UNIVERSITY OF CALIFORNIA, SAN DIEGO

Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

Kitty Pui Hang Cheung

Committee in charge:

Professor Ananda Goldrath, Chair
Professor Michael David, Co-Chair
Professor Stephen Hedrick
Professor Stephen Schoenberger
Professor Carl Ware
Professor Maurizio Zanetti

2009

The Dissertation of Kitty Pui Hang Cheung is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2009

DEDICATION

To my parents,

Rita Yuet Ming Ko and Wing Fu Cheung

You have always loved and supported me unconditionally under all circumstances. Thank you for trusting me enough to let me pursue my Ph.D.

EPIGRAPH

You gain strength, courage, and confidence by each experience in which you really stop to look fear in the face. You are able to say to yourself, “I have lived through this horror. I can take the next thing that comes along.” You must do the thing you think you cannot do.

Eleanor Roosevelt

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CURRICULUM VITA

Education

- **Ph.D, Biology (Molecular Biology Section)**
  University of California, San Diego (UCSD), 2009

- **B.A., Molecular and Cell Biology (Genetics)**
  University of California, Berkeley (UCB), 2000

Scholarships and Awards

- La Jolla Immunology Conference- T cell block speaker/Best Talk 2009
- The American Association of Immunologists (AAI) Trainee abstract award 2009
- AAI-T and B cell homeostasis block symposium speaker 2009
- AAI-CD4 and CD8 memory block symposium speaker 2008
- Keystone Symposia-Immunologic Memory- Scholarship recipient 2007
- Cellular and molecular genetics training grant- (UCSD) 2004-2007
- Chancellor's Scholarship recipient- (UCB) 1996-2000
- National Merit Scholarship recipient- (UCB) 1996-2000

Publications

Kitty P. Cheung, Edward Yang, and Ananda W. Goldrath. Memory-like CD8+ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells. Journal of Immunology, 2009 Sep 1;183(5):3364-72

Michael A Cannarile, Nicholas A Lind, Richard Rivera, Alison D Sheridan, Kristin A Camfield, Bei Bei Wu, **Kitty P Cheung**, Zhaoqing Ding and Ananda W Goldrath. Transcriptional regulator Id2 mediates $CD8^+$ T cell immunity. *Nature Immunology*. 2006 Dec;7 (12):1317-25

Ph.D. Dissertation: "Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells"
Advisor: Ananda W. Goldrath

Work Experience

Research Associate I Exelixis Inc. (Pharmacology)
10/2003-9/2004

Project: Evaluating the effect of drug compounds on various oncology models (in vitro/in vivo).

- Proficient with rodent handling including subcutaneous injections, transponder implantation, tumor processing, and thoracic puncture.
- Familiar in drug administration routes including oral dosing, intraperitoneal, and intravenous injections.
- Mammalian tissue culture of tumor cells for implantation and immunofluorescent analysis.
- Cell Cycle analysis
- Immunolfluorescent staining

Staff Research Associate I/II U.C. San Francisco
9/2000-5/2003

Project: Identifying the role of homeobox genes during pathologically induced angiogenesis, specifically the role of HoxA5 in the formation of hemangiomas.

- Cloning, transfection, and maintenance of stable cell lines.
- Basic molecular biology techniques including Western blotting, luciferase and beta galactocidase reporter assays.
- Chick Chorioallantoic Membrane (CAM) Assay.

URAP-Undergraduate Research Apprenticeship Program UCB
1/1999-6/2000

Project: Pollen mutants in *Arabidopsis thaliana*

Teaching Experience

Head Teaching Assistant	Immunology	UCSD	Spring Qtr. 2008
Head Teaching Assistant	Immunology	UCSD	Spring Qtr. 2007
Teaching Assistant	Intro to Biochem. Techniques	UCSD	Winter Qtr. 2006

Professional Activities

- AAI annual conference- poster presentation 2008 and 2009
- Keystone Symposia Immunologic Memory- poster presentation 2007
- La Jolla Immunology Conference- poster presentation 2006-2009
- Asilomar Midwinter Conference 2006
- AAI Advanced Immunology Course 2006

Professional Memberships

- AAI Trainee 2009

ABSTRACT OF THE DISSERTATION

Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells

by

Kitty Pui Hang Cheung

Doctor of Philosophy in Biology

University of California, San Diego, 2009

Professor Ananda Goldath, Chair
Professor Michael David, Co-Chair

Naïve T cells proliferate in response to lymphopenia and acquire the phenotypic and functional qualities of memory T cells, providing enhanced protection against infection. How well 'memory-like' T cells generated during lymphopenia-induced homeostatic proliferation (HP-memory) differentiate into secondary memory cells and compete with antigen-experienced 'true-memory' cells is previously unknown. We found that $CD8^+$ HP-memory T cells generated

robust responses upon infection and produced a secondary memory population comparable to true-memory cells in the absence of competition. However, when true-memory and HP-memory T cells competed during infection, HP-memory cells contributed less to the effector population, contracted earlier and formed fewer secondary memory cells than the true-memory cells, despite earlier expansion. The HP-memory T cells can in turn outcompete naïve T cells during the immune response, revealing the existence of a memory T cell response hierarchy. Furthermore, HP- and true-memory cells demonstrated distinct localization within the spleen during infection, indicating differential access to signals necessary for secondary memory formation. We attempted to rescue the HP-memory cells during the course of competition by administering saturating amounts of antigen and cytokine such as IL-7 and IL-15, but were not successful, indicating that they were not competing for those factors. Secondary memory derived from the HP-memory cells continued to defer to secondary memory derived from the true-memory cells in the course of the tertiary infection, indicating that the nature of the HP programming could not be overcome with antigen exposure. Thus, HP-memory T cells provide protection without compromising the true-memory population. This is of clinical relevance as HP-memory T cells can arise due to a multitude of medical causes. Their natural presence as part of the immune compartment will not contribute to the erosion of the established true-memory populations derived from vaccinations and prior infections. HP-memory cells are not an exact substitute for memory, but serve as

a viable source of protection from pathogenic invasion. Differences in HP-and true-memory T cells may reveal the basis for competition for limited resources within the memory T cell compartment.

INTRODUCTION

During acute infection, following encounter with their cognate antigen, $CD8^+$ T cells are induced to undergo extensive expansion and differentiate into cytolytic T lymphocytes (CTLs) able to kill infected cells (Fig. 1A). Following the clearance of infection, a portion of these antigen-specific lymphocytes (5%-10%) seeds the memory compartment, often providing the host with enhanced protection upon a subsequent encounter with that same pathogen. Antigen-derived or true-memory $CD8^+$ T cells mediate control of secondary infections due to their higher precursor frequency, rapid reacquisition of effector function, and access to peripheral sites of infection (1-3). $CD8^+$ T cell memory can be remarkably stable, providing life-time protection in some cases (4). The mechanisms that regulate the differentiation of the various T cell subsets within the memory T cell compartment and how heterogeneous populations of memory T cells change over time, and may compete with one another are not well elucidated.

Antigen-independent proliferation of naïve and memory T cells can occur when lymphocyte numbers drop below a certain threshold (5-11). This lymphopenia-induced expansion of lymphocytes is known as homeostatic proliferation (HP), which is a mechanism that contributes to the restoration of overall T cell numbers (Fig. 1B). This antigen-independent expansion by the majority of remaining peripheral T cell clones contribute to the regeneration of the

lymphocyte compartment, resulting in the preservation of the T cell specificities contained in the remaining naive and memory repertoire. The cells that result from HP (HP-memory cells) are unique in that they assume characteristics typical of memory cells derived from antigen exposure even though they have not encountered their cognate antigen (6, 12, 5, 13, 8, 3). Upon secondary infection, these HP-memory cells behave similarly to their true-memory cell counterparts by rapidly acquiring effector function upon re-stimulation, and also being able to facilitate clearance of pathogen (6, 12, 5).

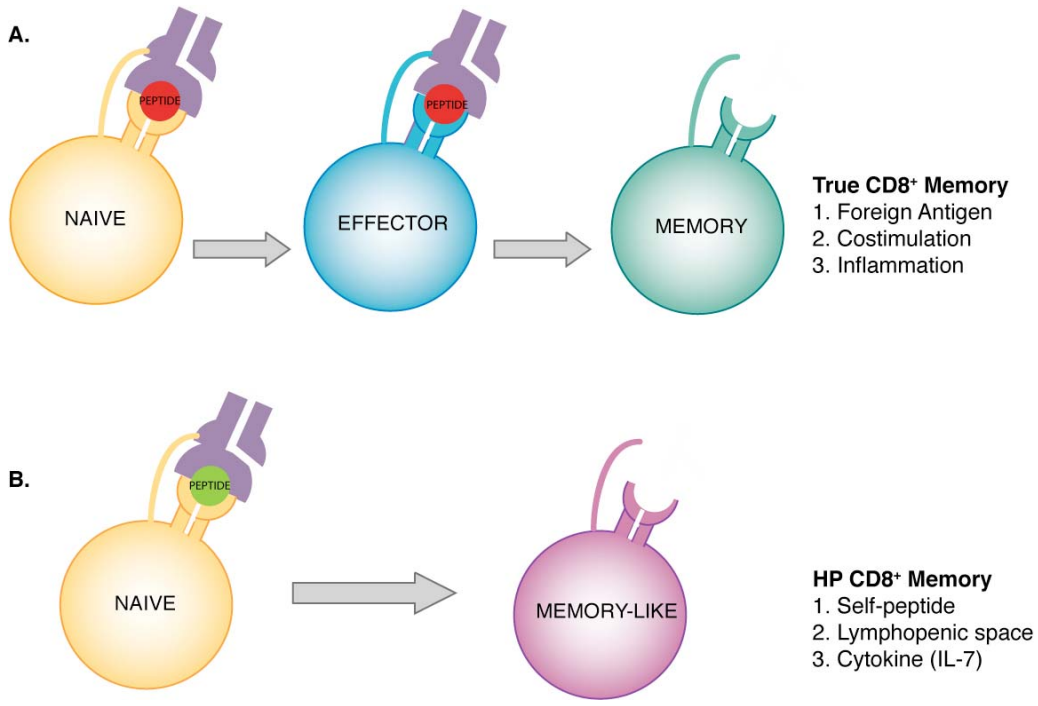

Figure 1. Comparison of CD8$^+$ true- and HP-memory formation.

(A) The three signals required for true-memory generation and the differentiation of the naïve CD8$^+$ T cell into an effector before contraction and leaving behind a population of true-memory cells. (B) HP-memory generation requires different signals and the naïve CD8$^+$ T cell never passes through an effector stage.

Memory cells play a central role in mediating protection upon re-infection. However, less understood are the benefits or dangers of the HP-induced memory response to infection and their overall impact on the health of an individual. In the process of anti-tumor therapy, patients often receive immunosuppressive drugs or chemotherapy that induce severe lymphopenia. Adoptive transfer of T cells used to introduce tumor specific CD8$^+$ T cells to their immune system and these cells often undergo HP (14). Furthermore, It has long been observed that the CD8$^+$ T cells undergoing HP show improved anti-tumor responses (15-18).

Besides simply an increase in numbers, HP also promotes enhanced activation and effector function of the $CD8^+$ T cells, even in presence of tumor cells which can also facilitate anergy of the $CD8^+$ T cells in a lymphoreplete individual (19, 20). The role of regulatory T cells (Treg) is to maintain self-tolerance but they are detrimental in the course of an anti-tumor response. For successful HP-memory cell therapy, Treg often have to be removed (21). $CD8^+$ T cells are only able to induce HP-mediated rejection of B16 tumor cells when combined with depletion of regulatory T cells (22). Thus, HP can enhance anti-tumor responses and immunotherapeutic approaches

In contrast, in a post-transplant setting, HP can contribute to the development of graft versus host disease (GVHD) due to the proliferation of donor anti-host T cells tissues (23, 24). This occurs because the post-transplant environment is more favorable to the generation of T cells by the graft rather than the host. It has been shown that the gain of functional activity associated with HP has proven to be a barrier for the acceptance of transplanted tissues (23, 24). Administration of Tregs in conjunction with the transplant can prevent GVHD (25).

Clinical autoimmune diseases such as Type 1 diabetes, rheumatoid arthritis, Sjögren's syndrome, systemic lupus erythematosus (SLE), Crohn's disease, and celiac disease are associated with lymphopenia (26-28). Often, lymphopenic hosts contain elevated levels of cytokines due to the dearth of lymphocytes (29). Naturally occurring lymphopenia in NOD mice is hypothesized to be an underlying cause of why these mice develop type 1 diabetes (30). IL-21

promotes inflammation during the immune response, but excessive levels of IL-21 leads to HP of auto-reactive $CD8^+$ T cells in the NOD mice. Increased levels of IL-21 are also linked to rheumatoid arthritis, inflammatory bowel disease, and systemic sclerosis (31). For patients with type 1 diabetes, islet transplantation has been used as a therapy to treat their condition. As this treatment involves use of immunosuppressive drugs, patients become lymphopenic due to the decreased numbers of B and T cells. As a result of the HP, autoreactive T cells are generated that result in a decrease in pancreatic islet numbers (32). However, it would appear that multiple factors in addition to HP alone would be necessary for an autoimmune reaction. The HP of potentially autoreactive $CD8^+$ T cells against pancreatic antigen is not enough to induce self-reactivity but require antigen-specific $CD4^+$ T cell help to elicit a response (28, 33).

Lymphopenia-induced HP remains a enigmatic process. This is especially true since the HP-memory cells appear to be double-edged sword, serving to enhance anti-tumor efficacy but at the same time promoting autoimmunity. What is exciting is that memory cells can be formed in a distinct manner from the conventional method of formation through antigen exposure. It remains to be determined whether the circumstances of HP-memory formation contribute to its effectiveness or lack thereof during an actual infection. Even if the HP-memory cells are effective, can they serve as a competent substitute for true-memory cells? A possibility remains that the HP-memory cells may even be detrimental to our health. If the HP-memory cells are not helpful, can their presence exert a

negative impact on the response of the true-memory cells? While the immune compartment expends resources to maintain a healthy and diverse population of memory cells, the mechanisms it uses to regulate the different populations are unknown. If this regulation is mediated through competition between the cells, what will be the result of the competition if the two memory cell types are truly identical?

Not all memory is created equal

While we are trying to ascertain whether the competitive fitness of the HP-memory cells is on par with that of the true-memory cells, it is important to note that even within the true-memory population itself, a heterogeneity of memory subsets exist. The function and long-term survival of these memory cells are quite variable. Different subsets of effector and memory cells can be generated from one infection, and indeed from even the same cell responding to (34-38, 2) (39). As a result, on an individual level, memory cells have a diverse repertoire of responses to re-infection. There is much debate over the characteristics that define a “true” memory cell.

Sallusto et al. was the first to report the existence of the effector memory (T_{EM}) subset characterized by $CD62L^{LO}CCR7^{LO}$ cells, and the central memory (T_{CM}) subset characterized by $CD62L^{HI}CCR7^{HI}$ cells following infection (35). The T_{CM} subset was initially characterized as having a greater capacity for replication and a tendency to home to the secondary lymphoid tissues. They were destined to seed the memory compartment, but assumed to have little effector capacity

(35-37). The T_{EM} subset on the other hand was reported to have greater effector function, localized to the peripheral tissues, but was not thought to be long lived. Many questions soon arose regarding this strategy of classification, namely whether effector capability was limited to T_{EM} cells. Among many experiments, Bannard et al. used a transgenic mouse in which the fate of granzyme-B expressing EYFP effector cells could be tracked to show that effector capability was not limited to only T_{EM} cells but was found in T_{CM} cells as well (40). Aside from debate over the functional differences between T_{EM} and T_{CM}, controversy remains surrounding their origin. There are many hypotheses regarding the derivation of these memory cells including the linear differentiation model, where the T_{CM} cells are the source of T_{EM} cells but also serve to maintain the T_{CM} population by self-renewal, or vice versa where the T_{EM} cells slowly convert to T_{CM} cells over time (37, 38, 2). This contrasts with the divergent differentiation model which suggests that the T_{EM} and T_{CM} subsets are of distinct lineages with no inter-conversion with the T_{CM} population eventually outgrowing the T_{EM} to populate the memory compartment. The signals that conclusively skew the memory population towards being T_{EM} or T_{CM} have still not yet been fully determined (41).

Another potential classification of effector and memory $CD8^+$ T cells has also been described by Joshi et al. (42). Increased expression levels of killer cell lectin-like receptor G1 (KLRG1) was found to correspond to what was termed as short-lived effector cells (SLECs). These cells were characterized as $KLRG1^{HI}CD127^{LO}$ (IL-7Rα) (43, 42, 34). This was in contrast to $KLRG1^{LO}$ $CD127^{HI}$

cells which were characterized as memory precursor effector cells (MPECs) corresponding to the cell population most likely to survive and become long-lasting memory. Similar to T_{CM} and T_{EM}, it has not been fully elucidated how the SLEC vs. MPEC cell fate is determined, how each may be incorporated into subsequent immune response or how each population may impact the other memory subsets.

Size of the memory compartment: Does competition exist?

The size and number of cells that the memory compartment can accommodate remains a conundrum. If the memory compartment has limitless capacity, then it can continue to provide for the maintenance of all previously formed memory cells following multiple infections. However, if the immune compartment is of limited size, then attrition of previously formed memory cells would need to occur to make space for the newly formed memory cells.

The predominant paradigm has been that the memory compartment is of limited space and that formation of new memory would cause an erosion of previously formed memory. Selin et al. has shown in multiple contexts that successive infections result in the attrition of previously formed memory cells due to the inflammation caused by each new infection (44, 45). The increase in type 1 IFN production during an infection leads to the death of pre-existing memory cells, presumably to make room for the new memory cells being created (44). This would prevent a domination of the immune response by the existing memory cell repertoire and leave room for a more diverse memory compartment. Contrary

to this viewpoint of the memory compartment consisting of a finite space, Vezys et al. showed that the memory compartment is not fixed in size, and that it is flexible enough to accommodate the new memory cells formed as a result of infection without necessarily resulting in a precipitous decline in the pre-existing memory (46).

Whether this regulation of cell numbers within the memory compartment exists is important because it determines how many memory cells can be formed during an infection, and how many of them can be retained to provide future protection. In addition to the number of memory cells generated, the rate at which the effectors acquire “memory” is also very relevant. Elevated numbers of memory cells are generated as a result multiple booster vaccinations, but successive boosters cannot be given until the memory cells generated previously have stabilized. Thus, vaccine efficacy could be improved if the interval between booster vaccinations can be shortened (47). Regardless, the process of competition does not just begin when the memory cells enter the memory compartment, but also can occur during the initial response to infection itself. Responding $CD8^+$ T cells must compete for factors that are required for a successful immune response and the subsequent memory formation that follow. To shed light on some of these underlying factors, an overview on the requirements for the formation of a true-memory cell versus a HP-memory cell will be presented. This will provide some insight on how their derivation may

predispose them to behave in during secondary infection particularly when in competition with other memory cells.

Why does lymphopenia induced HP occur?

While lymphopenia-induced HP occurs on a regular basis and has been repeatedly demonstrated in many different systems, the molecular mechanisms regulating HP are not fully elucidated. Presented here are models that have been postulated to explain the spontaneous proliferation of T cells induced in a lymphopenic environment (Fig. 2). The first model involves competition for available resources (Fig. 2A). Under lymphoreplete conditions, cytokines, growth factors, peptide/MHC etc. are limiting and the proliferation of cells is controlled because cells have to compete for the few resources. However, the onset of lymphopenia removes many of the cells, and the remaining cells that remain have exposure to elevated amounts of cytokine and access to self-peptide/MHC allowing them to undergo HP (29).

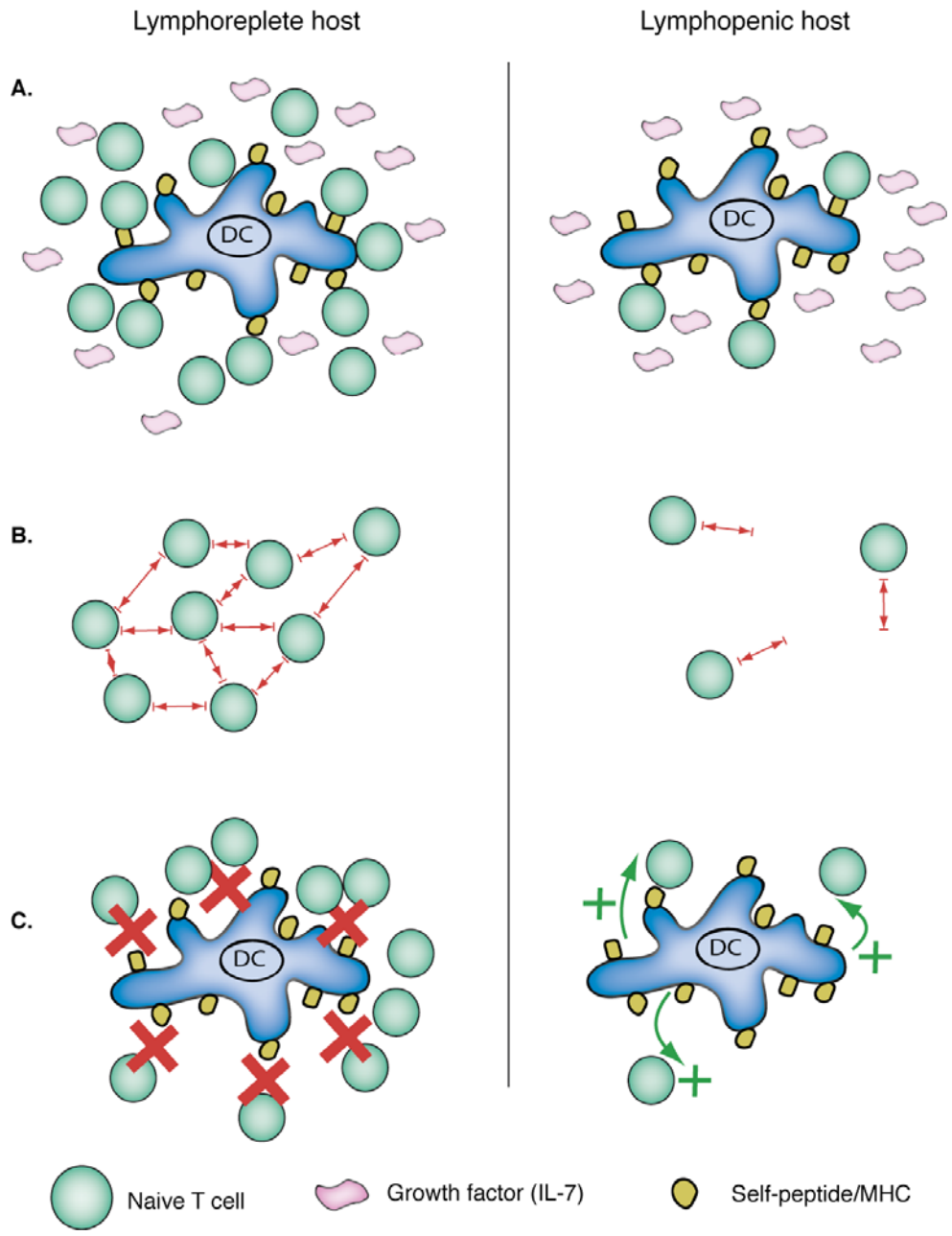

Figure 2. Possible models of lymphopenia-induced HP.

(A) In a lymphopenic environment, there is less competition for growth factors. Access to growth factors under saturating conditions allows proliferation to occur in response to self-peptide/MHC. (B) Auto-inhibitory signals from each T cell limit the amount of proliferation that other cells can undergo to prevent overpopulation. In a lymphopenic environment, the effect of auto-inhibitory signals are mostly negated due to the increase in space. (C) The proliferation of T cells under lymphoreplete conditions is mediated by a non-T cell that exerts inhibitory factors to control expansion. Under lymphopenic conditions, these cells can sense space and upregulate factors that promote the proliferation of T cells.

The second model involves autoregulation by the cells themselves. In a lymphoreplete host, overall cell numbers are maintained through homeostastic mechanisms. The T cells can actively exert inhibitory signals that affect the potential to divide by their other T cell counterparts. This therefore prevents proliferation in a host that is already lymphoreplete. Upon induction of lymphopenia, these inhibitory signals are diminished because fewer cells remain in close enough proximity to provide the negative signals. The third model involves trans-regulation of the T cell population by a non-T cell such as a DC or stromal cell. Under lymphoreplete conditions, this cell does not promote T cell proliferation and could rather serve in an inhibitory capacity. However, under lymphopenic conditions, this cell would upregulate a signal that promotes HP such as IL-7 secretion, causing proliferation of the naïve T cells. In this scenario, it is the APC that senses “space” and reacts to it, rather than the T cell.

At this point, the data support the first scenario, where severe lymphopenia results in proliferation by the remaining cells due to the excess of growth factors and the lack of cells to compete for them. It has not yet currently been proven that cells can exert inhibitory signals upon one another or that trans-regulation by another cell type occurs because there is a sensing of increased “space”.

A comparison: Infection induced CD8$^+$ T cell memory and HP induced memory

The antigen-experienced memory population sets the standard upon which all other "memory" subsets are judged. This true-memory population forms as a result of antigenic exposure in the context of infection. True-memory cells are more efficacious at clearing antigen upon secondary infection due to their shorter response time, increased numbers, and localization to peripheral sites allowing them quicker access to the invading pathogen (1). Additionally, true-memory cells are long lived and can be maintained for a lifetime (4). The requirements for the differentiation of true-memory from a naïve CD8$^+$ T cell are: antigen (signal 1), costimulation (signal 2), and inflammation (signal 3). Variations in the strength and duration of these signals can greatly alter the resulting size of the memory compartment as well as the type of memory cells that populate it (48). Can any other processes generate memory cells that are equivalent to the ones induced by infection? The process of HP does result in the differentiation of naïve CD8$^+$ T cells to memory phenotype cells with many characteristics of antigen-induced memory cells. The unusual method by which these HP-memory cells are generated will be compared with the more conventional ways of memory generation.

Different types of HP-induced proliferation

An example of one of the mechanisms used by the immune compartment to maintain population homeostasis is the spontaneous proliferation of

lymphocytes in the absence of infection. In a parallel to an acute infection, hosts with acute lymphopenia typically recover and eventually become lymphoreplete again. Acute lymphopenia is observed following radio- or chemo-therapy, after certain infections such as influenza (49), HIV (50), and Ebola (51), and also postnatally during the initial population of the secondary lymphoid organs (52). The main focus of the introduction will be on the HP-memory cells derived from naïve cells transferred into irradiated hosts, but the derivation of other types of HP-memory cells as well as the HP of true-memory cells will be also discussed.

Impact of antigen (quantity and quality) on the formation of true-memory

For a naïve $CD8^+$ T cell to undergo expansion, it must encounter a peptide fragment derived from cognate antigen presented by a specific MHC class I molecule. Increasing the amount of infectious pathogen usually results in an elevated immune response due to the corresponding increase in inflammation (48). However, there are limits to the degree of expansion that the $CD8^+$ T cells can undergo as there is a not an infinite supply of resources the expanding cells can draw upon for continued growth. The immune compartment also works to prevent a runaway inflammatory response through negative feedback mechanisms. Nevertheless, during the peak of an immune response, $CD8^+$ T cells of any one specificity can at most comprise 50%-70% of the entire $CD8^+$ T compartment (53). Interestingly, excessive antigen presentation actually results in an inferior cytolytic memory population (54). Thus, optimal memory generation would maximize the $CD8^+$ T cell response without exposing the cells to excessive

antigen. Wherry et al. demonstrated this by boosting the density or the overall number of available epitopes of an antigen and, the magnitude of the resulting $CD8^+$ T cell response was augmented. In this way an elevated $CD8^+$ T cell response can be achieved without an overwhelming amount of antigen (55). This indicates that memory generation is not reliant upon the amount of inflammation or antigen exposure.

Impact of antigen/MHC on lymphopenia induced HP

It has been established that two key signals required for lymphopenia-induced HP of naïve $CD8^+$ T cells are self-peptide presented by the MHC class I molecule and the cytokine IL-7 (8, 56, 57, 11, 58). These elements are similar to what are necessary for naïve $CD8^+$ T cell survival in a full compartment. Naïve $CD8^+$ T cells require contact with MHC class I molecules for survival (59, 60). IL-7 has been shown to enhance naïve $CD8^+$ T cell survival in vivo and has a role in regulation of their homeostasis (61, 62). It is startling that while the signals are the same, the effects they induce on T cells vary depending on the host. In a lymphopenic host, the T cells undergo rapid division, but in a lymphoreplete host, only slow basal turnover occurs. Upon transfer of naïve $CD8^+$ T cells into a host devoid of MHC class I expression, such as that found in TAP- deficient mice, lymphopenia-induced HP cannot occur (57, 58, 63, 60, 64). In a lymphopenic environment, cells are dividing in response to the self-peptides of similar affinity as required for their initial positive selection in the thymus (58, 57). In these experiments, HP occurs in the mouse expressing the peptide/MHC combination

that induces weak/low affinity interactions with the T cell receptor (TCR); high affinity interactions were not necessarily required (58, 57). However, HP did not occur in a mouse expressing an irrelevant peptide/MHC combination. This parallels what occurs in the thymus during positive selection where cells expressing TCRs that have lower affinity interactions with self-peptide are promoted to survive. While self-peptides were found to be responsible for the HP in irradiated hosts, another subset of cells can undergo proliferation in lymphopenic conditions but in response to foreign antigen (65). This type of proliferation is not HP as we have defined it as it is much more rapid and occurs upon transfer of naïve cells into syngeneically T-depleted hosts such as nude, SCID, or RAG-deficient mice. The immunodeficiency of these mice leaves them prone to infections, perhaps increasing the amount of foreign antigen in the mice. Housing these mice in germ-free environments greatly reduced the formation of these rapidly dividing HP cells and instead fostered the formation of the slower dividing HP-memory cells that were generated in response to self-peptide.

In contrast to naïve cells, interaction with self-peptide/MHC class I complexes is not necessary for the lymphopenia-induced HP of antigen-induced memory cells (60). This is not surprising as antigen experienced memory cells can persist and survive in hosts lacking MHC class I expression, while naïve T cells cannot (60). Thus, memory cells have different requirements for HP in a lymphopenic host compared to the transfer of naïve cells. This is most likely a result of the differentiation programs that are initiated when they are first primed.

Generally, transfer of naïve cells into an irradiated host will result in proliferation and generation of HP-memory cells but this not always the case. Transfer of high numbers of cells will result in a decrease in HP, an indication that competition exists for factors necessary for growth during periods of lymphopenia-induced HP (57). During infection, the most high affinity clones for the pathogen are selected for affinity maturation and formation of memory (66). It is most advantageous to use these high affinity clones for memory formation because upon re-infection they are much more likely to be efficacious responders. This has been demonstrated when multiple successive immunizations drive the selection of the high affinity clones over their lower affinity counterparts (66). Following multiple infections, the low-affinity clones gradually disappear, while the higher affinity clones predominate.

In an acute infection, the naïve $CD8^+$ T cells that ultimately respond to infection are the clones that are specific for a given antigen, while non-specific T cell clones are unable to be activated. Specificity for the antigen dictates whether a clone will proliferate during infection. In HP, the affinity of the TCR governs whether a cell will proliferate to self-peptide (67). Kieper et al. showed that TCR affinity positively correlated with the amount of CD5 expressed on the cells. CD5 negatively regulates TCR signaling and is expressed at a level proportional to the affinity of the TCR for self-peptide/MHC. OT-I cells express a high levels of CD5, which make them good candidates for HP because they have a high affinity for self-peptide/MHC class I. Other transgenic cells such as 2C, HY, and OT-II $CD4^+$

T cells, divide much less if at all in a lymphopenic environment due to their low affinity TCRs. Intrinsically, OT-I cells express the high levels of CD5, while HY cells express little (67).

Impact of costimulation on true-memory formation

Costimulation is one mechanism utilized by the immune system to prevent an auto-reactive T cell from responding to self-peptides it may encounter. Naïve $CD8^+$ T cells are activated by mature dendritic cells (DCs) presenting peptide antigens. This initiates a signaling program leading to differentiation of the naïve cells into CTLs and memory cells. Presentation of peptide by an immature DC without costimulation generally results in tolerance and non-reactivity upon re-encounter (68-70). Inflammation concurrent with infection is initiated by innate recognition of pathogen. This typically contributes to the upregulation of costimulatory molecules.

CD28 and 4-1BB are two of the most well known costimulatory molecules to be directly involved in $CD8^+$ T cell activation (71). CD28 was the first costimulatory molecule to be discovered as necessary for $CD8^+$ T cell activation in mice (72). Since then, the number of costimulatory molecules found to be capable of providing signal 2 continues to increase including members of the CD28 family, TNF family members, and other cytokines that all work synergistically in conjunction with TCR activation (73). Some members of the CD28 family include CTLA-4, ICOS, PD-1, and BTLA (73). TNF family members and their corresponding receptors that have costimulatory function include

OX40:OX40L, CD27:CD70, 4-1BB:4-1BBL, CD30:CD30L, GITR:GITRL and HVEM:Light (73). The three signals required for $CD8^+$ T cell activation are not independent of one another. Inflammation can enhance the signaling of costimulatory molecules (74). It is often difficult to discern the role of individual costimulatory molecules as their signaling pathways often overlap and can often compensate for one another.

Impact of costimulation on HP-memory

Unlike the response to foreign antigen, naïve $CD8^+$ T cells dividing in response to lymphopenia do not require costimulatory signals such as CD28, CD40, and 4-1BB (6, 75). Transfer of CD28-deficient lymph node cells into a lymphopenic host resulted in HP comparable to that of the WT cells (6, 75). While not directly required for lymphopenia-induced HP (6), CD28 expression was necessary for the antitumor response mediated by the naïve cells that undergo HP (15). The one costimulatory molecule found to be necessary for HP is CD24 (76, 77). CD24 deficiency by T cells limits their HP in a lymphopenic host. However, transfer of WT cells into a CD24-deficient host results in massive proliferation and death of the host (76). The CD24-deficient DCs in the hosts were found to be excessively stimulatory, and in the absence of regulation by the CD24-deficient DCs, the host succumbed to the massive T cell proliferation.

$CD4^+$ T cell help in true-memory formation

$CD4^+$ T cell help is dispensable for some primary responses to infection, but required for the formation of competent $CD8^+$ T cell memory (78-80, 71).

During the initial exposure to antigen, $CD4^+$ T cell help appears unnecessary during infections such as *Lymphocytic Choriomeningitis Virus* (LCMV), *Vesicular stomatitus virus* (VSV) or *Listeria monocytogenes* (Lm), because the $CD8^+$ T cells expand and mediate effector function due to the intense inflammatory milieu created by these infectious agents (81-83). In contrast, $CD4^+$ T cell help is required in situations for priming of $CD8^+$ T cell responses with non-inflammatory antigens (84, 85, 79). While $CD4^+$ T cell help may or may not be required for the primary response, the lack of it results in poor quality memory $CD8^+$ T cells. Without $CD4^+$ T cells present during the priming, the resulting memory $CD8^+$ T cells cannot respond to secondary infection and die via a TRAIL-mediated mechanism (Tumor-necrosis-factor-apoptosis-inducing-ligand) (80, 83). These unhelped $CD8^+$ memory T cells secrete TRAIL and commit activation-induced-cell-death upon antigen re-encounter. Due to this phenotype, the $CD8^+$ T cell memory formed in the absence of $CD4^+$ help are known as "helpless" $CD8^+$ T cells.

The nature of the help provided by $CD4^+$ T cells to the $CD8^+$ T cells during the primary infection remains elusive. It is now generally accepted that through the upregulation of CD40L by the $CD4^+$ T cells, DCs are activated though CD40L-CD40 pathway, and then they go on to activate the $CD8^+$ T cells (79). IL-2 has also been proposed as the source of help provided by the $CD4^+$ T cells. Priming $CD8^+$ T cells in the absence of IL-2 results in memory cells that exhibit defective expansion upon secondary challenge, similar to priming the absence of $CD4^+$ T

cells (86). Besides their role in priming, CD4 T cells also have a role in some settings in maintaining the survival and function of memory $CD8^+$ T cells (87). Thus, $CD4^+$ T cells play a major role in the generation of functional $CD8^+$ T cell memory and also in their long-term maintenance.

$CD4^+$ T cell help in HP-memory formation

One of the similarities of lymphopenia-induced memory formation to true-memory is the requirement for $CD4^+$ T cell help (88). HP-memory cells formed in the absence of $CD4^+$ T cells are unable to respond effectively to secondary infection. During conventional memory generation, CD40L expressing $CD4^+$ T cells are thought to license DCs through interaction with CD40 expressed on the DC surface. The activated DCs in turn go on to activate the $CD8^+$ T cells (85, 84).

It was also shown that lymphopenia induced HP in a CD40 deficient host does not generate functional HP-memory cells capable of responding effectively to a secondary infection (89). The formation of HP-memory can be rescued in a CD40 deficient host with the use of agonistic anti-CD40 antibodies. Thus, while the CD40L-costimulatory molecule does not necessarily need to be expressed on the $CD8^+$ T cell themselves, costimulation in form of $CD4^+$ T cell help to the DCs is needed to generate functional HP-memory. Furthermore, in the same study, IL-12, which is released by activated DCs, compensated for the lack of $CD4^+$ T cell help during HP.

Impact of inflammation on true-memory formation

An important and necessary consequence of infection is the release of inflammatory cytokines such as IL-12 and type-I IFNs (IFN-1) secreted by activated innate cells such DCs and phagocytes (90) which have pattern recognition receptors that recognize conserved molecular structures on bacteria, viruses, and parasites. Adjuvant is added to vaccines for the sole purpose of inducing the inflammatory response, without which poor memory responses are generated. The function of inflammation and the release of IL-12 serve to promote the clonal expansion and differentiation of $CD8^+$ T cells into CTLs (91). IL-12 induces production of IFN-γ, which is needed for cytolytic effector formation (92, 93). Although expansion occurs upon exposure to antigen without inflammation, in the absence of IL-12, $CD8^+$ T cells fail to make granzyme-B or become competent effectors (92, 93).

While one cannot underestimate the importance of IL-12 in inducing an appropriate immune response, the lack of IL-12 does not necessarily result in defective memory formation and can lead to a better recall response (94). The level and timing of exposure to inflammatory signals contributes to the rate of memory $CD8^+$ T cell differentiation. The caveat is that while inflammation is needed for a successful immune response, lower levels of inflammation actually promote accelerated memory formation (42, 95, 96). Attenuating the inflammation either by the reduction of stimulation or increasing intraclonal T competition with higher number of T cells to eliminate the pathogen can lead to

the preferentially formation of effector cells that are phenotypically $CD127^{HI}CD62L^{HI}KLRG1^{LO}$, which after contraction form long-lived memory cells (42, 95, 96). Joshi et al. showed that the cell fate decision between MPEC and SLEC formation was based upon the amount of inflammation present, where higher levels of inflammation skewed $CD8^+$ T cells toward the SLEC phenotype while lower levels induced formation of MPEC $CD8^+$ T cells (42).

The timing of exposure to inflammation can also affect the outcome of memory formation. During infection, responding cells can be exposed to inflammatory signals at any point before, during, or after their interaction with activating APCs. Not only do lower levels of inflammation promote memory formation, they also promote accelerated memory formation, where responding $CD8^+$ T cells acquired memory characteristics earlier (96). Increased inflammation exposure during the window of time when proliferation occurs results in a delay of the $CD8^+$ T cells converting from an SLEC to MPEC phenotype, with more cells being initially skewed toward a more SLEC phenotype.

Type I IFNs also have a role in inducing $CD8^+$ T cell effector differentiation, but how much they contribute to the differentiation process varies as the amount of type 1 IFN generated depends upon the type of pathogen. During LCMV infection, IFN-I receptor deficient $CD8^+$ T cells expanded much less than WT, but during Lm infection, the lack of IFN-I affected the response very little (97). If high levels of IL-12 are present, as occurs during a Lm infection, the role of IFN-I is

much less important (97). One inflammatory factor can often compensate for another and the signaling pathways they induce overlap much like the costimulatory pathways.

Maintenance of true-memory by cytokines

The role of IL-7 and IL-15 in the homeostasis of memory $CD8^+$ T cells has been well documented. True-memory cells require exposure to both IL-7 and IL-15 for survival (98, 3, 99, 100, 11), and IL-15 plays a major role in mediating the basal turnover of memory $CD8^+$ T cells (101). IL-15 acts as a bridge between the innate and adaptive immune systems because its secretion is induced by the release of IFN-I, which is involved in the inflammatory response (102, 100, 103). Memory cells transferred into an IL-15-deficient host fail to proliferate (98, 3, 99, 100, 11) even after injection IFN-I inducing agents such as TLR ligands are introduced (13). Memory cells in an environment lacking IL-15 fail to undergo basal proliferation and disappear (98, 3, 99, 100, 11, 13).

Along with IL-15, IL-7 also affects memory $CD8^+$ T cell homeostasis (104, 60, 7). IL-7R-deficient OT-I $CD8^+$ T cells can respond to infection, but fail to differentiate into memory cells (7). The background levels of IL-7 alone in an IL-15-deficient host are not sufficient enough to sustain the memory population (101). However, upon crossing the IL-15 deficient mice to an IL-7 transgenic, memory survival is restored, indicating that increased levels of IL-7 can compensate for the lack of IL-15 (105). For memory homeostasis, the accepted

role for IL-7 is to mediate cell viability and for IL-15 to promote basal homeostasis and turnover (3).

It is worth noting the circumstances of IL-15 presentation. The IL-15 receptor shares two components with the IL-2 receptor, namely the IL-2Rβ chain (CD122) and IL-2Rγ chain (CD132) (99). Unlike other cytokine receptors, IL-15Rα is not upregulated on the T cell that needs to receive the signal, but rather on the cell that is producing the IL-15. IL-15Rα is bound to IL-15 and is trans-presented to the receiving cell. IL-15 is thought to be induced on a variety of APCs following activation by type I IFNs (102).

Cytokine requirements for lymphopenia-induced HP

IL-7 is required for lymphopenia-induced HP as division is blocked in hosts treated with anti-IL-7R antibody, does not occur in IL-7KO hosts, an dIL-7R-defeiicinet cells cannot undergo HP (11, 8, 7). Unlike the survival of true-memory cells, IL-15 was not found to be necessary for naïve cells to undergo HP. In patients with severe $CD4^+$ T cell lymphopenia due to HIV infection, IL-7 levels were found to be increased in the blood and lymph nodes (29). The increase in IL-7 levels is thought to be due either a compensatory feedback loop by the immune compartment to enhance T cell differentiation during lymphopenia, or the result of the lack of lymphocytes competing to utilize the cytokines. It is this increased availability of IL-7 that is thought to enable the low-affinity TCR

interactions with self-peptide to induce proliferation when they normally would only promote survival (106).

Naïve and memory cells have different requirements for basal turnover and likewise, $CD8^+$ memory T cells that undergo HP have different cytokine requirements than naïve $CD8^+$ T cells. Memory cells are able to utilize either IL-7 or IL-15 for lymphopenia-induced HP. Only when access to both cytokines is removed that HP is prevented (11, 107).

Other factors to consider-Asymmetric cell division

Asymmetric cell division occurs when a naïve $CD8^+$ T cell starts to divide in response to TCR ligation (39). This finding changes the way we think about the derivation of $CD8^+$ T cell memory. Often there are conflicting arguments about how cell fate is derived, whether cells can inter-convert or retain their stable phenotype. Chang et al. showed that one cell can produce progeny that are of two different cell fates. During division, each daughter cell received unequal amounts of protein and mRNA related to cell signaling, fate specification and asymmetric division. The “proximal” daughter (because it was closer to the microtubule organizing center) assumed a more T_{EM} phenotype, containing more granzyme-B and IFN-γ mRNA. This daughter also contained high levels of activation molecules such as CD69, CD43, CD25, and CD44, with low CD62L expression. The distal daughter exhibited a more T_{CM} phenotype with low levels of CD69, CD43, CD25, and CD44, but high levels of CD62L. They also had higher levels of IL-7Rα which is an indication that these could be the cells meant

to perpetuate the lineage of this clone. The distal/T_{CM} daughter mediated more effective clearance of infection 30 days after transfer, showing they were able to persist long-term. Asymmetric division indicates that there is another level of regulation in memory formation.

Symmetrical cell division in lymphopenia induced HP

Asymmetric division undergone by cells exposed to antigen is different from that which happens to cells that undergo lymphopenia induced HP (39). Upon cell transfer into irradiated mice, division is symmetric and each daughter cell receives uniform amounts of proteins and mRNA. These experiments imply that sustained TCR contact with DC mediate asymmetric division, but without antigenic peptide or the sustained ability to adhere to the DC, symmetric division will occur. It has yet to be determined how the asymmetrical division and unequal partition of cell components affect the function of the memory subsets, and whether one type of division favors formation of functional memory has yet to be reconciled

Competition between cells

All the requirements previously mentioned above such as antigen, costimulation, cytokines, etc. necessary for the generation of HP- and true-memory cells are limiting in a normal immune compartment. This prevents uncontrolled expansion of the $CD8^+$ T cells during the immune response. This natural curtailment of the response facilitates competition between the responding cells. Competition has been well documented to occur between T cell

populations (108-112). High precursor transfers of $CD8^+$ transgenic T cells inhibited the endogenous response (113). Likewise, transgenic $CD4^+$ T cells must compete for resources with each other during a high frequency transfer (112). The result of the competition between two subsets could also be affected by their affinities for peptide/MHC or immunodominance (110, 114). Most of these experiments postulate that competition revolves around contact with the APC but the multiple factors that they are competing for remain undetermined, as well as the time frame in which they are needed (109).

Competition for antigen-peptide/MHC class I complexes

Access to APCs plays a major role during competition because they can influence the level or duration of antigen exposure (109). Competition can also exist for DC-derived factors such as costimulation, growth factors, or cytokine availability (109). All this contributes to the degree of stimulation and subsequent formation of secondary memory cells (1).

One of the factors that cells can specifically compete for on the APC is access to peptide/MHC-I. During LCMV infection, it was noted that the main responding subsets were specific for 3 epitopes: gp33, np396 and gp276 because cells specific for these epitopes were able to outcompete all other clones (108). Competition is also not only for physical space on the APC, but also for specific epitopes on the APC as increasing the precursor frequencies of gp33 specific cells did not affect the number of responding np396 or gp276 specific cells(108). Another experiment showing cells compete for peptide/MHC class I

complexes is where $CD8^+$ T cells were shown to sequester their own target epitopes on the surface of the APC following interaction with them for activation (111). This prevented $CD8^+$ T cells with the same specificity from being activated and served to promote a broader spectrum of responses by other cells since other peptide/MHC class I molecules are not sequestered. In this manner, cells with the same specificity will be limited without inhibiting cells of other specificities resulting in an overall more balanced response (108).

Access to APCs

Competition for epitope is different than competition for DC access, as lack of APC access implies lack of access to antigen and any additional signals that the DCs produce in the form of cytokines, inflammatory factors, or costimulation. In many situations, competition between T cells occurs due to the scarcity of APCs and abundance of T cells, which can be corrected by the addition of increased numbers of DCs (110). Competition for access to APC is most apparent across cells with different specificities (115, 116). Some competition between $CD8^+$ T cells specific for dominant and subdominant epitopes can only be rectified if the different peptides are presented on separate APCs, an indication that competition is not solely for peptide/MHC class I molecules but for the APCs themselves (115, 116). Thus for some competition, it is not enough to transfer more APCs, but the APCs have to bear different peptides. Access to APCs is of the utmost importance to a responding $CD8^+$ T

cells because antigen-bearing DCs persist for 2-6 days only, so cells have to compete for access or risking having no stimulation at all (117).

The outcome of competition determines what cells go on to form the memory pool, and also which memory cells are retained in the memory pool. HP occurs because the limited amount of resources usually available to cells in the immune compartment is disturbed, resulting in proliferation in the absence of antigen.

Cytokine- and chronic lymphopenia induced HP

Thus far, the focus of this intro has primarily been on HP-memory cells derived from naïve cells transferred into hosts rendered lymphopenic through irradiation, I will now cover other conditions under which HP can occur, even in lymphoreplete hosts: (1) if the host is lymphoreplete but receives exogenous/extra doses of cytokine, or (2) if the host is chronically lymphodepleted as found in SCID and nude mice. The requirements for these two conditions as well as the function of the resulting HP-memory cells derived from each of the conditions are discussed below.

Homeostatic proliferation due to excess cytokine

Proliferation of naïve $CD8^+$ T cells can occur in a full compartment with minimal TCR signaling if there are excessive levels of a cytokine, from either exogenous administration, over-expression, or deficient expression of the receptors able to utilize the cytokine (3). Among the cytokines that have shown to have an effect on HP are IL-2, IL-15, and IL-7 (71, 118-121).

During infection, IL-2 is required for proliferation and differentiation of the naïve $CD8^+$ T cells (71). In the absence of infection, elevated levels of IL-2 can induce HP. Addition of IL-2/anti-IL-2 complex promoted the differentiation of naïve $CD8^+$ T cells into effector cells in a lymphoreplete host (122). The HP-memory cells that resulted from excess IL-2 levels had no prior exposure to antigen but were able to effectively clear a Lm.OVA infection. However, in spite of their ability to clear antigen, the HP-memory cells that were induced by the exogenous IL-2/anti-IL-2 complex did not exhibit complete cellular fitness; they produced less IFN-γ, TNF-α and IL-2 upon restimulation.

Rapid homeostatic proliferation also occurs upon transfer of naïve T cells into mice deficient in expression of one of the subunits of the IL-2R, including CD25 (IL-2Rα), CD122 (IL-2Rβ), or CD132 (IL-2Rγ) (118, 119). These mice are lymphopenic and have elevated levels of IL-2 and IL-15 because the host cells are unable to utilize the cytokines. Unlike proliferation that occurs in a lymphopenic B6 host which is due to elevated levels of IL-7, the HP that occurs in a IL-2R-subunit-deficient mouse is much more rapid. This HP is almost as rapid as proliferation in response to antigen stimulation and appears to be dependent on IL-15 and partially on IL-2. The HP-memory cells that result are also capable of mounting a response to secondary challenge upon transfer into another B6 host. Of note, HY, 2C and SMARTA transgenic cells also underwent rapid proliferation in the CD132-deficient hosts despite undergoing poor HP in

irradiated B6 hosts. Cytokine-induced homeostatic proliferation is TCR dependent and also relies on the presentation of self-peptide MHC (118, 119).

In the same manner, excessive IL-7 levels can lead to the formation of HP-memory cells. Addition of IL-7/IL-7R complexes can induce HP of $CD8^+$ T cells in lymphoreplete hosts (120, 121). Similarly, IL-7 transgenic mice that express elevated levels of IL-7 contain a high number of HP-memory cells (105). These cells upregulated expression of CD44, similar to cells derived from lymphopenia-induced HP. Self-peptide/MHC complexes were also required for their division. All this illustrates that the disturbance of the immune compartment due to excess cytokines can stimulate the proliferation of naïve T cells in the absence of lymphopenia resulting the generation of memory-phenotype cells.

Chronic lymphopenia-induced homeostatic proliferation

Chronic lymphopenia is a condition found in a number of hosts that are T cell deficient, such as SCID, RAG-deficient, or nude mice. Unlike acute lymphopenia, mice that are chronically lymphopenic will never become lymphoreplete. These host environments are not necessarily devoid of all lymphocytes, but do not have normal lymphocyte numbers. Upon transfer of cells into chronically lymphopenic hosts, two cell populations undergo HP. One subset undergoes slow proliferation as observed under lymphopenic circumstances, but there is an additional subset of cells undergoing fast proliferation believed to be driven in response to foreign antigen (123, 65). That this occurs only upon transfer of polyclonal T cells into chronically lymphopenic environments further

supports that the fast proliferation is due to foreign antigen. Furthermore, this faster dividing population is also decreased when mice are grown in a germ-free environment (65). Chronic lymphopenia-induced HP is costimulation dependent, and also results in the upregulation of activation markers, and enhanced effector function by the resulting memory cells (123, 65).

Since raising the mice in a germ-free environment decreased this faster dividing population, it is speculated that the polyclonal cells are responding to the commensal microflora, which is reduced in a germ-free environment. It is still unclear why naïve cells transferred into a sublethally irradiated host do not undergo HP in response to commensal bacteria, but only transfer of cells into a chronically lymphopenic host does. It has been proposed that the integrity of the gut wall is maintained in mice that have been irradiated, excluding bacterial entry into the bloodstream (3). However, in chronically lymphopenic mice this integrity is breached, allowing cells transferred into this environment to undergo proliferation in response to commensal antigen in addition to self-peptide. It is speculated that the release of IL-22 promotes the integrity of the gut lining (124). Interestingly, HP-memory cells that are derived from RAG-deficient hosts cannot clear infection with the same efficiency as HP-memory derived from irradiated hosts. In terms of effector function, they produce less IFN-γ and TNF-α upon restimulation (88, 89). While it still remains unclear why the HP-memory cells formed in chronically lymphopenic hosts are not as effective as cells derived from acutely lymphopenic hosts, Hamilton et al. propose an explanation similar to the

one provided for the fast HP cells undergo in a chronically lymphopenic environment, albeit with a twist. They hypothesize that the irradiation causes a transient appearance of bacterial products in the bloodstream, DC activation, and production of inflammatory cytokines, which enhance the effector function of HP-memory cells derived from irradiated hosts (89, 125). Nonetheless, the cells in the irradiated hosts still undergo slow HP, an indication that they are dividing in response to self-peptide, and that the bacterial products in the bloodstream perhaps serve only to enhance inflammation.

While the focus of my research has been on the HP of $CD8^+$ T cells, this process is not unique to just to T cells. $CD4^+$ T cells, NK cells, NKT cells, and B cells have all been shown to be capable of undergoing lymphopenia-induced HP in recipients that have been irradiated or lack that specific cell subset (57, 126-128).

Memory characteristics of HP-memory cells

The most interesting and potentially therapeutically applicable portion of this HP process is that naïve $CD8^+$ T cells progressively acquire the phenotypic and functional characteristics of memory T cells. These HP-memory cells have surface expression of activation markers such as CD44, CD122, CD127, and Ly6C similar to true-memory cells (5, 6, 12, 57).

They also have the ability to rapidly make cytokines such as IFN-γ and lyse infected cells upon stimulation (88, 6, 10, 5, 12). Memory cells generated during HP provide protection against bacterial infection *in vivo* with comparable

efficiency as antigen-experienced $CD8^+$ T cells (88), showing that they are a good surrogate for the true-memory cells during secondary infections. The dependence of HP-memory on $CD4^+$ T cell help for functional protection also serves to emphasize their similarity to antigen-experienced memory cells (88).

Notably, the HP-memory gene-expression pattern is much more similar to the true-memory cell pattern in comparison with the naïve cells especially in reference to genes involved in cell cycling (129). The upregulation of markers associated with true-memory cells progressively increased over time after transfer to a lymphopenic host. However, perhaps as a reflection on the HP process whereby a naïve cell attains memory-like characteristics without differentiation into an effector cell, genes pertaining to effector function such as granzyme-B, perforin, and IFN-γ were not as highly upregulated on the HP-memory cells as compared to the true-memory cells (129). Importantly, examination of the microarray data did not reveal a unique gene-expression pattern that defined the HP-memory cells.

HP-memory cells do not solely arise as a side-effect of medical treatment or infection-they can also occur naturally. Natural memory-phenotype cells can be found in unimmunized mice and have the functional traits of true-memory cells (130).

The ability to induce immunological memory in the absence of antigen or infection represents significant therapeutic potential. This may be particularly advantageous during the recovery phase of immunocompromised individuals, as

the benefit of a rapid response to antigen could compensate for low lymphocyte numbers.

It is important to consider how memory subsets are regulated by the immune compartment. Since multiple memory populations may be mobilized to deal with an infection, how does the activation of the HP-memory cell subset impact the response of the true-memory cell subset? There is also an issue of competition by the activated memory subsets for limited space and resources. While it has been shown that competition for these resources between memory T cells of different specificities can result in attrition of previously formed memory (45, 131, 132), whether this occurs with HP- and true-memory cells is not known.

In this study we examined how these two distinct memory subsets generated in response to either infection or lymphopenia accumulate and form secondary memory cells following re-infection. There has been conflicting evidence on whether the HP-memory cells act in a beneficial way to improve the immune response to secondary infection (89, 88), or by promoting an anti-tumor response (15-18), but are also capable of acting detrimentally on the immune compartment by facilitating autoimmunity or GVHD and whether their presence would inhibit normal memory formation (32, 23, 24). Underlying the study of clinical relevance of the HP-memory cells are more basic questions such as how these HP-memory cells are regulated once they are formed, and whether they occupy the same niche as the true-memory cells. If these two memory subsets are part of the same niche, what factors are they competing for and do the HP-

memory cells negatively impact the true-memory cells during an immune response? Competition can result in the attrition of previously formed memory (131, 132, 45), so does the existence of one subset imply that the other subset would have to be eliminated?

Importantly, we found that these two populations make equivalent responses when they were sole memory T cell subset transferred, but when in competition with each another, the true-memory T cells dominated the response and formed more secondary memory. Interestingly, the defect in memory formation by HP-memory cells could not be rescued by exogenous cytokines or antigen exposure. Furthermore, we found that HP-memory cells showed disorganized trafficking patterns within the spleen, indicating that HP-memory cells may fail to receive the necessary signals to form secondary memory cells efficiently in the presence of true-memory cells. Thus, HP-memory cells provide improved protection over naïve cell responses in the absence of competition and do not compromise responses of the 'tried-and-true' antigen-experienced memory population.

Chapter 1 contains portions of the material as it appears in the Journal of Immunology, "Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells." Cheung, Kitty P., Yang, Edward, Goldrath, Ananda W., September 2009 Sep 1;183(5):3364-72. The dissertation author is the first author of this paper.

RESULTS

True-memory cells out-compete HP-memory CD8$^+$ T cells during secondary infection.

In order to determine the effect of competition on the regulation of memory populations during the immune response to infection, we set up a dual adoptive transfer system. Each population was congenically distinct, which allowed us to compare concurrent HP- and true-memory T cell responses to antigen. The co-transfer enabled us observe the response of memory CD8$^+$ T cells with identical antigen specificity under the same infection conditions (Fig. 3A). The HP-memory cells were generated by sorting 10^6 naïve CD44^{LO} OT-I TCR transgenic CD8$^+$ T cells (CD45.1) and transferring them into sublethally irradiated B6 mice (CD45.2). To allow for the formation of HP-memory cells, the transferred cells were allowed to normalize in the irradiated host for a period of 30-60 days before use. During this time, the naïve cells acquired “memory-like” characteristics and differentiated into HP-memory cells. Alternatively, true-memory cells were generated by transferring naïve OT-I cells (CD45.1.2) into B6 hosts and subsequently infecting with recombinant Lm expressing ovalbumin (Lm.OVA). The cells in the infected hosts were also allowed to normalize for a period of 30-60 days in parallel with the HP-memory cells. The OT-I CD8$^+$ T cells express the Vα2Vβ5 TCR heterodimer which recognizes a peptide derived from ovalbumin (OVAp 257–264) presented by the H-2K^b molecule.

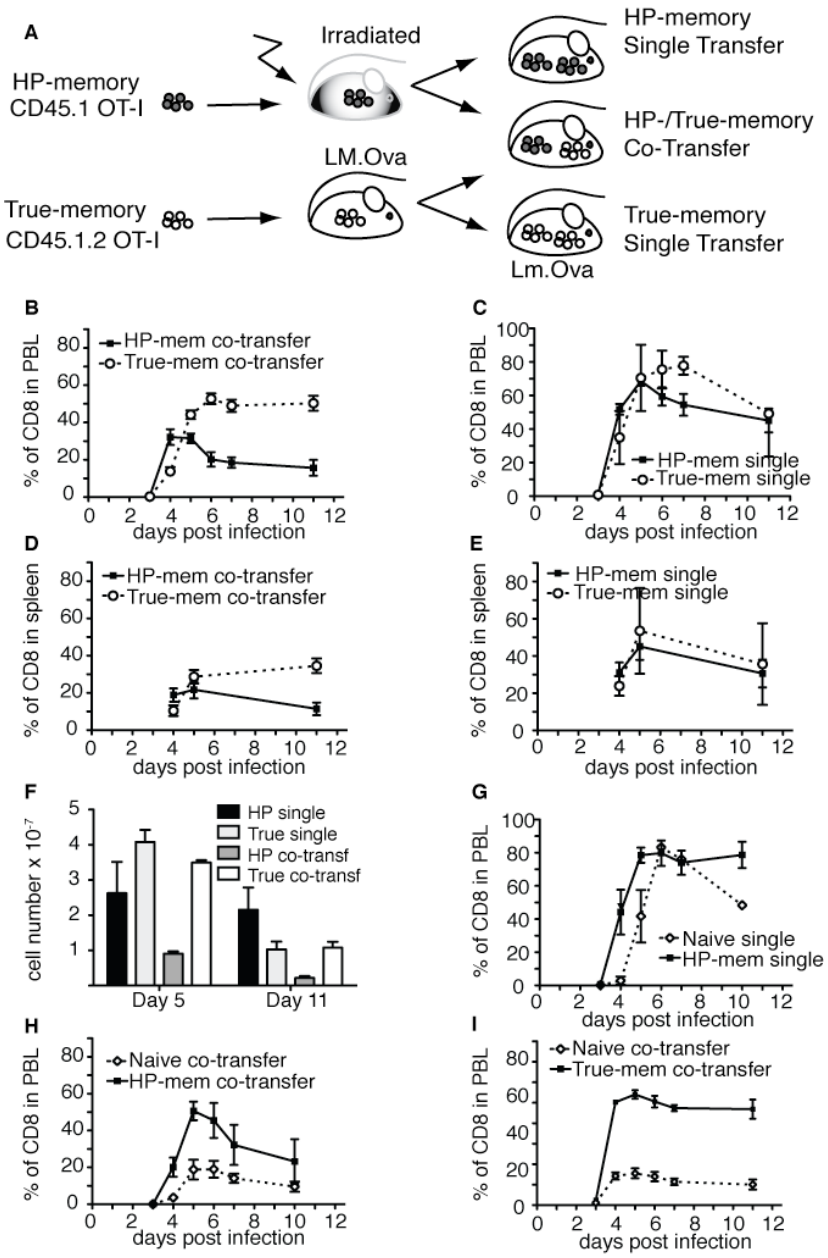

Figure 3. True-memory CD8⁺ cells outcompete HP-memory cells during secondary infection.

(A) Generation of memory T cell subsets and experimental design. Immune response of the donor memory subsets was measured as a percentage of the total CD8⁺ cells in the indicated tissue. (B) Co-transfer: HP- and true-memory, PBL. (C) Single transfer: HP- and true-memory, PBL. (D) Co-transfer: HP- and true-memory, spleen. (E) Single transfer: HP- and true-memory, spleen. (F) Total cell numbers recovered from spleen for indicated transfer conditions. Co-transfer: naïve OT-I and HP-memory, PBL. (G) Single transfer: naïve OT-I and HP-memory, PBL. (H) Co-transfer: naïve and HP-memory. Representative of > 3 experiments (n=3). Error bars indicate SD. (I) Co-transfer: naïve and true-memory.

To study the behavior of the HP- and true-memory cells during infection, three experimental groups (a 1:1 mix of both populations, HP-memory alone, or true-memory alone) were transferred into new B6 hosts (Fig. 3A), and infected with Lm.OVA one day later. For all three groups, the total number of transferred OT-I T cells was equal. Each of the single transfers received HP- or true-memory cells only. The mixed transfer received half the number of HP- and true-memory cells each for the same total number of cells as the single transfer. The co-transfer system allowed us to observe how well the HP- and true-memory cells competed for resources and space during infection. The accumulation of each subset was monitored by FACS in the peripheral blood (PBL) and spleen.

Following infection, both subsets underwent significant expansion (Fig. 3B-E) in the course of the single as well as the co-transfer. This expansion occurred in the PBL as well as the spleen. However, in the case of the co-transfer, the true-memory cells displayed enhanced expansion and formed more secondary memory compared to the HP-memory cells (Fig. 3B). This dramatic difference in the response by the HP-memory cells in the presence of the true-memory cells led to a closer examination of the response kinetics. The true-memory cells consisted of the majority of the overall OT-I population in spite of the earlier expansion by the HP-memory cells. Nonetheless, this early accumulation did not persist and the HP-memory cells ultimately generated fewer secondary memory cells. Of note, this defect was not due to any discrepancies

caused by the expression of different congenic markers by the different memory subsets. The HP-memory cells still deferred to the true-memory cells when the congenic markers were interchanged on the memory subsets, with the HP-memory cells being CD45.1.2 and the true-memory cells being CD45.1 (data not shown).

Comparing the average number of cells recovered from spleen on day 5 of infection we found that $3.5x10^7$ true-memory cells were recovered from the mixed transfers and $4.1x10^7$ true-memory cells were recovered from the single transfers (Fig. 3F). This indicated that similar or greater expansion by the true-memory cells in the mixed transfers occurred since the single transfer recipients received twice the number of true-memory precursors as the mixed transfers (Fig. 3F). At day 11 similar numbers of memory cells derived from true-memory donor cells were found in both the mixed and single transfer groups (~$1x10^7$ cells), an indication that the presence of HP-memory cells did not negatively affect the expansion of the true-memory cells. In contrast, HP-memory cells co-transferred with true-memory cells expanded less by day 5 when compared to the single transfer group ($0.9x10^7$ for mixed transfer versus $2.2x10^7$ cells for single transfer). This was reflected in the number of HP-memory cells on day 11 ($0.2x10^7$ for mixed transfer and $2.2x10^7$ for single transfer) (Fig. 3F). Thus, the expansion of the true-memory cells was not altered significantly in the presence of HP-memory cells, but the HP-memory cells were ultimately out-competed in the presence of true-memory cells.

The previous data were startling because these two memory subsets were thought to behave similarly. Though derived through different processes and environmental conditions, both the HP- and true-memory cells initially possessed similar surface phenotypes prior to infection. This includes upregulation of surface markers such as CD122, Ly6C, CD44, CD127, and CD27 which are molecules typically upregulated by $CD8^+$ memory T cells and also found to be upregulated by the HP-memory cells (Fig. 4).

Adjustment of the precursor transfer numbers to obtain primarily $CD62L^{HI}$ true-memory $CD8^+$ T cells was required. Since our experiments involved a comparison of the two memory populations, it was necessary that they be as phenotypically equivalent as possible. The HP process consistently generated memory cells that were mostly $CD62L^{HI}$. This was not the case during the course of an acute infection, where the CD62L expression of the true-memory cells generated can vary from high to low. To ensure that the majority of the true-memory cells generated would be $CD62L^{HI}$, a high number transfer of OT-I T cells (10^6) was used followed by Lm.OVA infection (95). Besides CD62L, no other phenotypic markers expressed by the true-memory cells were affected at this high precursor transfer number (data not shown). The protective capacity of the memory cells generated is also not affected by the naïve transfer numbers so we used these true-memory cells with confidence (133, 134). It has been postulated that T_{EM} localize primarily in the peripheral lymphoid tissues, as well as non-lymphoid tissues, and act as the early responders to infection, but their T_{CM}

counterparts are responsible for the later response and are ultimately responsible for the generation of protective immunity (35).

In recent years, other markers have also come to define the true-memory population besides the classical ones mentioned above. The expression of CD43, PD-I, KLRG1, and CXCR3 were also similar between the HP- and true-memory subsets. $CD27^{HI}/CD43^{LO}$ memory cells have been found to dominate the memory population and mediated the strongest response upon rechallenge, in contrast the $CD27^{LO}/CD43^{LO}$ cells slowly declined over time (135). Both the HP- and true-memory have low expression levels of PD-1, a marker of cell exhaustion (136). PD-1 is a member of the CD28 family of costimulatory receptors and is upregulated on exhausted $CD8^{+}$ T cells found during chronic viral infection (137).

While most surface markers examined had similar expression levels between the two memory subsets, CD49d, also known as VLA-4, was the exception. VLA-4 was more highly expressed on true-memory cells (Fig. 4), in agreement with to other reports (130). VLA-4 is an integrin involved in the recruitment of cells to the site of infection and is upregulated on primed T cells (138).

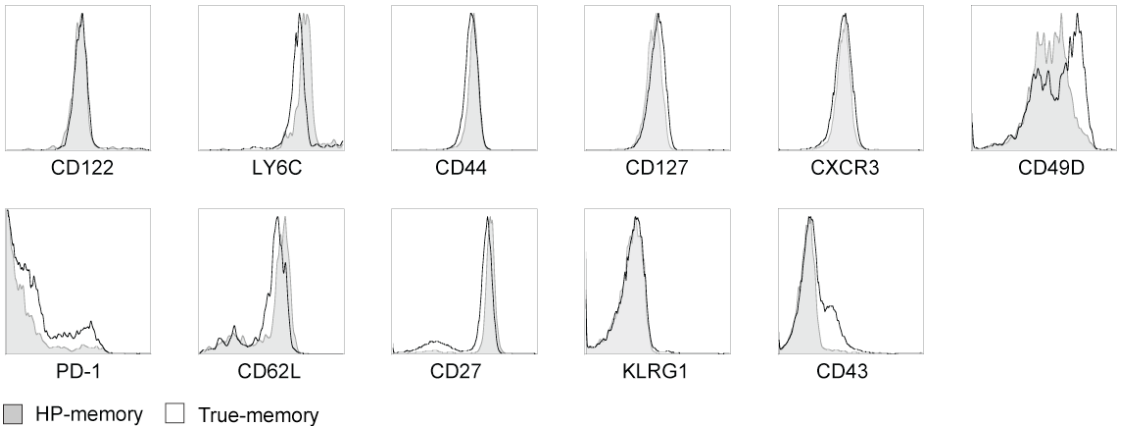

Figure 4. Characterization of the memory subsets prior to co-transfer.

The phenotype of the memory subsets was examined by flow cytometry prior to co-transfer. (A) Comparison of the indicated cell surface markers for the HP- and true-memory cells from pooled spleen and lymph nodes. Representative of 5 or more experiments (n=2-6).

Our experimental system relied on Lm.OVA infection, which led to differences in the HP- and true-memory responses in the presence of competition. Similar results were obtained during i.v. infection with VSV expressing OVA (VSV.OVA) (Fig. 5A). It had been shown that the primary and recall $CD8^+$ T cell responses to VSV.OVA were lymph node dependent, in contrast to Lm.OVA where priming occurs in the spleen (139, 140). Changing the location of priming did not alter the HP-memory cell response during competition.

Changing the pathogen did not alter the kinetics of the immune response or the outcome of the competition between the HP- and true-memory cells. In the same way, use of the P14 TCR transgenic HP- and true-memory $CD8^+$ T cells specific for LCMV (Fig. 5B) yielded similar results to those obtained from the OT-I

cells responding to Lm.OVA infection. Thus, the inability of HP-memory to compete with true-memory was not pathogen, route or TCR specific.

TRAIL, a secreted TNF family member is upregulated by “helpless” memory $CD8^+$ T cells, was also investigated as a possible cause for the HP-memory defect in the face of competition with the true-memory cells. Helpless $CD8^+$ memory cells are unable to undergo expansion upon secondary infection due to the lack of $CD4^+$ T cell help during the primary infection (83). A possibility was that following irradiation, the transferred naive $CD8^+$ T cells proliferated in the absence of $CD4^+$ T cell help (due to the lymphopenia), resulting in the formation of “helpless” HP-memory. The secondary response of the helpless memory $CD8^+$ T cells can be restored if TRAIL deficient $CD8^+$ T cells are used (80). We then also attempted to rescue the defective secondary response by the HP-memory cells in our competition model system with the use of TRAIL deficient HP-memory cells. Even with the use of TRAIL deficient $CD8^+$ T cells, the HP-memory cells still deferred to true-memory cells (Fig. 5C).

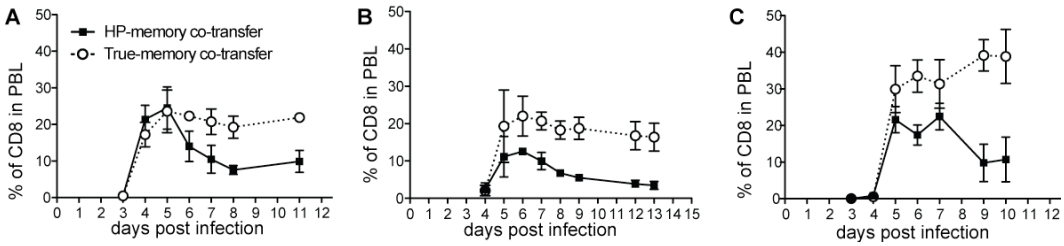

Figure 5. Competition between HP- and true-memory cells is observed in alternate infection systems and is not TRAIL dependent.

Co-transferred populations were identified by congenic markers. The percentage of donor cells among total $CD8^+$ cells in the PBL were measured. (A) HP- and true-memory cells generated as described in Fig. 3 were co-transferred into B6 hosts followed by infection with VSV.OVA. (B) HP- and true-memory P14 cells were co-transferred into a CD45.1 host followed by LCMV infection. (C) TRAIL-deficient HP- (CD45.2) and true-memory (CD45.1.2) OT-I were co-transferred into CD45.1 hosts followed by infection with Lm.OVA. Representative of 2 or more experiments (n=3). Error bars indicate SD.

In the absence of a competing memory subset, the HP-memory response largely mirrored that of the true-memory cells, accumulating to similar levels and forming equivalent amounts of secondary memory in the PBL (Fig. 3C, E, F). There were minor differences in the contraction phase, where the HP-memory peaked earlier than the true-memory cells; however they consistently formed robust “secondary” memory. The differences observed in the PBL between the single and competing memory cell transfers were reflected in the splenic analysis (Fig. 3D, E).

These results raised the possibility that the HP-memory cells were not able to compete well with true-memory cells in spite of their ability to expand, protect, and form secondary memory when they serve as the sole antigen-specific population (Fig. 3C, E). To assess the competitive fitness of the HP-memory cells compared with other T cell subsets besides the true-memory cells,

we next compared their ability to respond to infection with naïve $CD8^+$ T cells bearing a TCR with the same specificity (Fig. 3G, H). As was previously shown by Hamilton et al., the HP-memory cells had an improved response compared to the naïve cells and outcompeted them upon co-transfer (88). The HP-memory cells expanded first, and to a greater degree, and ultimately formed more secondary memory than naïve T cells in both single and co-transfer experiments (Fig. 3G, H). Interestingly, the ability of HP-memory cells to out-compete naïve T cells was not as robust as the true-memory subset, which formed a greater percentage of secondary memory when mixed with naïve T cells (Fig. 3I). Thus, the HP-memory out-compete naïve cells of the same specificity by providing more rapid expansion, greater secondary memory formation (Fig. 3G, H), and enhanced protection (88). The presence of naive OT-I cells did not hinder the ability of the HP-memory cells to respond to infection.

The presence of the true-memory cells appeared to dampen the expansion of the HP-memory cells. What HP-memory cell defects surface in presence of the true-memory population? To determine the cause behind the absence of the HP-memory cells from the blood and spleen, we examined the HP-memory cells for a proliferation or apoptotic defect in the presence of the true-memory cells.

A decrease in proliferation by the HP-memory cells would indicate that competition with the true-memory cells suppressed their division. Proliferation of both memory subsets was monitored by BrdU incorporation on days 4-7 after

infection, which incorporated the expansion and contraction of the immune response. During this period no significant difference was observed between the two memory subsets, but in both the spleen and lymph nodes, the true-memory cells consistently tended to incorporate more BrdU than their HP-memory counterparts (Fig. 6A, B). While moderate, this difference could translate into the significant decrease in accumulation seen in the PBL by the HP-memory cells (Fig. 3B). The initial discrepancy would be magnified as effector cells proliferate quite extensively (4-6 divisions per day) (141).

In addition to decreased proliferation, the HP-memory cells could also be subjected increased signals for apoptosis in the presence of competition with the true-memory cells. Death of donor cells was measured by TUNEL and Annexin-V flow cytometry assays (Fig. 6C-F) in the spleen and lymph nodes. During the process of apoptosis, nuclear DNA is fragmented by endonucleases. In the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) assay, the broken ends of the DNA are labeled with biotin conjugated dUTP using the enzyme deoxynucleotidyl transferase. In this way, the amount of fragmented DNA corresponding to apoptosis, can be quantified with FACS. To confirm the results of the TUNEL assay, Annexin-V staining was performed. Annexin-V binds the membrane lipid phosphotidylserine (PS), which is normally found on the inner face of the plasma membrane. However, in cells undergoing apoptosis PS is exposed on the outer surface. An annexin-V positive cell would indicate that it is apoptotic. However, as a cautionary measure, annexin-V

staining is usually done in conjunction with 7-AAD staining, which stains DNA. A cell that is both annexin-V and 7-AAD positive is an indication that it is in the late apoptotic stage. Though it may appear that there is a certain skewing towards the true-memory having increased annexin-V staining, these results were not consistent. Overall both the TUNEL and annexin-V staining did not reveal any significant trends in apoptosis differences between the memory subsets.

In essence, the presence of the true-memory cells had a slight negative effect on the proliferation of the HP-memory cells, which was magnified by the number of divisions undergone by the memory subsets. This defect was reflected by the kinetics measured in the blood and spleen where there was a decrease but not an elimination of expansion by the HP-memory cells, as the death rate of the HP-memory cells was not affected by the presence of the true-memory cells.

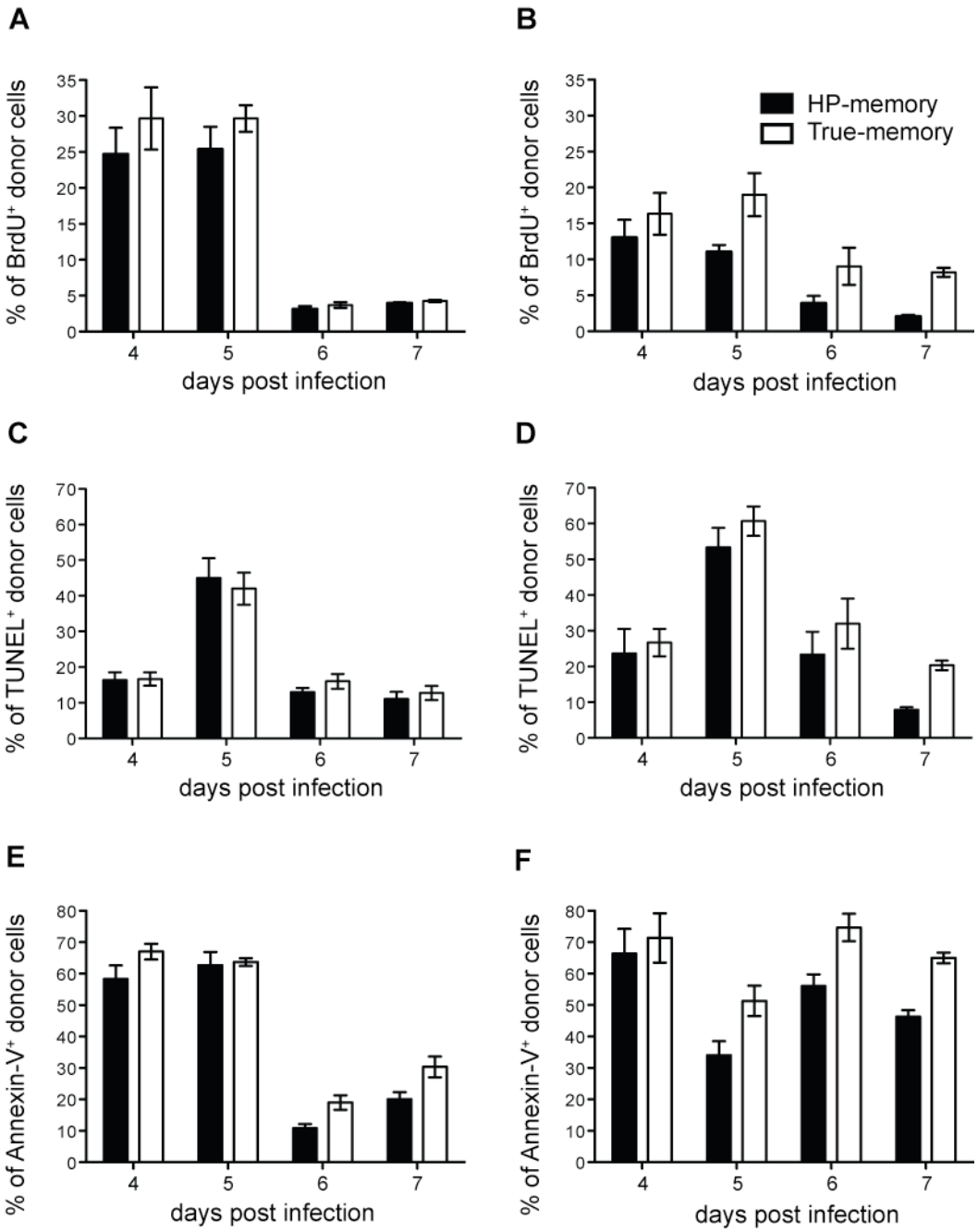

Figure 6. Comparison of the HP- and true-memory cell death and proliferation rates following co-transfer and infection.

BrdU incorporation, Annexin-V staining, and TUNEL staining were measured for the memory subsets in the co-transfer experiment described in Fig. 3. The percentage of donor cells that were: (A) $BrdU^+$ in the spleen, (B) $BrdU^+$ in the lymph nodes, (C) $TUNEL^+$ in the spleen, (D) $TUNEL^+$ in the lymph nodes, (E) Annexin-V^+ in the spleen, (F) Annexin-V^+ in the lymph nodes. Representative of 5 or more experiments. Error bars indicate SD.

Taken together, these data suggest that during infection, the HP-memory cells are at a disadvantage when competing with their true-memory counterparts for shared resources, in spite of their ability to out-compete naïve T cells. The HP-memory cells divided less in the presence of true-memory cells although the mere presence of a true-memory subset does result in the death of the HP-memory cells. Thus, we find that while the HP-memory population is phenotypically and functionally similar to true-memory, it is not an exact substitute.

Localization of HP-memory versus true-memory $CD8^+$ T cells during infection.

In the light of the previous data which showed that the HP-memory cells did not exhibit a dramatic defect in either proliferation or cell death, altered localization by the HP-memory cells was explored as a possible means to account for their failure to accumulate in spleen and blood during co-transfer.

We examined multiple tissues for the presence of both subsets following co-transfer. The first time-point examined was 24 hours after transfer but prior to infection where it was observed that the HP- and true-memory cells had similar access to peripheral tissues, displaying a 1:1 ratio in all tissues examined (Fig. 7A). This implies that at least initially, the HP-memory subset had equal access to antigen in all tissues examined compared with the true-memory cells.

Interestingly, after infection we observed that the ratio of the transferred cells began to favor the true-memory subset by 3-4 fold in all tissues evaluated with the exception being that of the lymph nodes (Fig. 7A, B). By day 4-5 of infection, it became evident that the true-memory cells had an advantage over the HP-memory cells. This increased population of true-memory cells left behind an increased number of secondary memory cells, so the advantage of the true-memory cells over the HP-memory cells was sustained through day 30 of infection and beyond. As mentioned, in striking contrast to the other tissues, the HP-memory cells were the predominant population in the lymph nodes early in the response (Fig. 7A, B) and this advantage persisted into the memory phase (Fig. 7A).

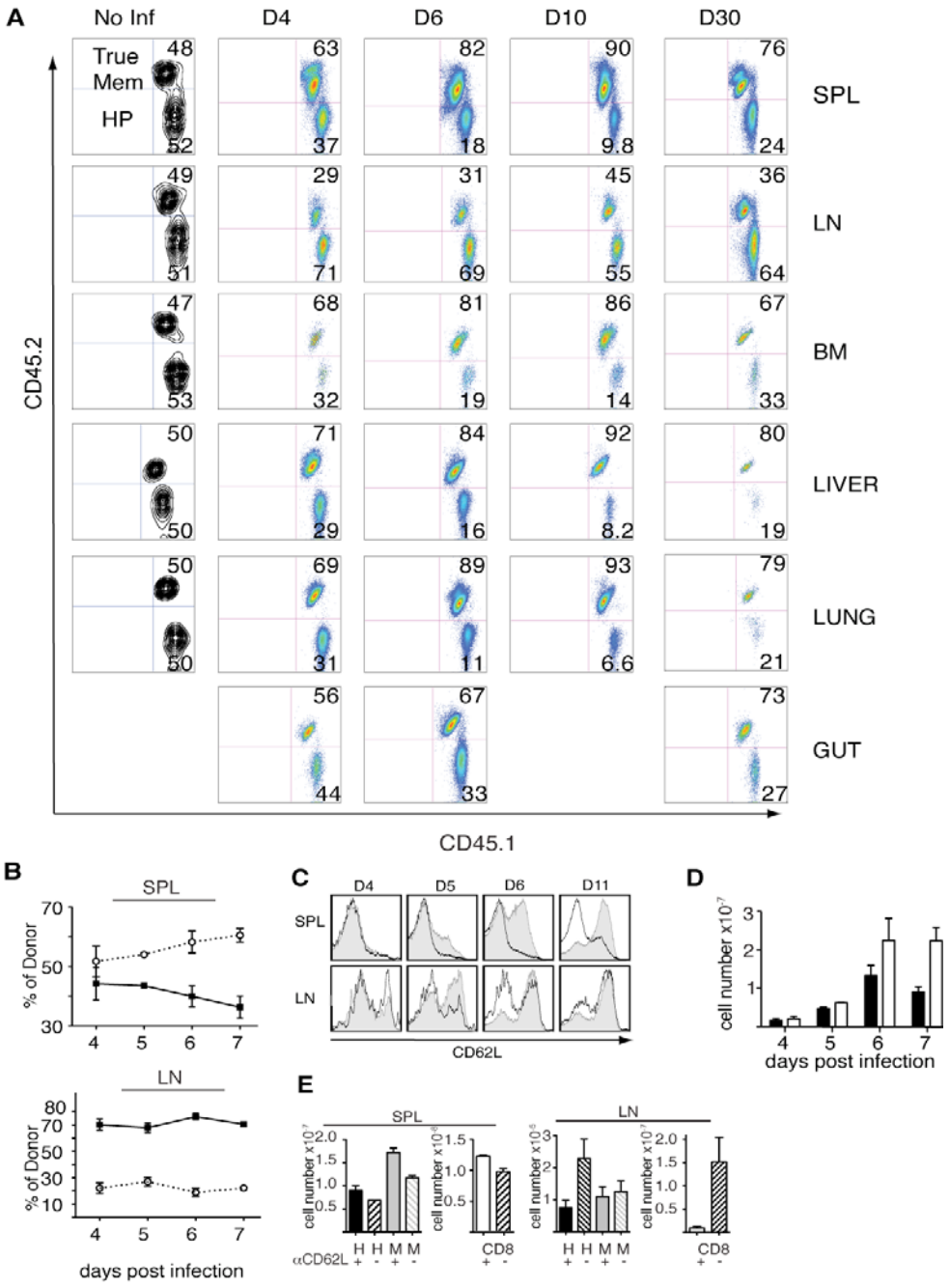

Figure 7. Defect in HP-memory cell accumulation is not due to peripheral localization.

(A) Relative accumulation of the donor subsets in the spleen (SPL), lymph nodes (LN), bone marrow (BM), liver, lung and gut after infection. (B) % of each donor subset (HP solid, True-mem dotted). (C) CD62L expression on co-transferred subsets (HP gray, True-mem white). (D) Average cell numbers from pooled spleen and lymph nodes (HP black, True-mem white). (E) Total cell numbers for HP- or true-memory cells from the SPL (left) and all recovered LN (right) following treatment with anti-CD62L or PBS. Host $CD8^+$ T cell numbers in separate graphs (right). Rep. of >3 expts. (n=3). Error bars indicate SD.

The preference of the HP-memory cells for homing to the lymph nodes correlated with their rapid upregulation of CD62L, a selectin that mediates entry into the lymph node (142). Upregulation of CD62L serves to facilitate trafficking to the peripheral lymph nodes where activation by APCs can occur (143, 144). Notably, prior to infection, the co-transferred HP- and true-memory subsets displayed equivalent trafficking to peripheral tissues (Fig. 7A) and possessed similar CD62L levels (Fig. 4).

After infection, both HP- and true-memory cells initially expressed low levels of CD62L (Fig. 7C), an indication that both cell types encountered antigen, as CD62L is downregulated upon $CD8^+$ T cell activation and differentiation to effector cells (145). However, by day 6, more than 50% of the HP-memory cells were $CD62L^{HI}$ compared to only ~10% of the true-memory cells (Fig. 7C). Of note, both $CD62L^{HI}$ and $CD62L^{LO}$ true-memory populations can out-compete HP-memory cells with similar efficiency (data not shown). This suggests that relative ability to compete is not dependent on differences between the T_{CM} and T_{EM} memory subsets.

In the lymph nodes, a higher percentage of the HP-memory population consistently expressed higher levels of CD62L compared with the true-memory cells from days 4-6 after the infection (Fig. 7C). The percentage of true-memory cells that expressed CD62L was not equivalent to that of the HP-memory cells until day 11 after infection. In tissues other than the lymph nodes such as the

spleen, the levels of CD62L expression between the two subsets never achieved parity, with the HP-memory cells always expressing higher CD62L levels than their true-memory counterparts. Furthermore, the HP-memory cells appeared to exhibit accelerated acquisition of CD62L when compared to the true-memory cells (146).

Additionally, on day 6 of infection, the surface phenotype of the effector cells generated from HP-memory cells showed a moderate increase in CD127 and CD27 as well as a decrease in KLRG1 expression compared to effector cells derived from true-memory cells (Fig. 8). As noted, prior to infection, the memory subsets expressed dissimilar levels of CD49d, but following infection there was no longer significant differences in CD49d levels (Fig. 8).

After an extensive examination of the phenotype following infection of the two memory cell types, we conclude they overall had similar phenotypes with the major exceptions of KLRG1, CD127, and CD62L. Whether the expression of these markers are an indication of the defect in competition by the HP-memory cells or contribute to the inability of the HP-memory cells to compete remains to be clarified.

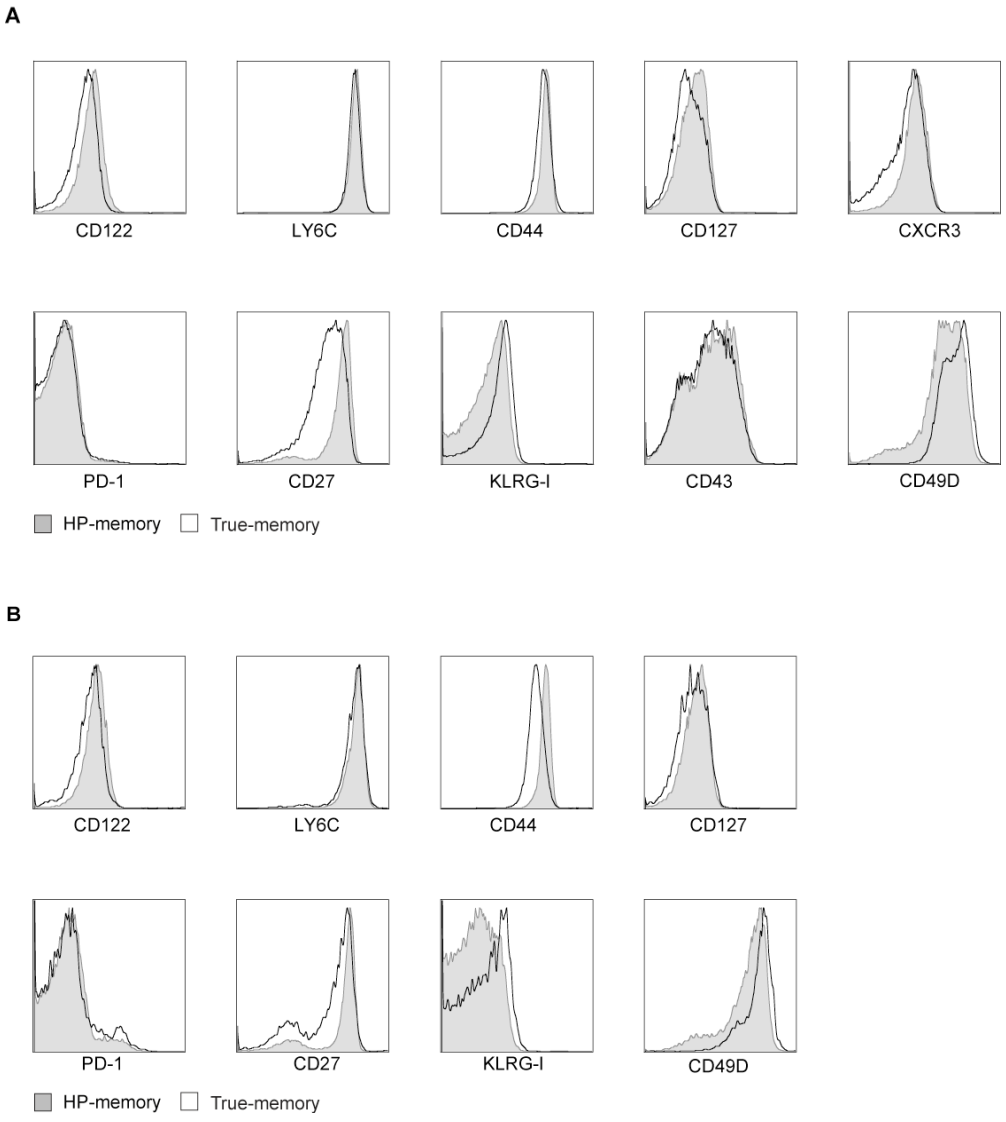

Figure 8. Phenotype of the co-transferred memory subsets on day 6 after infection.

Phenotype of memory subsets from co-transfer recipients on day 6 of Lm.OVA infection. Comparison of indicated cell surface markers in the (A) spleen (B) lymph nodes. Representative of 5 or more experiments (n=3-5).

To determine whether the increased numbers of HP-memory cells in the lymph nodes offset the loss of cell numbers in spleen and peripheral tissues, we compared donor cell recovery after co-transfer and infection. Compared with prior

lymph node and spleen extractions for cell number counting (Fig. 3F), this experiment was much more comprehensive. All physically obtainable lymph nodes were extracted including the cervical, axillary, mediastenial, mesenteric, and inguinal nodes to ensure that the increased number of HP-memory cells in the all lymph nodes could be accounted for. Even so, at day 7 of infection, more than 2-fold more true-memory than HP-memory cells accumulated in spleen and all recoverable lymph nodes (Fig. 7D), arguing that the loss of HP-memory in the spleen and tissues represented diminished accumulation and formation of secondary memory rather than lymph node relocation.

Nevertheless, it was a possibility that some lymph nodes were overlooked during the extraction process. In order to eliminate the chance that we were simply not recovering all of the HP-memory cells located in the other lymph nodes, we treated mice with anti-CD62L to block all trafficking of lymphocytes to the lymph nodes during our experiment. Following treatment, total numbers of $CD8^+$ T cells decreased for both memory subsets in the lymph nodes (Fig. 7E, left), indicating effective antibody blocking. However, prevention of homing to the lymph node by the HP-memory cells did not result in an increased recovery in the spleen. The numbers of HP-memory cells increased slightly in the spleen, but were still not restored to the levels of true-memory cells (Fig. 7E right). While CD62L expression may have played a role in the alternative localization pattern of the HP-memory cells to the lymph nodes, it did not explain the systemic failure

of HP-memory cells to accumulate in all of the other tissues and form an equivalent number of secondary memory cells.

HP-memory cells have a distinct localization pattern in the spleen after infection.

The distinct localization exhibited by the HP-memory cells in the various tissues led us to examine their localization within the spleen in more detail. The spleen consists of three main compartments: the red pulp (RP), marginal zone (MZ), and white pulp (WP). The WP consists of the T cell zone or periarteriolar lymphoid sheath (PALS) enclosed by the B cell zone. These compartments are not separated by strict boundaries and are not fixed in size but expand and contract in response to infection. The resident cells in the MZ depend on each other to maintain the integrity of the MZ through chemokine gradients (147).

Figures 9-11 depict cross sections of the spleen with emphasis on the PALS following co-transfer and infection. Anatomically, the RP surrounds the MZ and WP and forms the majority of the spleen. Interactions between the T cells and DCs occur in the T cell zone, leading to the activation of the T cells. During Lm.OVA infection, DCs migrate from the RP to the PALS to present antigen (148). From the histology of the PALS in the spleen from Figure 9, we observed that early during infection (day 2), HP- and true-memory cells were localized to the PALS. This was indicated by co-localization with $CD4^+$ T cells. Anti-CD4 was used to delineate the PALS as the $CD4^+$ T cells also occupy the T cell zone along with $CD8^+$ T cells (Fig. 9A-C). The localization of the two memory cell subsets

corresponded to what has been previously observed for a $CD8^+$ memory response where upon Lm infection, memory CD8+ T cells rapidly migrate to the PALS and undergo proliferation (149).

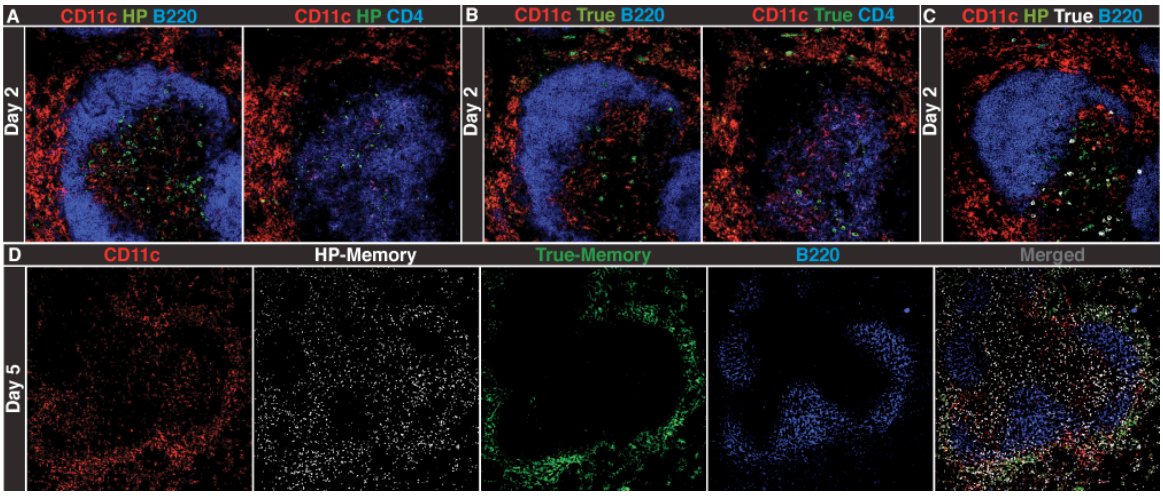

Figure 9. Localization of HP- and true-memory cells relative to $CD11c^+$ cells.

Confocal images of serial spleen sections from co-transfer recipients. (day 2 of infection). Sections were stained for CD11c, B220 or CD4, and congenic markers for: (A) HP-memory, (B) True-memory, (C) HP- and true-memory. (D) Spleen (day 5) was stained for CD11c, both memory subsets, and B220, 10X. Images are taken at 20X magnification unless noted. Representative of 3 experiments (n=2-3) per time point.

In our experimental system, we have designated the $CD11c^+$ cells as being the DCs even though it is found on other cells (150). This is because during Lm infection, DCs are the target population of Lm infection and are responsible for carrying the Lm.OVA to the spleen (139). From the histology, the $CD11c^+$ cells were found in the PALS, but also accumulated peripheral to the MZ in the RP.

We did not observe any noticeable differences in localization by the HP- and true-memory cells between days 1-2 of infection (Fig. 9, 11). This indicated that the HP-memory cells were initially located appropriately in the PALS for antigen recognition, consistent with Figure 3A, where no defect in expansion was observed during the first 3 days of infection, and Figure 7C, where the HP- and true-memory cells expressed lower levels of CD62L, an indication that the cells have been activated.

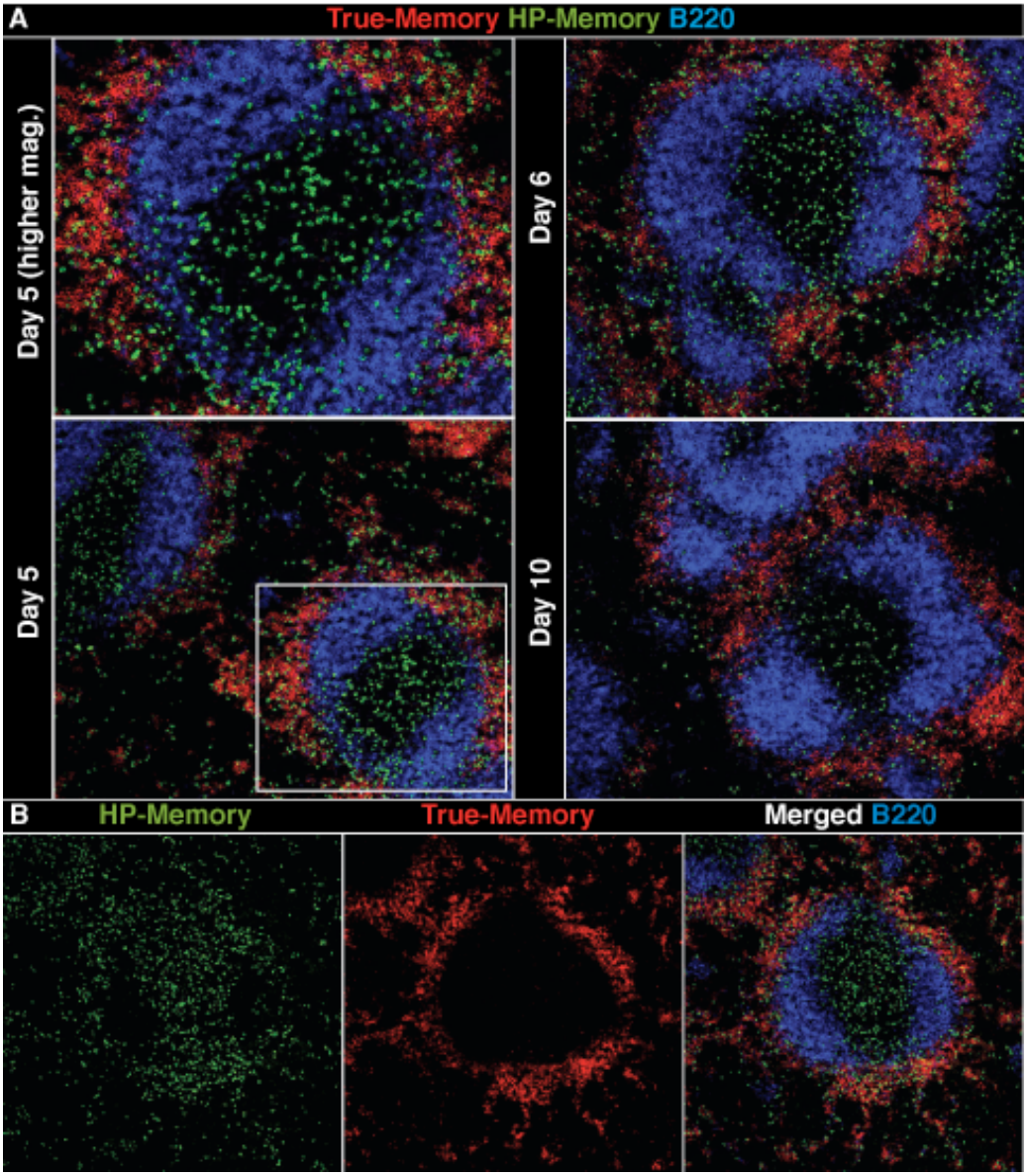

Figure 10. HP- and true-memory cells display distinct localization and clustering in the spleen.

(A) Spleen sections of co-transfer recipients on days 5, 6 and 10. Stained for both memory subsets and B220. (B) Panels with the indicated stains and merged image of spleen (day 5). Images are taken at 10X. Representative of 3 experiments (n=3) per time point.

Strikingly, it was evident by day 5 of infection that the true-memory cells had localized to the MZ/RP with the $CD11c^{+}$ cells, apparently exiting through the

bridging channels, similar to previously reported observations (Fig. 9D, 11) (149). In contrast, the HP-memory cells remained largely in the PALS. This striking difference between HP- and true-memory cell localization within the spleen was accentuated as the infection progressed (Fig. 10, 11). By day 5 of infection, the true-memory cells were already largely absent from the PALS and accumulated in the RP/MZ along the edge of the MZ (Fig. 10A, 11). This could be partly due to the fact that the T cells were migrating to the site of infection, since it has been shown that the Lm infected cells are maintained in the MZ (148). Alternatively, T cells also need continuous signals from the DCs, of which we observed significant clusters in the MZ. In contrast, the HP-memory cells were still abundant in the PALS and also scattered throughout the RP and even the B cell zone (Fig. 9-11).

The localization of the HP-memory cells to the B cell zone is unusual because T cells do not normally traffic there. The germinal centers in the B cell zone are the locations where B cells undergo clonal expansion, isotype switching, and somatic hypermutation. Interestingly, $CD8^+$ memory T cells (~30 days post Lm.OVA re-infection) eventually localize to the B cell follicles (149) and this corresponds with the HP-memory cells acquiring a memory phenotype earlier than the true-memory cells and localizing to the B cell follicles (Fig. 10).

Of the HP-memory cells that exited the WP, there was not a specific area where they localized. HP-memory cells were rarely found in clusters of more than 2-3 cells and only a portion co-localized with the true-memory cells along the

MZ/B cell follicle edge (Fig. 10A, B). At 10 days after infection, significant numbers of HP-memory cells remained in the PALS while true-memory cells were observed mainly in the RP/MZ regions (Fig. 10A, 11).

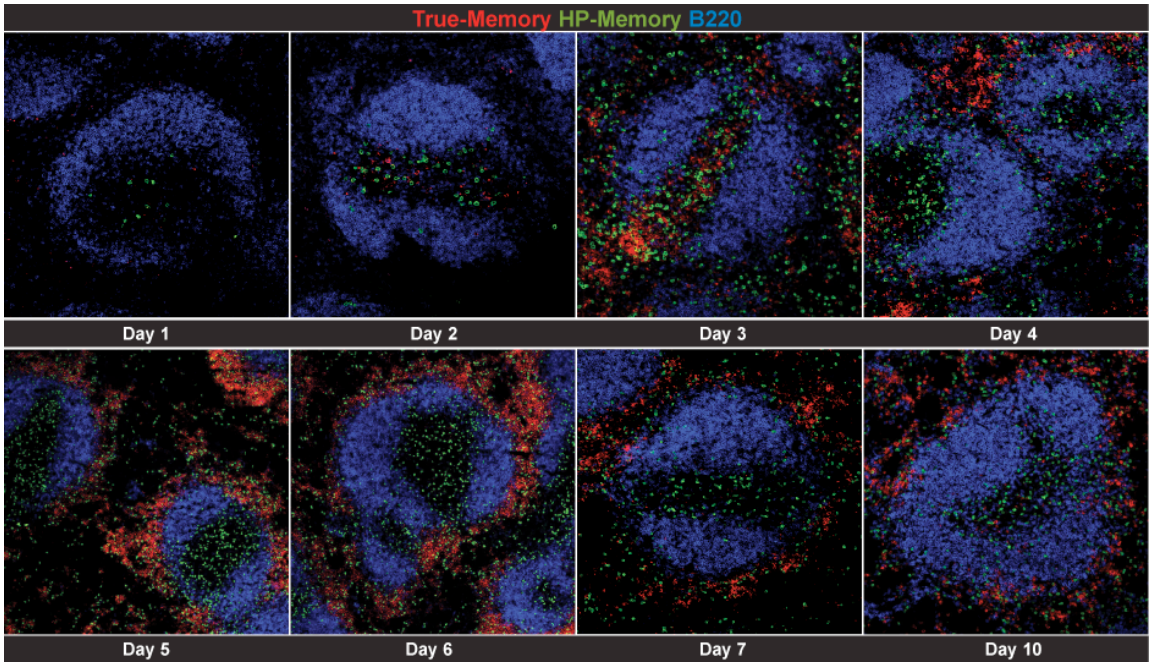

Figure 11. Timecourse of HP- and true-memory cell localization following co-transfer.

HP- and true-memory were co-transferred into B6 hosts and infected with Lm.OVA. Localization of donor cells in the spleen on days 1-10 after infection was evaluated by staining for CD45.1, Thy1.1, and B220. Representative of 5 or more experiments (n=3).

Under conditions of competition, the HP-memory cells still localized appropriately to the PALS at the beginning of the response. However, trafficking and localization of the HP-memory cells differed dramatically from true-memory cells later in the infection, indicating that the HP- and true-memory cells received distinct signals at some point in the response. Single transfer of HP-memory cells not competing with true-memory cells demonstrated an intermediate phenotype,

with increased numbers of cells in the RP along the MZ/B cell follicle edge during the peak of the response but not to the extent exhibited by the true-memory cells (single and co-transfer) and also with cells located in the PALS (Fig. 12). This would indicate that the competition from the true-memory cells exacerbated a defect in the HP-memory cells, as their aberrant trafficking is even more noticeable during competition. It is undetermined whether the altered trafficking is behind the decreased secondary memory formation by the HP-memory cells.

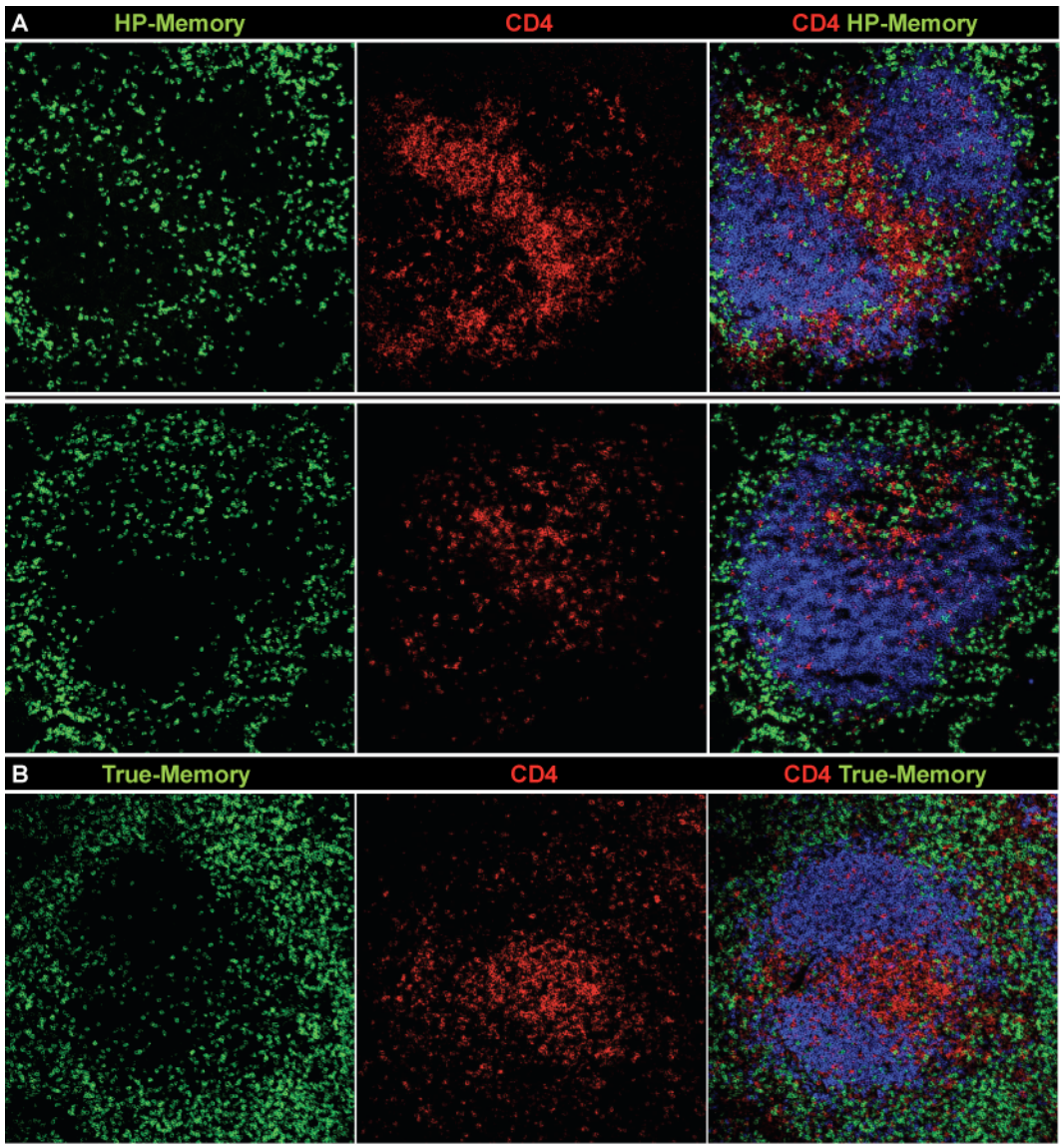

Figure 12. Localization of the HP- or true-memory subsets following single transfers.

HP- or-true memory cells were transferred separately into B6 hosts as shown in Fig 3A. Localization of donor cells in the spleen was evaluated by staining for CD45.1, CD4 and B220. (A) Single transfer of HP-memory cells on day 6 of infection. (B) Single transfer of true-memory cells on day 5 of infection. Images are taken at 20X and are representative of 2 or more experiments (n = 2-3).

Differential mRNA expression for chemokine receptors by HP-memory cells.

To determine the cause of the differential trafficking by the HP-memory cells (Fig. 9-11), we investigated possible differences in chemokine receptor expression by the two memory subsets. We used a chemokine/chemokine receptor qPCR array and tested mRNA extracted from cells on day 6 of infection, when there were clearly significant differences between the localization of the subsets (Fig. 3, 9-11). HP- and true-memory cells were sorted from pooled spleens of co-transfer recipients. Relative mRNA levels for 84 genes, comprised primarily of chemokines and chemokine receptors, were evaluated by qPCR. Figure 13 summarizes the target genes that have 2-fold or greater difference in expression between HP- and true-memory cells. Of particular interest were the chemokine receptors CXCR4, CXCR5, CXCR7, and CCR7, which have known roles in lymphocyte homing in secondary lymphoid organs (Fig. 13).

CXCR4 and CXCR7 both bind to CXCL12, a chemokine found in the splenic RP and LN medullary cords (151 , 152). These two chemokine receptors are co-expressed on T cell subsets, but are not characteristically expressed by $CD8^+$ T cells (151). Plasma cells, B cells and DCs typically express high levels of CXCR4 (153). CXCL12 signaling plays a role in mediating plasma cell positioning in the spleen through the heterodimerization of CXCR4 and CXCR7 (151). CXCL11 also binds to CXCR7, but with lower affinity than CXCL12 (154).

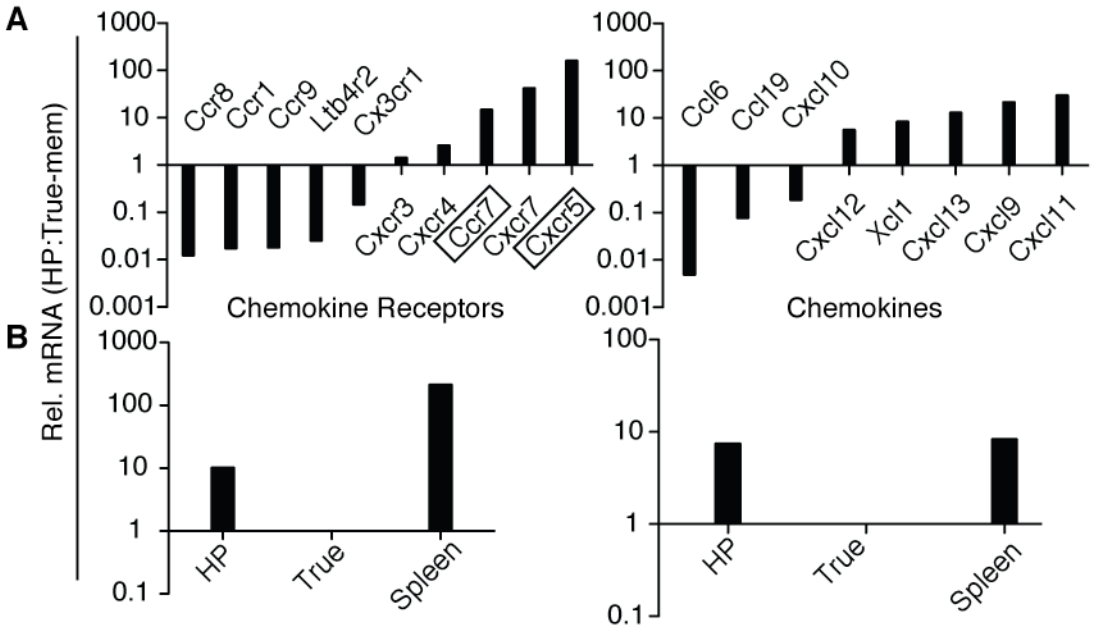

Figure 13. mRNA levels of chemokines and chemokine receptors by HP- and true-memory cells.

cDNA was generated from co-transferred HP- and true-memory cells sorted from pooled spleen (day 6). Relative mRNA levels for indicated genes were determined with a qPCR array and normalized to GAPDH. (A) The transcripts displaying a 2-fold or greater difference in expression were listed and further divided into receptors (left) and chemokines (right). (B) qPCR verification of CXCR5 and CCR7 mRNA levels. mRNA from total spleen used as a reference. Representative of 2 co-transfers tested >3 independent times.

The HP-memory cells expressed ~10-fold more CXCR5 mRNA than the true-memory controls (Fig. 13A, B left). While the function of CXCR5 on $CD8^+$ T cells is not well characterized, CXCR5 expression on $CD4^+$ T helper cells mediates their localization to the B cell follicle (155). CXCR5 is also expressed on B cells and skin derived DCs. The CXCR5 ligand CXCL13 is secreted by follicular DC (FDC) and stromal cells located in the B cell zone (156). CXCL13 typically works to attract B cells to the B cell follicles. Upregulation of CXCR5 could explain why the HP-memory cells were found to traffic there. CXCR5 signaling

induces the expression of lymphotoxin-$\alpha_1\beta_2$ (LT-$\alpha_1\beta_2$) on the B cell surface. LT-$\alpha_1\beta_2$ in turn induces the differentiation of FDC which produce more CXCL13, resulting in a positive feedback loop (156, 147).

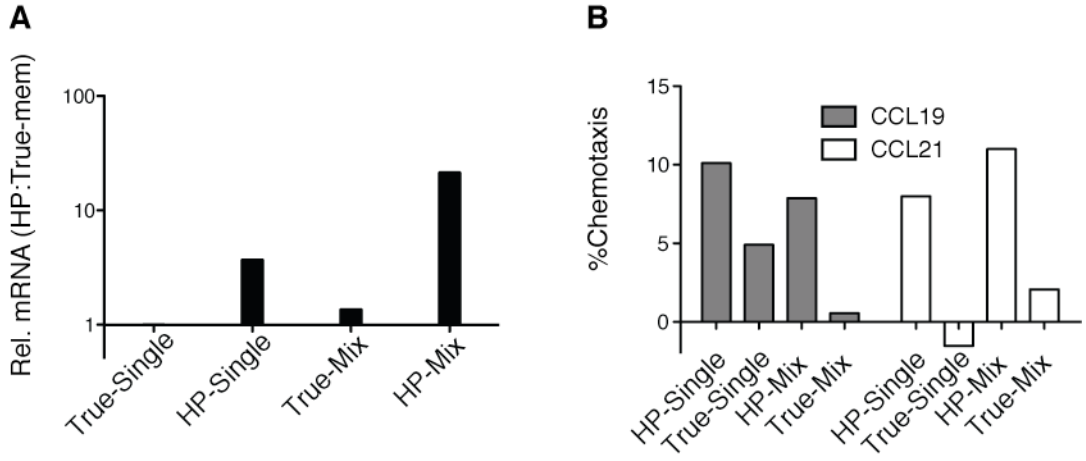

Figure 14. CCR7 mRNA expression and chemotaxis for memory subsets.

(A) Expression of CCR7 mRNA by memory subsets from single or co-transfers. Memory subsets were sorted from indicated transfers on day 6 of infection. Relative expression of CCR7 mRNA are shown compared to the true-memory single transfer population. (B) Percent of absolute cell numbers for each memory subset that migrated in response to CCL19 (1 µg/mL), CCL21 (3 µg/mL) in 3 hr at 37°C: HP-memory single transfer, true-memory single transfer, HP-memory co-transfer and true-memory co-transfer.

CCR7 was also upregulated on the HP-memory cells (Fig 13A, B right, 14A). CCR7 and its ligands CCL19 and CCL21 mediate T cell and DC homing and positioning in the T cell zone. CCL19 and CCL21 are secreted by DC, macrophages, and stromal cells in the T cell zone (155). Increased expression of CCR7 and chemokine receptors that mediate homing to the B cell follicle may explain why a significant portion of the HP-memory cells were retained in the PALS and B cell zone and why they displayed disorganized localization compared to the true-memory cells. After priming, downregulation of CCR7

occurs so that effector $CD8^+$ T cells can leave the WP and enter the blood stream for migration to the peripheral site of infection. Higher expression of CCR7 on the HP-memory cells was validated through a chemotaxis assay (Fig. 14B) using CCL19 and CCL21, chemokines that are specific for CCR7. The HP-memory cells did migrate more heavily towards these chemokines than the true-memory cells. This indicates that they do have higher expression of CCR7 and corroborates the qPCR data. The HP-memory cells also have higher levels of CCR7 expression as a single transfer compared with the true-memory single transfer (Fig. 14A). A comparison of the CCR7 expression levels on the HP-memory single transfer versus the HP-memory mix transfer shows that CCR7 expression is slightly higher on the single transfer (Fig. 14A).

Competition between HP- and true-memory cannot be fully rescued by provision of excess antigen or survival cytokines.

While many signals are necessary to support the expansion of T cells during infection, antigen and cytokines were obvious candidates for the basis of competition between the HP- and true-memory subsets, particularly considering the differential localization of the two cell types. Thus we attempted to rescue the HP-memory cell defect during competition by providing them increased resources through different means.

Since the true-memory cells colocalized with the DCs, the possibility existed that they had better access to the antigen being presented and to costimulation by the DCs. In order to alleviate this advantage by the true-memory

cells, we immunized the mice following mixed transfer with OVAp and LPS administered i.v. This allowed us to provide antigen in excess and without bias of professional APCs. The OVAp immunization provided antigen that would coat many different APCs not only DCs, and the HP-memory cells would have equal access to antigen even if they could not access the DCs. Secondly, immunization results in a surplus of antigen, which the HP-memory cells may not have access to if the true-memory cells kill the infected APCs (157). The expansion of the HP- and true-memory cells in response to OVAp and LPS was measured in the PBL (Fig. 15A). Here, we found that the HP-memory cells were still at a disadvantage compared to true-memory cells, indicating that the defect was not the result of a failure to compete for antigen in the early phase of the immune response.

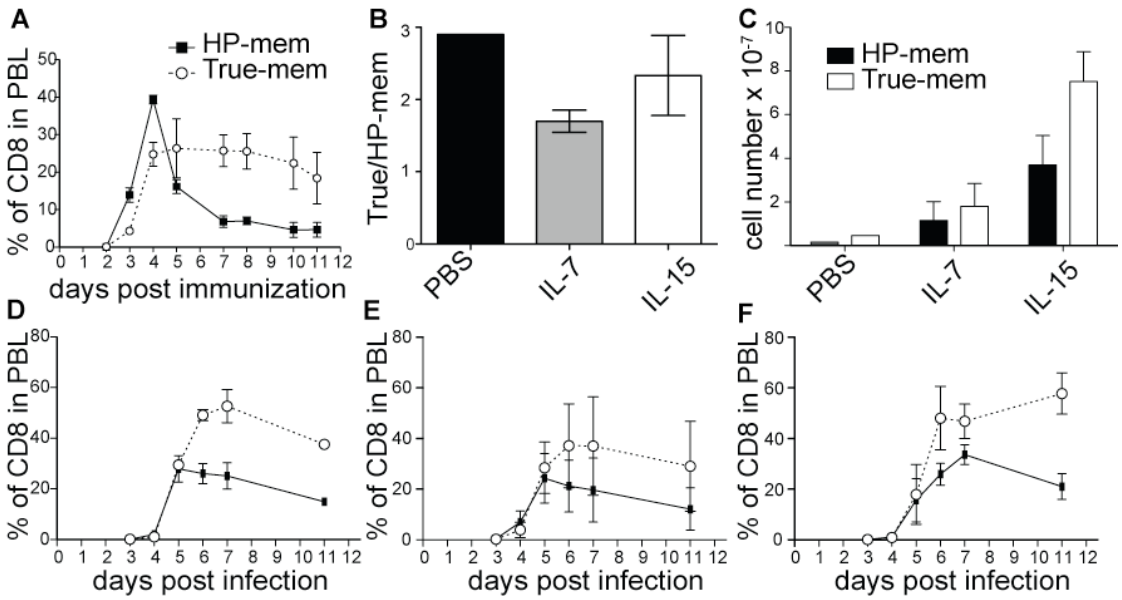

Figure 15. The role of antigen and cytokines in the defective competition by HP-memory cells.

(A) Percentage of donor cells among total CD8$^+$ cells, PBL: OVAp and LPS treated. (B) Ratio of the percent of true-memory to HP-memory cells in spleen on day 10 of infection, recipients treated with PBS, IL-7/anti-IL-7 mAb or IL-15/IL-15Rα complexes. (C) Total cell number of each memory subset (day 10 Lm.OVA) treated with (D) PBS (E) IL-7/anti-IL-7 mAb (F) IL-15/IL-15Rα complexes. Representative of at least 3 experiments (n=3). Error bars represent SD.

Besides antigen, other factors that the two cell types could compete for are cytokines. The common γ-chain cytokines IL-7 and IL-15 both are known to support accumulation of CD8$^+$ T cells during contraction of the immune response (158, 121). Thus, we tested whether providing cytokines in excess by administering IL-7/anti-IL-7 mAb or IL-15/IL-15Rα complexes could rescue the HP-memory cells (121). Cytokine complexes because IL-7/anti-IL-7 mAb and IL-15/IL-15Rα complexes have enhanced activity over their solo cytokine counterparts (121, 120, 159). Addition of exogenous IL-7/anti-IL-7 mAb and IL-15/IL-15Rα complexes led to an increase in numbers of both memory subsets

compared to PBS-treated controls, an indication that the cytokine treatments were effective (Fig. 15B, C). While we typically observed a three-fold difference between the true- and HP-memory, following IL-7/anti-IL-7 mAb treatment the fold difference decreased to two-fold. However, neither treatment altered the overall kinetics of the HP-memory expansion or secondary memory formation (Fig. 15D-F). In the case of the IL-7/anti-IL-7 mAb complex, the differences in the ratio, percentage and number of true- versus HP-memory cells revealed a partial rescue in the accumulation of the HP-memory cells following treatment (Fig. 15B-E). Otherwise, this correlated with what was previously shown with IL-7 complex treatment since its provision favors the survival of the KLRG1^{LO}CD127^{HI} CD8^{+} T cell subset (121) and the HP-memory cells at day 6 post infection had a similar phenotype (Fig. 8). Since IL-7 resulted in only a partial rescue, this would indicate other cytokines were involved in the maintenance of the HP-memory subset.

We next administered IL-15/IL-15Rα complexes from day 3 to 7 post infection (Fig. 15B, C, F). IL-15 has been shown to contribute to the magnitude of the CD8^{+} T cell expansion during the primary response (56). The addition of the IL-15/IL-15Rα complexes boosted cell numbers of both memory subsets, but the HP-memory subset still expanded less than the true-memory. While HP- and true-memory cells displayed similar expression of the receptors for these cytokines (Fig. 8), it was possible that the HP-memory cells did not compete well for limiting amounts of cytokines or gain access to the cytokine signals in light of their altered localization. Administration of the IL-15/IL-15Rα complexes also

resulted in an increase of cells following contraction. The enhanced survival of the cells post contraction correlates with what has been previously reported but did not increase the amount of secondary memory formation by the HP-memory cells (121). The effects of the IL-15/IL-15Rα complexes appear to be skewed in favor of the survival of the true-memory cells.

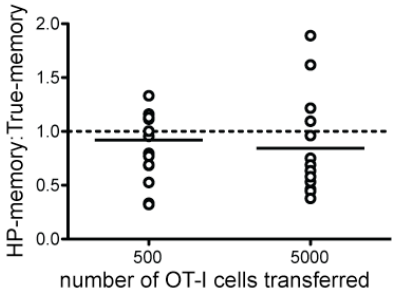

Figure 16. The role of precursor numbers in the HP-memory immune response.

The ratio of HP-memory to true-memory following transfer of 500 and 5000 memory cells day 10 post transfer and infection with Lm.OVA.

In addition to the lack of IL-7 or IL-15 and lack of access to antigen, many other factors such as inflammatory factors, other cytokines, growth factors, or environmental resources could serve as the limiting resource that the HP-memory cells lack during the immune response (160). While it is difficult to identify the specific factor involved, we provided the HP-memory cells with increased resources overall by decreasing competition. This was done by lowering precursor transfer frequencies. Badovinac et al. showed that a high transgenic precursor frequency suppresses the endogenous response (113).

However, there is also intra-clonal competition between the transferred transgenic cells that occur. If the endogenous cell response is altered by increased precursor frequency, then the response of the transgenic cells could also be too. To decrease the competition for resources between our memory subsets, we transferred decreasing numbers of cells, more similar to “physiological" levels of memory cells- 500 or 5000, though it has been shown that memory precursors are found at a much higher frequency than naïve cells (161). We performed a co-transfer at a 1:1 mixture of HP- and true-memory cells, and followed the response to infection with Lm.OVA in the PBL (data not shown). Despite high variability, which is likely due to differential take of transferred cells at low numbers, our results suggest that HP- and true-memory cell populations were nearly equivalent with a ratio near 1:1 (Fig. 16B). With increasing transfer numbers, even as low as the increase from 500 to 5000, the HP-memory cells are outcompeted by the true-memory cells. Thus, diminished numbers of competitors resulted in an HP-memory response more similar to what was observed when they were the sole responding population, supporting the idea that the HP-memory cell competition defect is not due to a dominant, inhibitory signal delivered by the true-memory cells. Instead of directly acting on the HP-memory cells to suppress their expansion, the true-memory cells may compete indirectly; by more efficiently utilizing resources and preventing the HP-memory cells from expanding to their full capacity. These data show that in cases of

diminished numbers of competitor cells, HP-memory cell expansion and memory formation can be rescued.

As for whether our original memory precursor transfer numbers (100,000-800,000) were physiologically relevant, as Seedhom et al. showed that a mouse previously immunized with vaccinia virus contained ~1,538,462 specific memory precursors (161). A titration has not been done for Lm infection, but we would argue that the amount of memory precursors that we transfer into each mouse is physiologically relevant.

Infection does not reprogram HP-memory cells to compete in subsequent immune responses

Our results indicate that a memory cell derived from HP does not receive full "programming" provided by antigenic stimulation when exposed to lymphopenia. This is supported by the microarray performed on the HP- and true-memory cells (129) which showed that the HP-memory cells expressed lower levels of genes related to effector function such as granzyme B, IFN-γ and perforin. How important is it for HP-memory cells to undergo this effector phase and how this affects their ability to compete effectively with the true-memory cells which have undergone this phase is not known.

During the generation of HP-memory cells, they lack exposure to infection and differentiation into a CTL. We were interested in whether antigenic exposure would rescue the HP-memory defect when competing with true-memory in a recall response. HP- and true-memory cells were mixed at a 1:1 ratio and then

transferred to a B6 host followed by Lm.OVA infection. More than 30 days later, the memory populations were re-sorted and normalized to a 1:1 ratio and then retransferred to a B6 host that was subsequently infected (Fig. 17A). The kinetics of the immune response was followed in the PBL of infected hosts. Even after exposure to antigen, the HP-memory cells gave rise to “secondary” memory cells that did not compete effectively with secondary memory cells derived from the true-memory subset, expanding less and forming fewer tertiary memory (Fig. 17B).

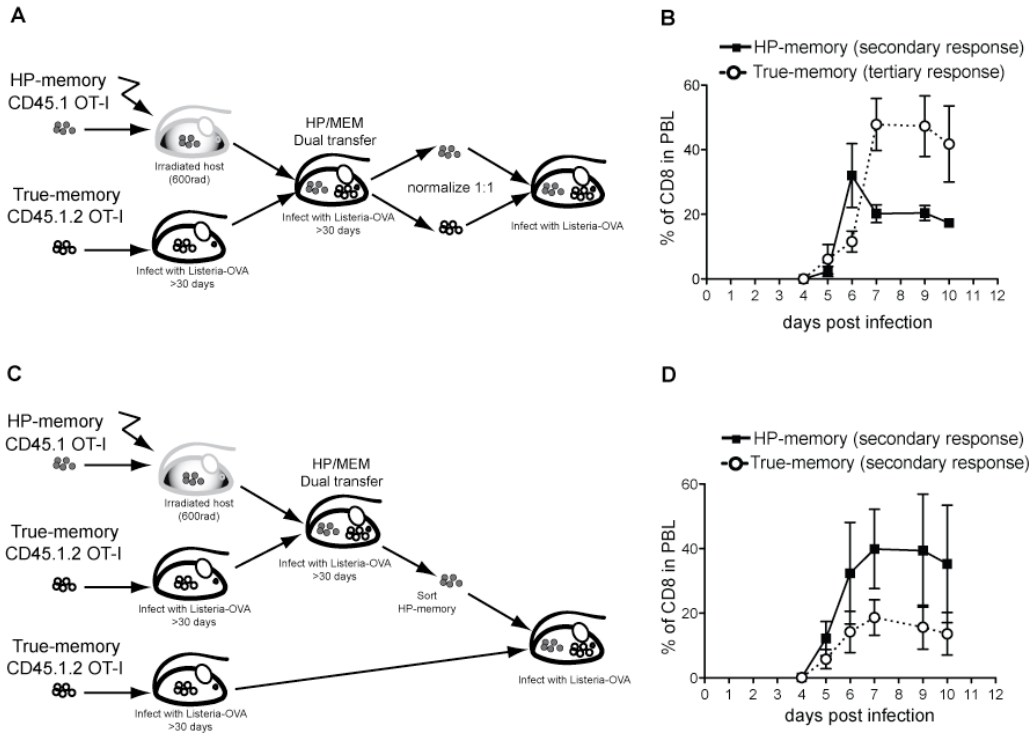

Figure 17. Re-exposure to antigen does not rescue defective competition by the HP-memory cells in the tertiary response.

(A) The co-transferred subsets were infected and allowed to normalize before sorting and re-transfer into a new host. (C) HP-memory cells from co-transfer and infection were sorted and mixed with true-memory cells generated from a primary infection. The immune response of the following memory subsets was measured as a percentage of donor cells among total $CD8^+$ cells: (B) tertiary true-memory and secondary HP-memory and (D) secondary true-memory and secondary HP-memory. Data are representative of 3 experiments (n=3). Error bars indicate SD.

We wanted to determine whether additional antigen exposure opportunities would remedy the HP-memory cell defect in competition with the true-memory cells. We next conducted experiments using the secondary memory formed from the HP-memory. As diagramed in Figure 17A, the true-memory cells were undergoing their tertiary challenge with antigen while the HP-memory cells were undergoing their secondary challenge (Fig. 17A, B). Since it has been shown that multiple antigenic exposures can enhance the memory response to

subsequent infections (146, 162), we sought to equalize the number of antigenic exposures each subset would encounter before rechallenge (Fig. 17C). In this scenario, HP-memory cells were sorted from a co-transfer with true-memory following infection and have been exposed once to antigen. True-memory cells derived from a primary infection, which have also seen antigen one time, were co-transferred into a new host and re-challenged. Under these conditions, the HP-memory cells out-compete the true-memory cells, with the true-memory population expanding less and forming fewer secondary memory cells (Fig. 17D).

These data indicate that secondary memory cells derived from HP-memory cells following challenge with Lm.OVA were still defective in some manner, which exposure to antigen could not rectify. The initial programming underwent by the HP-meory cell persists for the life of the cell (Fig. 17B). This emphasizes that a hierarchy exists between memory subsets, with the number of exposures to antigen enhancing a cell's ability to compete (146, 162). It also indicates that the HP process confers an advantage to a responding memory subset. This enhanced function however, is not equal to actual antigen exposure, but is better than no exposure at all as seen in the naïve $CD8^+$ T cell response. (Fig 3F)(88).

HP-memory cells undergo diminished basal homeostatic proliferation.

As memory T cells survive for extended periods of time while undergoing a slow turnover in the absence of antigen, we were also interested in the longevity of HP-memory and also how efficiently the HP-memory cells competed with true-

memory cells during this process of basal homeostatic proliferation. In a lymphoreplete environment without antigen, basal levels of IL-7 and IL-15 promote survival and homeostatic turnover (158). To test if HP- and true-memory compete equally for the limiting IL-7 and IL-15 in a basal homeostasis environment, we transferred HP- and true-memory cells at a ratio of 1:1 or alone into naïve, congenically distinct hosts, and the percentage of donor cells among the PBL was monitored for >100 days (Fig. 18). In mixed transfers, the true-memory subset slowly accumulated and accounted for the majority of the donor cells (Fig. 18A). The two subsets started off at a ~1:1 ratio, but by days 40-60 after transfer, the true-memory cells began to show an advantage over the HP-memory cells. By 120 days post transfer, CFSE dilution by transferred memory cells in the spleen showed that both populations underwent multiple rounds of division, but the true-memory underwent 2-3 more divisions than the HP-memory cells over the course of the experiment (Fig. 18B). This difference in basal homeostatic proliferation was reflected by the increased proportion of true-memory cells in the donor population (Fig. 18D), while the HP-memory subset remained relatively stable (Fig. 18C).

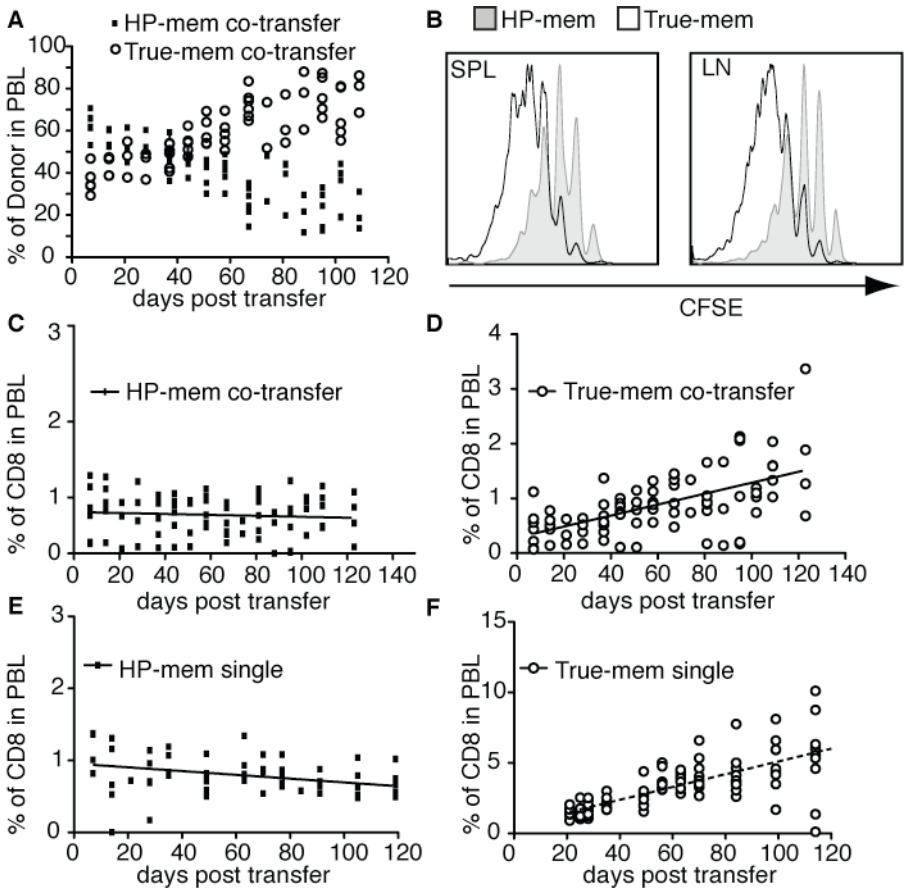

Figure 18. True-memory cells accumulate and show increased basal homeostatic proliferation compared to HP-memory cells in the absence of infection.

Monitoring of the HP/true-memory subsets (PBL) after transfer into naïve B6 hosts without infection. (A) Percent of donor cells in PBL after co-transfer. (B) CFSE detection of the co-transferred memory cells in the spleen and lymph nodes at 120 days post transfer. Percent of the indicated subset among total $CD8^+$ T cells: (C) Co-transfer: HP-memory (D) Co-transfer: true-memory (E) Single transfer: HP-memory (F) Single transfer: true-memory. Representative of 2 experiments (n=5). Error bars indicate SD.

Interestingly, a similar accumulation by the HP-memory cells was observed when the subsets were transferred separately (Fig. 18E, F). This data serves to emphasize that the presence of the true-memory subset does not affect the survival of HP-memory cells. The HP-memory cells in both the context of a

single transfer and mixed transfer divided and accumulated in a similar manner. Likewise, the true-memory cells accumulated in a similar manner whether it was in the context of a single or mixed transfer. The presence of a competing memory subset does not seem to affect the viability of the other memory subset in a negative manner.

Thus, in the absence of infection, the HP-memory cells possessed the ability to survive and compete for resources when in competition with true-memory cells, but they did not divide and accumulate to the levels observed by the antigen-experienced memory T cells. The ability of HP-memory cells to survive in the absence of infection is not affected by the presence of co-transferred true-memory cells (Fig. 18C, E), perhaps because they were required to compete with host cells within the memory compartment for cytokines or niches irrespective of whether OT-I true-memory cells were present.

Chapter 2 contains portions of the material as it appears in the Journal of Immunology, “Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells.” Cheung, Kitty P., Yang, Edward, Goldrath, Ananda W., September 2009 Sep 1;183(5):3364-72. The dissertation author is the first author of this paper.

DISCUSSION

The memory T cell compartment is heterogeneous, comprising of various phenotypic and functional subsets, including cells that acquired these properties not only through antigen exposure but also as a result of HP. Lymphopenia-induced HP occurs early in neonatal life, after some acute and chronic infections, and can be the result of lymphotoxic therapies. These memory-cell "doppelgangers" provide improved protection over the naïve response (88), but their integration into the memory T cell compartment and possible interference with the immune response of true-memory cells has not been investigated. Acting as the sole antigen-specific population, HP-memory cells respond to infection and form a 'secondary' memory population much like true-memory cells (Fig. 3C, E). However, HP-memory cells were out-competed in both basal conditions and secondary infection if true-memory cells of the same specificity were present (Fig. 3B, D and Fig. 18).

Early on, HP-memory cells co-localized with and expanded similarly to their true-memory counterparts, but contracted earlier and to a greater extent (Fig. 3, 9-11). Coinciding with this contraction, HP-memory cells displayed altered localization compared to true memory cells; remaining in the PALS, infiltrating the B cell follicle, and exhibiting a diffuse localization pattern (Fig. 9). Thus, the HP-memory subset contributed less to the 'secondary' memory population than the true-memory subset, revealing a competitive hierarchy whereby true-memory

cells respond more efficiently than HP-memory cells and both memory subsets out-compete naïve T cells of the same specificity during infection (Fig. 3G-3I).

Attempts were made to rescue the HP-memory cell response in the presence of true-memory cells through the addition of exogenous IL-7 and IL-15, and excess antigen in the form of peptide/LPS immunization, but neither were successful (Fig. 12). Additionally, the secondary memory population formed from these two memory subsets was examined for defects in the context of competition. Secondary memory cells derived from HP-memory cells following Lm.OVA infection still deferred to the secondary memory derived from true-memory cells, indicating that exposure to antigen and infection did not enhance the ability of HP-memory cells to compete (Fig. 14B). However, in the course of competition between secondary HP-memory cells and primary antigen-induced memory cells, secondary HP-memory cells were successful at outcompeting the true-memory. These data further support the existence of a $CD8^+$ memory response hierarchy. The HP process leaves a lasting imprint on naïve $CD8^+$ T cells, as well as the secondary memory cells generated from them, allowing them to outcompete true-memory cells that have not undergone HP.

The impact of initial programming on the memory response

The process of HP confers an advantage to HP-memory cells, allowing them to outcompete naïve $CD8^+$ T cells during infection, but this same HP process does not promote programming that allows HP-memory cells to compete fully with true-memory cells. This likely derives from the fact the signals

promoting HP- and true-memory cell differentiation are quite distinct. The initial naïve $CD8^+$ T cell response to infection and subsequent formation of a memory population requires TCR-mediated recognition of high affinity pathogen-derived peptide/MHC-I molecules on professional APCs, costimulatory signals and inflammatory cytokines (Fig. 1A) (158). In contrast, acquisition of a memory phenotype by naïve cells during lymphopenia-induced HP is triggered by low-affinity interactions with self-peptide/MHC class I complexes and IL-7 and is independent of costimulatory signals (Fig. 1B) (163, 75, 6). Nonetheless, previous experiments found that HP-memory cells share a similar gene-expression profile with true-memory cells (129) and exhibit true-memory phenotypic markers and functional characteristics, indicating that these disparate signals received during formation can initiate many common differentiation pathways (88, 6, 163). Thus, it was surprising that HP-memory cells deferred to the true-memory cells during infection, clearly highlighting the fact that these two memory subsets are distinct.

During its initial priming phase, a naïve $CD8^+$ T cell undergoes a programming regimen that determines its effector response and ultimately its commitment to a memory lineage (164). Many factors impact T cell programming including the type of infection, amount of inflammation, degree of costimulation, $CD4^+$ T cell help, and the cytokine environment (164). T cell programming has far reaching circumstances, affecting the proliferation, function, and trafficking of memory cells formed. In light of the severe trafficking differences observed

between our two memory subsets, how does the location of their initial priming affect their secondary response?

It has been repeatedly demonstrated that the location of priming affects the subsequent trafficking patterns of the memory cells formed. $CD4^+$ T cells primed in the mesenteric lymph nodes have been shown to preferentially home to the intestinal mucosa, while $CD4^+$ T cells primed in the inguinal and brachial lymph nodes home primarily to the skin (165, 166). In our experimental system, true-memory cells were derived from naïve $CD8^+$ T cells primed in the spleen (148). This is in contrast to lymphopenia-induced HP where efficient priming requires the presence of the lymph nodes (17, 167). Dummer et al. showed that HP can occur in the spleen in the absence of lymph nodes, but the HP-memory cells generated cannot mediate anti-tumor immunity (17). Since the location of priming affects the trafficking of the subsequent memory cells, the initial priming responsible for the formation of the HP- and true-memory cells can also impact their later trafficking. We see evidence of this in the preferential localization of the HP-memory cells to the lymph nodes (Fig. 7) and also their altered trafficking in the spleen (Fig. 9-11). While the exact location of priming with self-peptide during the HP process in the spleen is unknown, it may have an impact on the HP-memory formed.

A large portion of programming involves $CD8^+$ T cell interaction with the DCs. Under typical inflammatory conditions involving infection with a foreign pathogen, DC maturation requires the engagement of TLRs with pathogen and

costimulatory interactions with $CD4^+$ T cells for the generation of an effective $CD8^+$ T cell response (168). Whether all these elements occur during irradiation to generate appropriately licensed DCs is a matter of debate. While it has been shown that total body irradiation exerts pro-inflammatory effects by inducing the secretion of cytokines such as TNF-α, IL-12, and IL-18, IL-1β as well as the release of LPS into the bloodstream (125, 169, 170), this inflammatory effect on the activation of DCs is less clear. Are the levels induced by irradiation equivalent to the levels found during infection and what effect does that have on the DCs? According to the "Goldilocks" model put forth by Masopust et al. (164), too little inflammation may result in memory attrition, while too much stimulation may result in deletion. Additionally, the naïve cells formed during HP are proliferating in response to low-affinity self-peptide rather than high-affinity foreign antigen (58, 57). All this results in HP-memory cells that can respond to secondary infection, but are unable to compete as well with true-memory cells, which were formed under more optimal conditions.

Additionally, the apoptosis mediated by irradiation differs from what occurs during infection. Following irradiation, most apoptosis occurs as a result of either free radical-mediated DNA damage and stress caused by lipid membrane damage (171). Infection induced apoptosis is the result of a myriad of mechanisms among them: activation-induced cell death mediated by CD95 and TNFRI, expression levels of IL-7R, and IFN-γ exposure, are all implicated in modulating contraction (48). We cannot eliminate the possibility that the dying

cells during irradiation exert a negative effect on the surviving cells and affect the function of the HP-memory cells that result.

Secondary response: the effects of programming

We previously discussed some of the factors that play a part in mediating the programming that naive $CD8^+$ T cells undergo upon initial activation but what is response of the alternately programmed cells during competition? To summarize, there are three main differences in the kinetics of the HP-memory versus the true-memory response to Lm.OVA: (1) HP-memory cells are initially at a higher percentage in the PBL, (2) HP-memory contraction occurs before the true-memory cell contraction, and (3) HP-memory cells form less secondary memory cells (Fig. 3B, D). Interestingly, the kinetics of the immune response exhibited by the $CD8^+$ T cells stimulated with low-affinity ligands reflect the behavior of HP-memory cells during competition with true-memory cells following infection. Zhen et al. showed that $CD8^+$ T cells stimulated with a lower-affinity ligand appear in the blood and start contraction earlier compared to cells that had been stimulated with the high-affinity ligand. Meanwhile, the cells stimulated with the high affinity ligand also expand to a higher magnitude (172).

These parallels between the HP-memory cells and $CD8^+$ T cells stimulated with low-affinity ligand pose a conundrum for us: both of the memory subsets we examined express the same TCR and should have equal affinity for Lm.OVA, and both memory populations were in the same recipient and infected concurrently. So why do HP-memory cells exhibit a shortened response

compared to true-memory cells? One possibility is that the program induced upon rechallenge is different relative to the responding memory subsets. While the TCR affinity of each of the memory subsets may not change, the functional avidity of the cells could change (173), upon reinfection, the HP-memory cells then behave as if they have experienced priming with lower-affinity antigen, resulting in an inferior response compared to that of true-memory cells.

The above discussion provides a partial explanation for the behavior of HP-memory cells- that their initial programming during the HP process predisposed them to a less than optimal response during reinfection. Oftentimes, defects are difficult to detect due to compensatory signals from other pathways, such as when the lack of costimulation can be overcome with increased amounts of inflammation (174, 175).

We recognize that there is an improved response to antigen exhibited by the HP-memory cells during single transfer infection. This would imply that competition with the true-memory cells during the response affects their behavior. In the absence of competition, the HP-memory cells still showed signs of chaotic localization (Fig. 12) but received adequate signals for secondary memory formation (Fig. 3C, D). The intermediate localization pattern exhibited by the HP-memory cells in the single transfer agreed with the findings that CCR7 mRNA expression was not fully rescued and chemotaxis toward CCR7 ligands was retained in the single transfer (Fig. 14).

Several lines of evidence suggested that the HP-memory cells do not undergo the full memory program during lymphopenia-induced proliferation; their altered kinetics, localization, and expression of chemokine receptors, all of which occur during competition with true-memory cells during infection. Regulation of the HP-memory cells may not be antigen or TCR specific, since we have tried various pathogens and routes of infection to enhance cell access to antigen but none were successful. The HP-memory cells may have been compromised during their formation, upregulating a molecule that designates them for regulation by the host. This was observed during the basal homeostatic proliferation of the HP-memory cells which underwent slower turnover than the true-memory cells, comprising less of the memory compartment over time (Fig. 18).

The basis of competition: Peptide/MHC class I complexes and DC access.

HP- and true-memory cells function differently even if they are phenotypically indistinguishable. We performed multiple experiments attempting to elucidate the mechanism behind how HP-memory cells fail to compete with true-memory cells. A major source of competition for the cells during infection is for access to antigen. However, a differentiation must be made between whether the access is for antigen or the APC itself. The HP-memory cells could theoretically have access to the DCs but no access to the peptide/MHC class I molecules if they were hoarded on the surface of the DC by the true-memory cells (108). It would appear from the kinetics in the PBL and tissue data (Fig. 3B,

D) and the histology sections (Fig. 9-11), that HP-memory cells have access to cognate peptide/MHC class I as they are initially activated. Examination of our data show that both the memory $CD8^+$ T cell subsets appear to have equal access to the DCs presenting antigen (Fig. 9A), but it is unclear whether they have access to the antigen itself. Staining with an anti-OVA antibody would be illuminating, but we were unable to obtain adequate staining with the OVAp/MHC class I complex antibody. However, there also appears to be evidence of suboptimal activation of the HP-memory cells, as some cells remain in the T cell zone, while other cells are able to leave but not to the appropriate locations (Fig. 9-11). Thus, while we did not observe differences in DC access, HP-memory cells could still be lacking access to other factors needed for optimal priming.

Lack of access to the DCs would imply that the HP-memory not only lack access to antigen, but to growth factors that the DC produces in the form of cytokines, costimulation or inflammatory signal. To ensure that the HP-memory cells had DC access in its entirety, we provided excess DCs through peptide-pulsed DC to immunize and activate competing HP- and true-memory cells, but the results were inconclusive (data not shown). Another method that we uses to ensure that HP-memory cells had adequate access to antigen and DCs was to increase the dose of Lm.OVA used for infection. Mice were originally infected with 5000 pfu and at this dose we were able to observe the inability of the HP-memory cells to compete with the true-memory cells (data not shown). For all successive experiments, we increased the dosage to 10^5 pfu/mouse because this

should also lead to a proportional increase in the number of activated DCs. The increased antigen led to an enhanced response by both subsets, but the HP-memory cells still deferred to the true-memory cells. While the increased dosage should theoretically induce more DCs, we realized that a live infection is difficult to control and quantify. We cannot determine the numbers, locations, and the nature of the APCs that were generated through infection, so it is difficult to quantify the immune response. Due to the nature of the live infection, it was difficult to conclude whether using increased antigen dosage enhanced HP-memory access to the DCs.

We also used peptide/LPS immunization in an attempt to rectify the APC access problem. Immunization solves two issues: first, access to peptide is no longer limiting because the immunization will result in saturating amounts of peptide. Second, since many cell types are coated with peptide and not just DCs, the HP-memory cells should not have impaired access to antigen. The administration of LPS would also ensure both memory subsets had equal access to inflammatory signals. Following peptide/LPS immunization (Fig. 15A), we saw that the expansion and secondary formation of the HP-memory cells was still decreased in comparison with the true-memory cells. While immunization provides a way to ensure all cells have antigen access, the inflammation induced is not equivalent to the amount produced by a live infection (48).

Duration of DC access

HP-memory cells might have equal access to the DCs, but a possibility exists that they could be unable to maintain sustained contact with them, and therefore not receive the appropriate signals for activation. It has been shown that sustained contacts between the T cell and APC influence how efficiently memory formation occurs (176). $CD8^+$ T cell memory formation, but not expansion, is dependent on ICAM-1 expression by DCs. We found that the HP-memory cells expressed normal levels of LFA-1, the ligand for ICAM-1 (data not shown) (176). We did not test other molecules that may be involved in sustaining TCR contact with the APC. However, regardless of the duration of contact, the function of the responding cells remains intact (177). This implies that although less secondary memory will form from HP-memory cells, they will still be effective.

Integrins were another potential factor that could alter trafficking and adherence between the two cells. Prior to infection, the HP-memory cells expressed lower levels of CD49d, an integrin expressed by activated T cells, which mediates cell adhesion and trafficking. (130). However, blocking CD49d (178) did not eliminate the true-memory advantage or significantly alter localization (Fig. 19). This was perhaps not surprising because multiple factors and integrins are needed to facilitate trafficking and localization.

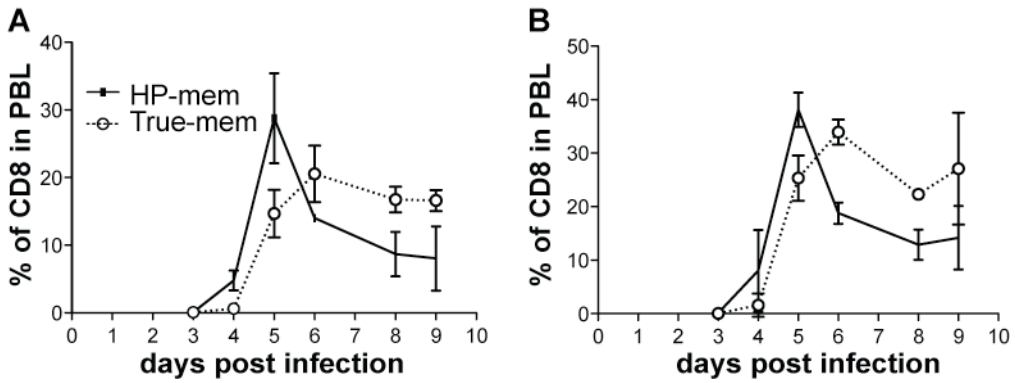

Figure 19. Treatment with anti-VLA4 does not rescues the HP-memory cells.

Percentage of donor cells among total $CD8^+$ cells measured in the PBL following Listeria.OVA infection and treatment with (A) PBS and (B) anti-VLA4 (CD49d) blocking antibody. Representative of at 2 experiments (n=3). Error bars represent SD.

Precursor frequency can alter competition patterns

Among the many elements that enable memory cells to be much more efficient during reinfection are that they exist at higher precursor ratios than naive cells and require a much lower threshold for activation. Competition for antigen is likely to be fostered among the higher numbers of memory cells compared to the naïve T cell compartment. To decrease the likelihood of competition, we transferred lower numbers of memory precursors into the naïve B6 host. We do not believe that our original transfer numbers of 100,000-800,000 were excessively high because the memory compartment can contain high numbers of memory cells specific for different pathogens (161), but the increased numbers do promote competition. By decreasing the transfer to a total of 500-5000 cells, we attempted to restore the ability of the HP-memory cells to expand equally to the true-memory cells. The decrease in precursor numbers did appear to restore

the 1:1 ratio that the cells were first transferred with (Fig. 16B). As the number of precursors transferred increased to 5000 and above, the ratio very quickly began to be skewed toward the true-memory cells (Fig. 16B).

The only caveat with this experiment was that at such low precursor transfer frequencies, it was difficult to ensure that the HP- and true-memory cells were initially at an equal 1:1 ratio. The cell numbers could very easily be skewed towards one population if the cells were not adequately mixed and affected by the differential take of each cell subset. With a higher number transfer, we were able to look at the tissues for confirmation of equivalent ratios before infection (Fig. 7A), which was not possible with lower precursor numbers. This resulted in a very large spread, of which the average is close to 1:1 (Fig. 16). Due to the difficulty in confirming the equal transfer numbers during a low precursor transfer, it is hard to concretely conclude that decreased numbers affect the outcome of competition but this trend is there. These results show that the true-memory cells are not directly acting on HP-memory cells by exerting an inhibitory signal to prevent their expansion, as HP-memory cells appear to expand equally well to true-memory cells at these lower transfer numbers.

Competition for cytokines

Both IL-7 and IL-15 mediate memory cell survival during contraction and were possible candidates responsible for the advantage observed in true-memory cells (121, 158). Provision of either cytokine during infection was unable to fully rescue HP-memory accumulation, however, IL-7 complexes did improve

the accumulation of HP-memory cells (Fig. 15D-F). Thus, the paucity of IL-7 or IL-15 is not the sole basis for competition, and the signals they mediate fail to completely overcome the HP-memory defect.

Administration of IL-15/IL-15Rα complexes during contraction leads to the accumulation of KLRG-1^{HI}CD127^{LO} (SLEC) CD8^{+} T cells while IL-7/IL-7mAb complexes resulted in the accumulation of KLRG1^{LO}CD127^{HI} (MPEC) CD8^{+} T cells (42, 121). To alleviate the competition for IL-15, we administered IL-15/IL-15Rα complexes during the expansion and contraction phase of the immune response. Here, HP-memory cells tended towards the MPEC phenotype, while the true-memory cells were characterized by the SLEC phenotype, so one prediction would be that true-memory cells would be affected by an excess of IL-15 levels (Fig. 8). We have shown that administration of IL-15/IL-15Rα complexes during expansion did not alter the kinetics of the response, although it resulted in increased overall cell numbers (Fig. 15C, D, F). Confirming our hypothesis, it did appear that during the contraction phase, administration of IL-15/IL-15Rα complexes enhanced the survival of the true-memory cells (Fig. 15D, F). While the administration of IL-15/IL-15Rα complexes did not reveal what the two memory subsets were competing for, it did indicate that the true-memory cells were more affected by IL-15/IL-15Rα complexes than the HP-memory cells (121). This may explain why true-memory cells were able to generate more secondary memory than HP-memory cells. However, the memory subsets did not

appear to be actively competing for it as the HP-memory cells were not rescued by increased IL-15 levels.

We also provided excess IL-7 to alleviate possible competition for this cytokine by the two memory subsets. Administration of IL-7/IL-7mAb complexes resulted in a partial rescue of the HP-memory cells (Fig. 15B, E). From this, we hypothesize that IL-7 could serve as one of the potential factors that the HP-memory cells are lacking, but because a full rescue did not occur, this indicated that more than one factor was needed to restore the HP-memory cells to full expansion potential. The HP-memory phenotype on day 6 after infection shows that they have slightly higher expression levels of CD127 (IL-7Rα) (Fig. 8) indicating tendency by HP-memory cells towards the MPEC phenotype because they also were KLRG1^{LO} (42). This upregulation in turn may render HP-memory cells more sensitive to the increased levels of IL-7.

Besides the T cell compartment, IL-7/IL-7mAb complexes also promote the expansion of the B cell compartment (121). This may affect the expansion of both memory subsets since they must now to compete with an increasing B cell compartment. This is supported by the cell numbers as well as the kinetics of the infection with IL-7/IL-7mAb administration which show that while cell numbers expand, they did not expand as much as when IL-15/IL-15Rα complexes were added (Fig 15C, E). Interestingly, true-memory cells may be competing with the expanding B cell subset rather than HP-memory cells, and therefore their expansion is less effected by the IL-7/IL-7mAb complexes. In this way, true-

memory cells could actually be competing for different factors with the B cells and the HP-memory cells.

The designation of $CD8^+$ T cells as either SLECs or MPECs also raises some issues. HP-memory cells tend toward the MPEC phenotype, but we see that they do not respond as well as the true-memory cells to secondary infection during competition. This would imply that the HP-memory cells have an accelerated memory phenotype though they could not compete effectively with true-memory cells. These phenotypes may reflect a more intermediate phase that the effector cells pass through and more definitive work is needed to determine other markers that truly define cells destined to seed the memory compartment.

Role of inflammation

Inflammatory signals have a role in mediating T cell expansion. As previously reported and supported by our data, live infections generate more robust T cells expansions than immunizations with adjuvants (Fig. 3B, C, 15A) (179). This suggests that the increased inflammatory environment in the presence of foreign antigen facilitates much more expansion compared to that induced by vaccination. Some of the major inflammatory signals involved in the $CD8^+$ T cell response are IL-12, IFN-I, and IFN-γ (48). These signals can affect the APC, as well as the $CD8^+$ T cell itself. $CD8^+$ T cells exposed to IL-12 are enhanced in their ability to proliferate and mediate effector function (94, 180). IFN-γ is required for the expansion and formation of CTLs (92). Inflammatory signals also influence the ability of DCs to cross-prime (181). The amount of

inflammation a $CD8^+$ T cell is exposed to can skew it towards a particular memory phenotype. Attenuation of inflammation results in skewing of effector cells towards the MPEC phenotype and the T_{CM} phenotype (37, 42). While cells do not typically compete for inflammatory signals, the altered trafficking of the cells could result in them being exposed to different amounts of inflammation. Lm is able to spread from the MZ to the PALS by the migration of the DCs (148). The initial target of Lm infection is thought to be the macrophages in the MZ, and $CD11c^+$ DCs (182). The DCs are responsible for the delivery of the intracellular bacteria to the PALS and initiation of the T cell response by secretion of IL-12 (90). However, it is noted that the true-memory cells eventually co-localize with the DCs in the MZ/RP of the spleen, in comparison to the HP-memory cells that remain in the PALS (Fig. 9-11). The localization of the true-memory cells in the RP/MZ allows them access to inflammatory signals released by the infected macrophages and DCs not only at early stages of the infection in the T cell zone but also during the later stages of the response. It is possible that true-memory cells received increased inflammatory signals that skew them towards the SLEC phenotype, while the HP-memory cells could be skewed towards the MPEC phenotype and T_{CM} phenotype as they remain in the T cell zone.

Altered trafficking- a symptom or a cause?

While it is difficult to determine what variety of factors the two memory subsets are competing for, the outstanding result from this competition is that the two memory populations localize to different areas of the spleen, observable only

during the latter part of the response. The altered trafficking can contribute to the decreased capacity of HP-memory cells to expand and form secondary memory, or maybe simply a symptom of the HP-memory cell's inability to compete.

From histology, we observed that early after infection, HP- and true-memory cells co-localized in the PALS along with the DCs (Fig. 9A). This co-localization allowed for HP-memory cells to be primed, as $CD11c^{+}$ cells mediate Lm.OVA transit into the PALS (148). Indeed, the HP-memory cells adopted phenotypic changes typical of antigen exposure (Fig. 8) and their initial expansion rivaled the true-memory cells (Fig. 3B). However, localization of HP-memory cells within the spleen was dramatically altered by day 4 of infection (Figs. 9B, 10-11). The true-memory cells were located primarily in the RP, where significant CD11c staining was also observed (Fig. 9B). In contrast, HP-memory cells were scattered throughout the PALS, B cell zone and RP (Fig. 9-11), likely due to aberrant expression of chemokine receptors that direct cells to the B cell zone (CXCR4, CXCR5, CXCR7) or retain cells in the T cell zone (CCR7) (Fig. 13A, 13B right, 14).

We observed that the competition results in the divergence of the expansion and secondary memory formation patterns of the two subsets. Mechanistically, the trafficking of these two memory cell types in the spleen could contribute to the inability of the HP-memory cells to compete, or could just be the result of the HP-memory cells' inability to compete.

We propose two models to explain what the trafficking of the memory cells may indicate and how that may play a role in the expansion and formation of secondary memory cells. In the first model, HP-memory cells fail to receive signals that alter chemokine receptor expression early in the response due to competition from the true-memory cells (Fig. 20A). True-memory cells traffic to the MZ/RP and the HP-memory cells remain in the T cell zone. Their retainment in the PALS results in poor secondary memory formation because they lack of access to cytokine or other growth factors that the true-memory cells may receive when they traffic to the edge of the B cell follicle/MZ (Fig. 9-11). The DCs appear to extravisate along with the true-memory cells to the MZ while the HP-memory cells remain in the T cell zone for the entirety of the response after day 4 (Fig. 9-11 and data not shown). DCs are capable of storing antigen for up to several days, so while initial activation is important in priming, DCs can still continue to exert an impact on $CD8^+$ T cells several days later (183). Therefore, the HP-memory cells fail to receive signals needed for continued expansion and secondary memory formation due to their improper localization.

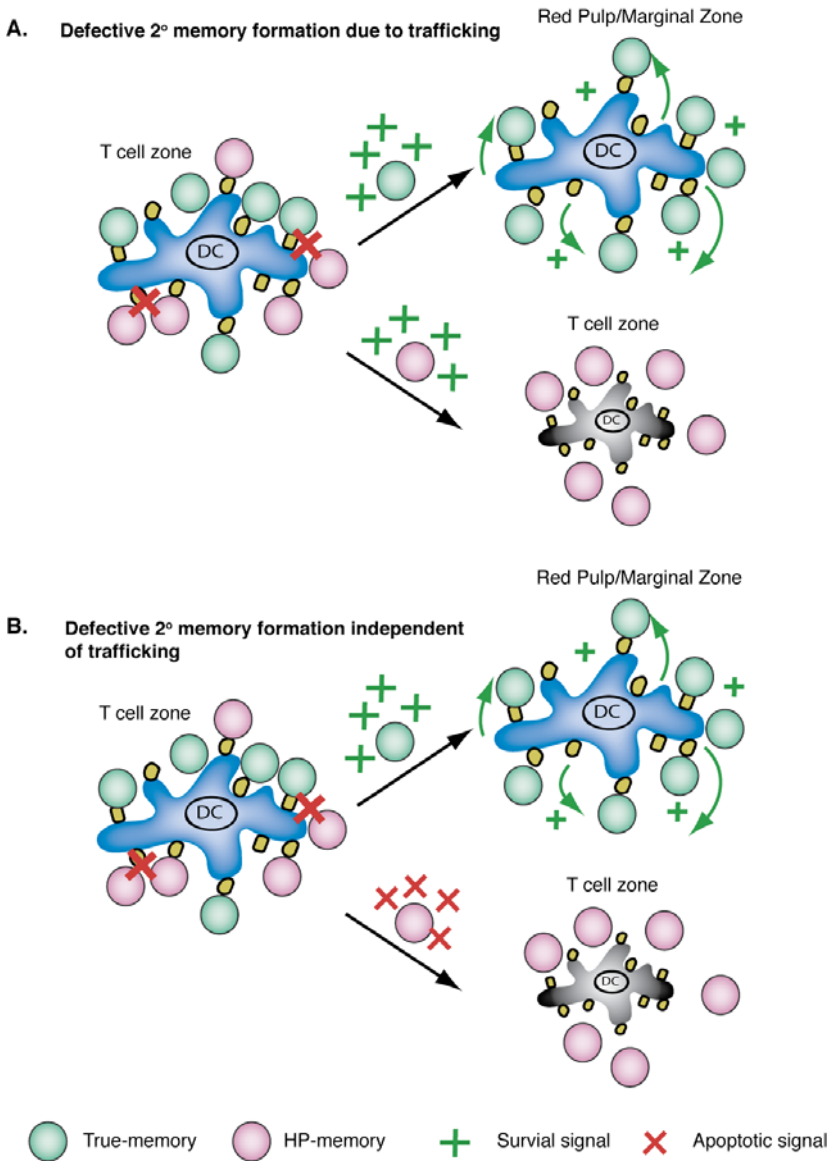

Figure 20. Models of HP- and true-memory cell trafficking and its effect on survival.

(A) Trafficking dependent survival. True-memory cells expand and generate secondary memory due to continuous survival signaling from the DCs. (B) Trafficking independent survival. Due to competition, the HP-memory cells were not selected to expand prior to trafficking to the T cell zone.

Alternatively, the second model proposes that HP-memory cells fail to compete efficiently with true-memory cells during priming, but the altered

trafficking is not what directly causes their inability to expand and form secondary memory (Fig. 20B). Rather, the true-memory cells receive the signals necessary for them to expand and form secondary memory, while the HP-memory cells do not. This occurs during the early phase of priming when both cells are located in the PALS. The true-memory cells then proceed to enter the RP/MZ to expand. It is however, not their localization in the RP/MZ that enables them survive and expand but rather the initial signals received from the DCs during activation. This is in contrast to the HP-memory cells that did not receive the appropriate signals from the DCs due to competition and therefore cannot continue to expand and form secondary memory. They then traffic to their respective location in the T cell zone. In this second model, the altered trafficking of the HP-memory cells is the result of competition but their inability to expand was determined prior to their continued localization in the T cell zone.

To test the validity of our model, we would need to alter the trafficking of either memory subset. If the altered trafficking leads to a rescue of their defective phenotype, then it can be concluded that the signals received from the DCs in the RP/MZ later in the immune response are responsible for sustaining their continued expansion. However, if the HP-memory cells inability to expand and form secondary memory still persists even if they display alternate localization, then it would be the signals they received initially from the DCs in the T cell zone that determine their ability to survive, which would not change regardless of their location.

Together, these data indicate that the presence of true-memory cells exacerbates a defect in the ability of HP-memory cells to directly access signals which regulate chemokine receptor expression and/or secondary memory formation. The defect in the HP-memory cells may be intrinsic but is not apparent to same extent in the absence of competition. Taken together, our results show that HP-memory T cells provide improved protection over naïve T cell responses in the absence of competition, but importantly do not compromise responses of the 'tried-and-true' antigen-experienced memory population. Thus, homeostatic mechanisms ensure that those memory-like cells arising during lymphopenia are not retained at the expense of pathogen- or vaccine-induced memory cells, suggesting that generation of these cells following lymphotoxic therapies will not erode recall responses.

Chapter 3 contains portions of the material as it appears in the Journal of Immunology,"Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells." Cheung, Kitty P., Yang, Edward, Goldrath, Ananda W., September 2009 Sep 1;183(5):3364-72. The dissertation author is the first author of this paper.

MATERIALS AND METHODS

Mice and adoptive transfers

All mouse work was performed in an AAALAC accredited facility according to the UCSD Institutional Animal Care and Use Guidelines. C57BL/6J (B6) mice were obtained from The Jackson Laboratory and bred in our facility along with CD45.1 and CD45.1.2 congenic mice on a B6 background. OT-I RAG-/- TCR-transgenic mice (CD45.1 or CD45.1.2) express a Vα2Vβ5 TCR heterodimer that recognizes a peptide derived from ovalbumin (257–264) (OVAp) presented by H-2Kb.

To generate HP-memory populations, 10^6 CD44^{lo} OT-I cells (CD45.1) were sorted and adoptively transferred into B6 hosts rendered lymphopenic by sub-lethal irradiation (600 rads) 24 h prior. Cells were allowed to undergo homeostatic proliferation for at least 30 days before subsequent transfers. For generation of true-memory, 10^6 OT-I (CD45.1.2) cells were adoptively transferred into naïve mice and infected with 5000 cfu Lm.OVA i.v.; 30 days were allowed to pass before subsequent transfers. Before the second adoptive transfer into naïve B6 mice, lymphocytes from spleen and lymph nodes were pooled and depleted of B and CD4$^+$ T cells (MACS). 1 x 10^5 - 8 x 10^5 cells were transferred per mouse unless otherwise specified; similar results were obtained for this range. Each of the different experimental groups received the same total number of OT-I T cells. Mice were re-challenged with 10^5 cfu Lm.OVA, immunized with 100μg OVAp/50μg LPS, or left uninfected. Where indicated, lymphocytes from pooled

spleen and lymph nodes were labeled with CFSE (10μM final concentration, Molecular Probes) for 10 min at 37°C in PBS 0.1% BSA.

To inhibit CD62L-mediated entry into lymph nodes, 200 mg of anti-CD62L (MEL-14) was administered i.p ~4 h before adoptive transfer of memory cells. The next day 200 mg of antibody was administered i.p ~4 h before infection and thereafter each day. Rat IgG2a κ isotype or PBS was administered concurrently to control mice with similar results to untreated hosts. For cytokine complexes, IL-7 was pre-complexed with an anti-IL-7 mAb (500-M07 PeproTech) and IL-15 was pre-complexed with its soluble IL-15-receptor-α as previously described and administered i.p on days 3-7 of infection (159, 121).

Lymphocytes isolated from lung and liver, as previously described (184) with minor modifications. Mice were euthanized with CO2 and perfused with PBS. Following collagenase digestion, cells were resuspended in HBSS, 5mM EDTA, and 2% BGS, and layered on Ficoll-Paque Plus solution (Amersham Bioscience) and separated according to manufacturer's instructions. Intraepithelial lymphocytes (IEL) were isolated as previously described (184) with a modified protocol. After incubation with 1mM dithioerythritol, tissue was incubated at 37°C with HBSS, 1.3mM EDTA for 30 min and layered over Ficoll.

Flow Cytometry

Following secondary challenge, single cell suspensions were prepared from spleen and lymph node lysates. Fc receptors were first blocked with unconjugated mouse antibody to CD32/16 (2.4G2). The following antibodies were

used for phenotypic analysis: CD44 (IM7), CD62L (Mel-14), CD122 (TM-β1), CD127 (A7R34), LY6C (AL-2), CD43 (1B11), CXCR3 (R&D Cat# FAB1585P), CD27 (LG.759), CD49d (R1-2), KLRG1 (2F1), PD-1 (J43), CD132 (4G3), CD45.1 (A20), CD45.2 (104), and CD8α (53-6.7). All antibodies are available commercially from Ebioscience or BD Pharmingen unless otherwise noted. To detect apoptosis, APC-conjugated annexin V/annexin buffer (Invitrogen) and 7-AAD (Invitrogen) were used. TUNEL staining was performed using the FragEL DNA fragmentation detection kit (Calbiochem) according to manufacturer's instructions. In short, cells were stained with surface antibodies then fixed in PBS with 1% paraformaldehyde. Following a wash in PBS and 0.2% Tween, fluorescent TdT mix was added and incubated for 1.5 h. To measure in-vivo proliferation, 1 mg BrdU (Sigma-Aldrich) was injected i.p into mice 15 h prior to sacrifice on indicated days. Splenocytes or lymph node cells were stained according to instructions from the BrdU Flow kit (BD Biosciences). All samples were run on BD's FACSCalibur or FACSAria. FlowJo software (TreeStar) was used for analysis.

mRNA Array

SuperArray®'s qPCR array (Cat No. PAMM-022) was used to compare the relative levels of cDNA between HP- and true-memory OT-I cells 6 days post infection from sorted pooled spleen cells. mRNA was extracted using TRIzol (Invitrogen) and cDNA was generated using the RT2 First Strand Kit (Superarray). Primers for mouse CXCR5 and CCR7 and the RT2 SYBR

Green/ROX PCR Master mix were obtained from SuperArray. For each set of triplicates, the mean value of each gene was calculated using the ΔΔCt method in comparison with the housekeeping gene.

Immunofluorescence

Freshly harvested tissues were soaked in 30% sucrose overnight and embedded in OCT. 6∝m thick tissue sections were cut and fixed in acetone (-20°C). Sections were blocked in a solution of 10% bovine serum albumin, 2.5% normal goat serum, 2.5% normal donkey serum, and fish scale gelatin. Tissue sections were then incubated with combinations of conjugated anti-mouse CD45.1, anti-rat Thy1.1/CD90 (OX-7), anti-mouse B220 (RA3-GB2), anti-mouse CD8a, anti-mouse CD4 (GK1.5) or anti-mouse CD11c (N418) followed by incubation with secondary mAb Alexa Fluor 546 streptavidin. Sections were mounted using Invitrogen ProLong Gold antifade reagent. Images were taken with an Olympus FV1000 confocal microscope with 5 laser lines at wavelengths of 405, 458, 488, 515, 543, and 647nm, using 10X and 20X air objectives. Images were analyzed using ImageJ.

Chapter 4 contains portions of the material as it appears in the Journal of Immunology, “Memory-like $CD8^+$ T cells generated during homeostatic proliferation defer to antigen-experienced memory cells.” Cheung, Kitty P., Yang, Edward, Goldrath, Ananda W., September 2009 Sep 1;183(5):3364-72. The dissertation author is the first author of this paper.

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