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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 Animal Care and Use Committee at the University of Utah. LCMV animal experiments were conducted with the approval of the Institutional Care and Use Committee at the University of Utah (30). All

OD at 600 nm (OD of 1 = 1 μm). Mice were infected 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 (gp61-80), along with a subdominant class I-restricted epitope (gp77-77) (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. gp61-80–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. gp61-80–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
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We previously observed that the most long-lived Th1 memory cells skewed to a higher functional avidity (8). Additionally, secondary challenge was shown to induce the selective outgrowth of high-
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 gp61–80, 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 1. Heterologous rechallenge results in the stable maintenance of secondary Th1 memory cells.](http://www.jimmunol.org/)

![FIGURE 2. Homologous rechallenge results in poor maintenance of secondary Th1 memory cells.](http://www.jimmunol.org/)
found that long-lived Th1 memory cells that are maintained most stably also skewed 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 gp61–80 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.](http://www.jimmunol.org/)

**FIGURE 3.** Homologous boosting results in a loss of Ag sensitivity by secondary Th1 effector cells. Splenocytes were restimulated with gp61–80 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.
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
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 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.

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 higher 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

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