T CELL RECEPTOR-DRIVEN DIFFERENTIATION,

MAINTENANCE AND DYNAMIC RECALL

OF CD4$^+$ MEMORY T CELLS

by

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ABSTRACT

During acute infection, a CD4$^+$ T cell response begins with the interaction between the T cell receptor (TCR) and its cognate antigen presented by major histocompatibility complex (MHC) class II on antigen presenting cells. The nature of these interactions impacts various aspects of T cell fate. Here we find that TCR signals influence the generation of Th1 memory cells. The transition of activated CD4$^+$ T cells from effector to memory is associated with a significant decrease in TCR repertoire diversity. Particularly, a slow dissociation rate of TCR-antigen interactions, but not TCR avidity, corresponds to memory potential. Thus, long-lived TCR interactions with antigen during priming are a determinative factor in promoting Th1 memory differentiation.

Once generated, memory T cells are maintained at stable levels. However, CD4$^+$ memory T cells gradually decline in some mouse models of acute infection. We find that heterologous rechallenge of Th1 memory cells with a pathogen sharing only a CD4$^+$ T cell epitope, which allows for robust boosting of memory T cells without rapid antigen clearance, leads to the generation of highly stable secondary Th1 memory cells that do not decline. Importantly, enhanced memory stability corresponds to the acquisition of high antigen sensitivity, often referred to as functional avidity, at the peak of the recall response. In contrast, homologous rechallenge of Th1 memory cells, where memory

cells are weakly stimulated due to the limited antigen persistence, does not enhance function and stability of secondary Th1 cells.

Upon heterologous rechallenge, the recall response of Th1 memory cells is characterized by the early emergence of secondary responders with high functional avidity, followed by functional avidity decay to the level similar to the parent memory cells. Unexpectedly, responding secondary effectors progressively lose their functional avidity when secondary infection is prolonged, which corresponds to the generation of poorly stable secondary memory cells. Functional avidity decay requires an extended period of both antigen presentation and infectious inflammation and correlates with the diminished magnitude of TCR signaling. Together, the recall response of Th1 memory cells is functionally dynamic, and the nature of secondary stimulation influences function and stability of secondary Th1 cells.

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CHAPTER 1

INTRODUCTION

Memory CD4^{+} T cell development during acute infection

During acute viral and bacterial infections, rare antigen-specific naïve CD4^{+} T cells interact with peptide antigens presented by major histocompatibility complex (MHC) class II on mature dendritic cells (DCs) in secondary lymphoid organs. This interaction leads to their activation, rapid expansion (up to 10,000-50,000-fold) and effector differentiation [1,2]. Depending on the infection-induced inflammatory environment, CD4^{+} T cells can differentiate into distinct effector T helper (Th) cell subsets, such as Th1, Th2, Th17, T follicular helper (Tfh) and regulatory T cells (Treg), as defined by canonical transcription factors and effector cytokine production [3]. These effector T helper cells provide help for other immune cells including macrophages, B cells and CD8^{+} T cells, mediate direct effector functions and suppress immunopathology, which collectively coordinate protective immunity. Following antigen clearance, most effector cells die by apoptosis, but a small proportion (~5-10%) survives and becomes long-lived memory cells [4]. Whether these effector subsets retain their polarized status in the memory pool is, however, still being defined [1,5].

Memory T cells have several features that distinguish them from naïve T cells. For example, they are present at higher frequencies than naïve counterparts and persist long term via slow homeostatic turnover in the absence of cognate antigen. They also have a decreased activation threshold and acquire effector functions more rapidly than naïve T cells. Furthermore, some memory T cells preferentially localize to peripheral sites of infection due to expression of tissue-specific trafficking molecules. Combined, these properties allow memory T cells to provide rapid and enhanced protection during secondary exposure to the same or a related antigen [1,4].

While many factors involved in activation and effector differentiation have been studied extensively [6], less is known regarding the signals that lead to memory CD4^{+} T cell development. Evidence indicates that memory T cells arise from antigen-experienced effector T cells through linear differentiation [7-9]. In studies of CD8^{+} T cells during acute infection, cytotoxic T lymphocytes (CTL) fated to become memory cells can be identified at the peak of the primary response based on the expression of certain cell surface molecules, such as interleukin-7 receptor α (IL-7Rα, expressed on memory precursor CTL) and killer cell lectin-like receptor G1 (KLRG1, expressed on effector CTL fated to die) [10,11]. Similarly, recent studies showed that memory precursor CD4^{+} T helper cells are also a component of the early effector pool [12]. Therefore, effector cells fated for memory differentiation are predetermined during the primary response, indicating that cell fate decisions are largely made at the early stages of T cell activation. Numerous studies have attempted to identify the signals that drive these decisions and have focused on the role of extrinsic environmental cues, such as inflammatory cytokines and growth factors, as well as intrinsic signals delivered through T cell receptor (TCR) [13].

Extrinsic cues for effector and memory T cell differentiation

Extrinsic environmental cues impact effector and memory T cell differentiation. For example, inflammatory adjuvants promote the recruitment of antigen-specific T cells with a wide range of avidity during peptide immunization [14]. Infection-induced stimulatory factors also facilitate clonal expansion, whereas limiting early inflammation favors memory generation [15,16]. Specifically, inflammatory cytokines, such as IL-12

and type I interferons, induce optimal development of effector and memory T cells [17]. However, prolonged exposure to these cytokines promotes the development of end-stage effector CD8^{+} T cells lacking memory potential [11,18]. Similarly, while IL-2 was initially characterized as a T cell growth factor, studies showed that IL-2 signals during priming preferentially promote short-lived effector CTL differentiation [19-21]. Conversely, T cell activation in the absence of IL-2 signals leads to dysfunctional memory CTLs that are defective in secondary expansion upon rechallenge [22].

IL-7 is a survival cytokine for T cells, and IL-7Rα expression on activated CD8^{+} T cells is associated with memory precursor cells, though its expression is not sufficient for memory differentiation [10,23,24]. For CD4^{+} T cells, prior works suggest a critical role for IL-7 signals in the survival of effector cells during the contraction phase [25,26]. However, more recent studies showed that constitutive IL-7 signals failed to enhance CD4^{+} memory T cell generation, indicating that similar to CD8^{+} T cells, IL-7 signals are not a predominant factor for CD4+ memory T cell differentiation [27,28].

Inflammatory cytokines induce the expression of several key transcription factors, and recent studies have revealed their important roles in the fate decision between short-lived versus memory CTL. In brief, high levels of T-bet and Blimp-1 lead to terminal differentiation into short-lived effector CD8^{+} T cells [11,29]. STAT5 activation by IL-2 drives Th1 effector memory differentiation [30,31]. Conversely, STAT3 activation through IL-10 and IL-21 promote formation of CD8^{+} memory precursors [32], and the transcriptional repressor Bcl-6 is also involved in CD8^{+} and CD4^{+} memory development [30,33].

The role of TCR signals in effector and memory

CD8^{+} T cell differentiation

Along with environmental cues, intrinsic signals delivered through the TCR also influence various aspects of T cell differentiation. Because of the diverse nature of the TCR repertoire, during activation, naïve T cells receive varying levels of TCR signals, and the magnitude of those signals is determined by several factors, including TCR avidity for antigen, amount of antigen, costimulatory molecules and duration of interactions between T cells and antigen presenting cells (APCs) [34]. Currently, two models have been proposed to describe the relative role for TCR signals in driving the T cell recruitment, expansion and differentiation during infection. First, a progressive differentiation model proposes that prolonged antigenic stimulation progressively promotes clonal expansion, effector differentiation, survival and memory formation [35,36]. A second model, programmed differentiation, proposes that differentiation of T cells is programmed upon a brief antigen encounter in the absence of further antigenic stimulation [37]. While numerous studies have supported both models [13], evidence suggests that TCR signals are incorporated differently during the differentiation of CD4^{+} and CD8^{+} T cells in response to acute infection.

For CD8^{+} T cells, only a short period (6-24 hours) of interaction with antigen is sufficient to recruit T cells into the response. Once recruited, CD8^{+} T cells are able to undergo all phases of subsequent T cell differentiation such as expansion, acquisition of effector function and memory formation without further antigen encounter [38-41]. While a short exposure to antigen limits the magnitude of the primary expansion, effector function and memory development are not affected by the duration of TCR stimulation

[42]. Similarly, while TCR avidity across a 700-fold range for a given antigen impacts the kinetics of clonal expansion, the daughter clones of all CD8^{+} effector T cells that were recruited into the response compete equally for entry into the memory pool [43].

The role of TCR signals in memory differentiation has also been determined by analysis of TCR repertoire usage in the effector and memory pool, and several prior works have suggested that during acute infection, the TCR repertoire of CD8^{+} effector T cells is highly similar to that of memory cells as well as secondary effectors following rechallenge [44-47]. Together, these studies support a programmed differentiation model and suggest that TCR signals are not a determining factor in effector/memory fate decisions by activated CD8^{+} T cells. Instead, they play a key role in the recruitment of T cells into the response, and subsequent differentiation steps appear to be driven by environmental factors [37].

The role of TCR signals in effector and memory

CD4^{+} T cell differentiation

The role of TCR signals is more complex during CD4^{+} T cell differentiation. While inflammatory stimuli obviously impact the acquisition of distinct effector functions, prior work showed that antigenic signals through TCR also play a role in effector subset differentiation, such as Th1, Th2, Tfh and regulatory T cells (Treg) [48-51]. By tracking the progeny of a single naïve T cell in vivo, a recent study also found an instructive role for intrinsic TCR signals in the Th1/Tfh fate decision early during acute infection [52].

Along with effector differentiation, the nature of TCR-antigen interactions also impacts CD4^{+} memory T cell differentiation. Unlike CD8^{+} T cells that need only a brief antigen encounter for initiating programmed effector and memory differentiation, naïve CD4^{+} T cells require prolonged antigenic stimulation for optimal activation and expansion as well as full differentiation into effector and memory cells [53-55]. In addition, restimulation of CD4^{+} memory T cells enriches secondary effectors with higher TCR avidity for antigen as compared to primary counterparts [56]. Thus, these findings support progressive differentiation of CD4^{+} T cells, with increasing antigenic signals driving T cell differentiation throughout the immune response.

The requirement for a strong antigenic stimulus in CD4^{+} memory T cell development is supported by the finding that increasing precursor frequency of antigen-specific naïve CD4^{+} T cells during priming corresponds to a defect in memory differentiation [57,58]. Clonal competition also impacts long-term maintenance of CD4^{+} memory T cells [59]. Intravital two-photon imaging studies clearly observed the prolonged interactions between T cells and peptide-loaded DCs at a low precursor frequency during priming, whereas the duration of this interaction became much shorter when abnormally high frequency CD4^{+} T cell clones were adoptively transferred [60]. These findings suggest that clonal competition for available antigen inhibits the prolonged stimulation of CD4^{+} T cells, which negatively impacts subsequent memory differentiation.

Analysis of TCR repertoire usage also supports a role for TCR signals in memory differentiation. In a mouse model of peptide immunization, the TCR repertoire of antigen-specific CD4^{+} T cells narrows to higher avidity during primary and secondary

responses, which was not observed for CD8^{+} T cells, implying that antigen-driven selection for high avidity clones may occur [56,61]. Additionally, prior work from our laboratory suggests that not all CD4^{+} T cells that undergo robust expansion and effector differentiation can equally differentiate into memory T cells [62]. Failure in memory development is associated with both low TCR avidity and poor antigen sensitivity, often termed functional avidity, by activated CD4^{+} T cells at the peak of the effector phase [62]. Furthermore, the transition of CD4^{+} effector T cells to memory as well as the development of very long-lived memory T cells are characterized by the emergence of memory populations with higher functional avidity [62]. Together, these findings suggest that strong TCR signals during priming are required for memory CD4^{+} T cell differentiation [13].

Long-term stability of memory CD4^{+} T cells

Once established, memory T cells can persist for a long time via slow homeostatic turnover. In humans, both CD8^{+} and CD4^{+} memory T cells specific for the smallpox vaccine are detectable for up to 75 years postimmunization with a half-life of 8-15 years [63,64]. In mouse models of acute infection, CD8^{+} memory T cells also maintained in the long-term with no observable decline throughout the life of the mouse [4]. In contrast, memory CD4^{+} T cells tend to gradually decline over time in some mouse models [62,65,66]. Therefore, it appears that the mechanism controlling maintenance of CD4^{+} memory T cells is different from that of CD8^{+} memory T cells.

CD8^{+} and CD4^{+} memory T cells rely on the contact with both IL-7 and IL-15 for their long-term maintenance under normal physiological conditions [25,67-69]. While

TCR signals from contact with MHC II have been thought to be required for the maintenance of antigen-specific CD4$^+$ memory T cells, it is now evident that similar to CD8$^+$ T cells, their homeostasis is largely MHC independent. For memory CD8$^+$ T cells, signaling through IL-7 receptor (IL-7R) delivers survival signals, and IL-15 induces basal homeostatic proliferation [69]. In the case of memory CD4$^+$ T cells, IL-7 appears to play a bigger role than IL-15 in homeostasis, as they express much lower levels of IL-15 receptor than memory CD8$^+$ T cells. This suggests that memory CD4$^+$ T cells compete less effectively than memory CD8$^+$ T cells for available IL-15 [68], and this may be a factor in the differential maintenance of CD8$^+$ and CD4$^+$ memory T cells.

As described above, CD4$^+$ T cells progressively acquire high functional avidity in the transition from effector to memory and during long-term memory maintenance [62]. In terms of memory stability, although CD4$^+$ memory T cells have been noted to decline over time, the rate of memory decline also becomes progressively slower [62]. Importantly, this coincides with corresponding enrichment of a memory population with higher functional avidity [62]. Additionally, prior work also demonstrated that while the entire memory population, including both high and low functional avidity T cells, decreased over time, memory cells with high functional avidity, as measured by restimulation of T cells with a low amount of peptide antigen, did not decline during the same time period [62]. This finding suggests that initial TCR signal strength during priming may impact not only entry into the memory pool but also their long-term stability as memory T cell populations over time.

Recall responses of memory T cells

As described above, rapid recall responses and efficient clearance of recurrent infection are attributed to distinct properties of memory T cells, including higher precursor frequency, lower activation threshold, immediate effector function and wide tissue distribution [4]. However, little is known about the molecular mechanisms that control the rapid recall response. Elevated expression of proximal TCR signaling molecules, such as tyrosine kinase Zap70, in resting memory T cells has been reported [70]. One study also suggested that memory T cells have more and larger oligomeric TCR complexes on the cell surface than their naïve counterparts, enhancing T cell responsiveness to lower antigen doses [71].

While protective functions of memory CD8^{+} T cells correlate to their capability of specific killing of infected target cells, how memory CD4^{+} T cells provide enhanced protective responses following secondary challenge is not fully understood [72]. It has been shown that memory CD4^{+} T cells are better than naïve counterparts in providing help for B cells, which accelerates robust B cell antibody response [73]. Memory CD4^{+} T cells also enhance early inflammatory responses and rapidly activate APCs, which contributes to early control of infection [74,75]. For Th1 memory cells, the ability to make multiple effector cytokines, including IFN-γ, tumor necrosis factor α (TNF-α) and IL-2, strongly correlates to protective capacity [76,77]. More recently, some memory CD8^{+} and CD4^{+} T cells that persist at tissue sites of infection for long periods have been defined as a distinct memory population, termed tissue-resident memory T cells. Emerging evidence indicates that they provide a first line of defense by facilitating more rapid recruitment and activation of innate and adaptive immune cells [78-81].

Several prior works have shown that the phenotypes and protective capacity of memory T cells are changed during secondary responses. For CD8^{+} T cells, infection-induced inflammatory cytokines such as IL-12 enhance antigen responsiveness of memory CTLs [82]. Repetitive antigen stimulation results in the development of secondary memory CD8^{+} T cells that display more effector-like gene expression signatures, altered trafficking into lymph nodes and decreased proliferative capacity [83]. Depending on the nature of subsequent pathogen challenges, these changes were either beneficial or detrimental for protection. For example, as compared to primary memory, secondary memory cells are more protective against certain types of infection such as *Listeria monocytogenes*, whereas they are more susceptible to functional exhaustion following chronic antigen exposure [84,85]. Overall, these findings indicate that the context of secondary challenge markedly influences the functional properties of memory CD8^{+} T cells.

Remarkably, far less is known regarding the secondary recall response of memory CD4^{+} T cells and their functional capacity. Both naïve and memory CD4^{+} T cells have a similar lag phase (~3 days) before the onset of cell proliferation upon viral infection [86]. Unlike CD8^{+} memory T cells, CD4^{+} memory T cells need an extended period of antigen exposure for robust secondary expansion, though they also have an intrinsic limit in the magnitude of secondary expansion [87,88]. In the context of influenza A virus, a recent study found that secondary effector CD4^{+} T cells display distinct functional and phenotypic characteristics as compared to primary effectors, including enrichment for producers of multiple cytokines, enhanced trafficking to tissue sites of infection and

greater contribution to viral clearance [89]. However, how functions of secondary

effectors are controlled during secondary responses remains to be addressed.

Summary of dissertation

Unlike CD8$^+$ T cells, CD4$^+$ T cell differentiation is hierarchical, and the strength

of antigen-driven TCR signals during priming progressively promotes T helper cell

recruitment, clonal expansion, acquisition of effector functions and survival [13]. In

Chapter 2, we determine whether the nature of TCR signals has a determining role in

effector/memory fate decisions by activated CD4$^+$ T cells following acute viral or

bacterial infection. To test this, we track the antigen-specific TCR repertoire during the

transition from effector to memory by performing deep sequencing of the third

complementarity-determining region (CDR3) of the TCR. We find that memory

development is associated with a significant decrease in TCR repertoire diversity.

Further analysis of binding properties of individual T cell clones to peptide-MHC II

tetramers indicates that TCR avidity for antigen does not correlate to memory

differentiation potential. Instead, entry into memory pool significantly corresponds to a

slow antigen off-rate. Thus, we conclude that stable and sustained interactions with

antigen during activation are a determining factor in promoting Th1 memory

differentiation.

As described above, long-term survival of CD4$^+$ memory T cells corresponds to

their high functional avidity [62]. In Chapter 3, we address the hypothesis that secondary

challenge of Th1 memory cells would enrich responders with high functional avidity,

which in turn results in more stable maintenance by ensuing secondary memory cells as

compared to primary memory. We find that heterologous rechallenge of Th1 memory cells with a pathogen sharing only a CD4$^+$ T cell epitope, which allows for robust boosting of memory T cells without rapid antigen clearance mediated by pre-existing antibodies and memory CTLs, results in the expansion of secondary responders displaying high-level functionality as well as development of very stable secondary Th1 memory cells that do not decline. However, when Th1 memory cells receive a weak homologous rechallenge, which results in extremely rapid clearance of infection, secondary responders display poor expansion, loss of high functional avidity and decay of ensuing memory cells with kinetics similar to primary memory. Therefore, these findings suggest that robust secondary challenge can enrich highly functional secondary Th1 cells that persist stably without decay. Furthermore, the nature of secondary stimulus profoundly influences the function and stability of secondary Th1 memory cells.

Chapter 4 addresses the factors regulating function of secondary Th1 effectors during the recall response in more detail. We find that upon heterologous rechallenge, secondary Th1 responders rapidly acquire extremely high functional avidity, followed by functional dematuration to the level similar to resting memory cells at the peak of the recall response. This functional characteristic is strikingly different from the primary response, as both naïve CD8$^+$ and CD4$^+$ T cells undergo functional maturation throughout the primary response [90,91]. Furthermore, responding secondary Th1 responders progressively lose their high antigen sensitivity as well as long-term stability in the secondary memory pool when infection is abnormally prolonged. Importantly, we report that both antigen-driven TCR signals and inflammatory mediators are required for this step. Finally, we demonstrate that high-level functionality was associated with enhanced

proximal TCR signaling. Thus, these findings highlight dynamic functional modulation of Th1 memory cells during early recall responses and suggest that the duration of the secondary stimulus shapes secondary Th1 effector and memory differentiation, function and survival.

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CHAPTER 2

SUSTAINED INTERACTIONS BETWEEN T CELL

RECEPTORS AND ANTIGEN PROMOTE

THE DIFFERENTIATION OF

CD4^{+} MEMORY T CELLS

Chulwoo Kim, Theodore Wilson, Kael F. Fischer and Matthew A. Williams

Article

Sustained Interactions between T Cell Receptors and Antigens Promote the Differentiation of CD4+ Memory T Cells

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SUMMARY

During CD4+ T cell activation, T cell receptor (TCR) signals impact T cell fate, including recruitment, expansion, differentiation, trafficking, and survival. To determine the impact of TCR signals on the fate decision of activated CD4+ T cells to become end-stage effector or long-lived memory T helper 1 (Th1) cells, we devised a deep-sequencing-based approach that allowed us to track the evolution of TCR repertoires after acute infection. The transition of effector Th1 cells into the memory pool was associated with a significant decrease in repertoire diversity, and the major histocompatibility complex (MHC) class II tetramer off rate, but not tetramer avidity, was a key predictive factor in the representation of individual clonal T cell populations at the memory stage. We conclude that stable and sustained interactions with antigens during the development of Th1 responses to acute infection are a determinative factor in promoting the differentiation of Th1 memory cells.

INTRODUCTION

After their activation, CD4+ T cells undergo a period of clonal expansion that coincides with the acquisition of specific effector cell functions. Once the antigen is cleared, a small subset of effector CD4+ T cells survives and populates the long-lived memory T cell pool (van Leeuwen et al., 2009). The differentiation steps that lead to the formation of effector T helper 1 (Th1) cells have been studied extensively. Less is known about the signals that enable a subset of effector Th1 cells to differentiate into memory cells, although CD4+ T cells fated to become memory cells can be identified during the effector response to acute infection (Marshall et al., 2011). Identification of the signals that promote memory cell differentiation is key to understanding how activated T cells make fate decisions as well as to the design of better vaccination and immunotherapeutic strategies aimed at enhancing CD4+ memory T cell formation and function.

External environmental cues, including cytokines, control the expression of transcription factors that promote T helper subset differentiation; such transcription factors include T-bet, Blimp-1,

STAT3, STAT4, and Bcl-6 (Eto et al., 2011; Johnston et al., 2012; Johnston et al., 2009; Nakayamada et al., 2011; Pepper et al., 2011). The extent to which these factors promote effector or memory T cell fate decisions is less clear. Some recent articles have implied potential roles for Bcl-6 and IL-21 in the differentiation and formation of CD4+ central memory T cells, along with an opposing role for interleukin-2 (IL-2)-driven STAT5 activation in driving effector-memory Th1 cell differentiation (Crotty et al., 2010; Johnston et al., 2012; Lüthje et al., 2012; Pepper et al., 2011; Weber et al., 2012a).

Cell-intrinsic differentiation cues, in particular those dependent on T cell receptor (TCR) binding and signaling, also play a clear role in many aspects of CD4+ T cell differentiation. For CD4+ T cells, the strength of TCR-mediated signaling progressively drives effector differentiation and survival (Gett et al., 2003), and repeated stimulation selectively enriches for responding CD4+ T cells with high-avidity TCRs (Savage et al., 1999). Additionally, several days of exposure to antigens in vivo are required for full differentiation of effector (Obst et al., 2005; Williams and Bevan, 2004) and memory (Jelley-Gibbs et al., 2005) CD4+ T cells. The nature of the TCR stimulus also influences the differentiation of T helper subsets, including Th1, T helper 2 (Th2), T follicular helper (Tfh), and regulatory T (Treg) cells (Brogdon et al., 2002; Fazilleau et al., 2009; Lee et al., 2012; Leitenberg and Bottomly, 1999; Moran et al., 2011; Olson et al., 2013). Low immunizing doses can result in the generation of CD4+ memory T cells with high-affinity TCRs (Rees et al., 1999), and secondary responses are characterized by the emergence of secondary CD4+ T cell responders with high avidity for antigen (Savage et al., 1999). An additional study reports defects in memory cell formation related to naive precursor frequency (Blair and Lefrançois, 2007). On the basis of the combined evidence, one can reasonably conclude that high-avidity CD4+ T cells are progressively selected in the presence of antigens. However, it is unknown how TCR-mediated differentiation signals during the primary T cell response might influence long-term fate once antigens are cleared. The role of sustained TCR interactions with antigenic peptide bound to MHC class II molecules (pMHCII) in the specification of memory T cell fate has not been directly determined.

We previously showed that not all clones that participate in the effector Th1 response to acute infection are equally represented in the subsequent Th1 memory cell population (Williams et al., 2008). Instead, memory T cell differentiation potential corresponds to the development of high antigen sensitivity during

TCR-Driven Selection of CD4+ Memory T Cells

the primary response, and stable maintenance of the memory state is associated with the emergence of Th1 memory cells with high functional avidity (Kim et al., 2012; Williams et al., 2008). These findings suggest the hypothesis that strong TCR-pMHCII interactions are a key element in Th1 memory cell fate decisions. To test this hypothesis, we generated a deep-sequencing-based model system that allowed us to track TCR repertoire evolution during effector and memory Th1 cell differentiation, as well as characterize the binding of pMHCII molecules by individual TCRs, thus relating the potential for memory differentiation to the kinetic and equilibrium binding properties of individual TCRs. TCR repertoire diversity substantially decreased as CD4+ memory T cell populations emerged after infection with either lymphocytic choriomeningitis virus (LCMV) or recombinant *Listeria monocytogenes* expressing the immunodominant MHC class II (MHCII)-restricted epitope, GP_{61-80}, derived from the LCMV glycoprotein (Lm-gp61). However, when the binding properties of individual TCRs were assessed, memory T cell differentiation potential did not correspond to apparent K_d measurements as determined by MHCII tetramer binding. Instead, memory T cell differentiation was predicted by tetramer-binding off rates, suggesting that in settings where the amount of antigen is not limiting, such as during a robust viral or bacterial infection, antigen off rates might be a better predictor of the biological consequences of sustained TCR-pMHCII interactions. Furthermore, the differing potential of monoclonal populations of T cells to differentiate into memory T cells could be predicted by tetramer off rates. Overall, we conclude that sustained TCR-mediated signaling during priming is a key element in the specification of CD4+ memory T cell fate.

RESULTS

### Generation and Characterization of a Fixed Single-Chain TCR Transgenic Mouse

To track virus-specific CD4+ T cell repertoires after acute viral or bacterial infection, we generated a single-chain TCR transgenic mouse that expressed the *Tcra* chain cloned from the SMARTA TCR (Oxenius et al., 1998). Because SMARTA TCR transgenic mice are specific for the immunodominant class-II-restricted epitope of LCMV glycoprotein, GP_{61-80}, polyclonal expression of the TCRβ chain allowed for efficient tracking of antigen-specific TCRβ repertoires paired to a known TCRα during antigen-specific T cell responses. We crossed the fixed SMARTA *Tcra* chain transgenic mouse (SMα) to a TCRα-deficient background, ensuring the selection of only TCRβ chains that pair with the SMARTA TCRα (Figure S1).

SMα mice generated readily detectable populations of CD4+ T cells in the spleen and lymph nodes, albeit at frequencies marginally lower than those of WT B6 mice. Surface expression of the SMARTA TCRα chain was comparable to that of polyclonal endogenous CD4+ T cells in WT B6 mice (Figures 1A and 1B). As previously described (Moon et al., 2007), we used magnetic sorting to enrich CD4+ T cells from the spleens of B6, SMα, or B6 immune (>42 days after infection with LCMV) mice that were capable of binding MHC II GP_{66-77} tetramers. We spiked the splenocyte population (Thy1.2+) with a known number (1×10^3) of congenically marked (Thy1.1+) SMARTA CD4+ T cells prior to enrichment as a positive control to normalize total

tetramer-binding cell counts. We also isolated tetramer-binding cells from the spleens of LCMV immune mice to control for the efficiency of tetramer enrichment. We calculated that the total number of GP_{66-77}-specific naive CD4+ T cells in the spleens of SMα mice ranged from 1×10^3 to 2×10^3, a 10- to 12-fold increase over naive precursor frequencies in wild-type mice (Figure 1C). As a second approach, we employed a previously described competitive inhibition assay (Whitmire et al., 2006) by transferring increasing numbers of Thy1.1+ SMARTA cells into Thy1.2+ SMα or B6 mice, then infected mice with LCMV 1 day later. By measuring relative inhibition of the endogenous polyclonal response by using either tetramers or ex vivo peptide-stimulated IFN-γ production, we calculated naive precursor frequencies in SMα mice to be ∼1×10^3, 8- to 10-fold higher than those found in B6 mice (Figure 1D and data not shown).

Although earlier studies have indicated that artificially elevating precursor frequency can lead to intraclonal and interclonal competition, these observations have typically taken place when precursor frequencies are an order of magnitude or more higher than those observed in our model system (Blair and Lefrançois, 2007; Foulds and Shen, 2006). Because we observed only modest increases in naive precursor frequency in SMα mice, we analyzed their response to direct LCMV or Lm-gp61 infection. Whereas SMα mice cleared the Lm-gp61 challenge with kinetics similar to those of WT mice, ∼50% of SMα mice failed to clear the LCMV challenge (data not shown). Because all T cells in these mice bear a TCRα chain specific for an MHC II-restricted epitope, we hypothesized that MHC I-restricted responses were defective. Transfer of 5×10^6 naive CD8+ T cells enabled SMα mice to clear acute LCMV infection with similar kinetics to those of WT mice even at early time points (days 3 and 5) after infection (data not shown). Therefore, we conducted subsequent studies by infecting SMα mice that had received a CD8+ T cell adoptive transfer 1 day previously.

SMα mice generated robust CD4+ T cell responses to both LCMV and Lm-gp61 infection. By day 8 post-infection with LCMV, >60% of the CD4+ T cells in the spleen produced IFN-γ upon ex vivo restimulation with GP_{61-80} peptide. The response was also robust after Lm-gp61 infection of SMα mice: ∼25% of CD4+ T cells made IFN-γ upon restimulation at the peak of the effector response (Figure 2A). Although the size of the responses was expected on the basis of the elevated precursor frequencies in SMα mice, the overall kinetics and magnitude mirrored CD4+ T cell responses to the same epitope in B6 mice (Figures 2B and 2C). The magnitude of primary expansion, estimated on the basis of our calculations of precursor frequency (Figure 1C), was not significantly different in B6 and SMα mice (Figure 2D). Furthermore, after LCMV infection, readily detectable memory T cell populations were generated in SMα mice and persisted with similar kinetics to B6 mice, although memory Th1 cells in SMα mice were more stably maintained after Lm-gp61 infection (Figures 2A and 2C). Importantly, Th1 effector and memory cells induced in SMα mice displayed the same cytokine-producing profile as polyclonal Th1 cells generated in B6 mice (Figure 2E). In all, these data indicate that SMα mice are a robust model for analyzing the evolution and distribution of antigen-specific CD4+ T cell TCR repertoires after acute infection in vivo.
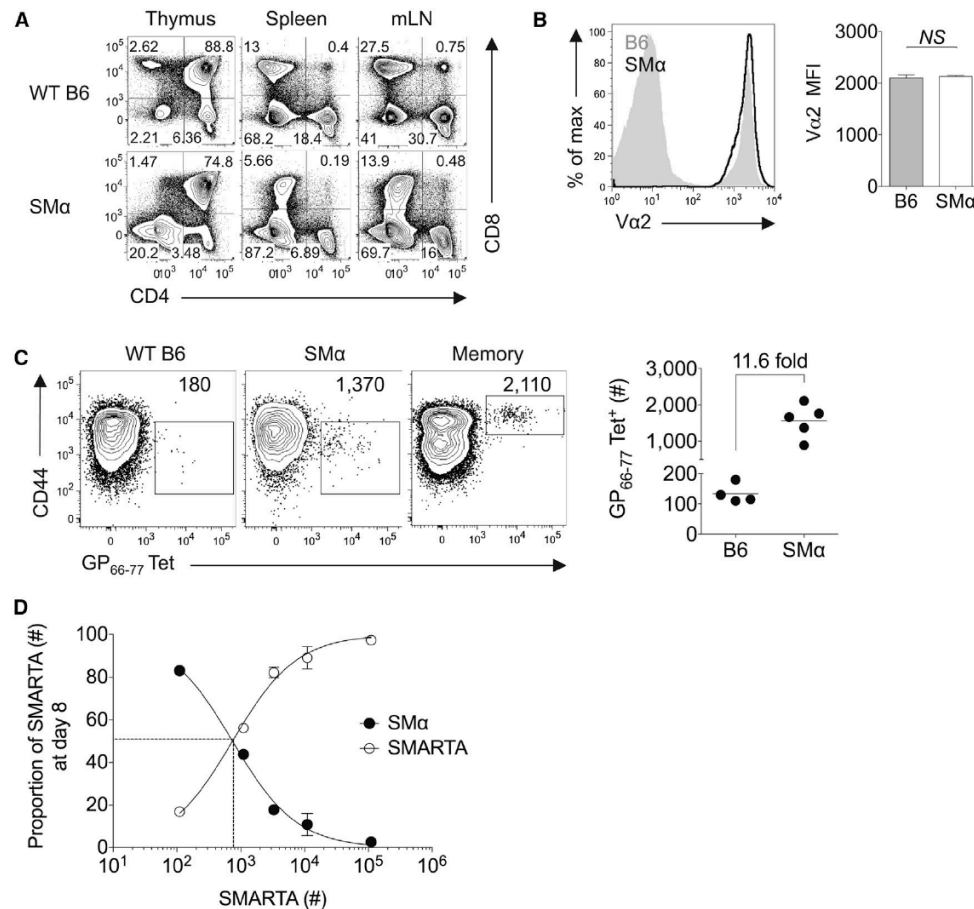
Figure 1. SMα Mice Generate a Readily Detectable Population of Polyclonal Naive Precursors Specific for LCMV GP$_{61-80}$
(A) Representative flow plots indicate the frequency of CD4+ and CD8+ T cells in the spleen, mesenteric lymph nodes, and thymus of B6 and SMα mice.
(B) A representative flow plot shows TCR surface expression (Vα2+) in B6 or SMα mice. A bar graph indicates the Vα2 MFI of Vα2+CD4+ cells in the spleens of B6 or SMα mice (n = 4/group).
(C) Representative flow plots show tetramer staining after magnetic enrichment of tetramer-binding cells in B6, SMα, or B6 immune (>6 weeks after infection with LCMV) mice; numbers indicate the estimated number of tetramer-binding cells per spleen after internal normalization to "spiked" Thy1.1+ SMARTA cells (1 × 10^3). The graph indicates the estimated frequency of tetramer-binding CD4+ T cells in individual mouse spleens and the estimated n-fold difference in precursor frequency between B6 and SMα mice.
(D) SMARTA cells were adoptively transferred in various numbers into SMα mice, and LCMV infection followed 1 day later. The plot indicates the relative proportion of SMα and SMARTA responders at day 8 after infection, in comparison to the estimated SMARTA precursor frequency in the spleen at day 0 (estimated as 10% take of the initial transfer). Error bars indicate the standard error of the mean (SEM) (n = 4 mice/group). Results are from two independent experiments. See also Figure S1.

Skewed Vβ Usage by Th1 Memory Cells Corresponds to Differences in Functional Avidity

As an initial broad approach to measure TCR repertoire usage by effector and memory Th1 cells after LCMV or Lm-gp61 infection, we analyzed Vβ usage of antigen-specific responders by antibody staining. We focused primarily on the three predominant Vβ subsets (Vβ7, Vβ8.1, and Vβ14) utilized by SMα CD4+ T cells during the response to the GP$_{61-80}$ epitope (data not shown). MHCII tetramers might bind different TCRs with variable efficiency. Additionally, a recent report showed that a large proportion of IFN-γ-producing Th1 cells induced by LCMV fail to

bind tetramers at all (Sabatino et al., 2011). Therefore, we measured Vβ expression by IFN-γ-producing cells. When we compared the peak of the effector response (day 8) to memory time points (≥42 days after infection), the Vβ7 and Vβ8.1 subsets significantly decreased as a proportion of the overall antigen-specific repertoire after both LCMV and Lm-gp61 infection (Figures 3A, 3B, 3D, and 3E). We observed a corresponding increase in the representation of the most dominant subset, Vβ14, at memory time points (≥42 days after infection) after Lm-gp61 infection (Figure 3F). We did not observe the same increase in the Vβ14 subset after LCMV infection (Figure 3C), possibly

reflecting variable and inconsistent participation of other Vβ subsets, including Vβ8.3 and Vβ3, in the memory T cell pool (data not shown). While antigen-specific polyclonal Th1 cells in B6 mice showed some changes in the distribution and usage of Vβ subsets, we observed a similar decrease in the proportion of Vβ7- and Vβ8.1-expressing memory Th1 cells (Figure S2A).

We previously observed that the differentiation of memory Th1 cells is accompanied by an increase in their overall functional avidity, or antigen sensitivity, as measured by the production of IFN-γ in response to stimulation with decreasing concentrations of antigen (Williams et al., 2008). Similarly, effector Th1 cells in SMα mice displayed an increase in functional avidity as they transitioned to become memory Th1 cells (data not shown). We considered at least two possibilities to explain these observations. First, the population increase in functional avidity could represent a selective loss of low-functional-avidity responders in the formation of the memory T cell pool. Second, acquisition of higher antigen sensitivity might represent a normal facet of memory T cell differentiation that broadly applies to all individual clones within the response. To distinguish between these possibilities, we measured the functional avidity of the Vβ7, Vβ8.1, and Vβ14 subsets at the peak of their response (day 8) and at memory time points (days 42 and 75 after infection) after Lm-gp61 infection. Functional avidity was assessed on the basis of the ability of each subset to make IFN-γ, as measured by intracellular cytokine staining after ex vivo peptide restimulation. Importantly, although in vitro restimulation can result in TCR downregulation, surface expression of TCR after restimulation was sufficient to be readily detectable with antibodies (Figure S2B). Additionally, Vβ antibody staining of tetramer-binding Th1 cells at day 8 after LCMV infection in SMα mice was similar to that of IFN-γ-producing cells, (data not shown).

At day 8 after infection, Vβ7+ and Vβ8.1+ IFN-γ-producing responders showed significantly lower functional avidity than did Vβ14+ responders. They required 4- to 5-fold higher concentrations of peptide to induce a half-maximal response than did the Vβ14 subset. After the emergence of memory Th1 cells (≥42 days after infection), both the Vβ7 and Vβ8.1 subsets showed an increase in functional avidity, whereas the Vβ14 subset, which began at higher functional avidity during the effector response, maintained its high functional avidity and did not demonstrate an additional increase after memory Th1 cell differentiation (Figures 3G–3I). Overall, these findings demonstrate that the TCR repertoire of memory Th1 cells shows broad skewing, and they highlight a role for TCR-driven differentiation events in the selection of the memory T cell pool. Additionally, the memory T cell differentiation potential of each subset corresponded with the subject's functional avidity at the peak of the effector response, suggesting that the increased functional avidity of memory Th1 cells represents the selection of high-functional-avidity effector cells into the memory T cell pool and not broad functional avidity maturation of all responders.

Deep-Sequencing Analysis Reveals a Loss of Repertoire Diversity by Memory Th1 Cells

Although Vβ analysis can provide a broad overview of TCR repertoires, we sought to analyze the evolution of SMα TCR repertoires in a more specific and comprehensive way. As an initial approach to studying the evolution of TCR repertoires, we transferred large numbers of naive CD4+ T cells (5×10^6) from SMα mice (Thy1.1+) into B6 hosts (Thy1.2+) and followed this with LCMV infection 1 day later. The response of adoptively transferred SMα Th1 cells resembled that of the B6 host in terms of estimated expansion, as well as the onset and kinetics of contraction (Figures S3A–S3C). After fluorescence-activated cell sorting (FACS) of IFN-γ-producing SMα Th1 cells and RT-PCR-based cloning and sequencing of expressed TCRβ transcripts within the Vβ14 subset, we observed the emergence of a variety of clones and an apparent loss of clonal diversity in the memory Th1 population (Figures S3D and S3E). Although these preliminary studies confirmed that CD4+ T cells derived from SMα mice differentiated normally in a wild-type setting, they also presented a key caveat. Because of the relatively low precursor frequency of antigen-specific T cells in SMα mice, even transfer of large numbers of CD4+ T cells did not guarantee adequate representation of the full naive TCR repertoire in individual recipients. Therefore, we employed a more powerful deep-sequencing approach for the characterization and analysis of TCR repertoires.

We purified IFN-γ-producing effector (day 8 after infection) and memory (days 42, 75, and 150 after infection) Th1 cells from the spleens of LCMV or Lm-gp61 infected SMα mice by FACS (>95% purity), then used RT-PCR to amplify a small region of the Vβ7, Vβ8.1, or Vβ14 TCRβ chains encompassing the CDR3 region. Amplicons were multiplexed and sequenced with the Illumina HiSeq 2000 (Figure S3F). We minimized likely noise as a result of PCR error rate or contamination with nonspecific T cells during FACS isolation by choosing a rate cut-off (0.1%) that excluded all amino acid sequences that were observed only once (Figure S3G). We analyzed 2.6 million to 4.9 million nucleotide sequences per mouse and identified 275 unique CDR3 amino acid sequences across all mice, time points, and infections. A number of these sequences (57) were shared by at least 75% of mice (3/4) 8 days after either LCMV or Lm-gp61 infection (Table S1). We defined this group of TCR sequences as the "public" repertoire and the remaining sequences, observed in fewer mice at day 8 after infection, as the "private" repertoire.

A substantial number of public TCRs (33) were unique to LCMV infection, whereas few (7) were unique to Lm-gp61 infection (Figure S3H). These findings confirm that the public T cell repertoire recruited by Lm-gp61 is a subset of the repertoire recruited by LCMV and that the repertoire recruited by LCMV is broader overall. We next divided public clones into two groups: those whose representation within the overall T cell repertoire significantly declined between days 8 and 75 after infection and those whose representation increased or remained unchanged (Figure 4A; see also Table S1). After LCMV infection, 37% of public clones underwent at least a 50% decrease in frequency during the transition to the memory state, and for 24% of public clones, this decrease was statistically significant (Figure 4A; see also Table S1). Similarly, after Lm-gp61 infection 38% of public clones underwent a decrease of more than 50% in frequency as they entered the memory Th1 phase, and for 29% of public clones,
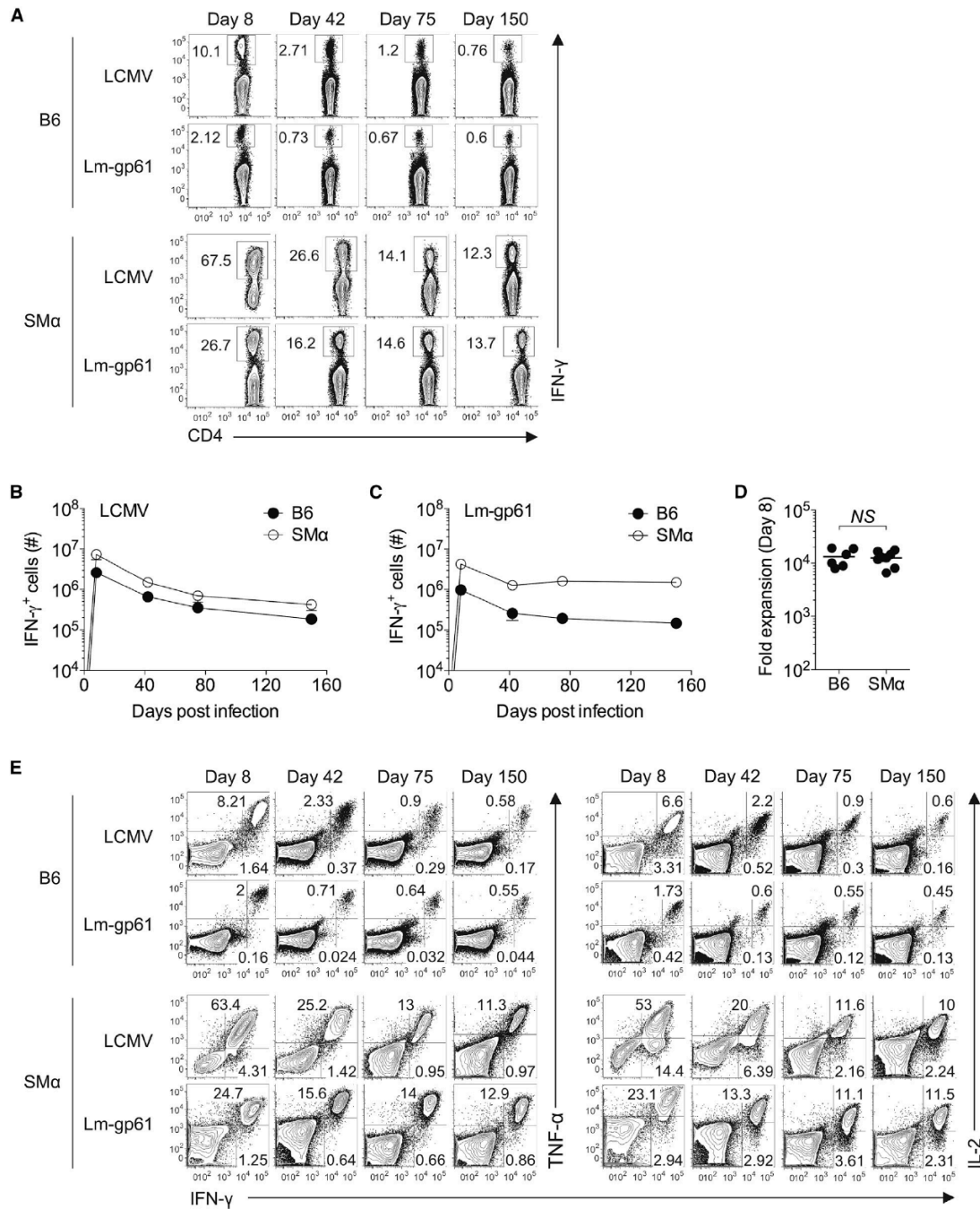
Figure 2. **Activated CD4⁺ T Cells in SMα Mice Undergo Physiological Expansion and Differentiation after Infection with LCMV or Lm-gp61**
B6 or SMα mice were injected with 5 × 10⁶ naive CD8⁺ T cells isolated from the spleens of B6 mice. One day later, mice were infected with LCMV or Lm-gp61.
(A) Representative plots indicate the frequency of IFN-γ-producing CD4⁺ T cells in the spleen at the indicated postinfection time points after ex vivo restimulation with GP₆₁₋₈₀ peptide in the presence of Brefeldin A.
(B and C) Graphs indicate the number of IFN-γ-producing cells in the spleen of B6 or SMα mice in a time course after infection with either LCMV or Lm-gp61. Error bars indicate SEM (n = 4 mice/group).

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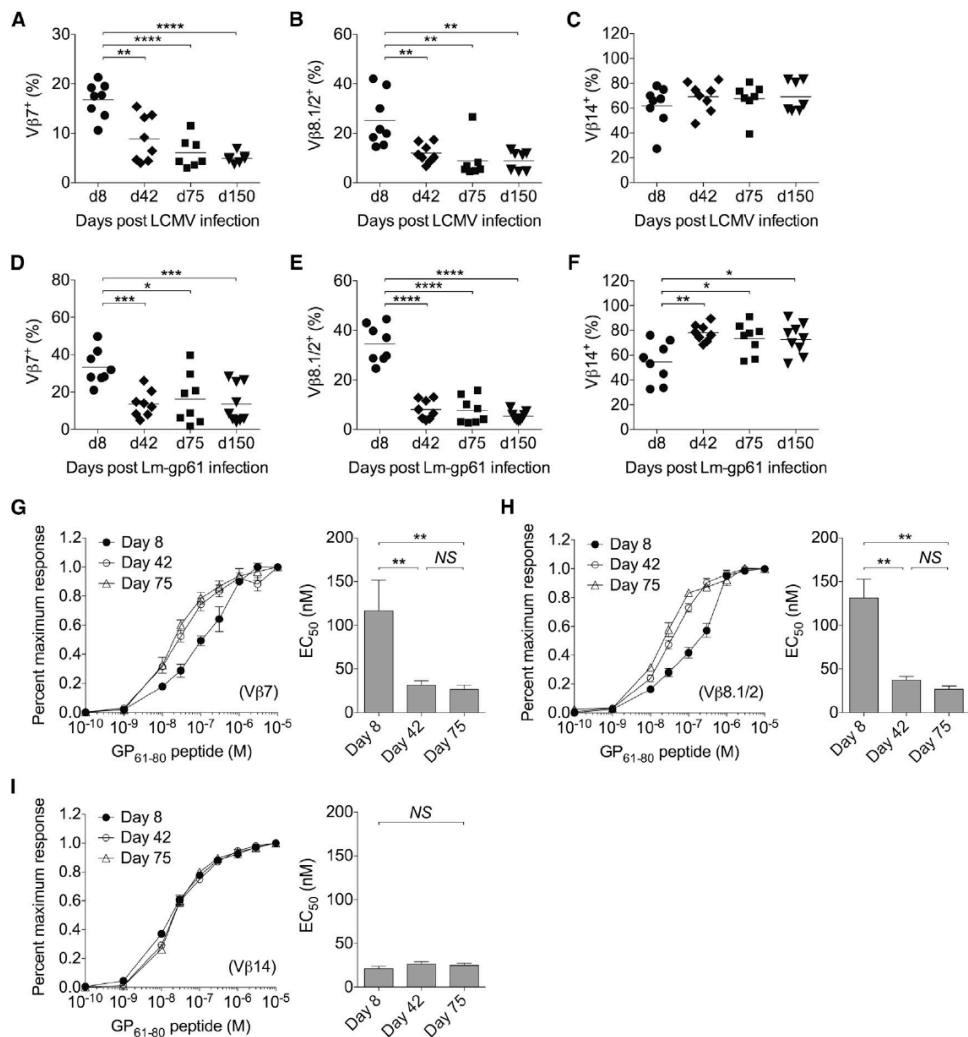
Figure 3. TCR Repertoires Skew in the Transition from Effector to Memory T Cells

(A–F) Graphs display the frequency of Vβ subsets among GP$_{61–80}$-specific IFN-γ$^+$CD4$^+$ T cells in the spleen for Vβ7, Vβ8 (Vβ8.1 and Vβ8.2), and Vβ14 at the indicated time points after (A–C) LCMV infection or (D–F) Lm-gp61 infection.

(G–I) Functional avidity, as measured by the percent maximal number of IFN-γ-producing cells at the indicated concentrations of peptide restimulation, was calculated for individual Vβ subsets after Lm-gp61 infection. Functional-avidity peptide-dose response curves are plotted at days 8, 42, and 75 after infection for (G) Vβ7 (H) Vβ8.1, and (I) Vβ14 subsets. Line plots display functional avidity maturation, and bar graphs indicate the effective peptide concentration required to elicit a half-maximal response (EC$_{50}$) for each subset at the indicated time points. Error bars indicate SEM (n = 4 mice/group). As determined by a Student's t test: *p < .05; **p < .01; ***p < .001; ****p < .0001, and NS = not significant (p > .05). See also Figure S2. Results are from two independent experiments.

this decrease was statistically significant. Overall, decreases in frequency ranged from 50% to almost 99% (Figure 4A; see also Table S1).

To determine whether changes in the frequency of individual clones within the memory cell pool could be observed on a global level, we calculated changes in clonal diversity among

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(D) The plot displays estimated n-fold expansion of GP$_{61–80}$-specific CD4$^+$ T cells during the first 8 days after infection on the basis of our calculations of naive precursor frequency in B6 or SMα mice. "NS" indicates "not significant," as measured by a two-tailed Student's t test (p > .05).

(E) Representative flow plots indicate the frequency of IFN-γ$^+$TNF-α$^+$ and IFN-γ$^+$IL-2$^+$ CD4$^+$ double producers in the spleen at the indicated time points after infection for B6 and SMα mice. Results are from three independent experiments.
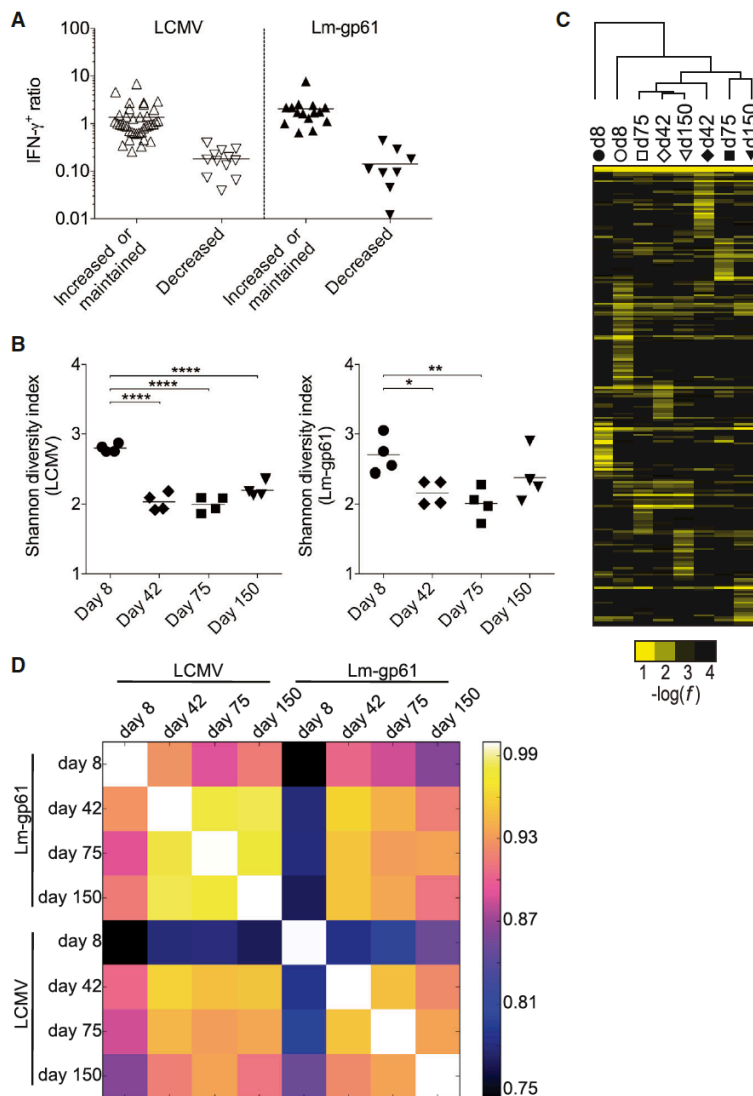
Figure 4. Deep-Sequencing TCR Repertoire Analysis Reveals a Loss of Clonal Diversity upon Emergence of Memory Th1 Cells

After deep sequencing, public clones were identified as those CDR3 sequences present at frequencies of >0.1% in at least 75% of mice at effector time points.

(A) Public TCRs after LCMV or Lm-gp61 were separated into two groups: (1) those whose frequency within the antigen-specific repertoire increased or was maintained and (2) those whose frequency within the antigen-specific repertoire decreased after memory Th1 cell differentiation. Plots indicate the ratio of memory (day 42–150) to effector (day 8) Th1 cells. Clones were placed in each group on the basis of the presence or absence of a statistically significant decrease in frequency within the total TCR repertoire between days 8 and 75 after infection (p < .05). Only mice in which the clone was present (n = 3–4/group) were included.

(B) Shannon's diversity index was used for calculating changes in TCR distribution and diversity at effector (day 8) and memory (days 42, 75, and 150) time points after infection with LCMV or Lm-gp61.

(C) Hierarchical cluster analysis of average rates (n = 4 mice per column) for each CDR3 sequence (open, LCMV; filled, Lm-gp61).

(D) Pairwise Pearson correlation coefficients of average repertoire profiles. As measured by a Student's t test: *p < .05; **p < .01; and ****p < .0001. See also Figure S3.

for the sets of sequence frequencies from each time point versus every other time point. In this analysis, the higher the coefficient, the greater the degree of similarity. Again, whereas memory T cell populations were similar to each other regardless of the infection model, they diverged dramatically from the effector T cell populations from which they arose (Figure 4D). Effector T cell populations also diverged strongly from each other, highlighting the diverse nature of the effector Th1 cell repertoire (Figure 4D). These data demonstrate selective representation of some effector T cell clones but not others within the memory T cell pool and suggest a role for TCR signals in memory T cell fate specification.

Entry into the Memory T Cell Pool Corresponds with MHCII Tetramer Off Rates

We next sought to determine the TCR-binding characteristics that corresponded to enhanced memory T cell differentiation. On the basis of the public TCR sequences obtained in our deep sequencing (Table S1), we cloned the *Tcrb* gene of 16 different public clones (Table 1). We cloned *Tcrb* chains with the SMARTA *Tcra* chain by conducting fusion PCR into a retroviral expression vector with a GFP reporter. We separated the *Tcra* and *Tcrb* genes by a P2A sequence to allow for bicistronic

all public and private TCRs by using Shannon entropy analysis (Singh et al., 2010; Stewart et al., 1997). We found that memory Th1 differentiation was accompanied by a significant decrease in overall diversity (Figure 4B) for both LCMV and Lm-gp61 infection. In large part this decrease in diversity took place in between days 8 and 42 after infection, whereas during the long-term maintenance of Th1 memory cells, TCR repertoire diversity remained stable (Figure 4B). Additionally, we performed hierarchical cluster analysis based on the average frequency of TCR sequences at each time point. In general, memory T cell populations were more similar to each other than to the effector T cell populations from which they arose, even if they arose from disparate infections (Figure 4C). We also calculated the Pearson correlation coefficient

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Table 1. Tetramer-Binding Properties of Cloned Public TCRs

Clone ID	CDR3β	Length	Vβ	Jβ	K_d (nM)	Off Rate (min)
clone 1	ALQGDNNQAPL	11	TRBV31*01	TRBJ1-5*01	5.52	824.4
clone 2	AWRPGTANSDYT	12	TRBV31*01	TRBJ1-2*01	88.34	709.4
clone 5	AWSRDSSDYT	10	TRBV31*01	TRBJ1-2*01	7.43	803.3
clone 7	AWSLPNYAEQF	11	TRBV31*01	TRBJ2-1*01	7.93	591.9
clone 10	ASSDFGQGAERLF	13	TRBV13-3*01	TRBJ1-4*02	2.77	54.2
clone 13	ASSDQNNQAPL	11	TRBV13-3*01	TRBJ1-5*01	15.58	620.5
clone 18	AWSLWEYAEQF	11	TRBV31*01	TRBJ2-1*01	92.37	212.0
clone 19	AWSPGLGVNYAEQF	14	TRBV31*01	TRBJ2-1*01	116.20	501.6
clone 20	AWSLIEVF	8	TRBV31*01	TRBJ1-1*01	2.33	466.5
clone 22	ASSDHNQANTEVF	13	TRBV13-3*01	TRBJ1-1*01	10.86	379.0
clone 24	ASSEMGTGIETLY	13	TRBV13-3*01	TRBJ2-3*01	3.31	124.4
clone 25	ASSLAGTGGYEQY	13	TRBV29*01	TRBJ2-7*01	6.32	151.3
clone 26	ASSSPGTANYAEQF	14	TRBV29*01	TRBJ2-1*01	4.50	38.2
clone 27	ASSPSGTGGYEQY	13	TRBV29*01	TRBJ2-7*01	3.57	43.3
clone 28	ASSLHNSGNTLY	12	TRBV29*01	TRBJ1-3*01	8.71	288.8
clone 29	AWSLPNSYEQY	11	TRBV31*01	TRBJ2-7*01	5.46	265.7

Sixteen TCRβ sequences were cloned into a retroviral expression vector and paired to the SMARTA TCRα by fusion PCR. The CDR3β amino acid sequence, length, Vβ usage, and Jβ usage for each clone are displayed. Retroviral vectors expressing cloned TCRs were transfected into 293T cells, and tetramer staining and calculation of apparent K_d and tetramer off rates followed. See also Table S1.

expression, as previously described (Holst et al., 2006; Osborn et al., 2005).

We transfected 293T cells with TCR retroviral expression vectors, along with multi-cistronic retroviral vectors encoding *Cd3d*, *Cd3e*, *Cd3g*, and *Cd247*, in order to permit surface TCR expression (Holst et al., 2006). Because the absence of CD4 does not enhance the avidity of tetramer binding to MHCII (Crawford et al., 1998; Hamad et al., 1998), we directly stained transfected 293T cells with decreasing concentrations of GP_{66-77} tetramer, normalized to GFP and surface TCR expression in equilibrium binding assays. We then generated Scatchard plots and calculated apparent K_d values (Figures S4A–S4C). The panel of TCRs displayed K_d values across a 50- to 100-fold range (Figure 5A). Next, we performed tetramer decay assays to determine the tetramer dissociation rate for each TCR (Figure 5B; see also Figure S4D). Tetramer off rates and apparent K_d values for each TCR were discordant (Figures 5A and 5B). Some clones demonstrated high-avidity binding to tetramers but quick off rates, whereas others demonstrated low-avidity binding but extremely slow off rates (Table 1; see also Figure S4E). When each of these parameters was compared to the survival of individual TCR clones between days 8 and 42 after infection, the only significant predictor of memory T cell potential was the tetramer off rate (Figure 5C). We obtained similar results when we compared tetramer off rates to the total numbers of IFN-γ-producing cells in the spleen as a ratio between days 8 and 42 after infection (Figure 5D). One example of this phenomenon was TCR clone 2, a dominant Vβ14+ clone present at enriched frequencies within the memory Th1 population. Although clone 2 TCR bound tetramer with low avidity at equilibrium binding concentrations, it maintained extremely slow off rates in tetramer decay assays (Table 1; see also Figures S4D and S4E).

Sustained TCR-pMHCII Interactions Promote Memory Fate Specification

To directly assess the role of sustained TCR-pMHCII interactions in promoting the differentiation of Th1 memory cells, we measured the impact of individual TCRs on Th1 memory cell differentiation potential in the context of infection in a wild-type mouse. We used the above-described TCR retroviral vectors to transduce RAG-deficient bone marrow and to subsequently generate TCR retrogenic bone marrow chimeras, as previously described (Holst et al., 2006). Eight to ten weeks after bone marrow transplantation, naive CD4+GFP+ T cells were harvested from their spleens, transferred into naive B6 mice in small numbers (1 × 10^4 cells per recipient), and stimulated by LCMV infection 1 day later. All TCRs that we tested expanded potently and produced cytokines in response to GP_{61-80} peptide stimulation after LCMV infection (Figure S5 and data not shown), verifying that our model system and our criteria for establishing cut-offs for individual sequences effectively identified antigen-specific TCR clones.

We selected four clones for further analysis on the basis of their similar surface TCR expression both before and after activation (Figure 6A). These clones (clones 2, 7, 25, and 27) exhibited a range of tetramer off rates and tetramer binding avidity (Table 1). After LCMV infection of host B6 mice, each clone expanded and formed memory T cells, albeit to varying extents, and produced IFN-γ upon restimulation at both day 8 and day 42 after infection in the spleen (Figures 6B and 6C). The memory potential of each effector Th1 cell population, as measured by the overall decline in numbers in the spleen between days 8 and 42 after infection, varied widely: two clones (2 and 7) underwent minimal contraction, a third clone (25) underwent moderate contraction, and a fourth clone (27) underwent extensive contraction (Figure 6C). The memory potential of each clone did not correspond to primary expansion (Figure 6D) but rather
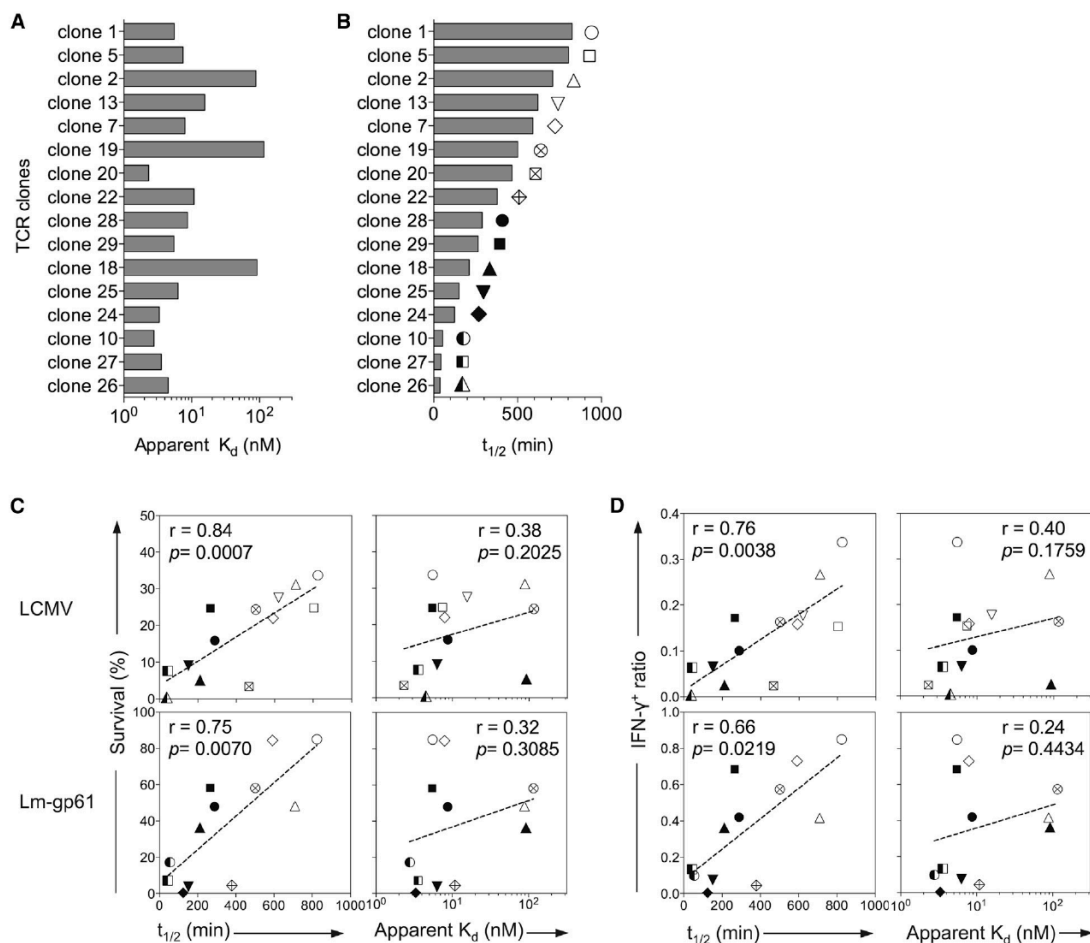
Figure 5. CD4+ Memory T Cell Differentiation Is Driven by Slow TCR-pMHCII Dissociation Rates

293T cells were transfected with retroviral vectors expressing the indicated TCR clone and a GFP reporter. Samples were subsequently stained with tetramer and normalized to GFP and cell-surface TCR expression.

(A) The bar graph displays the apparent K_d for each clone, as determined by the intensity of tetramer binding under equilibrium binding conditions for varying tetramer concentrations.

(B) For each clone, bar graphs display the tetramer binding half-life, determined from tetramer binding decay (normalized fluorescence) after the addition of high concentrations of MHCII-blocking antibody. Apparent K_d and half-life measurements are representative of 2–3 separate transfections for each clone.

(C and D) For each clone, plots display tetramer-binding half-life or apparent K_d on the x axis and, on the y axis, (C) the percent survival between day 8 and day 42 or (D) the ratio of the total number of IFNγ-producing cells in the spleen at memory (day 42) versus effector (day 8) time points of individual TCR clones observed by deep sequencing. Dotted lines indicate the best fit by linear regression. Correlation and significance were calculated by a two-tailed Spearman's rank correlation. See also Figure S4.

corresponded to tetramer off rates (Figure 6E). These findings validate and recapitulate the results derived from our deep-sequencing data for individual clones. We conclude that sustained TCR-pMHCII interactions are a key component in promoting decisions about Th1 memory cell fate in vivo.

DISCUSSION

Overall, these findings demonstrate that sustained TCR-pMHCII interactions are a key component of the memory T cell differen-

tiation signal for CD4+ T cells. Although TCR-pMHCII interactions play important roles in T cell activation, function, and survival, we report here that the kinetics of TCR-pMHCII interactions can differentially discriminate between end-stage effector and memory differentiation programs in T cells. Previous studies of the role of antigens in the emergence of high-avidity secondary responders have largely concluded that this event occurs as a result of antigen-driven selection of high-avidity clones throughout the primary and secondary response (Savage et al., 1999). In contrast, we found that specific TCR binding properties

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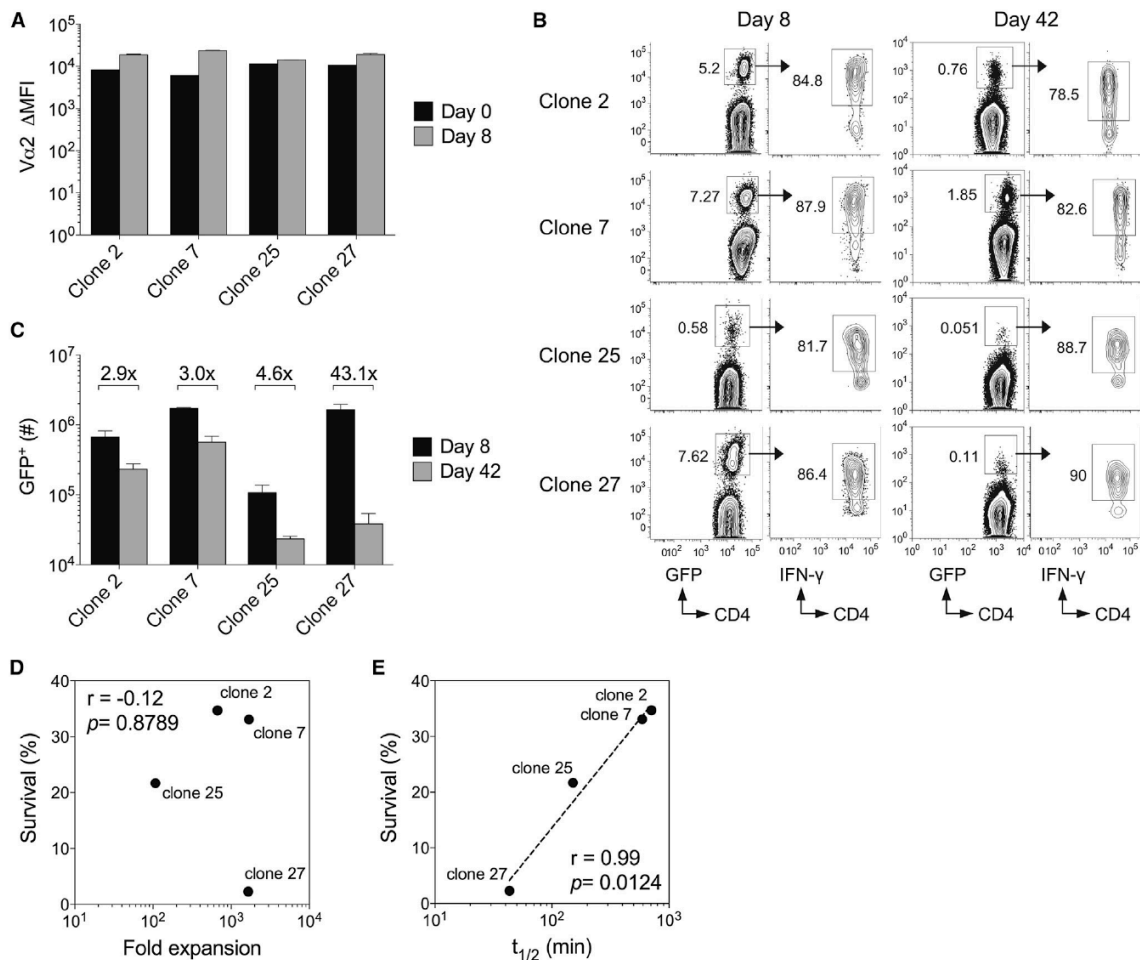
Figure 6. Sustained TCR-pMHCII Interactions Promote CD4+ Memory T Cell Differentiation

TCR "retrogenic" T cell clones (GFP+) (1 × 10⁴) were adoptively transferred into B6 mice, and LCMV infection followed 1 day later.

(A) The bar graph indicates Vα2 surface expression as determined by antibody staining and calculated as the shift in mean fluorescence intensity as compared to that in Vα2-negative CD4+ T cells in the same host.

(B) Representative flow plots indicate the frequency of GFP+ retrogenic T cells within the CD4+ T cell population in the spleen, as well as IFN-γ production by gated GFP+ T cells at days 8 and 42 after infection with LCMV.

(C) The bar graph indicates the total number of GFP+ retrogenic T cells in the spleen at days 8 and 42 after infection for the indicated clones. Numbers indicate the n-fold difference in absolute numbers between days 8 and 42. Error bars indicate the SEM (n = 4–5 mice/group).

(D) The plot indicates n-fold expansion between days 0 and 8 for each clone on the x axis and the percent survival between days 8 and 42 on the y axis.

(E) The plot indicates the tetramer off rate for each clone on the x axis and the percent survival between days 8 and 42 on the y axis. A dotted line indicates the best fit by linear regression. Correlations and their significance were calculated by two-tailed Spearman's rank correlation. Results are from two independent experiments. See also Figure S5.

and the signals they deliver promote a CD4+ memory T cell differentiation program that takes place once antigens are cleared.

A simple model of TCR-pMHCII interaction would suggest that long-lived interactions between a single TCR and its MHC-restricted antigen are a key step in the initiation and amplification of the T cell signaling cascade required for robust activation and differentiation. In support of this, different occupation rates of phosphorylation sites of CD3 subunits have been associated

with peptides over a defined range of affinities and agonist activity for a fixed TCR (Kersh et al., 1998; Rabinowitz et al., 1996). However, a strictly quantitative model of TCR signaling does not fully predict biological outcomes after T cell activation as they relate to memory T cell development and function. Recent studies have found that the polyclonal CD4+ T cell response to LCMV is populated with clones that are unable to bind pMHCII tetramers at the peak of the effector response and after viral

clearance (Huang et al., 2010; Sabatino et al., 2011). Another study has found that the variable ability of monoclonal populations to either expand during the primary response or generate effective secondary responses is not necessarily determined by TCR affinity for cognate antigens, suggesting that the polyclonal response could be populated with clones that have highly variable and complex fates (Weber et al., 2012b). Our own findings suggest that representation within the Th1 effector cell compartment is not necessarily indicative of memory potential (Williams et al., 2008). Importantly, a recent report has also found that the duration of TCR-pMHCII interactions can influence CD4⁺ T cell responses during commitment to Th1 or Tfh cell differentiation (Tubo et al., 2013), providing additional evidence that sustained interactions between the TCR and antigen provide a unique signal for cellular differentiation independent of recruitment and expansion.

A variety of other factors most likely influence biological outcomes related to TCR binding of pMHCII; such factors include TCR surface expression and the clustering of TCRs and CD3 subunits on the cell surface, a factor that has previously been shown to enhance antigen sensitivity (Kumar et al., 2011). Deciphering the interplay of these factors, along with the actual kinetics of TCR-pMHCII interactions, is key to our understanding of how T cells incorporate activation signals to initiate distinct differentiation programs. For example, although quantitative differences in the magnitude of the TCR signal might play a role in differentiating between effector and memory T cell fate, TCR signals delivered in short bursts and with quick dissociation rates may also be qualitatively distinct from those characterized by more sustained signaling events and slow dissociation rates.

Although we identify here a role for the TCR in promoting effector versus memory Th1 cell differentiation, the differentiation, function, and survival of Th1 memory cells is driven by TCR-independent factors as well. For example, SMARTA TCR transgenic T cells exhibit a range of functional avidities at the peak of their effector response and an increase in functional avidity during the transition to the memory state (Williams et al., 2008). Factors such as the inflammatory microenvironment, the activation status of the APC, and the amount of antigen presented could influence the acquisition and maintenance of high antigen sensitivity. One possibility is that effector Th1 cells that acquire higher antigen sensitivity during the primary response and are therefore better able to initiate sustained TCR activation compete more effectively for entrance into the memory T cell pool. A key focus of future studies will be to delineate how T cell intrinsic and extrinsic factors cooperate to initiate a memory differentiation program.

Most prior studies have relied on the analysis of a fixed TCR binding to altered peptide ligands or on genetic alterations to the TCR itself to adjust binding properties. Here, however, we have relied on analysis of TCRs during a biological response to an infectious pathogen. All TCRs included in our analysis have passed thresholds of activation, differentiation, and effector function, allowing us to compare differences in agonist-driven T cell activation. Given the availability of a large panel of naturally derived TCRs with known antigen-binding properties, our future studies will focus on the qualitative and quantitative nature of TCR signaling as it relates to off rates and memory T cell fate determination.

EXPERIMENTAL PROCEDURES

Mice and Infections

C57BL/6, Rag1-deficient, and TCRα-deficient (6- to 8-week-old) mice were purchased from Jackson Laboratories. SMARTA mice (Oxenius et al., 1998) were maintained in our colony at the University of Utah. SMα mice were generated on a C57BL/6 background at the University of Utah Transgenic Core Facility by standard microinjection techniques involving a T-cell-specific expression vector, VA-hCD2, in which the SMARTA Tcra gene was placed under the control of the human Cd2 promoter and a 3' locus control region of the Cd2 gene (provided by M. Bevan, University of Washington, Seattle) (Zhumabekov et al., 1995). LCMV Armstrong 53b was grown in baby hamster kidney cells, titered in Vero cells as described (Ahmed et al., 1984), and injected intraperitoneally into recipient mice at a dose of 2×10^5 plaque-forming units. Recombinant Listeria monocytogenes expressing the $GP_{61–80}$ epitope of LCMV (Lm-gp61, provided by M. Kaja-Krishna, Emory University, Atlanta) was grown to log phase in brain heart infusion broth, and the concentration was determined by measurement of the O.D. at 600 nm. Mice were injected intravenously with 2×10^5 colony-forming units. All mouse experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Utah.

Cell Preparations and Flow Cytometry

Splenocyte and lymph node cell suspensions were placed in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, streptomycin, and L-glutamine. For cell-surface stains, cells were incubated with fluorescently conjugated antibodies (eBiosciences or BD Biosciences) diluted in antibody staining buffer (PBS containing 1% fetal bovine serum) at 4°C. For intracellular cytokine assays, splenocytes were restimulated for 4 hr with 10 μM (or indicated dilutions in functional avidity assays) $GP_{61–80}$ peptide from LCMV (GLKGPDIYKGVYQFKSVEFD) at 37°C in the presence of Brefeldin A (GolgiPlug, 1 μl/ml) per the manufacturer's instructions (BD Biosciences). Samples were then stained with cell-surface antibodies in antibody staining buffer, and permeabilization with a kit (BD Biosciences) and staining with fluorescently labeled antibodies specific to the indicated cytokines followed.

Adoptive Transfers and TCR Sequencing

Untouched naive (Thy1.1⁺CD44ˡᵒ) CD4⁺ T cells were isolated from SMα mice via magnetic beads (Miltenyi) and injected intravenously into B6 mice, and mice were infected with LCMV 1 day later. For direct infection of SMα mice, untouched CD8⁺ T cells were isolated from the spleens of B6 mice via magnetic beads (Miltenyi) and injected intravenously into SMα mice 1 day prior to infection. MHCII-tetramer-based enrichment of naive antigen-specific T cells was performed with magnetic beads (Miltenyi) as described elsewhere (Moon et al., 2007). Live IFN-γ-producing, antigen-specific CD4⁺ T cells were isolated from the spleens of infected mice at the indicated time points after infection with the use of a kit (Miltenyi), and fluorescence-activated cell sorting (FACS) (FACSAria II, BDBiosciences) followed. We purified RNA (RNEasy, QIAGEN), generated a cDNA template (Superscript III, Invitrogen), and performed RT-PCR. For adoptive-transfer experiments, we amplified the entire Tcrb molecule by using primers specific for Vβ14 and then used TA cloning to place it in the pCR2.1-TOPO vector by using a kit (Invitrogen). We then isolated plasmids from individual colonies and sequenced Tcrb molecules at the University of Utah DNA Sequencing Core Facility. For deep-sequencing studies of TCRs derived from direct infection of SMα mice, we used primers designed to amplify a small ~110 base pair portion of Tcrb encompassing the CDR3 region. The primer sets for each of Vβ subsets encompassed the following sequences: Vβ7, 5'-GACATCTGTGTACTTCTGTGC-3'; Vβ8.1, 5'-ACAGCTGTATATTTCTGTGCC-3'; Vβ14, 5'-TCTGGCTTCTACCTCTGTG CC-3'; and Cβ-specific reverse, 5'-CTTGGGTGGAGTCACATTTCTCAGA TCC-3'. Amplicons were multiplexed and underwent single-end 50 base pair sequencing performed with the Illumina HiSeq 2000 at the University of Utah Microarray and Sequencing Core Facility. Data were segregated on the basis of barcode as well as sequences corresponding to specific Vβ regions, and low-quality reads (Phred score < 38 for 20 of 50 bases) were excluded from the analysis.

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Analysis of TCR Repertoires

After trimming the primer sequence from each read, we translated nucleotide sequences to determine the CDR3 amino acid sequence and calculated intra-subset frequencies for each distinct amino acid sequence for every mouse. We converted these to global frequencies for each CDR3 sequence in each mouse by multiplying them by relative Vβ subset frequency, as determined by flow cytometry. To reduce the introduction of sequencing artifacts into the analysis, we required that an amino acid sequence be present in at least two mice at a frequency greater than an empirically determined cut-off. On the basis of the distribution of sequence frequencies (Figure S3G), we chose a cutoff of 0.1% to exclude singleton observations from the analysis of public and private sequences. We performed hierarchical clustering on average pathogen-specific subset-sequence frequencies by combining the four mice at each time point and applying the 0.1% cutoff to the average values. Clustering was performed with the Pearson correlation and Cluster 3.0 (Eisen et al., 1998) and visualized with JavaTreeview (Saldanha, 2004). Pairwise correlations were calculated with NumPy and visualized with Matplotlib (Hunter, 2007). The IMGT database and the IMGT/V-QUEST tool (IMGT, The International Immunogenetics Information System, http://www.imgt.org) were used for identification and verification of TRBV, TRBJ, and CDR3 sequences. Shannon's diversity index, which reflects both abundance and richness, was used for evaluation of TCR sequence diversity (Stewart et al., 1997). Shannon's diversity index was calculated as $H' = -\Sigma[p_i \times \ln(p_i)]$, where p_i is the proportion of TCR sequence i.

TCR Cloning and Retrogenic Bone Marrow Chimeras

Tcrb genes were cloned by fusion PCR and expressed in a retroviral vector (MigR1) along with the SMARTA *Tcra*. In this vector, the *Tcra* and *Tcrb* coding regions were separated by the picornavirus-derived P2A sequence, a *cis*-acting hydrolase element that allows for bicistronic expression (Szymc-zak et al., 2004). The vector additionally contained a GFP reporter under the control of an IRES. To analyze TCR binding properties, we transfected TCR expression vectors, along with a retroviral vector driving expression of the *Cd3d*, *Cd3e*, *Cd3g*, and *Cd247* subunits (provided by D.A. Vignali, St. Jude Children's Research Hospital, Memphis) (Holst et al., 2006) into 293T cells by using FuGENE (Promega). We transduced TCR-expressing retroviruses into Rag1-deficient bone marrow cells by using described methods (Holst et al., 2006; Yun and Bevan, 2003), then injected 1×10^6 bone marrow cells intravenously into irradiated (450 rads) Rag1-deficient hosts. Eight to ten weeks later, GFP^{+}TCR^{+}CD4^{+} T cells harvested from the spleens of the retrogenic chimeras were intravenously transferred (1×10^4 cells per recipient) into B6 hosts. Recipient mice were infected with LCMV 1 day later.

Tetramer Staining and Analysis

MHCII monomers bound to GP$_{66-77}$ were expressed by stably transfected S2 cells, purified, and converted into fluorescently tagged tetramers via previously described methods (Pepper et al., 2011; S2 cells were provided by M. Pepper, University of Washington, Seattle). Staining was performed at 25°C for 1 hr in RPMI containing 2% fetal calf serum and 0.1% sodium azide, and washing and cell-surface staining followed. Tetramer fluorescence was normalized to samples stained with control hCLIP tetramer (NIH Tetramer Core Facility). Scatchard plots and apparent K_d were calculated as described (Savage et al., 1999). Fluorescence units (bound) were plotted on the x axis, and fluorescence units divided by tetramer concentration (bound/free) were plotted on the y axis. K_d was determined as the inverse of the slope. For tetramer decay assays, after extensive washing of tetramer-stained cells and cell-surface staining, cells were incubated in high concentrations (100 μg/ml) of I-A[b] blocking antibody (BioLegend). For the determination of tetramer-binding half-life, total fluorescence of tetramer binding at various time points after MHCII blockade was normalized to the total fluorescence at the zero time point, as described elsewhere (Savage et al., 1999).

ACCESSION NUMBERS

The Sequence Read Archive (SRA) accession number for the TCR sequences reported in this paper is SRA100070.

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CHAPTER 3

STABILITY AND FUNCTION OF SECONDARY

TH1 MEMORY CELLS ARE DEPENDENT

ON THE NATURE OF THE

SECONDARY STIMULUS

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Stability and Function of Secondary Th1 Memory Cells Are Dependent on the Nature of the Secondary Stimulus

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Following acute infection in some mouse models, CD4$^+$ memory T cells steadily decline over time. Conversely, in humans, CD4$^+$ memory T cells can be maintained for many years at levels similar to CD8$^+$ T cells. Because we previously observed that the longevity of Th1 memory cell survival corresponded to their functional avidity, we hypothesized that secondary challenge, which enriches for high functional avidity Th1 responders, would result in more stable Th1 memory populations. We found that following a heterologous secondary challenge, Th1 memory cells were maintained at stable levels compared with primary Th1 memory cells, showing little to no decline after day 75 postinfection. The improved stability of secondary Th1 memory T cells corresponded to enhanced homeostatic turnover; enhanced trafficking of effector memory Th1 cells to tissue sites of infection, such as the liver; and acquisition or maintenance of high functional avidity following secondary challenge. Conversely, a weaker homologous rechallenge failed to induce a stable secondary Th1 memory population. Additionally, homologous secondary challenge resulted in a transient loss of functional avidity by Th1 memory cells recruited into the secondary response. Our findings suggest that the longevity of Th1 memory T cells is dependent, at least in part, on the combined effects of primary and secondary Ag-driven differentiation. Furthermore, they demonstrate that the quality of the secondary challenge can have profound effects on the longevity and function of the ensuing secondary Th1 memory population. *The Journal of Immunology*, 2012, 189: 2348–2355.

A key feature of memory T cells is their ability to self-renew and persist at stable levels for long periods of time. In mouse models of acute infection, CD8$^+$ memory T cells, once established, are maintained with no observable decline throughout the life of the mouse (1, 2). CD8$^+$ and CD4$^+$ memory T cells specific for the smallpox vaccine in humans persist for many years, with population half-lives estimated in the range of 8–15 y (3, 4). Smallpox survivors demonstrate equally robust long-lived immunological memory (5). In contrast, mouse models of acute viral or bacterial infection suggest that the mechanisms that control the stability of CD4$^+$ memory T cell populations are distinct, because, in certain cases, they were noted to decline over time (6–8). In at least one study, CD4$^+$ memory T cells became virtually undetectable by 2 y postinfection (6), although the rate of memory decay may decline over time (8). Understanding the mechanisms that control the generation and survival of CD4$^+$ memory T cell populations that are stable at high frequencies is of critical importance in generating more effective vaccination and immunotherapeutic strategies.

Several factors were shown to regulate the homeostatic turnover and survival of memory T cell populations. Of these, the best

described are the cytokines IL-7 and IL-15. Both CD4$^+$ and CD8$^+$ T cells receive signals via these cytokines that regulate cell division and survival; presumably, the relative rates of each process determine the overall stability of the memory population (9–11). It is also possible that the activation and differentiation signals delivered during initial T cell priming also play a key role in regulating the long-term fate of memory T cells. For example, various aspects of CD8$^+$ memory T cell survival and function are programmed through the influence of CD4$^+$ T cell help (12–14) and IL-2 (2, 15, 16).

The differentiation of CD4$^+$ T cells differs from that of CD8$^+$ T cells in several key ways. First, although CD8$^+$ effector and memory T cell differentiation is programmed after a short period (6–24 h) of Ag exposure (17–19), CD4$^+$ T cells require longer periods of Ag stimulation (3–4 d) for optimal expansion and differentiation (20–22). Second, CD4$^+$ T cell effector differentiation is dependent, at least in part, on the strength of the antigenic stimulus (23–27). Third, CD4$^+$ T cell repertoires skew to higher-avidity responders upon successive antigenic challenges (28, 29). Last, we recently observed that the transition of CD4$^+$ effector T cells into the memory pool, as well as the emergence of very long-lived CD4$^+$ memory T cells, coincided with an increased ability of surviving memory cells to respond to low concentrations of Ag (8). Collectively, these findings suggest that, in comparison with CD8$^+$ T cells, CD4$^+$ T cells are subject to a prolonged period of selection on the basis of their ability to bind Ag and that the nature of the antigenic signal impacts all subsequent phases of CD4$^+$ effector and memory T cell differentiation and survival.

Because the emergence of CD4$^+$ memory T cells that are highly sensitive to Ag stimulation corresponds to a decrease in the rate of memory decay (8), and high-avidity CD4$^+$ responders are enriched following secondary challenge, we hypothesized that secondary challenge of Th1 memory T cells would result in stable secondary Th1 memory populations that did not undergo decay. To test this hypothesis, we used a model of heterologous rechallenge using lymphocytic choriomeningitis virus (LCMV) and a recombinant *Listeria monocytogenes* expressing the immunodominant MHC

class II-restricted epitope from the LCMV glycoprotein, gp61–80 (Lm-gp61). This system allows for robust boosting of CD4$^+$ memory T cells without rapid Ag clearance mediated by broadly reactive CD8$^+$ T cells or Ab. Although primary memory cells declined for several months postinfection with LCMV or Lm-gp61, a strong secondary stimulus induced by heterologous secondary challenge (i.e., LCMV-immune mice rechallenged with Lm-gp61 or Lm-gp61–immune mice rechallenged with LCMV) resulted in robust secondary expansion, retention of high-level functionality, and long-term stability of the resulting secondary memory populations. In contrast, a weaker secondary stimulus induced by homologous rechallenge (i.e., LCMV-immune mice rechallenged with LCMV or Lm-gp61–immune mice rechallenged with Lm-gp61) resulted in poor secondary expansion, a failure to achieve enhanced secondary function, and the decay of secondary memory populations with kinetics similar to primary memory cells. Furthermore, although heterologous rechallenge resulted in a relative increase in the distribution of long-lived Th1 memory cells to peripheral sites of infection, such as the liver, homologous rechallenge did not result in a similar enrichment. Secondary CD4$^+$ memory T cells induced by heterologous challenge expressed similar levels of homeostatic cytokine receptors and the prosurvival molecule Bcl-2 as did primary CD4$^+$ memory T cells. However, long-lived secondary memory cells induced by heterologous rechallenge turned over at a significantly more rapid rate than did both their primary memory counterparts and secondary memory cells induced by homologous rechallenge, suggesting an intrinsically enhanced capacity to respond to homeostatic signals from the host. Overall, our findings suggest that, although secondary challenge can result in the enrichment of highly functional and stable Th1 memory cells, their overall fate and function are heavily influenced by the nature of the secondary stimulus. Therefore, these findings are directly applicable in the design of vaccination strategies that target CD4$^+$ T cell responses and in validating their efficacy.

Materials and Methods

Mice and infections

Six- to eight-week-old C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Thy1.1$^+$ SMARTA TCR transgenic mice were maintained in our colony at the University of Utah (30). All animal experiments were conducted with the approval of the Institutional Animal Care and Use Committee at the University of Utah. LCMV Armstrong 53b was grown in BHK cells and titered in Vero cells (31). For primary challenges and heterologous rechallenges, mice were infected i.p. with 2×10^5 PFU. For homologous rechallenges, mice were infected with 2×10^6 PFU i.v. Lm-gp61 (M. Kaja-Krishna, University of Washington, Seattle, WA) was characterized previously and generated using described methods (8, 32, 33). Prior to infection, the bacteria were grown to log phase, and concentration was determined by measuring the OD at 600 nm (OD of $1 = 1 \times 10^9$ CFU/ml). For primary infections and heterologous rechallenges with Lm-gp61, mice were injected i.v. with 2×10^5 CFU. For homologous rechallenges, mice were injected i.v. with 2×10^6 CFU. All mice were initially infected when 8–12 wk of age, and secondary challenges occurred 60–75 d after primary infection in all cases.

Cell preparations and flow cytometry

Splenocytes were placed in single-cell suspension in DMEM containing 10% FBS and supplemented with antibiotics and L-glutamine. Liver lymphocytes were isolated from perfused whole livers following digestion in Collagenase B and DNAse I (Roche) for 1 h, followed by Percoll (Sigma-Aldrich) separation and resuspension in the same media as described above. For CFSE experiments, SMARTA splenocytes were labeled using the CellTrace CFSE Labeling Kit (Invitrogen), according to the manufacturer's instructions, followed by i.v. adoptive transfer (1×10^6 SMARTA/mouse). For cell surface staining, cells were incubated with fluorescent dye-conjugated Abs, with specificities as indicated (eBio-

sciences, San Diego, CA, or BD Biosciences, Mountain View, CA), in PBS containing 1% FBS. MHC class II tetramers presenting gp$_{66-77}$ in the context of I-A^b were obtained from the National Institutes of Health tetramer core facility (Atlanta, GA). Tetramers were incubated with cells in RPMI 1640 containing 2% FBS and 0.1% sodium azide at 37°C for 3 h, followed by cell surface staining in PBS with 1% FBS. Ab-stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences), and results were analyzed using FlowJo software (TreeStar).

Peptide restimulation and intracellular staining

Resuspended cells were stimulated for 4 h with 1 μM the gp$_{61-80}$ peptide (GLKGPDIYKGVYQFKSVEFD) in the presence of brefeldin A (1 μl/ml GolgiPlug). Cells were stained with cell surface Abs, permeabilized, and stained with cytokine Abs using a kit, per the manufacturer's instructions (BD Biosciences). In some cases, cells were also stained with Bcl-2 Abs (BD Biosciences) at the same time as the cytokine Abs. For functional avidity assays, cells were restimulated with a range of peptide concentrations (10 μM–0.1 nM) prior to cytokine staining, with the percentage of maximal response determined by calculating the frequency of IFN-γ–producing cells at any given concentration as a percentage of the frequency of IFN-γ–producing cells at the highest peptide concentration.

BrdU assays

BrdU (Sigma-Aldrich, St. Louis, MO) was added to the drinking water of mice at 0.8 mg/ml for 2 wk. Splenocytes were harvested and resuspended in media, followed by peptide restimulation, as described above. Cells were surface stained, permeabilized, treated with DNAse I, and costained with BrdU and cytokine Abs using a kit, per the manufacturer's instructions (BD Biosciences).

Results

Heterologous boosting results in stably maintained secondary Th1 memory cells

We infected B6 mice with either LCMV or Lm-gp61 to induce CD4$^+$ effector and memory T cells under distinct infectious conditions. These pathogens share a single MHC class II-restricted epitope (gp$_{61-80}$), along with a subdominant class I-restricted epitope (gp$_{67-77}$) (34). Following the establishment of memory (>60 d postinfection), LCMV-immune mice were heterologously rechallenged with Lm-gp61, and Lm-gp61–immune mice were heterologously rechallenged with LCMV. gp$_{61-80}$-specific primary and secondary IFN-γ–producing Th1 responders in the spleen were then measured at effector and memory time points over the next 200 d by ex vivo peptide restimulation, followed by intracellular cytokine staining. gp$_{61-80}$-specific CD4$^+$ T cells exhibited a vigorous expansion after either LCMV or Lm-gp61 infection, followed by contraction and the development of primary Th1 memory cells. In agreement with previous reports, primary Th1 memory cells gradually declined over time following either infection. Heterologous rechallenge also resulted in a massive expansion of primary Th1 memory cells during the first week of the recall response, followed by the development of secondary Th1 memory cells with a significantly increased frequency compared with that of primary memory cells (Fig. 1A, 1C).

To precisely compare their stability, we measured the rate of decline of primary and secondary Th1 memory cells. Although primary Th1 memory cell populations gradually decayed throughout the first 6–7 mo postinfection by 60–80%, there was no statistically significant reduction in the number of secondary Th1 memory cells during the same time period. Additionally, secondary Th1 memory cells showed elevated stability, regardless of the order of prime-boost infection (Fig. 1B, 1D).

A weak secondary challenge induced by homologous boosting results in poorly maintained secondary Th1 memory cells

We previously observed that the most long-lived Th1 memory cells skew to a higher functional avidity (8). Additionally, secondary challenge was shown to induce the selective outgrowth of high-

FIGURE 1. Heterologous rechallenge results in the stable maintenance of secondary Th1 memory cells. (**A** and **C**) We infected B6 mice with LCMV or Lm-gp61 and measured the number of gp_{61-80}-specific IFN-γ–producing cells in the spleen at the indicated time points. At day 75 postinfection, mice received a heterologous rechallenge with Lm-gp61 or LCMV, respectively, and we measured the number of gp_{61-80}-specific IFN-γ–producing cells in the spleen at the indicated time points. (**B** and **D**) The percentage contraction of IFN-γ–producing CD4$^+$ T cells specific for gp_{61-80} between days 75 and 200 postinfection was measured after primary challenge with LCMV or Lm-gp61 or after heterologous rechallenge with Lm-gp61 or LCMV. The error bars indicate the SEM, and p values were calculated using a Student t test (n = 4–5 mice/group). Results are representative of three separate experiments.
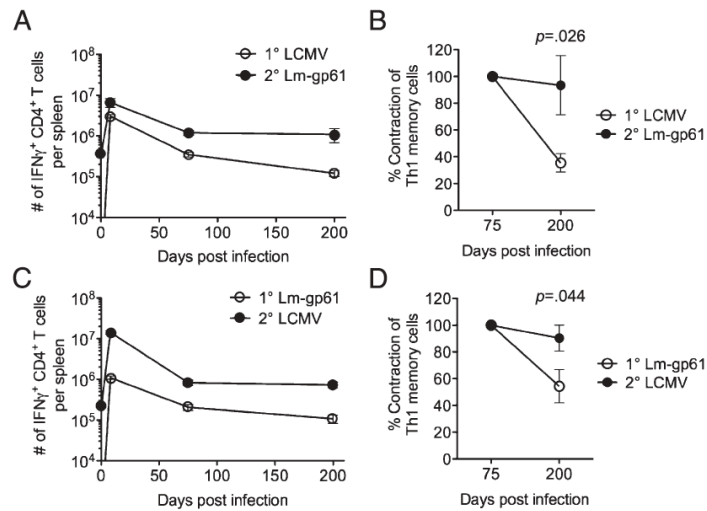
avidity clones (29). Therefore, we hypothesized that the strength of the secondary stimulus, as defined by its duration and antigenic load, could impact the differentiation and function of secondary Th1 memory cells. Compared with heterologous rechallenge, homologous rechallenge induces a relatively poor secondary Th1 response (21), presumably as the result of rapid clearance by pre-existing Abs and/or memory CTL. We confirmed that homologous rechallenge results in rapid Ag clearance compared with heterologous rechallenge. CFSE-labeled TCR-transgenic SMARTA cells, which are specific for LCMV gp_{61-80}, did not undergo cell division when transferred 2 or 3 d after homologous rechallenge. In contrast, SMARTA cells underwent several cell divisions when transferred into heterologously challenged hosts at similar time points (Supplemental Fig. 1). Therefore, although heterologous rechallenge boosts the response to a single class II-restricted and a single class I-restricted epitope, it provides a more robust boost than does homologous rechallenge, which is rapidly cleared by broadly acting CTL and Ab responses. Therefore, we used a model of homologous rechallenge (>60 d postinfection) to assess the maintenance and function of secondary Th1 memory cells following a weak secondary challenge.

Similar to what we reported previously (21), homologous rechallenge of either LCMV-immune or Lm-gp61–immune mice resulted in little boosting of the Th1 response at either effector or memory time points compared with the primary Th1 response to the same pathogen (data not shown). Furthermore, the resulting memory population declined with kinetics similar to the primary Th1 memory population. Between days 60 and 120 postchallenge, both primary Th1 memory cells and secondary Th1 memory cells generated by homologous rechallenge were reduced in number by 50–70% (Fig. 2). Importantly, the rechallenge doses used were sufficient to effectively induce a robust secondary CD8$^+$ T cell response (data not shown), highlighting the differences in antigenic requirements in the generation of primary and secondary CD4$^+$ or CD8$^+$ T cell responses. In sum, these data indicated that the strength or duration of the secondary stimulus influenced the long-term survival of secondary Th1 memory cells.

Strength of stimulus impacts function and localization of secondary Th1 responses

Previous studies showed that secondary challenge results in the selective expansion of responders with high avidity for Ag. We
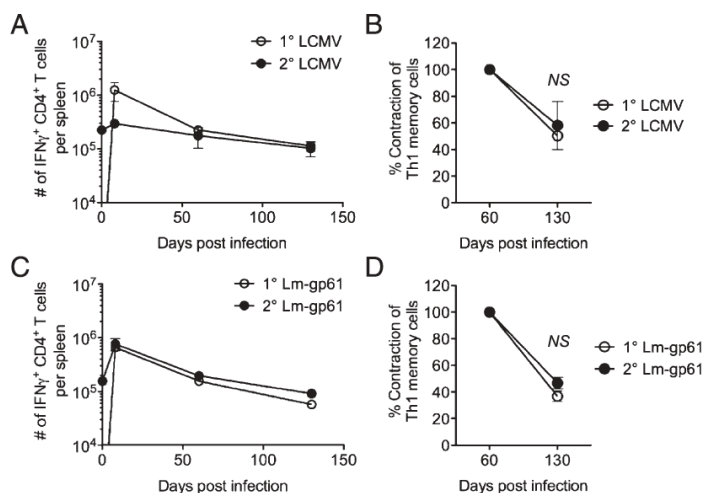
FIGURE 2. Homologous rechallenge results in poor maintenance of secondary Th1 memory cells. (**A** and **C**) We again infected B6 mice with LCMV or Lm-gp61 and measured the number of gp_{61-80}-specific IFN-γ–producing cells in the spleen at the indicated time points by peptide restimulation and intracellular cytokine staining. At day 75 postinfection, mice received a homologous rechallenge with LCMV or Lm-gp61, respectively, and we measured the number of gp_{61-80}-specific IFN-γ–producing cells in the spleen at the indicated time points. (**B** and **D**) The percentage contraction of IFN-γ–producing CD4$^+$ T cells specific for gp_{61-80} between days 60 and 130 postinfection was measured after primary challenge with LCMV or Lm-gp61 or after homologous rechallenge with Lm-gp61 or LCMV. The error bars indicate the SEM, and p values were calculated using a Student t test (n = 4 mice/group). Results are representative of two separate experiments.

found that long-lived Th1 memory cells that are maintained most stably also skew to a high functional avidity (as measured by the Ag dose required to elicit a functional response, such as IFN-γ production) (8). We hypothesized that the induction of secondary Th1 responses with high Ag sensitivity would correspond to increased stability of the ensuing memory population. We assessed the functional avidity of primary and secondary Th1 responders following either homologous or heterologous rechallenge. The development of long-lived Th1 memory in LCMV-immune mice was associated with an overall increase in functional avidity, as previously reported. Secondary challenge with Lm-gp61 resulted in secondary effector Th1 cells with similarly high functional avidity (Fig. 3A). However, a homologous rechallenge with LCMV led to an overall decrease in functional avidity compared with the memory population prior to rechallenge, leaving them with a relatively low antigenic sensitivity that was similar to primary Th1 responders (Fig. 3B, 3C). Although secondary Th1 memory cells eventually skewed once again to high functional avidity after homologous rechallenge (Fig. 3D, 3E), this corresponded to secondary Th1 memory decline that was similar to the decline seen in primary memory (Fig. 2). Therefore, the eventual reacquisition of high functional avidity by secondary Th1 memory cells induced by homologous rechallenge may come at a cost of decreased secondary memory maintenance. Reacquisition of high functional avidity may reflect cell-specific changes in functional avidity or the preferential population-based outgrowth of high functional avidity responders.

We also determined whether the differences in function were a T cell-intrinsic response to homologous challenge or whether their function was dictated by the stimulatory environment of the challenge itself. We transferred Lm-gp61–immune (Thy1.1^{+})

CD4^{+} memory T cells into Lm-gp61–immune or LCMV-immune secondary hosts (Thy1.2^{+}). The transferred CD4^{+} memory T cells were then given a "homologous" rechallenge with Lm-gp61. The functional avidity of the ensuing recall response depended on the environment of the rechallenge. Lm-gp61–induced memory cells maintained high functional avidity when rechallenged in LCMV-immune hosts (homologous challenge in a heterologous environment), whereas they demonstrated lower functional avidity when rechallenged in Lm-gp61–immune hosts (homologous challenge in a homologous environment) (Supplemental Fig. 2). Additionally, it is possible that newly arising naive cells with specificity for gp$_{61-80}$ could complicate the interpretation of the functional avidity assays following rechallenge. However, homologous and heterologous rechallenges were also given to B6 mice containing LCMV-induced memory SMARTA cells with similar results, indicating that differences in functional avidity were due to bona fide differences in recall responses and not the influence of newly arising naive cells (data not shown).

We next tested the impact of secondary challenge on the relative distribution of Th1 memory cells in the secondary lymphoid organs versus a peripheral site of infection for both LCMV and Lm-gp61, the liver. Although both primary and secondary Th1 memory cells in the spleen expressed similar levels of CD62L (Supplemental Fig. 3), the relative ratio of Th1 responders in the liver versus the spleen significantly increased following heterologous rechallenge. Following heterologous rechallenge of LCMV-immune mice with Lm-gp61 or Lm-gp61–immune mice with LCMV, Th1 responses in the liver were boosted significantly at both effector (data not shown) and memory (Fig. 4A) time points. Furthermore, the relative ratio of Th1 memory cells in the liver versus the spleen following secondary challenge also increased significantly com-
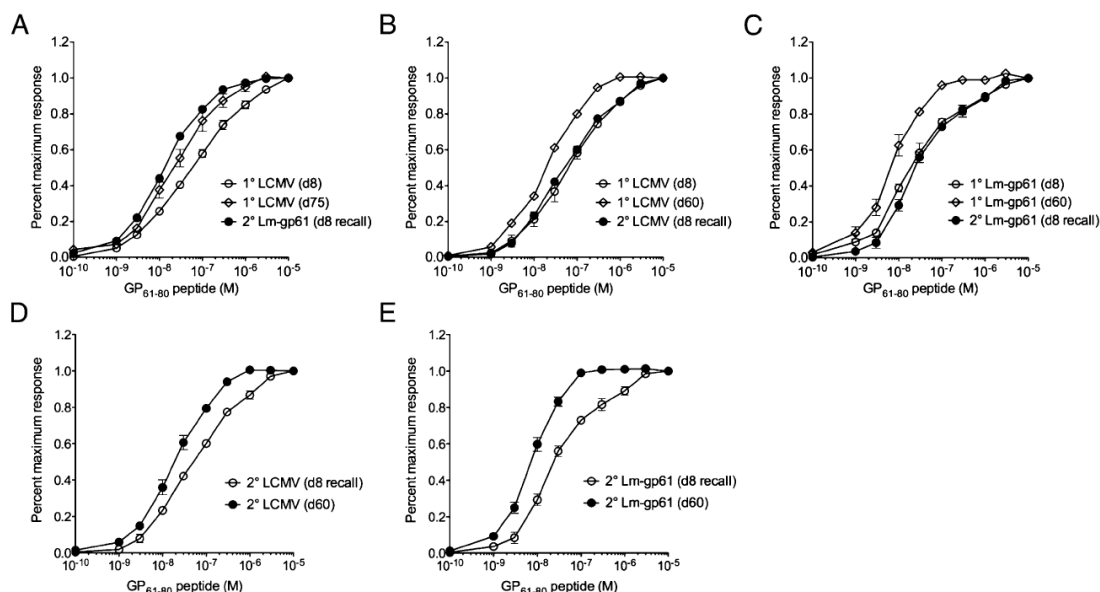
FIGURE 3. Homologous boosting results in a loss of Ag sensitivity by secondary Th1 effector cells. Splenocytes were restimulated with gp$_{61-80}$ peptide at the indicated concentrations and then stained for the production of IFN-γ. Results are represented as the percentage maximal response, with the maximal response defined as the frequency of CD4^{+} T cell responders at the highest peptide concentration. (**A**) Response across a range of peptide concentrations after primary infection with LCMV (days 8 and 75 postinfection) or after rechallenge with Lm-gp61 (day 8 postrechallenge). (**B** and **C**) Response at the indicated time points after primary challenge with LCMV or Lm-gp61 (days 8 and 60 postinfection) or after homologous rechallenge (day 8 postinfection). (**D** and **E**) Response at the peak of the secondary effector response (day 8 postrechallenge) and following the establishment of memory (day 60 postrechallenge) after homologous rechallenge with LCMV or Lm-gp61. Error bars indicate the SEM ($n = 4$–5 mice/group). Results are representative of two separate experiments.
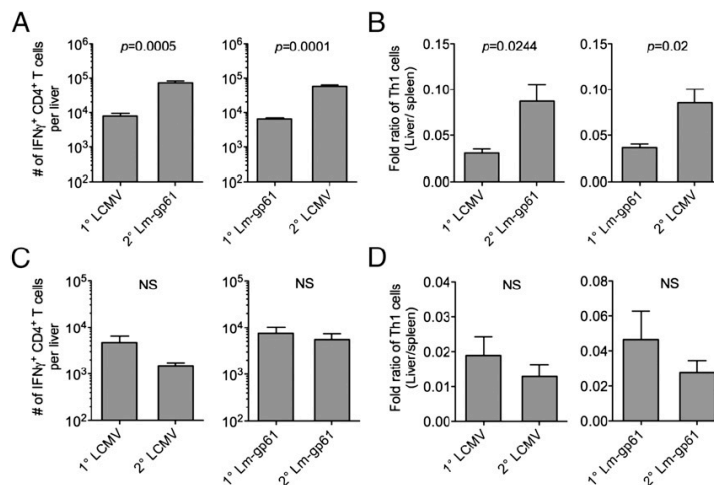
FIGURE 4. Heterologous rechallenge boosts the frequency of tissue-homing Th1 effector memory cells. Lymphocytes were isolated from perfused livers following digestion in Collagenase B and DNAse I. Total numbers of CD4$^+$ IFN-γ–producing cells were calculated following ex vivo restimulation with gp$_{61-80}$. (**A**) Number of IFN-γ–producing Th1 cells in the liver at day 75 after primary challenge or heterologous rechallenge. (**B**) Relative distribution of Th1 memory cells in the spleen and liver at day 75 after primary challenge or heterologous secondary rechallenge. Data represent the fold ratio of Th1 cells in the liver versus the spleen. (**C**) Number of IFN-γ–producing Th1 cells in the liver at day 60 after primary challenge or homologous rechallenge. (**D**) Relative distribution of Th1 memory cells in the spleen and liver at day 60 after primary challenge or homologous secondary rechallenge. Data represent the fold ratio of Th1 cells in the liver versus the spleen. Error bars indicate the SEM, and p values were calculated using a Student t test (n = 4–5 mice/group). Results are representative of two separate experiments.

pared with primary challenge (Fig. 4B). These findings suggest that robust boosting of Th1 responses can result in an increase in the overall number of Th1 memory cells, as well as a relative shift toward tissue homing effector memory Th1 cells. In contrast, homologous rechallenge failed to boost the number of Th1 memory cells in the liver or increase the ratio of Th1 memory cells residing there (Fig. 4C, 4D). In summary, although heterologous boosting resulted in enhanced numbers, survival, function, and tissue homing by secondary Th1 memory cells, homologous rechallenge resulted in neither boosting nor functional enhancement of Th1 memory populations. In fact, homologous boosting resulted in a loss of functional avidity by Th1 memory cells recruited into the response. In all ways that we measured, secondary Th1 responses induced by homologous rechallenge displayed functions characteristic of primary Th1 responses.

Stable maintenance of Th1 memory corresponds to enhanced homeostatic turnover

We next sought to identify the characteristics of the secondary response that might explain the ability of secondary Th1 memory populations induced by heterologous rechallenge to maintain themselves at stable levels long-term. Primary Th1 responses induced by either LCMV or Lm-gp61 are characterized by the expansion of CD4$^+$ T cells with the ability to produce multiple cytokines upon Ag stimulation, and the presence of multiple cytokine producers is a strong correlate of CD4$^+$ T cell-mediated protection (35, 36). At the peak of the response to either infection, >60% of IFN-γ–producing Th1 cells also produced IL-2 and TNF-α. This number was enriched to ~80% during memory maintenance. Heterologous rechallenge resulted in an initial enrichment of secondary Th1 effector cells producing only IFN-γ. However, the resulting secondary Th1 memory cells once again skewed toward multiple cytokine producers and were not significantly different in their cytokine production profile compared with primary Th1 memory cells (Fig. 5).

We next considered the hypothesis that secondary Th1 memory cells responded to homeostatic or survival signals more effectively than did primary Th1 memory cells, thus resulting in more stable maintenance. However, secondary Th1 memory cells expressed similar levels of the homeostatic cytokine receptors CD122 (IL-15Rβ) and IL-7Rα (Supplemental Fig. 4A, 4B). Similarly, primary and secondary Th1 memory cells expressed similar levels of the prosurvival molecule Bcl-2 (Supplemental Fig. 4C).

To address definitively whether secondary Th1 memory cells enjoyed a homeostatic advantage over primary Th1 memory cells, we administered the nucleotide analog BrdU into the drinking water of mice over a 2-wk time period and measured its incorporation into dividing cells. Following heterologous rechallenge, secondary Th1 memory cells incorporated BrdU at a significantly higher rate at days 75 and 200 postrechallenge compared with primary Th1 responders (Fig. 6A, 6B). Enhanced homeostatic turnover corresponded to memory stability, because secondary Th1 memory cells induced by homologous rechallenge, which are not maintained stably, demonstrated no increase in homeostatic turnover following either a homologous LCMV rechallenge (Fig. 6C, 6D) or a homologous Lm-gp61 rechallenge (data not shown). We concluded that secondary Th1 memory cells induced by robust heterologous rechallenge developed an enhanced intrinsic capacity to divide in response to homeostatic signals, despite the fact that they were present in much higher numbers and, therefore, were competing for a more limited supply of these signals.

Discussion

Our findings demonstrate that enhanced recall responses induced by robust secondary challenge improve the stability, size, and early acquisition of increased effector function by Th1 memory populations. They also suggest that the context of effector Th1 differentiation has profound consequences for the long-term fate of ensuing memory populations. Although the concept of early commitment by developing CTL to a memory differentiation

FIGURE 5. Secondary Th1 memory cells induced by heterologous challenge are highly functional. (**A**) We measured the polyfunctionality of Th1 effector and memory cells following infection with LCMV or Lm-gp61 or following heterologous secondary challenge with either Lm-gp61 or LCMV. After peptide restimulation in the presence of brefeldin A, cells were permeabilized and stained with Abs to IFN-γ, TNF-α, and IL-2. Representative flow plots at each time point indicate the frequency of CD4$^+$ T cells that costained with Abs to IFN-γ and TNF-α. (**B**) Frequency of IFN-γ–producing cells that costained with Abs to TNF-α and IL-2 at the indicated time points after primary or secondary challenge. Error bars indicate the SEM (*n* = 4–5 mice/group). Results are representative of three separate experiments.
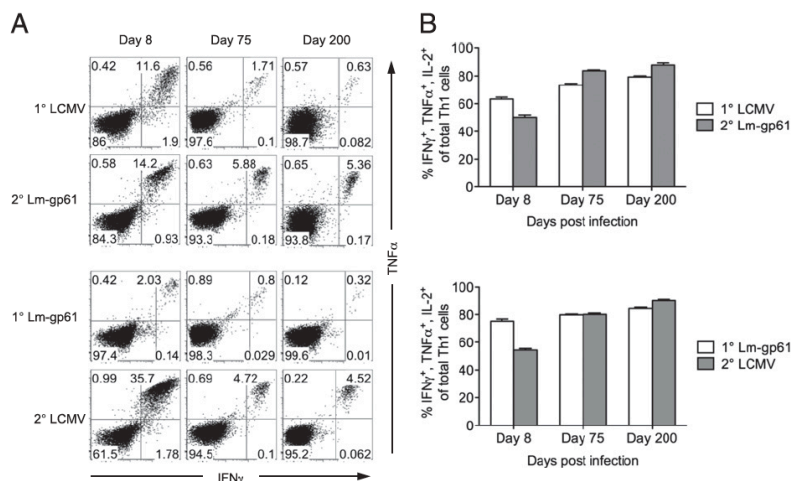
program has long been established, more recently, convincing evidence has begun to accumulate that subsets of effector CD4$^+$ T cells are similarly fated for subsequent memory differentiation (37). In this context, it seems likely that the ability to enter the memory pool, as well as the long-term survival of ensuing memory populations, is dependent on the nature of activation signals received during the primary response.

We previously showed that even while primary Th1 memory cells decline, as a population they acquire heightened sensitivity to Ag. Acquisition of a higher sensitivity to Ag, in turn, corresponds to a decrease in the rate of decline (8). One possible explanation for these observations is that Th1 memory cells acquire heightened sensitivity to Ag throughout memory maintenance. However, a second possibility is that T cell clones that successfully acquire a heightened ability to translate antigenic stimulation into a functional response during primary activation enjoy a selective survival advantage during memory maintenance. Prior data showed that secondary challenge results in the selective outgrowth of CD4$^+$ T cells with high TCR avidity for Ag (29). In our studies,

we found that long-lived Th1 memory cells (>75 d postinfection) were of high functional avidity compared with primary Th1 effector cells and that heterologous rechallenge resulted in the expansion of secondary Th1 effector cells whose functional avidity reflected that of the memory population from which they arose. Furthermore, high functional avidity during the secondary Th1 response corresponded to enhanced stability of the ensuing memory population, supporting a model in which the acquisition of high functional avidity during the effector response is predictive of long-term survival within the memory pool.

What, then, are the signals that induce the outgrowth of secondary Th1 effector and memory cells with high functional avidity? Importantly, a weak homologous rechallenge, even though it created an environment of increased competition for Ag, failed to result in a highly functional secondary Th1 effector response. In fact, the Th1 memory population displayed a decline in functional avidity after recruitment into the secondary response. The failure to acquire enhanced function corresponded to poor stability of the ensuing memory populations. These observations lead to two
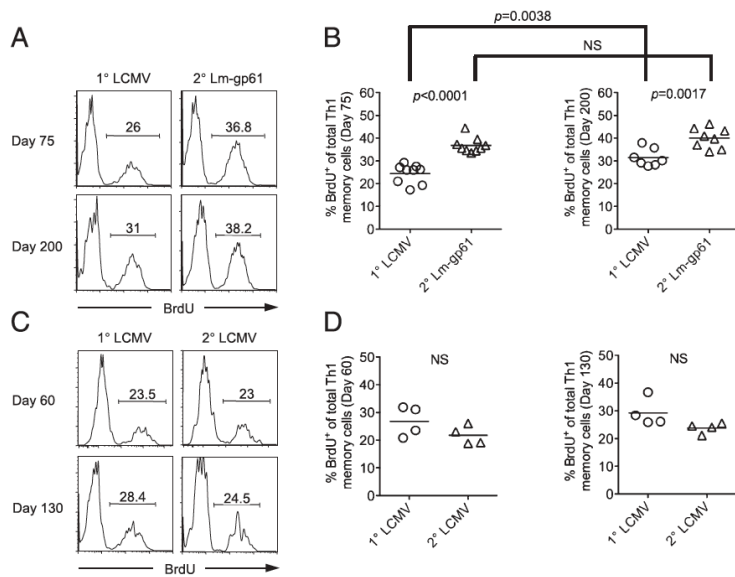
FIGURE 6. Secondary Th1 memory cells induced by heterologous rechallenge display an increased rate of homeostatic division. Mice were fed BrdU in their drinking water for 2 wk beginning at the indicated time points after primary or secondary challenge, after which splenocytes were restimulated with peptide and stained for expression of IFN-γ and BrdU. Representative flow plots (**A**) indicate BrdU staining by IFN-γ–producing Th1 memory cells at the indicated time points after primary infection with LCMV or heterologous rechallenge, whereas scatter plots indicate the frequency of BrdU$^+$ cells at day 75 or day 200 after primary or heterologous secondary rechallenge (**B**). (**C** and **D**) Similar plots display the results obtained after primary challenge with LCMV or homologous rechallenge. The *p* values were calculated using a Student *t* test; results are representative of two separate experiments.

conclusions. First, the context of secondary stimulation impacts the long-term fate and survival of the ensuing secondary memory population. Second, the generation of high functional avidity responders following secondary challenge cannot be entirely explained by the selection of high TCR avidity clones as the result of competition for limited amounts of Ag. Rather, the selection of effective secondary Th1 effector and memory cells likely depends on the quantity of the secondary stimulation, as well as its quality. This is supported by findings that both CD4^{+} and CD8^{+} monoclonal T cell populations can shift their functional avidity during the primary response (38, 39). We also found that monoclonal populations of CD4^{+} memory T cells can display a broad spectrum of functional avidity in response to Ag stimulation (8). We propose a model in which a weak secondary challenge results in poor-quality activation events and the subsequent decrease in Ag sensitivity by CD4^{+} memory T cells recruited into the secondary response. However, our findings do not rule out the possibility that selection of long-lived and stable secondary Th1 memory is, at least in part, clonal (e.g., dependent on the strength of TCR signaling), because a high-quality secondary stimulus may be required to provide the appropriate context for selective outgrowth of highly functional clones and mediate their entry into the memory pool.

Although the precise mechanisms allowing enhanced survival of secondary Th1 memory cells are unknown, we made two key observations. First, secondary Th1 effector and memory cells induced by heterologous rechallenge maintained a higher functional avidity phenotype than did either primary Th1 effector cells or secondary Th1 effector cells induced by homologous rechallenge. The stability of the ensuing memory populations corresponded directly to the emergence of high functional avidity Th1 memory cells, suggesting that those responders able to acquire high functional avidity also enjoyed a selective advantage for survival within the memory compartment. Second, secondary Th1 memory cells induced by heterologous rechallenge turned over at a higher rate than did primary Th1 memory cells. IL-15 and IL-7 are required for the maintenance and homeostatic proliferation of primary CD4^{+} memory T cells (9, 11, 40). Although primary and secondary Th1 memory cells express similar levels of the IL-15 and IL-7 receptors, it is possible that secondary Th1 memory cells are better equipped to transmit these cytokine signals into a biological response. Future studies are needed to determine the extent to which the stable maintenance of secondary Th1 memory cells is dependent on IL-7 and IL-15.

These studies have clear implications for the design of vaccination strategies aimed at the generation of protective CD4^{+} memory T cells. Additionally, it is likely that successful vaccination to a variety of infections, including hepatitis C virus and HIV, will require coordinated mobilization of all aspects of adaptive immunity, including CTL, B cells, Th1 cells, and follicular Th cells. Our findings suggest that the success of simultaneous boosting of CTL and Th1 responses may hinge on the ability to adequately stimulate the formation of stable and highly functional secondary Th1 memory cells.

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Disclosures

The authors have no financial conflicts of interest.

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CHAPTER 4

DYNAMIC FUNCTIONAL MODULATION OF

CD4^{+} T CELL RECALL RESPONSES IS

DEPENDENT ON THE NATURE OF

THE SECONDARY STIMULUS

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Running title: Functional modulation of secondary Th1 cells

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Abstract

The parameters that induce the generation of highly functional secondary Th1 effector cells are poorly understood. In this study, we employ a serial adoptive transfer model system to show that the functional sensitivity of secondary Th1 effector cells is dynamically modulated throughout the recall response in a manner dependent on the environment induced by the secondary challenge. Adoptive transfer of Th1 memory cells into lymphocytic choriomeningitis virus (LCMV)-immune hosts, followed by an efficiently controlled heterologous secondary challenge with recombinant *Listeria monocytogenes* recombinantly expressing a portion of the LCMV Glycoprotein (Lm-gp61), resulted in the rapid emergence of high functional avidity Th1 effector cells, limited contraction after clearance and stable maintenance of secondary memory populations. In contrast, transfer of Th1 memory cells into naïve hosts resulted in an extended exposure to a rechallenge environment that more closely resembled a primary infection. In these hosts, secondary Th1 effector cells up-regulated expression of phosphatases known to regulate TCR signaling and down-regulated expression of several TCR-associated tyrosine kinases. These changes corresponded to a decline in Th1 antigen sensitivity during the latter stages of infection, increased death of Th1 cells during the contraction phase and poor maintenance of Th1 secondary memory cells. We conclude that the functional sensitivity of secondary Th1 effector cells is dynamic and can be manipulated by environmental differentiation cues associated with the strength or duration of the secondary challenge.

Introduction

During acute viral and bacterial infections, antigen-specific naïve T cells clonally expand and acquire effector functions that contribute to pathogen clearance. Upon elimination of the pathogen, a small proportion of effector T cells survive and differentiate into long-lived memory cells that provide rapid and enhanced protection against secondary challenge. Activated T cells have been shown to integrate numerous signals during the primary response that impact downstream effector and memory T cell differentiation [1,2]. Identification of those signals that lead to the generation of functional memory T cells are major goals for the design of vaccines and immunotherapies.

The transition from effector to memory is marked by the acquisition of heightened sensitivity to low levels of antigen, often referred to as functional avidity [3]. Functional avidity is not fixed, and can change in a manner that is dependent on differentiation status and, in some cases, independent of the TCR. For example, both $CD8^+$ and $CD4^+$ TCR transgenic T cells undergo changes to their functional responsiveness throughout the primary effector response to infection [4,5], and we have previously reported that SMARTA TCR transgenic T cells, with monoclonal specificity to the LCMV-derived class II-restricted epitope GP_{61-80}, undergo functional avidity maturation in the transition from effector to memory [3]. Therefore, while TCR signals play a role in modulating functional responses, TCR-independent signals are also clearly important.

While most past studies have focused on the modulation and acquisition of functional avidity during the primary effector and memory T cell response to infection, far less is known about the mechanisms that control T cell functionality and the

protective capacity during the secondary response. For CD8^{+} T cells, repetitive reactivation of memory T cells resulted in the acquisition of more effector-like phenotype [6], a differentiation status associated with enhanced protection from some infections [7] but not others [8-10]. Additionally, infection-induced inflammatory signals such as IL-12 have also been shown to impact the functional capacity of secondary effector CTL [11,12]. As compared to primary memory, secondary CD8^{+} memory T cells exhibit enhanced cytolytic capabilities and provide enhanced protection against certain infections such as *Listeria monocytogenes*, whereas they are more prone to functional exhaustion following chronic antigen exposure [13,14]. Therefore, one can conclude that the functional characteristics of secondary effector CTL depend at least in part on the nature of the secondary stimulus.

Less is known about the mechanisms that control the functionality of secondary effector and memory CD4^{+} T cells. Both naïve and memory CD4^{+} T cells show a similar delay in the onset of cell proliferation after exposure to antigen, despite the fact that memory T cells become activated and produce effector cytokines within several hours [15]. In the context of influenza A virus, secondary effector CD4^{+} T cells display distinct functional and phenotypic characteristics as compared to primary effectors, including enrichment for producers of multiple cytokines, enhanced trafficking to tissue sites of infection and greater contribution to viral clearance [16]. The strength of pathogen rechallenge may also play a key role in mediating changes to the long-term fate and function of secondary Th1 responders. We previously found that, unlike CD8^{+} memory T cells, a homologous secondary challenge was insufficient to induce CD4^{+} memory T cells to engage in optimal secondary expansion and effector differentiation [17]. A

rapidly cleared homologous rechallenge of lymphocytic choriomeningitis virus (LCMV)-
or *Listeria monocytogenes* (Lm)-immune mice, resulted in poor acquisition of secondary
Th1 effector function and decreased survival within the memory pool, whereas reciprocal
heterologous rechallenge with a pathogen sharing only a single CD4^{+} T cell epitope
resulted in rapid acquisition of high secondary functional avidity and long-lived, stable
secondary Th1 memory [18]. Additional evidence of a role for infectious environment in
the differentiation of secondary Th1 effector and memory cells is that the functional
characteristics of secondary Th1 responders demonstrate plasticity that is dependent on
the nature of the secondary stimulus [18,19]. Overall, these findings suggest that the
function and longevity of secondary CD4^{+} T cell responders are not hardwired but are
dictated by the nature of the secondary stimulus.

In an effort to further define the TCR-independent infectious conditions that
regulate secondary effector and memory Th1 differentiation, we employed a serial
adoptive transfer system that allowed us to manipulate the nature of the secondary
challenge as well as the precursor frequency of memory Th1 cells. Initially, we injected
naïve mice with small numbers of T cell receptor (TCR) transgenic SMARTA CD4^{+} T
cells and infected them with LCMV. Following the differentiation of SMARTA memory
cells, they were isolated and parked in either an infection-matched LCMV-immune host
or a naïve uninfected host. Mice were then given a heterologous challenge with
recombinant *Listeria monocytogenes* expressing the MHC class II-restricted LCMV
GP$_{61-80}$ epitope (Lm-gp61). Whereas heterologous rechallenge of SMARTA memory
cells parked in a LCMV-immune secondary host resulted in secondary Th1 effector cells
with high functional avidity similar to the Th1 memory cells from which they were

derived, a secondary challenge of memory cells parked in a naïve host, which basically

replicates the features and kinetics of the primary acute infection, resulted in functional

avidity decay, increased death during contractions and poor survival of the resulting

secondary Th1 memory cells. Neither prolonging antigen presentation nor extending the

inflammatory period were alone sufficient to induce functional avidity decay, leading us

to conclude that both TCR-dependent and TCR-independent signals were required to

regulate modulation of functional avidity. Instead, functional avidity decay was

associated with increased expression of molecules associated with TCR signal regulation.

Overall, these findings define key parameters that regulate the acquisition of secondary

effector function following the rechallenge of Th1 memory cells and are highly

applicable to the development of vaccine and boosting strategies targeting the induction

CD4$^+$ T cell-mediated immunity.

Results

Functional avidity of secondary Th1 effectors is influenced

by recall stimulus

We previously observed that Th1 cells undergo functional avidity maturation

during the transition from effector to memory [3]. In support of this, we transferred small

numbers of naïve SMARTA T cells (1 x 10^4) into naïve B6 mice and infected them with

LCMV one day later. SMARTA cells harvested from the spleen following the

establishment of memory demonstrated superior functional avidity (defined as the ability

to make IFNg in response to decreasing concentrations of antigen) as compared to

SMARTA cells harvested at the peak of the effector Th1 response (Fig. 4.1A-B).

Figure 4.1. Adoptively transferred Th1 memory cells undergo functional avidity decay following heterologous boosting in naïve but not immune hosts. A) 1 x 10^4 naïve SMARTA cells were transferred into naïve B6 mice. Functional avidity of the SMARTA response after primary LCMV infection (day 8 and day 50 after infection) or after rechallenge of LCMV-immune memory with Lm-gp61 (day 8 after rechallenge) were measured by ex vivo restimulation with the indicated concentrations of GP$_{61-80}$ peptide, followed by intracellular IFNg staining. Graph displays the percent maximal response, calculated as the frequency of IFNγ–producing cells at any given concentration as a percentage of the frequency of IFNγ–producing cells at the highest peptide concentration. B) Bar graph displays the effective peptide concentration required for a half maximal response (EC$_{50}$), as determined by fitting the data to a sigmoidal curve (GraphPad Prism). C) LCMV-induced memory SMARTA cells (day 50) were isolated and adoptively transferred into either LCMV-immune or naïve hosts. Graph displays functional avidity of secondary SMARTA responses after rechallenge with Lm-gp61 (day 7 after infection), or of primary SMARTA responses after challenge with LCMV (day 8 after infection). D) Bar graph displays EC$_{50}$. E) LCMV-induced polyclonal Th1 memory cells (day 50) were isolated and adoptively transferred into either LCMV-immune or naïve hosts. Graph displays functional avidity of secondary Th1 responses in the indicated hosts after rechallenge with Lm-gp61 (day 7 after infection), or of primary Th1 responses after challenge with LCMV (day 8 after infection). F) Bar graph displays EC$_{50}$. Error bars indicate the SEM (n=4-5 mice/group). Results are representative of three separate experiments.
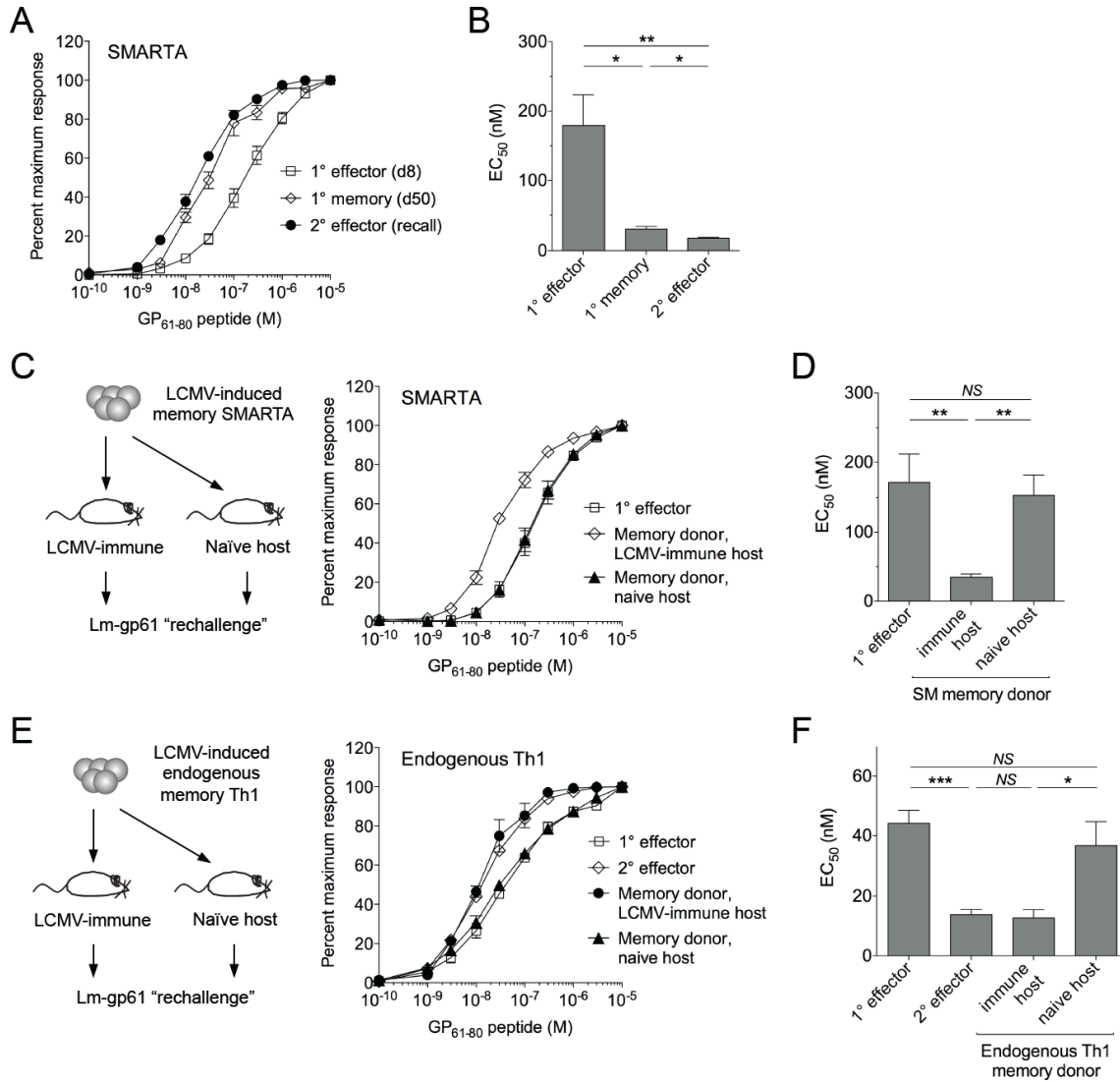
Following heterologous rechallenge of LCMV-immune mice with recombinant Lm-gp61, secondary effector SMARTA cells maintained their high functional avidity at the peak of the secondary effector response (Fig. 4.1A-B).

Because SMARTA cells, a monoclonal population, demonstrated remarkable plasticity in their functional avidity, we sought to establish a model system in which we could better define the TCR-independent factors controlling secondary effector and memory Th1 differentiation. To accomplish this we chose a serial adoptive transfer system in which LCMV-induced SMARTA memory cells (Thy1.1^{+}), generated as described above, underwent a second adoptive transfer into either naïve or infection matched LCMV-immune secondary recipients (Thy1.2^{+}), followed by secondary challenge with Lm-gp61. As expected, SMARTA memory cells parked in LMCV-immune hosts prior to rechallenge maintained a high functional avidity at the peak of their secondary response, comparable to that of the originating memory population as well as secondary responders following a heterologous challenge without secondary transfer (Fig. 4.1C-D). In contrast, SMARTA memory cells parked in naïve recipients prior to rechallenge demonstrated functional avidity decay following secondary activation (Fig. 4.1C-D), resulting in functional avidity similar to primary Th1 effector cells. This observation was applicable to polyclonal T cell populations as well, as endogenous Th1 memory cells isolated from LCMV-immune mice and parked in naïve recipients also displayed a loss of functional avidity following secondary Lm-gp61 challenge, as compared to those parked in LCMV-immune secondary recipients (Fig. 4.1E-F). These data taken together indicated that functional avidity, or antigen

sensitivity, was sensitive to extrinsic factors and that the immune environment of the secondary recipient shaped the functional response of secondary Th1 responders.

Secondary host immune environment impacts effector cytokine production and long-term memory maintenance of secondary Th1 cells

The generation of Th1 cells that can simultaneously produce multiple effector cytokines, particularly IFNγ, TNFα and IL-2 ("triple producers"), correlates to protective immunity to subsequent infections [20,21]. Therefore, we tested the cytokine-producing capabilities of secondary SMARTA effector cells derived from identical memory populations parked in either naïve or LCMV-immune hosts and rechallenged with Lm-gp61. Secondary SMARTA effector cells derived from challenge of naïve hosts showed a significant decline in the generation of triple producers at the peak of the secondary response, as compared to secondary SMARTA effector cells derived from challenge of LCMV-immune hosts (Fig. 4.2A-B), although these differences were not observed following the generation of secondary SMARTA memory. However, the rechallenge environment impacted the magnitude of secondary SMARTA contraction following pathogen clearance as well as the long-term stability of secondary SMARTA memory populations. SMARTA secondary effector cells derived from memory transfers into naïve hosts contracted rapidly during the transition to memory, and the resulting memory populations continued to steadily decline during the memory maintenance phase after day 75 postinfection (Fig. 4.2C-D). In contrast, SMARTA secondary effector cells derived from transfer into LCMV-immune hosts exhibited far less severe contraction between
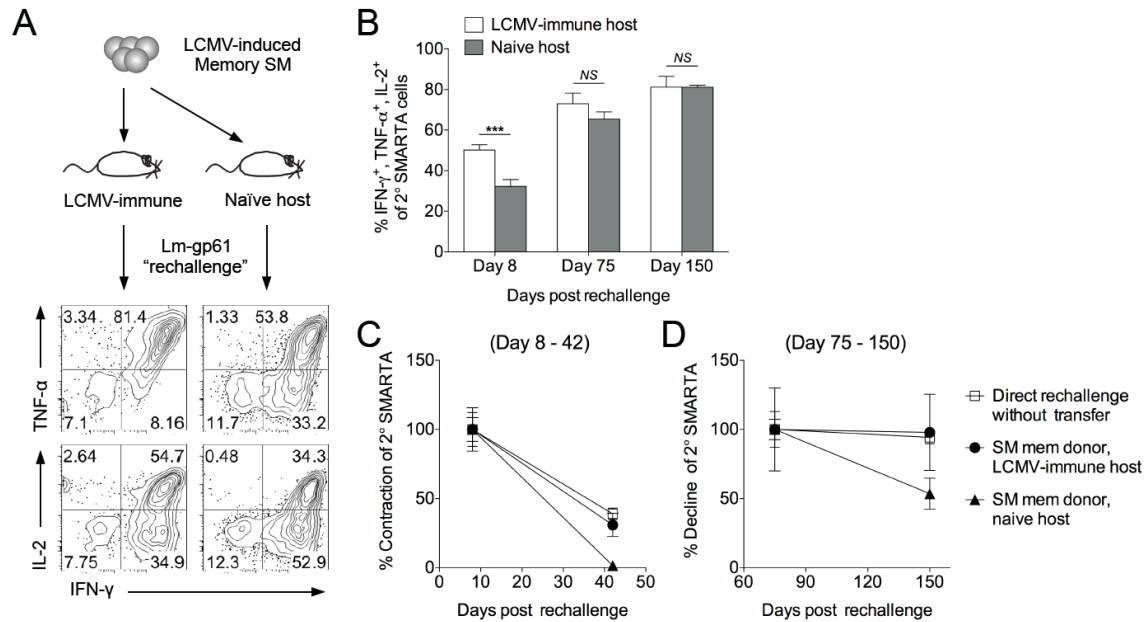
Figure 4.2. Secondary Th1 effectors responding in naïve hosts display poor polyfunctionality, memory differentiation and memory stability. LCMV-induced memory SMARTA cells were transferred into either LCMV-immune or naïve hosts and rechallenged with Lm-gp61 as previously described. A) Representative flow plots indicate the frequency of secondary SMARTA Th1 effector cells derived from either LCMV-immune or naïve hosts that produced IFNγ, TNFα, and IL-2 following ex vivo peptide restimulation. B) Bar graph indicates the frequency of secondary SMARTA Th1 effector cells that simultaneously produced TNFα and IL-2 at day 8, 75, and 150 after rechallenge with Lm-gp61. C-D) Graphs display the percent contraction of the secondary SMARTA effector cells between days 8 and 42 (C) and the percent decline of ensuing secondary memory cells between days 75 and 150 (D) after rechallenge with Lm-gp61. Error bars indicate the SEM (n=3-4 mice/group). Results are representative of two separate experiments.

days 8 and 42 after rechallenge, and the resulting memory populations remained stable during memory maintenance (Fig. 4.2C-D). The survival kinetics of secondary SMARTA memory cells derived from secondary transfer into LCMV-immune hosts mirrored that seen for secondary SMARTA memory cells derived from Lm-gp61 challenge of LCMV-immune hosts without secondary transfer (Fig. 4.2C-D). Interestingly, the kinetics of secondary contraction and memory maintenance of secondary SMARTA memory cells derived from transfer into naïve hosts mirrored our previously published observations of both primary Th1 memory cells as well as secondary Th1 memory cells derived from a weak homologous rechallenge [3,18]. Taken together with our current observations, our findings confirm that the function, survival and long-term fate of secondary Th1 effector and memory cells are highly dependent on the environment induced by the secondary challenge.

Functional avidity decay of secondary Th1 effector cells

corresponds to infection duration

Both intrinsic and extrinsic factors may influence the functionality of secondary Th1 responders following transfer and rechallenge of naïve or LCMV-immune mice. These factors include competition with endogenous Th1 cells for antigen and resources, differences in priming, alterations in the inflammatory cytokine environment and the duration of the secondary challenge. To better understand the infectious environment following rechallenge of SMARTA memory cells parked in either naïve or LCMV-immune hosts, we investigated the kinetics of pathogen clearance and antigen presentation in each setting. During the course of the Lm-gp61 challenge, LCMV-

immune mice exhibited more rapid clearance kinetics and significantly lower bacterial loads starting at day 3, as compared to challenge of naïve mice (Fig. 4.3A). Rapid clearance kinetics may be attributed either to the direct contribution of Th1-mediated secondary immunity or CTL-mediated immunity to a previously described class I-restricted epitope within GP$_{61-80}$ [22]. The rapid clearance of Lm-gp61 in LCMV-immune mice resulted in undetectable antigen presentation by day 5 after infection, whereas antigen presentation was still readily detectable following challenge of naïve mice (Fig. 4.3B).

To determine whether functional avidity decay was associated with defects in early activation events, we assessed the kinetics of functional avidity at the early stages of the recall response. Strikingly, at day 3 after rechallenge, secondary SMARTA effectors derived from rechallenge of both naïve and LCMV-immune hosts exhibited a massive increase in functional avidity as compared to memory SMARTA cells prior to rechallenge, requiring ~50-fold lower peptide concentration to induce a half-maximal response (Fig. 4.3C-E). By day 5 after rechallenge, the functional avidity of secondary SMARTA effectors derived from rechallenge of both naïve and LCMV-immune hosts had declined and was once again similar to the parent SMARTA memory population from which they were derived. Only secondary SMARTA effector cells derived from rechallenge of naïve hosts underwent continuous functional dematuration, with a further 5-fold reduction in antigen sensitivity by day 7 after infection, correlating to the period of time in which the secondary challenge persists in these mice (Fig. 4.3C-E). From these findings we concluded that functional avidity decay of secondary SMARTA effector cells derived from challenge of naïve mice was not likely to be a result of early differences in

Figure 4.3. Functional avidity decay of secondary Th1 responders is associated with a prolonged infectious period. A) Graph displays the kinetics of Lm-gp61 clearance in the spleen during either primary response in naïve mice or secondary response in LCMV-immune mice. LOD is limit of detection. B) Naïve or LCMV-immune mice were infected with Lm-gp61. At either day 5 or 8 after infection, they were injected with CFSE-labeled naïve SMARTA cells. Splenocytes were harvested 3 days later, and SMARTA cells were analyzed for CFSE dilution. C-E) LCMV-induced memory SMARTA cells were transferred into either naïve or LCMV-immune hosts that were then rechallenged with Lm-gp61. SMARTA functional avidity was determined at days 3, 5 and 7 in naive hosts (C) or LCMV-immune hosts (D) as described above. E) Bar graph displays EC_{50}. Error bars indicate the SEM (n=3-4 mice/group). Results are representative of two separate experiments.
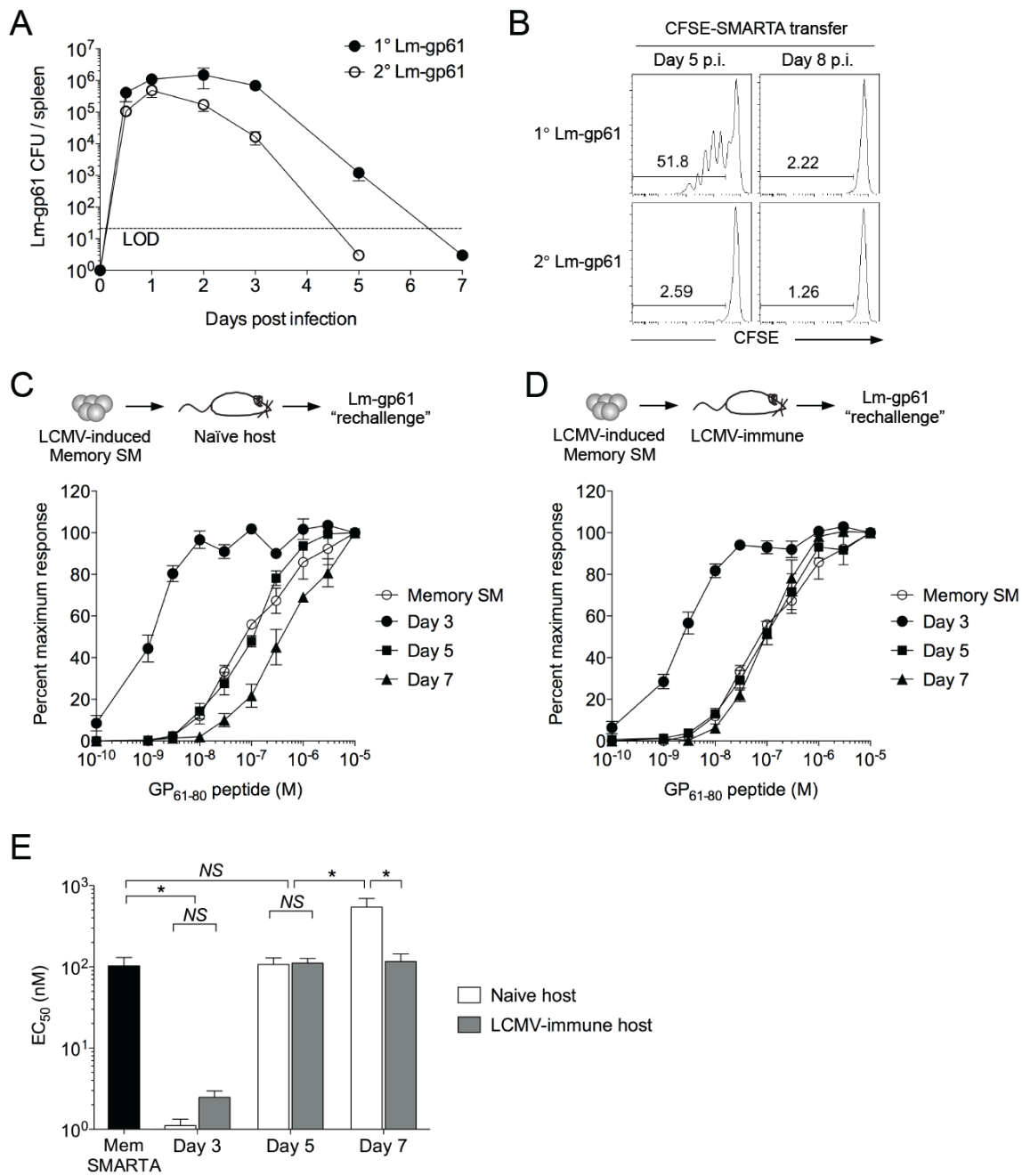
activation and function resulting from clonal competition or altered activation cytokines, but rather the persistence of infection in the later stages of the secondary challenge.

Neither antigen nor inflammation alone are sufficient to induce
the functional dematuration of secondary Th1 responders

As noted above, the rapid clearance of Lm-gp61 in rechallenged LCMV-immune mice correlated with the absence of detectable antigen by day 5 after infection, whereas we detected antigen following infection of naïve mice for a longer period of time. While the duration of the infection might allow for a more extended period of antigen presentation that could influence the functional avidity of secondary Th1 responders, we considered the alternative possibility that the persistence of the infection-induced inflammatory environment could promote functional avidity decay independently of antigen.

We first sought to generate a system in which antigen presentation could be extended independently of infectious inflammation following heterologous rechallenge. To accomplish this, we co-immunized mice with GP_{61-80} peptide-loaded DCs at days 2, 4 and 6 after a heterologous Lm-gp61 rechallenge. We designed the immunizations to coincide with the time period in which the Lm-gp61 challenge is cleared. Nevertheless, extending the period of antigen presentation in the context of a heterologous rechallenge was insufficient to induce functional avidity decay by secondary SMARTA Th1 cells (Fig. 4.4A-B).

To test the possibility that the inflammatory environment alone could modulate the functional avidity of secondary Th1 cells, we cochallenged LCMV-immune mice
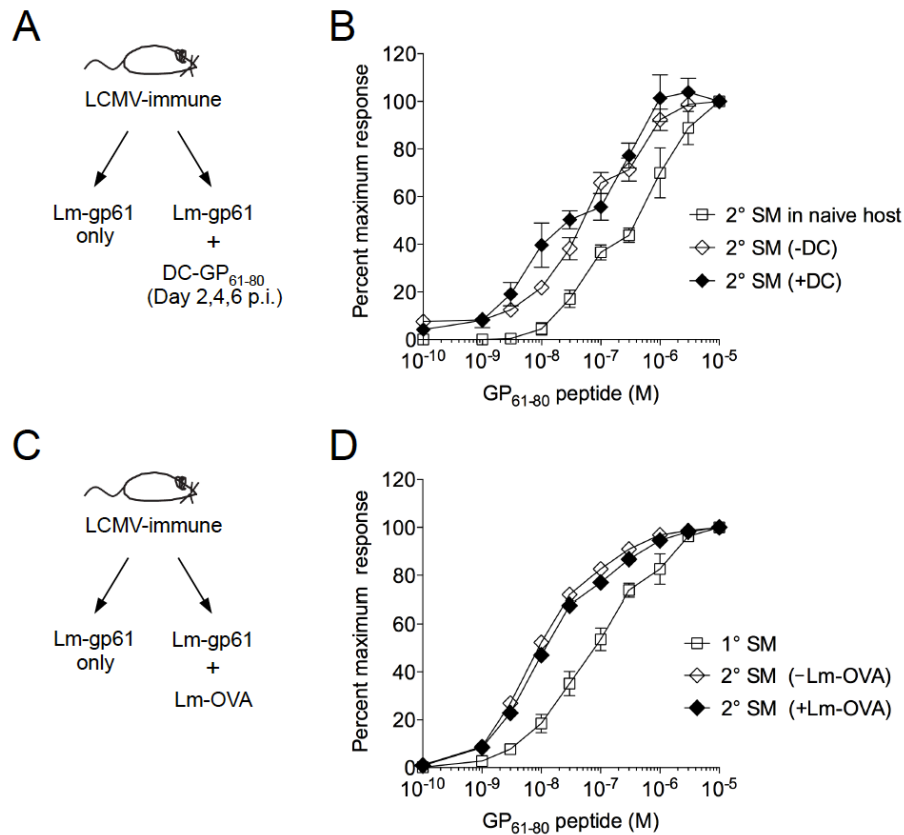
Figure 4.4. Neither extended antigen presentation nor inflammatory signals alone are sufficient to induce functional avidity decay. A) LCMV-induced memory SMARTA cells were transferred into either naïve or LCMV-immune hosts that were then rechallenged with Lm-gp61. Some of the rechallenged immune mice were given DCs loaded with LCMV GP$_{61-80}$ peptide on days 2, 4 and 6 after rechallenge. B) Graph displays the functional avidity of secondary SMARTA effectors at day 7 after Lm-gp61 rechallenge. C) Memory SMARTA cells in LCMV-immune hosts were rechallenged with Lm-gp61 alone or cochallenged with Lm-gp61 and Lm-OVA. D) Graph displays the functional avidity of secondary SMARTA effectors at day 7 after Lm-gp61 rechallenge, along with primary SMARTA effectors after LCMV infection. Error bars indicate the SEM (n=3-4 mice/group). Results are representative of two separate experiments.

with Lm-gp61 and a recombinant *Listeria* expressing the irrelevant antigen OVA (Lm-OVA), thus replicating the longer duration of the *Listeria* infection observed in naïve mice but without extending the duration of GP_{61-80} antigen presentation. Due to the fact that Lm-Ova is erythromycin resistant, we were able to measure the duration of both Lm-gp61 and Lm-Ova infection following cochallenge. Both Lm-gp61 and Lm-OVA reached similar bacterial loads by day 3 after infection, but at day 5, when Lm-gp61 was undetectable in the spleen, Lm-Ova persisted at levels similar to the bacterial burden observed in naïve mice infected with Lm-gp61 alone (data not shown). However, extending duration of infection-induced inflammation following heterologous challenge, without also extending antigen presentation, was insufficient to induce functional avidity decay by secondary SMARTA Th1 cells (Fig. 4.4C-D). We concluded that both antigen persistence and inflammatory milieu duration were required to induce functional avidity decay.

Loss of high antigen sensitivity corresponds to
differential TCR signaling

To understand the intrinsic differences between secondary effector Th1 cells responding within a previously naïve or pre-immune environment, differences in expression levels of TCR signaling molecules, survival factors, and signaling regulators were analyzed. We observed enhanced gene expression of several proximal TCR signaling molecules, including Zap70, Lck, and SLP76, in secondary SMARTA Th1 cells derived from heterologous rechallenge of LCMV-immune hosts, as compared to primary SMARTA Th1 cells. In contrast, secondary SMARTA Th1 effector cells derived from

challenge of a naïve host displayed expression levels similar to, or lower than, primary

effector SMARTA cells (Fig. 4.5A). This was not universally true, as expression of TCR

signaling molecules Fyn and PLCγ was similar between primary SMARTA Th1 effector

cells and secondary effector SMARTA Th1 effector cells induced in immune or naïve

recipients (Fig. 4.5A).

Concurrent with an increase in expression of TCR signaling molecules, SMARTA

Th1 effector cells induced following rechallenge of LCMV-immune hosts displayed

decreased expression of the TCR proximal phosphatases Src homology region 2 domain-

containing phosphatase-1 (SHP-1) and the TCR distal phosphatase dual specific

phosphatase 6 (DUSP6), as compared to primary SMARTA Th1 effector cells (Fig.

4.5B). In sharp contrast, secondary SMARTA Th1 effector cells derived from challenge

of a naïve host maintained high expression levels of these molecules, similar to primary

Th1 cells. SHP-1 is a well-described phosphatase regulator of TCR sensitivity that

negatively regulates the TCR proximal kinase ZAP70 and Lck [23-25]. DUSP6 is a

potent negative regulator of mitogen-activated protein kinase (MAPK) activity, and has

recently been implicated in regulating defects in antigen sensitivity among CD4^{+} T cells

in aging patients by dampening ERK signals following TCR stimulation [26,27]. In

contrast, we observed heightened expression of the E3 ubiquitin ligase Cbl-b, which

negatively regulates T cell activation by targeting proximal TCR signals, such as PLCγ

[28,29], in high functional avidity secondary responders. This suggests that the

regulation of antigen sensitivity in these cells is complex and distinct from mechanisms

that promote T cell anergy. Secondary effector SMARTA cells derived from challenge of

LCMV-immune mice exhibited an upregulation of Bcl-2, a well-known CD4^{+} T cell
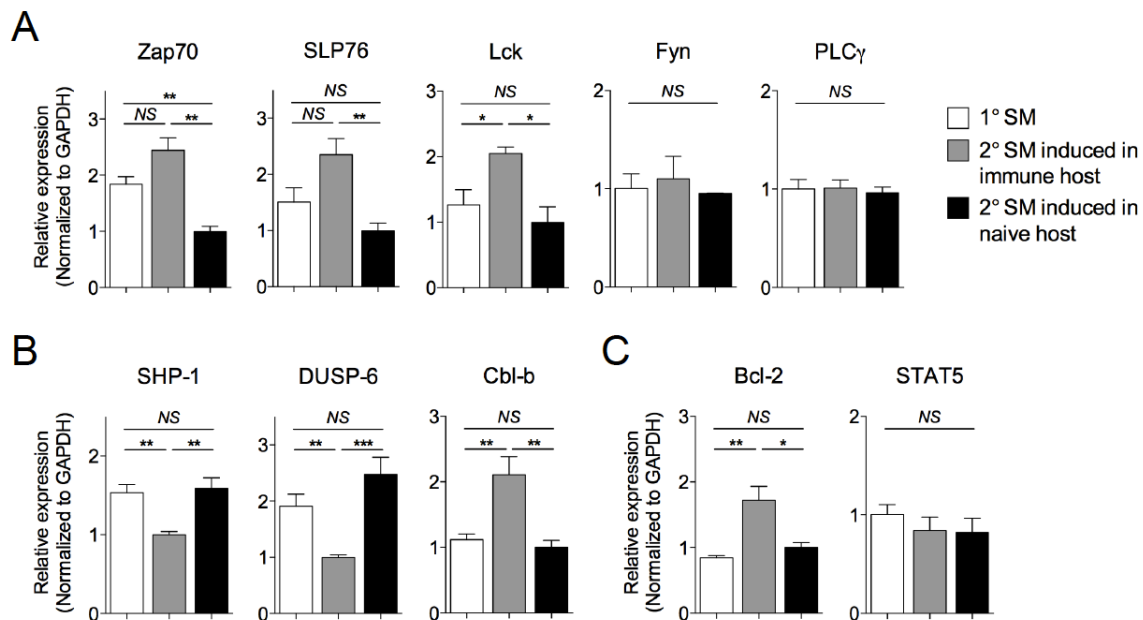
Figure 4.5. Maintenance of high functional avidity after secondary challenge is associated with enhanced expression of TCR signaling molecules. The relative gene expression of Zap70, SLP76, Lck, Fyn, and PLCγ (A), SHP-1, DUSP-6, Cbl-b (B), Bcl-2 and STAT5 (C) were evaluated by quantitative RT-PCR in primary and secondary SMARTA Th1 effector cells induced in either LCMV-immune or naïve hosts. Results were normalized to GAPDH. Error bars indicate the SEM (n=3-4 mice/group). Results are representative of two separate experiments.

survival factor, while observing no difference in STAT5 expression, a transcription factor

upstream of several prosurvival pathways (Fig. 4.5C). Overall, the expression profile of

secondary effector SMARTA cells in an immune environment is skewed towards being

prosurvival and pro-TCR signaling, whereas induction of secondary SMARTA Th1

effector cells in a naïve host results in an overall expression profile reminiscent of the

primary response.

Discussion

Our findings demonstrate that the antigen sensitivity, cytokine production

profiles, and survival of secondary Th1 responders are dependent upon the duration of the

secondary challenge. The persistence of antigen and the inflammatory environment

induced by rechallenge with a given pathogen were inseparable in our experiments and

ultimately determining the functional avidity and long-term fate of secondary Th1

responders. While it is well established that the context of the primary infection is

important for the differentiation, stability, and functional maturation of effector Th1 cells

[2,30], our findings show that the context of the secondary challenge can have profound

consequences for the functional maturation of responding secondary Th1 effector cells

and the long-term survival of subsequent Th1 memory populations. Functional attributes

are not permanently imprinted on Th1 cells during primary activation, but rather

secondary Th1 differentiation demonstrates functional plasticity that is dependent on the

context of the secondary challenge.

We have previously shown that in the context of a homologous rechallenge,

where memory Th1 cells are weakly stimulated due to the limited persistence of the

infection, secondary effector Th1 cells exhibit decreased functional avidity and diminished long-term survival [18]. As detailed in this study, we now report that when the infectious period of the secondary challenge is prolonged, secondary Th1 cells exhibit similar defects in functional avidity and survival. However, the functional maturation of secondary Th1 effector cells in these two settings may be quite different, as it is not known if secondary Th1 effector cells acquire extremely high functional avidity following homologous rechallenge in a manner similar to secondary Th1 effector cells following heterologous rechallenge in immune or naïve hosts, as observed here. Regardless, these studies together suggest that secondary Th1 effector and memory differentiation are acutely sensitive to the context of the secondary challenge, with "too much" or "too little" secondary stimulation resulting in suboptimal effector function and memory differentiation. Robust generation of highly functional secondary Th1 effector and memory cells instead seems to fall under a "Goldilocks" scenario in which the "just right" signal provided by heterologous rechallenge of immune hosts promotes both robust secondary Th1 effector cell differentiation and the stable persistence of long-lived secondary Th1 memory.

Recent findings have focused on the importance of inflammatory cytokines, including IFN-I, IL-12, and IL-18, in promoting the increased antigen sensitivity of local effector primary and secondary CD8$^+$ T cells independent of antigen or clonal selection [11,12]. In these studies, infection-induced enhanced antigen sensitivity of memory CD8$^+$ T cells was transient. The idea of antigen-independent induction of increased functional avidity in primary and secondary CD8 T cells is compelling, but whether this also applies to Th1 cells is unknown. While we observed an early enhanced antigen

sensitivity of secondary Th1 cells following rechallenge by day 3 postrechallenge infection, the increase was transient, returning to pre-immunization levels by day 5, and was unrelated to whether the host was immune or naïve prior to rechallenge. The early dynamic fluctuation of functional avidity by secondary Th1 cells may represent the influence of the inflammatory environment, antigen, or both.

Understanding the mechanisms by which memory Th1 cells maintain their function following secondary challenge will be a key to understanding the nature of the signals that regulate the generation of secondary immune responses. Other studies have suggested that enhanced antigen sensitivity by T cells correlates to the up-regulated expression of proximal TCR signaling molecules [31,32]. We find this to be true as well for secondary Th1 effector cells. Of particular interest is the correlation of TCR proximal phosphatases in secondary Th1 effector cells undergoing functional avidity decay. Their induction is TCR-dependent in other settings [24,25,27], and it is possible that they represent, at least in part, antigen-driven feedback in regulating the ongoing secondary response. Due to their capacity for inducing inflammatory disorders and autoimmunity, CD4$^+$ T cell responses are tightly regulated. One possible interpretation of the expression patterns that we observe are that they are a natural consequence of extended antigen presentation within an inflammatory environment and represent a normal regulatory mechanism for tuning down potentially damaging Th1 responses in settings of chronic stimulation.

Materials and methods

Ethics Statement

This study was carried out in accordance with the recommendations provided by the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This study was approved by the University of Utah Animal Care and Use Committee (PHS Assurance Registration Number A3031-01, Protocol Number 12-10011).

Mice and infections

Six to eight week old C57BL/6 (B6) mice were purchased from The Jackson Laboratory. SMARTA TCR transgenic mice [33] were maintained at the University of Utah. Lymphocytic choriomeningitis virus (LCMV) Armstrong 53b was grown in BHK cells and titered in Vero cells [34]. Mice were infected i.p. with 2×10^5 plaque-forming units (PFU). *Listeria monocytogenes* expressing the GP_{61-80} epitope of LCMV (Lm-gp61, M. Kaja-Krishna, University of Washington) and *Listeria monocytogenes* expressing OVA (Lm-OVA) were propagated in BHI broth and agar plates. Prior to infection, the bacteria were grown to log phase and concentration was determined by measuring the O.D. at 600 nm (O.D. of $1 = 1 \times 10^9$ CFU/ml). For primary infections or secondary rechallenge of LCMV-immune mice (>42 days after infection), mice were injected i.v. with 2×10^5 CFU Lm-gp61. For Lm-OVA, mice were injected i.v. with 1×10^4 CFU.

Adoptive transfers

To generate primary SMARTA memory cells, untouched CD4^{+} T cells were isolated from the spleens of SMARTA mice (Thy1.1^{+}) using a MACS CD4^{+} T cell isolation kit (Miltenyi Biotec). In addition, we added biotinylated anti-CD44 antibody (eBiosciences, San Diego, CA) to eliminate CD44^{hi} "memory phenotype" SMARTA as previously described [3]. Naïve SMARTA cells were re-suspended in PBS and injected i.v. into recipient mice (Thy1.2^{+}) 1 day prior to LCMV infection. For adoptive transfer of memory SMARTA cells, CD4^{+} T cells were isolated from the spleens of LCMV-immune B6 mice containing memory SMARTA cells (>day 42 after infection) and then injected i.v. into secondary recipients that were subsequently infected 1 day later. Similarly, for adoptive transfer of endogenous GP$_{61-80}$-specific Th1 memory cells, CD4^{+} T cells were enriched from the spleens of LCMV-immune B6 mice (>d42 days after infection), and 5 x 10^{6} CD4^{+} T cells were injected i.v. into secondary recipients prior to rechallenge.

Dendritic cell immunizations

DCs were expanded in B6 mice with a Flt-3L-secreting B16 mouse melanoma cell line as previously described [17,35]. DCs were enriched to 70-80% purity from the spleens and lymph nodes by transient adherence overnight. They were then pulsed with 1 µM LCMV GP$_{61-80}$ peptide for 2 hours in the presence of 1 µg/ml LPS. LCMV-immune mice (>d42 days after infection) were rechallenged with Lm-gp61 and subsequently injected with 1 x 10^{6} DCs i.v. on days 2, 4, and 6 after infection.

Cell preparations and flow cytometry

Splenocytes were placed in single-cell suspension in DMEM containing 10% FBS and supplemented with antibiotics and L-glutamine. For CFSE experiments, naïve SMARTA splenocytes were labeled using the CellTrace CFSE Labeling Kit (Invitrogen), according to the manufacturer's instructions, followed by i.v. transfer (1×10^6 SMARTA/mouse). For cell surface staining, cells were incubated with fluorescent dye-conjugated antibodies, with specificities as indicated (eBiosciences, San Diego, CA, or BD Biosciences, Mountain View, CA), in PBS containing 1% FBS. Antibody-stained cells were detected on a FACSCanto II flow cytometer (BD Biosciences) and results were analyzed using FlowJo software (TreeStar).

Peptide restimulation and intracellular staining

Resuspended cells were restimulated for 4 hours with 10 µM GP_{61-80} peptide (GLKGPDIYKGVYQFKSVEFD) in the presence of brefeldin A (GolgiPlug, 1 µl/ml). Cells were stained with cell surface Abs, permeabilized and stained with cytokine specific antibodies using a kit, per the manufacturer's instructions (BD Biosciences). For functional avidity assays, cells were restimulated with a range of peptide concentrations (10 µM–0.1 nM) prior to cytokine staining, with the percentage of maximal response determined by calculating the frequency of IFNγ–producing cells at any given concentration as a percentage of the frequency of IFNγ–producing cells at the highest peptide concentration.

RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) from FACS-sorted primary SMARTA effectors and secondary SMARTA effectors induced in either LCMV-immune or naïve hosts. cDNA was prepared from the RNA and real-time RT-PCR was performed on a Roche LightCycler 480 (Roche, Indianapolis, IN) using Superscript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Expression levels were normalized to GAPDH expression. Oligonucleotide primer sets used are as follows: Zap70: F-AGCGAATGCCCTGGTATCAC, R-CCAGAGCGTGTCAAACTTGGT; SLP76: F-AGAATGTCCCGTTTCGCTCAG, R-TGCTCCTTCTCTCTTCGTTCTT; Lck: F-TGGTCACCTATGAGGGATCTCT, R-CGAAGTTGAAGGGAATGAAGCC; Fyn: F-ACCTCCATCCCGAACTACAAC, R-CGCCACAAACAGTGTCACTC; PLCγ: F-ATCCAGCAGTCCTAGAGCCTG, R-GGATGGCGATCTGACAAGC; SHP-1: F-CCCGCTCAGGGTCACTCATA, R-CCCGAGTAGCGTAGTAAGGCT; DUSP-6: F-CCGTGGTGCTGTACGACGAG, R-GCAGTGCAGGGCGAACTCGGC; Cbl-b: F-GTCGCAGGACAGACGGAATC, R-GAGCTGATCTGATGGACCTCA; Bcl-2: F-GTGGTGGAGGAACTCTTCAGGGATG, R-GGTCTTCAGAGACAGCCAGGAGAAATC; STAT5A: F-CGCCAGATGCAAGTGTTGTAT, R-TCCTGGGGATTATCCAAGTCAAT; GAPDH: F-ATTGTCAGCAATGCATCCTG, R-ATGGACTGTGGTCATGAGCC.

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CHAPTER 5

CONCLUSION

In this dissertation, we find that TCR signals during priming have a determining role in CD4$^+$ T cell memory differentiation. Furthermore, during the recall response, the acquisition of protective function and subsequent long-term maintenance of secondary Th1 responders are profoundly influenced by the nature of the secondary challenge. These findings advance our understanding of the generation, maintenance and recall responses of memory CD4$^+$ T cells and have direct implications for the rational designs of better vaccination and immunotherapeutic strategies.

In Chapter 2, we find that following acute infections, the transition of activated CD4$^+$ T cells from effector to memory is associated with a significant loss of TCR repertoire diversity. Mechanistically, we demonstrate that sustained and stable interactions of the TCR with peptide-MHCs, as demonstrated by slow antigen off-rates, preferentially promote Th1 memory differentiation. These findings suggest an instructive role for TCR signals in the fate decisions made by activated CD4$^+$ T cells to become either end-stage effectors or long-lived memory cells.

TCR signal strength impacts T cell recruitment and activation and is regulated by the binding properties of TCR-antigen interactions [1]. However, binding parameters that correspond to T cell activation are controversial [2]. For example, several lines of evidence indicate that high TCR affinity for antigen promotes stronger T cell activation [3], whereas other studies suggest that slow antigen off-rates induce stronger TCR signals and higher T cell reactivity [4]. Recently, fast on-rates have been proposed to be a better predictor of robust T cell activation, as the TCRs with fast on-rates could accelerate TCR binding and rebinding to the same antigen, leading to unexpectedly longer TCR-antigen interactions [5,6]. However, none of these models can explain all functional outcomes.

Importantly, while most of the binding parameters have been experimentally measured based on the interactions between the TCR and peptide-MHC monomers/tetramers, a recent study found that in a mouse model of acute infection, a substantial proportion of antigen-specific polyclonal effector cells capable of making effector cytokines were not detected by MHC tetramers due to their extremely low TCR affinity for antigen [7]. Therefore, several other factors related to TCR-antigen interactions clearly impact the T cell activation in vivo. For example, the same TCR could interact with different APCs at different times in different locations throughout the immune response, which results in heterogeneous T cell activation. Furthermore, upon antigen encounter, T cells form an immunological synapse where the TCRs and other accessory molecules are clustered, which increases the local binding of the TCRs with antigens and thereby amplifies TCR signals [8]. A recent study showed that antigen stimulation of T cells resulted in an increase in oligomeric TCR complexes on the cell surface, enhancing antigen sensitivity [9]. Additionally, qualitatively distinct TCR signals could be delivered with a brief antigen contact. Therefore, all of these parameters could be incorporated via the TCRs during antigen encounter, which ultimately determines the various functional outcomes in vivo.

We find that sustained TCR-antigen interactions correlate to Th1 memory potential. This observation raises the question of what are the downstream molecular pathways associated with TCR binding kinetics and memory generation. A prior study showed that weak TCR signals during activation resulted in a decrease in the stability of anti-apoptotic molecule Mcl-1, thereby limiting the expansion and survival of low affinity T cell clones in the effector pool [10]. Recent work from our laboratory found

that pro-apoptotic molecule Bim was highly expressed in suboptimally activated Th1 effector cells and mediated effector cell death during the contraction phase [11]. Therefore, one possibility is that TCR binding kinetics may directly regulate the apoptotic machinery of activated $CD4^+$ T cells during early primary responses. In addition, we also find that Th1 effectors with lower functional avidity compete poorly for entry into the memory pool. Thus, the half-life of TCR-antigen interactions may control the expression of several genes involved in functional responses to antigen.

In Chapter 3 and 4, we find that during the recall response, various phenotypic and functional properties of secondary Th1 effectors, including secondary expansion, antigen sensitivity, effector cytokine production and trafficking to tissue sites of infection, are influenced by the nature of the secondary stimulus. Importantly, the acquisition of high-level functionality by secondary Th1 effectors at the peak of the recall response ultimately leads to the development of remarkably stable secondary Th1 memory cells. While activation signals during the primary response obviously impact downstream effector and memory T cell differentiation, our findings highlight that the context of pathogen rechallenge is also important for determining function and long-term fate of secondary Th1 effector and memory cells. Furthermore, our findings suggest that functional property of $CD4^+$ memory T cells is not fixed, but instead dynamically modulated in response to the secondary stimulus.

Our results suggest that the duration of the secondary challenge is one of the critical factors in the generation of highly functional secondary Th1 effectors. When Th1 memory cells receive either "too weak" secondary stimulation (following homologous rechallenge of immune mice, where infection is rapidly cleared) or "too strong"

secondary stimulation (following heterologous rechallenge of transferred memory cells in previously naïve mice, where the infectious period is prolonged, comparable to primary infection), recall responses are similarly defective, displaying a loss of high functional avidity by responding effector cells and diminished long-term survival by ensuing secondary memory cells. Thus, the secondary differentiation of Th1 memory cells seems to follow the "Goldilocks" principle in that they need just the right amount of secondary stimulation in order to generate highly functional as well as highly stable secondary effector and memory cells.

Remarkably, the secondary Th1 effector differentiation coincides with a massive increase in functional avidity, followed by functional dematuration to the level similar to the parent memory cells from which they arise. This dynamic functional modulation during early recall responses is strikingly different from that of the primary response, where naïve T cells undergo extensive functional maturation throughout the primary response [12,13]. Whether the early acquisition of extremely high function by secondary Th1 effectors is programmed and which factors regulate this functional modulation remains to be addressed. Interestingly, a recent study found a transient increase in antigen sensitivity of memory CD8^{+} T cells in a manner dependent on infection-induced inflammatory cytokines such as IL-12, IL-18 and type I IFN [14,15]. Whether this also applies to Th1 cells is unknown. Additionally, the early emergence of highly functional secondary Th1 effectors may be critical for the rapid control of pathogen challenge. In this regard, prior work has shown that memory CD4^{+} T cells provided enhanced protection by inducing an early innate response [16], suggesting a possible link between

the early acquisition of high functionality and induction of more rapid and robust innate inflammatory response for enhanced protection.

When recall stimulation is prolonged, we find that responding secodnary Th1 effectors progressively lose their high functional avidity as a result of reduced expression of TCR proximal molecules as well as concurrently increased expression of TCR proximal phosphatases, such as SHP-1 and DUSP6. Conversely, secondary Th1 effector cells with high functional avidity have opposite gene expression patterns. Whether the duration of secodnary challenge directly regulates transcription of these genes is not known. Nevertheless, these findings indicate that responding secondary effector cells intrinsically tune TCR activation threshold in response to secondary stimulation by regulating gene expression involved in TCR signaling.

Much progress has been made in our understanding of the generation, maintenance and secondary activation of CD4$^+$ memory T cells. It is now clear that the nature of activation signals profoundly influences subsequent CD4$^+$ T cell differentiation during both primary and secondary responses. However, several key issues still remain unanswered. Future studies will address the molecular mechanism, by which CD4$^+$ T cells sense varying nature of TCR signals fated for either effector or memory, how the functional plasticity of secondary Th1 responders is regulated during the recall response, and what is the biological relevance resulting from the early acquisition of high functionality by secondary Th1 effectors. The answers to these questions will advance our understanding of memory T cell biology as well as have direct implications for the design of better vaccination and immunotherapeutic strategies aimed at enhancing CD4$^+$ memory T cell formation and function.

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APPENDIX

NATURE AND NURTURE: T-CELL RECEPTOR-

DEPENDENT AND T-CELL RECEPTOR-

INDEPENDENT DIFFERENTIATION

CUES IN THE SELECTION OF

THE MEMORY T-CELL

POOL

Chulwoo Kim and Matthew A. Williams

IMMUNOLOGY REVIEW ARTICLE

Nature and nurture: T-cell receptor-dependent and T-cell receptor-independent differentiation cues in the selection of the memory T-cell pool

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Summary

The initiation of a T-cell response begins with the interaction of an individual T-cell clone with its cognate antigen presented by MHC. Although the strength of the T-cell receptor (TCR) –antigen–MHC (TCR-pMHC) interaction plays an important and obvious role in the recruitment of T cells into the immune response, evidence in recent years has suggested that the strength of this initial interaction can influence various other aspects of the fate of an individual T-cell clone and its daughter cells. In this review, we will describe differences in the way CD4^{+} and CD8^{+} T cells incorporate antigen-driven differentiation and survival signals during the response to acute infection. Furthermore, we will discuss increasing evidence that the quality and/or quantity of the initial TCR-pMHC interaction can drive the differentiation and long-term survival of T helper type 1 memory populations.

Keywords: CD4/helper T cells; memory; T-cell receptor; T cells; vaccines

Introduction

Following exposure to an intracellular pathogen, T cells undergo numerous rounds of cell division, expanding up to 50 000-fold in the course of about a week.[1,2] During this period of priming and expansion, they also differentiate into cytokine-producing and cytolytic effector cells and make fate decisions that govern their entry into the long-lived memory pool. While most effector T cells die in the weeks following antigen clearance, a small proportion survives and becomes long-lived memory T cells.[3,4] Because cells fated for either memory differentiation or death can be identified at the peak of the primary response,[5] numerous studies have attempted to identify signals delivered during the primary response that drive the effector/memory fate decision. Generally, these studies have either focused on the context, duration, quantity or quality of the antigen-specific signal delivered through the T-cell receptor (TCR) or the role of non-antigen-specific signals, such as growth or inflammatory factors, in driving differentiation of effector and memory T cells. The ability of any individual naive T cell to recognize peptide–MHC complexes (pMHC) via its TCR regulates its recruitment into the immune response but the subsequent role of TCR signals in driving expansion, effector differentiation and survival remains unresolved. We suggest that disparate results regarding TCR-driven T-cell differentiation can be largely attributed to differences in the way CD4^{+} and CD8^{+} T cells incorporate and respond to antigen-specific signals during the primary response to acute infection, and this review will focus particularly on the role of TCR signals in driving not only recruitment and expansion of CD4^{+} T cells, but also their acquisition of effector functions and ability to populate the long-lived memory compartment.

Extrinsic and intrinsic differentiation cues

Following exposure to antigen, T cells undergo a period of massive expansion, sometimes exceeding 50 000-fold, and acquire effector functions that enable them to co-ordinate pathogen clearance. After clearance, the majority of effector T cells die, leaving behind a population (5–10%) of long-lived memory T cells that provide enhanced protection from re-exposure to the same or a related pathogen.[3,4,6,7] Memory T-cell populations maintain the ability to both survive independently of cognate antigen[8] and self-renew in response to external homeostatic signals such interleukin-15 (IL-15) and IL-7,[9,10] hence maintaining themselves at stable levels for many years. However, it has become increasingly clear in recent years that crucial T-cell fate determination events occur

during the primary response, as short-lived effector T cells can be distinguished from memory precursor effector T cells during its later stages. For example, memory precursor T cells can be identified on the basis of expression of IL-7 receptor α,[5,11] although IL-7 signals themselves may not be absolutely required in the memory fate decision.[12–14] Furthermore, in the CD8$^+$ effector T-cell compartment expression of a variety of natural killer cell receptors, most particularly KLRG1, has been associated with short-lived effector/effector memory differentiation and inversely correlates to entry into the long-lived effector/central memory pool.[15]

In addition, the effector and memory differentiation of T cells can be remarkably heterogeneous. Memory T cells can be broadly divided into central and effector memory subsets based on cell surface phenotype and tissue localization; the relative roles of these subsets in protection from secondary pathogen exposures has only been understood in recent years.[16,17] For T helper type 1 (Th1) memory T cells, for example, one of the best predictors of protective capacity is the ability to make multiple cytokines [interferon-γ (IFN-γ), tumour necrosis factor-α and IL-2) immediately upon re-exposure to antigen.[18,19] Whereas central memory cytotoxic T lymphocytes (CTL) are thought to have the best proliferative and protective capacity,[20] in some models of acute infection and re-challenge, the activation phenotype of memory CD8$^+$ T cells has proved to be the best predictor of protection from secondary challenges, independent of central or effector memory phenotype.[21] These findings do not rule out a role for tissue-residing effector memory T cells in protection. Although they appear to be less stable as a population and have less proliferative capacity compared with central memory populations, they probably provide an important line of defence at the site of infection. Indeed, we would argue that the community dynamics of pathogen transmission would dictate that the most likely period of re-exposure to a pathogen are the months immediately following the initial exposure. The long-lived central memory compartment can 'remember' infectious history over the course of many years whereas the shorter-lived effector memory compartment provides a short-term memory of not only the nature of the pathogen but the site of the initial pathogenic challenge. Although we simplistically refer to effector/memory fate decisions in the current review, a full and accurate understanding of T-cell fate decisions will require a better understanding of the nature and function of memory T cells across the spectrum of their differentiation states. A primary goal in efforts to understand the biological processes that drive the simultaneous but asymmetric and heterogeneous differentiation of effector and memory T cells during the primary response should be to identify the nature of the fate determination signals, with obvious implications for a wide range of vaccination and immunotherapy strategies.

Efforts to understand why a few effector T cells go on to populate the memory pool while the majority die following pathogen clearance have focused on the role of external environmental cues (e.g. co-stimulation, inflammatory mediators, growth factors) as well as intrinsic differences in the ability of individual T-cell clones to respond (e.g. TCR avidity). In this, the differentiation of pathogen-specific CD4$^+$ and CD8$^+$ T cells differs in certain key respects. Once CD8$^+$ T cells are recruited into the immune response against a foreign pathogen, all aspects of differentiation are enabled. Clonal progeny from initially recruited CD8$^+$ T-cell responders differentiate into cytotoxic effector cells, cytokine producers and long-lived memory cells after only a brief period of selection[22–24] over a wide range of TCR avidities for antigen.[25] The repertoires of CD4$^+$ primary and secondary effector T-cell populations also appear to be dominated by the preferential expansion of clones with high avidity TCRs following peptide or protein immunization.[26–29] CD4$^+$ T cells, however, also appear to undergo multiple stages of differentiation, with progressively stronger activation signals promoting not only their recruitment and activation, but also differentiation and survival.[30,31]

Environmental cues and memory T-cell differentiation

External signals play a key role in the recruitment, expansion and differentiation of antigen-specific effector and memory T cells. One recent study found that clonal recruitment of CD4$^+$ T cells following peptide or protein immunization was acutely dependent on the presence and nature of an accompanying adjuvant.[32] Inflammatory adjuvants promoted the recruitment of a T-cell repertoire with a wider range of individual TCR avidities for antigen following peptide immunization.[32] Even though only a short period of antigenic stimulation (6–12 hr) is required *in vivo* for recruitment of antigen-specific CD8$^+$ T cells, their optimal expansion requires the presence of a robust, infection-induced inflammatory response.[23] Furthermore, in the context of a *Listeria monocytogenes* infection antigen-specific T cells were efficiently recruited into the immune response across a 700-fold range of TCR avidities. Interestingly, however, the extent and kinetics of expansion were proportional to the avidity of the TCR-pMHC interaction,[25] suggesting that whereas low-avidity TCR signals can mediate recruitment, stronger and/or prolonged TCR signals probably influence the final clonal composition of the effector CTL pool.

The inflammatory environment also profoundly impacts the differentiation of effector function and memory potential. For example, high levels of IL-12 or type I IFN signalling have been implicated in the preferential differentiation of effector CTL,[15,33,34] at least in part through the graded up-regulation of the T-box transcription factor T-bet.[15]

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Interleukin-2 is another cytokine implicated in driving effector and memory CTL differentiation.[35,36] In particular, activation of T cells in the absence of IL-2 results in a decrease in primary effector function in response to acute and chronic infection and a severe impairment in the ability of resulting memory T cells to generate secondary responses upon re-challenge.[37,38] A related cytokine, IL-21, has also been found to drive effector T-cell differentiation during chronic infection,[39–41] though whether it plays a similar role to IL-2 in promoting primary and secondary effector T-cell differentiation during acute infection remains to be determined.

Recent attempts to understand the fate decisions that T cells make during the primary response have revolved around the function of several transcription factors whose expression has been linked to exposure to growth and inflammatory cytokines. The T-box transcription factors T-bet and Eomes have been shown to promote CD8$^+$ effector T-cell differentiation,[15,42] and high levels of T-bet activity are associated with terminal differentiation into short-lived effector T cells.[15] Blimp-1, a zinc finger transcription factor required for the differentiation of antibody-secreting plasma cells,[43] has recently been found to play a role in the effector differentiation of T cells responding to either acute or chronic viral infection.[44–46] Conversely, the transcriptional repressor Bcl-6 has been reported to promote lymphocyte differentiation and survival of memory T cells.[47–49]

The role of these transcription factors is complicated by the observation that relatively small differences in activity can have large consequences for differentiation outcomes. For example, high levels of inflammatory cytokines such as IL-12 or type I IFNs have been shown to induce strong T-bet activity in CD8$^+$ T cells and promote entry into a terminal differentiation pathway. On the other hand, activation in the complete absence of T-bet results in dysfunctional memory T cells, perhaps partly because of poor expression of IL-15Rβ (CD122).[15] Similarly, whereas either strong or prolonged IL-2 signals preferentially promote CD8$^+$ T-cell effector differentiation,[35,36] the complete absence of IL-2 results in dysfunctional memory cells that are unable to re-enter the effector differentiation pathway upon reactivation.[37,38] In these and other cases, CD8$^+$ memory T-cell differentiation seems to follow the 'Goldilocks' principle in that they require just the right amount of effector differentiation stimuli. Certain activation stimuli are required for CD8$^+$ memory T-cell differentiation, whereas an over-abundance of these stimuli leads to committed effector CTL differentiation, and a complete absence of these stimuli leads to defects in both effector and memory T-cell differentiation. The full nature and timing of the stimuli remain controversial. One recent study proposed that CD8$^+$ memory T-cell differentiation might result from a first asymmetric division directly subsequent to priming events.[50] Another recent

report demonstrated that CD8$^+$ memory T cells capable of robust secondary replicative function develop from precursors that have undergone some level of effector differentiation.[51,52] In all, a pressing question is when during the response do CD8$^+$ T cells become committed to either a memory or effector differentiation pathway?

Remarkably, far less is known regarding the general nature of fate decisions that CD4$^+$ T cells make during the primary response to acute infection, and even less is known regarding the role of the accompanying inflammatory environment. T-bet has a well-described IL-12-dependent function in driving polarization of IFN-γ-producing Th1 responders.[53] However, remarkably little is known regarding the role of T-bet in the differentiation and survival of long-lived CD4$^+$ memory T-cell populations, or whether CD4$^+$ T cells themselves even undergo the same sort of terminal differentiation pathway that has been observed for plasma cells and now CD8$^+$ effector T cells. Blimp-1 plays a role in repressing genes required for Th1 differentiation, including T-bet,[54] and Bcl-6 may be important for Th1 memory differentiation,[55] but little is known regarding how the activity of these transcription factors in Th1 cells is modulated in response to their external environment and how they shape effector and memory Th1 cell differentiation.

TCR-driven differentiation: CD8$^+$ T cells

The relative roles of antigen-mediated signals through the TCR and non-specific signals such as inflammatory cytokines and growth factors in driving T-cell differentiation are still being defined, and compelling evidence exists for both scenarios. Two broad models have been proposed to describe the required duration of antigen presentation in driving the recruitment, expansion and differentiation of T cells during their response to intracellular pathogens. The first, termed progressive differentiation, posits that sequential encounters with antigen progressively promote cell division, enhanced survival and functional differentiation.[31] An extended period of antigen presentation would be required for the full differentiation. The second, termed programmed differentiation, proposes that the differentiation of T cells is programmed upon a brief initial period of antigen encounter.[56] Although there is substantial evidence for each model, neither comprehensively describes what has been observed for *in vivo* CD4$^+$ T-cell responses.

For CD8$^+$ T cells, a short encounter with dendritic cells presenting antigen is sufficient to induce downstream stages of differentiation. As little as 6–24 hr is sufficient to recruit CD8$^+$ T cells into the immune response directed toward a foreign pathogen. Additionally, once recruited, CD8$^+$ T cells are capable of undergoing dividing, developing effector functions and differentiating into memory cells independent of further antigen encounter.[22–24] One

recent study found that while the recruitment of antigen-specific CD8+ T cells into the immune response can be accomplished with a short exposure to antigen, optimal expansion is subsequently driven primarily by the inflammatory environment.[23]

Although TCR signals, as regulated by TCR avidity and the abundance of cognate antigen, drive recruitment and expansion of primary CTL, the precise role of TCR signals in promoting memory differentiation remains controversial. Studies in multiple model systems have suggested that the TCR repertoire of effector CTL is widely dispersed in tissues and similar to that of both memory and secondary effector CTL,[57–61] suggesting that once a clonal population is represented in the expanded effector CTL pool, it maintains no intrinsic competitive advantage in proceeding to the memory pool. Likewise, another recent study found that whereas changes in CTL TCR avidity for antigen across a 700-fold range impacted the kinetics and magnitude of clonal expansion, the size of the resulting memory population remained proportional to the peak of the effector pool.[25] Similarly, while altering antigen dose impacted CTL expansion, it did not alter the clonal composition of the effector pool.[62] Indeed, a single naive CD8+ T-cell precursor can give rise to heterogeneous effector and memory CTL differentiation that largely mimics that of polyclonal responders,[63,64] again suggesting that TCR signals do not play a dominant role in differentiation outcome. Conversely, one recent report found that disruption of TCR signalling could have differential effects on T-cell fate. In this study, mutations to the TCR-β transmembrane domain that prevented efficient synapse formation, and attenuated TCR signalling allowed efficient effector CTL differentiation but poor memory CTL differentiation.[65]

One question arising from these studies is whether the extent of expansion is influenced by a short period of high avidity interactions between the TCR and pMHC or a prolonged period of TCR-pMHC interaction throughout the primary expansion phase. At least two pieces of evidence suggest that the former possibility is likely. First, direct visualization of the first few days of the *in vivo* T-cell response suggests that stable T-cell–antigen-presenting cell (APC) interactions are largely confined to the first couple of days following antigen challenge.[66] Second, experiments in which antigen is presented to T cells for variable amounts of time in the presence or absence of inflammation indicate that the determining factor for robust clonal expansion is the persistence of the inflammatory environment following recruitment of T cells into the response.[23] Therefore, it seems likely that the ability to undergo robust clonal expansion is dictated by the quality or quantity of the TCR-pMHC interaction at the initiation of the response, whereas the infectious inflammatory environment plays a central role in driving that expansion. Conversely, CD8+ T cells recruited in a limited or non-

inflammatory environment undergo less initial expansion but retain or quickly recover the ability to undergo secondary expansion upon immediate re-challenge.[67–69]

TCR-driven differentiation: CD4+ T cells

For CD4+ T-cell responses the role of antigenic signal strength is more complex. The selection of Th1 precursors into the response is influenced by TCR avidity for peptide/MHC class II, and certainly at some level this influences the repertoire of the effector pool.[26] CD4+ T cells, in contrast to CD8+ T cells, also require extended or repeated contacts with antigen during the first few days of the response for full expansion.[70,71] Evidence also suggests that secondary stimulation of CD4+ memory T cells continues to skew the TCR repertoire towards higher avidity responders, an observation not seen for CD8+ T cells.[29] These observations suggest that CD4+ T cells are faced with a longer period of selection, during both primary and secondary responses, on the basis of their ability to bind antigen. Other studies have also supported a model in which antigen signals drive progressive differentiation of CD4 responders, with increasing signals driving first expansion, then effector function and survival.[30,31,72–74] Polyclonal CD8+ T cells, on the other hand, require a short window of antigenic stimulation *in vivo* (12–24 hr).[75] The magnitude of expansion is impacted by the duration or strength of the initial stimulus but T cells that are recruited into the response readily go on to form memory populations capable of robust secondary responses.[22] One interpretation of these results is that once CD8+ T cells reach a certain activation threshold, all phases of differentiation are enabled, with non-antigen-specific signals promoting differentiation of end-stage effector and memory precursor populations. CD4+ T cells, on the other hand, with their extended requirement for exposure to antigen, demonstrate a consistent skewing towards high-avidity TCRs throughout the effector response and following subsequent re-challenges.

Analysis of TCR repertoires has also suggested a role for the TCR in driving CD4+ T-cell differentiation. The TCR repertoire of antigen-specific T cells narrows to progressively higher avidity throughout the primary response and during subsequent re-challenges,[27,29] suggesting that CD4+ T-cell responses undergo selection for high-avidity clones in the presence of antigen. Furthermore, the avidity of CD4+ effector and memory T cells is dependent on the initial antigen dose.[28] On the other hand, the role of antigen in driving T-cell differentiation is not solely dependent on high avidity TCR. Clonal populations of CD4+ T cells can undergo functional avidity maturation throughout the primary response, resulting in the emergence of T cells with heightened sensitivity to antigen even as the TCR itself remains fixed.[2] One possible explanation for these results is that functional avidity is

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hardwired into the response on the basis of the quality of the initial interaction with APC. In this scenario, one could envisage heterogeneity in the abundance of antigen, the surrounding microenvironment, the type of APC and the activation status of that APC leading to a spectrum of functional avidity in a monoclonal T-cell population. Conversely, non-TCR-specific mechanisms may promote the acquisition of heightened TCR signal sensitivity throughout the primary response, leading to the selection of effector clones in a largely TCR-independent manner.

Recent work has demonstrated a role for TCR–antigen interactions in driving not only effector differentiation but also survival into the memory pool. The initial clues that this might be the case were derived from clonal competition experiments. Increasing the frequency of antigen-specific CD4^{+} T cells at the initiation of the response inversely corresponded to memory differentiation potential. At very high clonal frequencies of TCR transgenic T cells, presumably accompanied by fierce competition for available antigen, the differentiation of virus-specific Th1 memory was almost entirely impaired.[76] Clonal competition also impacted the long-term maintenance of Th1 memory cells.[77] Competition during the primary CD4^{+} T-cell response for factors other than TCR, such as IFN-γ, has also been shown to impact the quantity and quality of ensuing memory T-cell populations.[78]

Our recent findings have also indicated that not all CD4^{+} T-cell clones that undergo massive expansion and effector differentiation in response to acute infection are capable of populating the memory pool.[79] Small numbers of adoptively transferred SMARTA TCR transgenic T cells, which are specific for the lymphocytic choriomeningitis virus (LCMV) glycoprotein-derived immunodominant CD4^{+} T-cell epitope GP$_{61-80}$, effectively mimic endogenous CD4^{+} T-cell responses in the same host following LCMV infection. SMARTA cells respond quite differently, however, following challenge with recombinant *Listeria monocytogenes* expressing the same epitope (Lm-gp61). Although SMARTA cells expand robustly initially, the resulting Th1 effector cells demonstrate partially impaired cytokine-producing capabilities. Furthermore, they are unable to progress to the memory pool and disappear entirely in the weeks following pathogen clearance, despite the efficient development of polyclonal endogenous CD4^{+} memory T cells directed toward the same epitope in the same host.[79]

We found that memory differentiation potential corresponded to both structural and functional TCR avidity at the peak of the effector response. In LCMV-infected hosts, SMARTA effector cells demonstrated similar avidity to polyclonal responders in the same host. Conversely, in Lm-gp61-infected hosts SMARTA effector cells displayed lower TCR avidity than endogenous responders. One possibility, therefore, is that their failure to enter the memory pool was reflected in their poor sensitivity to antigen during the primary response. In support of this, polyclonal CD4^{+} memory T-cell populations skewed to a higher functional avidity in the transition from effector cells (1 week post-infection) to memory cells (6 weeks post-infection). Furthermore, in the months following infection, CD4^{+} memory T cells consistently skewed to a higher functional avidity.[79] It has been reported that CD4^{+} memory T cells decline over time,[80] an observation that we make in our own studies. However, we found that the rate of decline decreases over time (6 months to 1 year), corresponding to the emergence of CD4^{+} memory T cells with high functional avidity.[79] One possibility, therefore, is that CD4^{+} memory T-cell populations eventually stabilize following acute pathogenic challenge, resulting in highly functional high-avidity memory cells capable of long-term protection. This would be consistent with observations of human populations in which vaccine-induced CD4^{+} memory T-cell populations can persist for up to 75 years with a half-life of 8–15 years.[81]

While much of the focus regarding CD4 memory differentiation has been on the signals during the primary response that promote memory differentiation, another line of research has sought to identify the factors that promote massive cell death during the contraction phase. In our studies, we found that the pro-apoptotic molecule Bim was highly up-regulated in 'doomed to die' SMARTA Th1 cells following Lm-gp61 infection, providing a possible link between weak antigenic signals and effector cell elimination following pathogen clearance.[79] Bim and Bcl-2 play opposing roles in promoting T-cell survival during various stages of differentiation.[82] In particular, Bim activity has been shown to mediate the death of end-stage effector CD4^{+} and CD8^{+} T cells during the contraction phase of the response.[83–85] However, less is known about the factors controlling the activity of Bim. The decision to enter a Bim-mediated death pathway is probably made before the contraction phase, as IL-7 receptor α^{hi} memory precursor cells at the peak of the response are largely spared Bim-mediated apoptosis.[85] Our finding that Bim expression is associated with SMARTA responders that have low functional avidity compared with the endogenous CD4^{+} T-cell response suggests that TCR signals may influence the ability of the Th1 effector cells to survive into the memory phase of the response through regulation of Bim activity. We further found that 'doomed-to-die' SMARTA effectors induced by Lm-gp61 expressed higher levels of the transcription factor FoxO3a, compared with their LCMV-activated counterparts. FoxO3a has been shown to regulate the survival of CD4^{+} memory T cells and promotes apoptosis in T cells.[86–88] One target of FoxO3a is Bim, leading us to hypothesize that FoxO3a may also play a role in regulating the survival of CD4^{+} T cells in the transition from the effector to the memory pool in a Bim-dependent manner.

Hierarchical CD4+ T-cell differentiation

The above-described differences in the role of antigen-driven TCR signals in the expansion, differentiation and survival of CD8+ and CD4+ T cells responding to acute infection show that while all aspects of CD8+ T-cell differentiation are enabled, or 'programmed', following the initial activation/recruitment event, CD4+ T-cell differentiation is hierarchical, with increasing antigenic signals enabling progressively enhanced expansion, effector differentiation and survival. We hypothesize a model in which CD4+ memory T-cell differentiation and the ability of CD4+ memory T cells to survive long-term during the memory maintenance phase are enabled, at least in part, by the strength of the antigenic signal during the primary response (Fig. 1). Several studies have analysed the evolution of the TCR repertoire during the CD8 response to acute infection and found that that effector repertoire and the memory repertoire were similar.[57,61] However, even though a strong rationale exists for a higher dependence on antigen for the differentiation of CD4+ memory T cells, no analogous studies of *in vivo* infection-induced CD4+ memory T-cell repertoires as they develop and then decline over time have been performed. Furthermore, previous studies have largely used broad methods for analysing clonotype distribution, such as analysis of V-region or J-region subsets or spectratyping analysis of CDR3 length distribution, rather than analysis of the distribution of single clonal populations through individual CDR3 sequences, as measured by deep sequencing analysis. A primary focus of current research efforts should be to determine if, on a clonal level, high-avidity TCR-pMHC interactions during the primary response result in preferential differentiation of long-lived CD4+ memory T cells.

Several other possibilities exist. First, strong TCR-pMHC interactions may be important during the memory maintenance phase after antigen has been cleared rather than during the primary response. It is possible, for example, that clonotypes with strong avidity for cognate antigen presented by MHC class II also have higher avidity for self MHC class II molecules in general. Recent support for a role for MHC class II interactions in promoting CD4+ memory T-cell survival was provided by the finding that the half-life of CD4+ memory T cells was inversely proportional to their frequency, although how these findings apply to diverse polyclonal memory populations with presumably varying specificities for endogenous peptides remains unclear.[77] A second possibility is that the high functional avidity of CD4+ memory T cells compared with the effector populations that they are derived from is unrelated to actual TCR avidity but is instead a property acquired upon receipt of non-antigen-specific memory differentiation/maintenance signals. Monoclonal CD4+ and CD8+ T cells have been shown to acquire higher functional
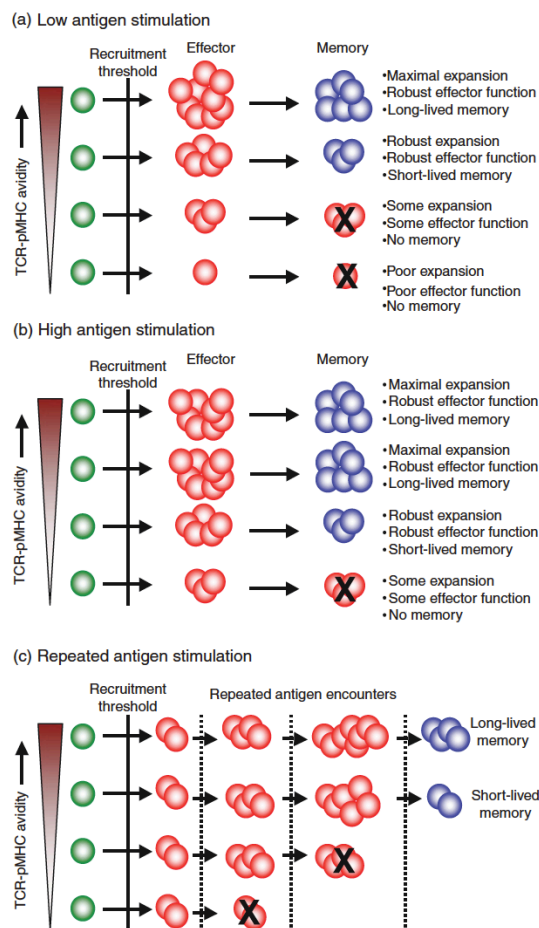

Figure 1. Hierarchical CD4+ T-cell differentiation is influenced by T-cell receptor avidity and antigen availability. (a) Under conditions of low antigen availability, only intermediate to high-avidity clones participate in the Th1 effector response, whereas only high-avidity clones populate the memory pool. (b) Under conditions of high antigen availability, clones with low, intermediate and high avidity participate in the effector response, whereas intermediate and high-avidity clones populate the memory pool. (c) Following repeated antigen stimulation, high-avidity clones might gain a competitive advantage and preferentially populate the effector and memory pools.

avidity throughout the effector response, and a cardinal feature of memory T cells is their lower activation threshold.[2,89] Finally, it is possible that antigen retained after pathogen clearance could continue to shape the TCR repertoire in ways similar to that seen during chronic antigen exposure. In support of this, it has been shown that antigen retained in germinal centres can continue to shape the CD4+ memory T-cell compartment for several weeks after clearance.[72] A careful and thorough analysis of Th1 TCR repertoire evolution during the effector response, in

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the transition from effector to memory and during the long-term maintenance of memory in the absence of continued antigen stimulation, is needed to ascertain whether and to what extent the strength of the initial TCR-pMHC interaction influences the long-term fate of individual T-cell clones and their daughters.

Disclosures

The authors declare no financial or conflict of interest.

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