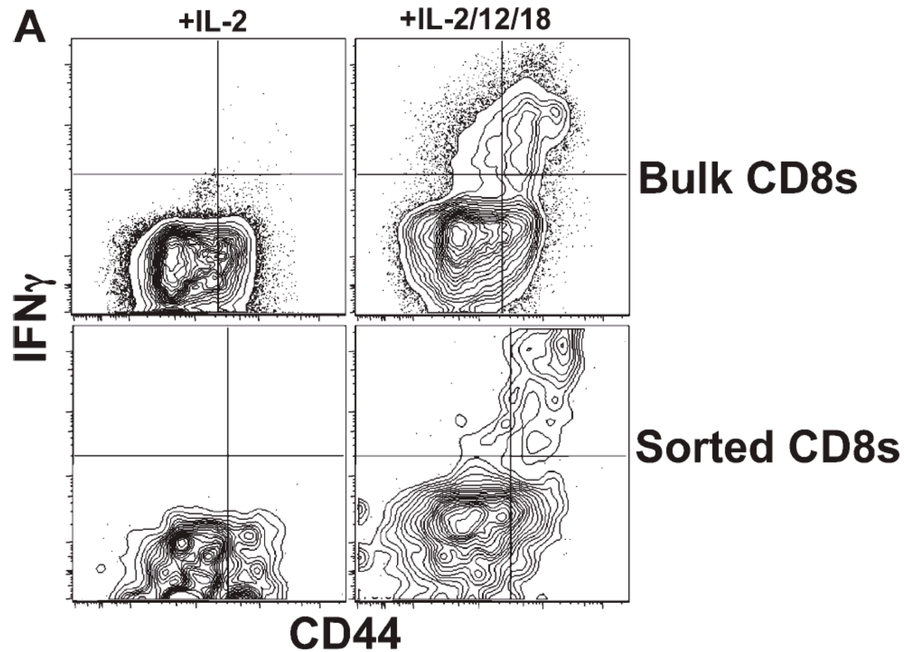


Figure 4-3. VM cells migrate faster and initiate immune response to infection in peripheral tissues. Congenically marked $CD44^{hi}$ and $CD44^{lo}$ $CD8^{+}$ T cells were transferred in 1:1 ratio into wild type B6 mice that were then primed with VV-WR. 4 days after priming, spleen and ovaries were harvested and stained for the congenic markers and with $B8R/K^{b}$ tetramer. Numbers of donor and host $CD8^{+}$ T cells in the spleen and ovaries (A) and numbers of $B8R/K^{b}$ positive cells for donors and host in the spleen and ovaries (B) are shown. Two experiments were done with 4 mice per experiment.

Tetramer-binding $CD44^{hi}$ $CD8^{+}$ T cells from unprimed animals rapidly produce IFN-γ in response to innate immune cues

Previous studies have indicated that antigen-driven memory $CD8^{+}$ T cells have the capacity to display certain innate immune functions. Specifically, in the absence of TCR engagement, memory $CD8^{+}$ T cells efficiently produce IFN-γ in response to TLR and cytokine stimulation (169-171). Receptors for IL-12 and IL-18 are upregulated on memory-phenotype $CD8^{+}$ T cells and binding of these cytokines leads to production of IFN-γ (169-171). This rapid production of IFN-γ has been shown to play an important role in the early resistance to bacterial infection (169). To investigate whether antigen-specific $CD44^{hi}$ $CD8^{+}$ T cells in unprimed mice could also perform this function, we

cultured splenocytes from B6 mice with IL-12, IL-18, and IL-2, conditions previously shown to stimulate IFN-γ secretion from antigen-driven memory CD8^{+} T cells (169).



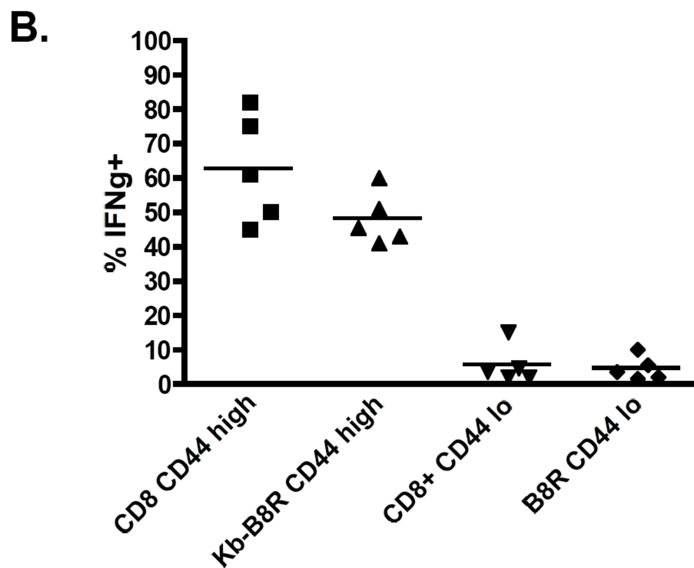


Figure 4-4. Antigen-specific CD44hi CD8 T cells make IFN-γ in response to innate cytokine stimulation. Spleen cells from B6 mice were cultured for 18 hours in the

presence of either IL-2 or a mixture of IL-2, IL-12, and IL-18. Brefeldin A was added for the last 4 hours of culture. Cells were then subjected to tetramer enrichment using B8R-K^b tetramers. (A) Representative staining of either bulk $CD8^+$ T cells (upper panels) or B8R-K^b tetramer binding $CD8^+$ T cells (lower panels) for IFN-γ. (B) The percentage of IFN-γ^+ cells in the $CD44^{hi}$ and $CD44^{lo}$ compartments of bulk $CD8^+$ T cells or B8R-K^b specific $CD8^+$ T cells. Each symbol represents an independent sample (n = 5), and the data are compiled from 3 experiments.

After the 18 hours stimulation period, B8R-K^b tetramer enrichment was performed followed by immediate intracellular staining for IFN-γ. We found that when cultured with IL-2 alone, $CD8^+$ T cells produced little IFN-γ (Fig. 4-4A), but with the addition of IL-12 and IL-18 we observed robust IFN-γ production by $CD44^{hi}$ (but not $CD44^{lo}$) B8R/K^b tetramer binding $CD8^+$ T cells (Fig. 4-4A and B). The same IFN-γ production pattern was observed when bulk $CD8^+$ T cells were examined, confirming that this response is non-antigen specific. These findings demonstrate that antigen-specific $CD44^{hi}$ $CD8^+$ T cells from unimmunized animals are capable of producing the effector cytokine IFN-γ in response to innate cytokine stimulation, and such cells may therefore be able to participate in both innate and adaptive phases of the “primary” immune response.

Discussion

It is known that the $CD8^+$ T cell compartment of unprimed mice not only contains naïve but memory-phenotype cells. Although these memory-phenotype $CD8^+$ T cells did not arise in response to foreign antigens, they have specificities for multiple foreign antigens and share certain phenotypic and functional features of conventional memory cells, memory cells generated by foreign antigen exposure (150 or chapter 2). Here we aimed to determine how these memory-phenotype cells respond to various stimuli in competition with naïve $CD8^+$ T cells.

We report here that VM cells respond to their cognate antigen. We found that antigen-specific memory-phenotype $CD8^+$ T cells proliferate vigorously in response to foreign antigen in lymphopenic hosts (Fig. 4-1). Although we did not compare the homeostatic proliferation of VM and naïve cells in this report, our data suggest that lymphopenia greatly enhanced the antigenic response of VM cells. Because we could not completely separate lymphopenia-induced proliferation from antigen-driven expansion in this system, we extended these studies to non-lymphopenic recipients and tracked the response of donor cells to antigen. We saw that VM cells outcompete naïve $CD8^+$ T cells early (day 4) during the immune response to infection as expected for memory cells (172) (Fig. 4-2A/B), and this early advantage was maintained throughout the primary and the recall responses (Fig. 4-2B). These data suggest VM cells may respond to antigen similarly to conventional memory cells, but how VM cells really compare to conventional memory cells is not known and will be assessed in the future.

Our previous observation that VM cells express high levels of certain adhesion markers as conventional memory cells suggested to us that both memory subsets would display similar trafficking patterns. This prompted us to determine whether VM cells would traffic to non-lymphoid peripheral sites of infections and initiate immune response faster than naïve cells. Indeed we found that polyclonal and antigen-specific memory-phenotype cells expanded faster in the ovaries of vaccinia virus-infected mice (Fig. 4-3A/B). Regardless of whether VM cells encountered antigen before accessing the ovaries or not, they are present in this peripheral non-lymphoid vaccinia reservoir in greater numbers than naïve $CD8^+$ T cells, suggesting that they can readily access and initiate immune responses in non-lymphoid sites of infections more efficiently than naive cells (110, 112). Preliminary data from our group also shows that VM cells traffic to inflamed lymph nodes faster than naïve $CD8^+$ T cells in a CXCR-3-dependent (CD44-dependent?) manner as was previously shown for effector and memory cells (173). Furthermore we also show that VM cells were competent to produce IFN-γ following stimulation with the pro-inflammatory cytokines IL-12 and IL-18 (Fig. 4-4).

Our report further reinforces our previous findings that VM cells share certain phenotypic and functional characteristics of conventional memory cells although VM cells are not generated by foreign antigen exposure. Now a question remains whether the enhanced proliferation and accumulation shown by VM cells in response to infections will translate into improved protection, especially during the neonatal period when these cells are generated.

Comment

The work discussed in this chapter is being continued by a graduate student in our laboratory and therefore incomplete. Because of missing pieces, interpretation of the data presented here is difficult and the link between the functional characteristics of VM cells shown here and any protection could not be made. One of the obstacles to answering the pertinent question of whether VM cells are protective is that cell numbers were a limiting factor since we were focusing on the endogenous antigen-specific memory-phenotype $CD8^+$ T cells that are present at low frequencies in unprimed animals. We are currently considering using transgenic mice in order to solve this problem.

Chapter 5

Discussion

Conclusions

The work presented here identifies a new population of memory $CD8^+$ T cells present in unprimed SPF and GF mice. Memory cells are known to derive from antigen-specific precursors from the naïve pool either in response to foreign antigens, homeostatic cues, or a recently identified IL-4 bystander effect in the thymus (22, 143-146, 60-68, 87-91), but the specificity, origin, and functions of memory-phenotype $CD8^+$ T cells present in unprimed SPF and GF animals were not known. Although there were hints in the literature regarding the possible origin of these memory-phenotype cells (74-77), to our knowledge work presented here is the first to provide direct evidence that these memory-phenotype $CD8^+$ T cells: 1) have specificity for various foreign antigens, 2) are generated during the neonatal period in response to physiologic lymphopenia with IL-4 contribution, and 3) bear certain functional properties of conventional and HP memory $CD8^+$ T cells.

This work began with the objective to determine the precursor frequency of antigen-specific cells within the $CD8^+$ T cell compartment of unprimed SPF mice. As described in chapter 2, we used a combination of peptide-MHC class I tetramers and magnetic bead enrichment protocol modified from Moon et al. (115), to enrich for antigen-specific $CD8^+$ T cells in unprimed mice. To our surprise, we found that not only we isolated $CD8^+$ T cells specific for multiple foreign antigens, but there were memory-phenotype cells within each specificity (150), contrary to published report from Obar et al. (116). These memory-phenotype antigen-specific $CD8^+$ T cells termed "virtual memory" (VM) cells were also present in GF mice, thus making it unlikely that they are

generated via exposure to foreign antigens and commensal microbes. Instead we found that the majority of VM cells express low levels of α-4 integrin, a phenotype associated with HP memory cells (150). Because neonatal mice were reported to be lymphopenic and to allow homeostatic proliferation (74-77), we proposed then that VM cells were generated in response to neonatal lymphopenia but not in response to foreign antigens (150 or chapter 2).

Recently, reports from our group and others showed that mature single positive CD8 T cells could become memory cells in the thymus of unprimed mice when exposed to IL-4 (these cells were termed "bystander memory" cells) (87, 88, 90). These "bystander memory" cells were first discovered in various gene knockout and BALB/c mice but it was not clear whether IL-4 exposure can also lead to the generation of memory cells in B6 mice. As shown in chapter 3, we analyzed the VM pool in the secondary lymphoid organs of IL-4R$^{-/-}$ B6 mice and saw a significant reduction in VM frequencies compared to wild type B6, despite similar numbers of B8R/K^{b} and HSVgB/K^{b} tetramer positive T cells. This reduction in antigen-specific memory-phenotype frequencies was also seen for the polyclonal CD8^{+} T cell pool in the thymus and secondary lymphoid tissues. These findings were a complete surprise because reports from our group and others suggested that the bystander effect of IL-4 was not prominent in B6 mice (88, 158). On the contrary our data suggested that IL-4 has a role in the generation of VM cells in B6 mice.

To determine whether VM cells are also generated in the thymus as bystander memory cells, we investigated whether they appear first in the thymus or the periphery in

B6 mice. We found that VM cells appear first in the periphery during the neonatal period before appearing at low frequencies (and low numbers) in the thymus; and that the peripheral VM pool is maintained long term, even during the immune response to unrelated antigens. All together our data in chapter 3 suggest VM cells to be a stable peripheral memory pool generated in response to neonatal lymphopenia and IL-4.

The mechanism we have identified for the generation of VM cells in chapter 3 would suggest that naïve single positive CD8 T cells are exposed to low levels of IL-4 in the thymus, remain naïve, and only become memory-phenotype cells after undergoing homeostatic proliferation in the physiologically T-cell-lymphopenic secondary lymphoid organs of neonatal mice. The role for IL-4 in the generation of VM cells in B6 mice is not well understood here, but we think that sensing IL-4 increases the ability of the cells to undergo HP. We also think that IL-4 is present and sensed in the secondary lymphoid organs to even push the cells further during HP (Fig. 3-4B and C show a sharp increase in the percentage and number of VM cells from 2 to 3 weeks), but more work needs to be done to determine if this is true or not and to determine the cells responsible for producing the IL-4 in the periphery and thymus during steady state conditions.

The observation that VM cells are generated in response to neonatal lymphopenia and IL-4 leaves us with a question as to whether VM cells have functional properties comparable to HP memory $CD8^+$ T cells. This question is of particular importance during the neonatal period when VM cells are generated. To approach this question, we sought to determine whether VM cells could even respond to their cognate antigens, because specificity for tetramers may not translate into antigen responsiveness. As described in

chapters 2 and 4, adoptively transferred memory-phenotype $CD8^+$ T cells responded to priming with peptide and microbes attesting to their specificity for foreign antigens. In chapter 4 we also showed that VM cells proliferate faster and outcompete naïve $CD8^+$ T cells, especially early during the response to both peptide and live pathogens. Furthermore we showed that VM cells could produce IFN-γ when cultured with IL-2/IL-12/IL-18, although they did not produce it in response to peptide stimulation (or at least not as fast as conventional memory $CD8^+$ T cells in chapter 2). Together these data suggest VM cells have certain properties inherent to conventional memory $CD8^+$ T cells, but at this time we do not know whether VM cells will contribute to protection against pathogens. If it turns out that VM cells show ability to enhance protection against infections, then more studies are required to determine how to take advantage of their protective properties especially during the neonatal period when newborns are very vulnerable to infections. We previously showed that protection again pathogens is enhanced following expansion of the VM pool with IL2/anti-IL2 cytokine complex treatments (48), but whether this applies to neonatal mice needs to be determined.

Memory cells including HP memory cells have also been associated with both autoimmunity and graft rejection (174-178) and since VM cells have properties of memory $CD8^+$ T cells, it will be interesting to know whether there are self- and/or allo-specific VM cells that could be responsible for these pathologies. It will also be important to know if VM cells reside in peripheral non-lymphoid tissues as tissue-resident memory cells (29, 30, 179) where they could either be a first line of defense against pathogens or mediate tissue rejection and autoimmune attacks.

In conclusion work described in this thesis adds another memory subset to the pool of memory $CD8^+$ T cells and provides data supporting the homeostatic origin of these cells. In addition we identified a role for IL-4 in the generation of VM cells, although this IL-4 effect does not manifest in the thymus but instead in the periphery of B6 mice. Also, this thesis provides data suggesting VM cells have functional similarities with HP and conventional memory cells. Memory-phenotype cells have been identified in human fetal spleen (161), but more careful studies need to be conducted in order to determine whether they represent a population of conventional, or bystander, or virtual memory cells. Also the specificities, functions, and tissue distribution of these memory-phenotype cells need to be determined before even considering manipulating the VM pool for therapeutic purposes.

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