Distinct Memory CD4⁺ T Cells with Commitment to T Follicular Helper- and T Helper 1-Cell Lineages Are Generated after Acute Viral Infection

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SUMMARY

CD4⁺ T follicular helper (Tfh) cells provide the required signals to B cells for germinal center reactions that are necessary for long-lived antibody responses. However, it remains unclear whether there are CD4⁺ memory T cells committed to the Tfh cell lineage after antigen clearance. By using adoptive transfer of antigen-specific memory CD4⁺ T cell sub-populations in the lymphocytic choriomeningitis virus infection model, we found that there are distinct memory CD4⁺ T cell populations with commitment to either Tfh- or Th1-cell lineages. Our conclusions are based on gene expression profiles, epigenetic studies, and phenotypic and functional analyses. Our findings indicate that CD4⁺ memory T cells “remember” their previous effector lineage after antigen clearance, being poised to reacquire their lineage-specific effector functions upon antigen reencounter. These findings have important implications for rational vaccine design, where improving the generation and engagement of memory Tfh cells could be used to enhance vaccine-induced protective immunity.

INTRODUCTION

Naive pathogen-specific CD4⁺ T cells respond to acute infections through robust proliferation and differentiation to generate effector cells with the capacity to provide help to the many and diverse branches of the immune system. Following antigen clearance, the majority of antigen-specific effector cells undergo apoptosis, leaving behind a population of memory CD4⁺ T cells. In addition to their ability to survive and undergo homeostatic proliferation in the absence of antigen, memory T cells retain the capacity to rapidly recall effector function, traffic to a wide range of tissues, and exist at much higher frequencies than naive cells specific for the same antigen. These features provide the host with a protective network of pathogen-specific memory T helper cells that are poised to swiftly respond upon a secondary challenge (Sallusto et al., 2010).

Naive CD4⁺ T cells have multiple fates and upon activation can develop into a variety of specialized subsets, such as T helper 1 (Th1), Th2, Th17, and regulatory T (Treg) cells. Each of these lineages has distinct gene expression programs that are regulated by specific STATS, transcription factors, and epigenetic mechanisms (O’Shea and Paul, 2010). More recently, an additional subset known as T follicular helper (Tfh) cells has been identified as the CD4⁺ T cell subset that provides help for antibody responses. Tfh cells provide the necessary signals to antigen-specific B cells to generate and maintain the germinal center reaction, thus facilitating efficient class switching and affinity maturation of antibodies, and the generation of long-lived antibody-secreting plasma cells (Crotty, 2011). Tfh cells were first characterized in humans by their expression of the B cell follicle homing receptor CXCR5 (Breitfeld et al., 2000; Kim et al., 2001; Schaerli et al., 2000), high ICOS and PD-1 expression, and the transcription factor Bcl6 (Crotty et al., 2010). Tfh cells can localize to the B cell follicle by sensing CXCL13 through CXCR5 (Ansel et al., 1999; Kim et al., 2001). Bcl6 has recently been identified as a Tfh lineage regulator (Johnston et al., 2009; Nurieva et al., 2009; Yu et al., 2009), and shares a reciprocal relationship with the transcriptional repressor Blimp-1, which suppresses Tfh differentiation (Crotty et al., 2010; Johnston et al., 2009). However, it remains unclear whether Tfh cells possess the capacity to further differentiate into the resting memory CD4⁺ T cell pool and retain their Tfh lineage commitment after antigen clearance (Crotty, 2011; Fazilleau et al., 2007; Liu et al., 2012; Lüthje et al., 2012; Marshall et al., 2011; Pepper et al., 2011; Weber et al., 2012).

To address whether Tfh memory cells exist within the pool of memory CD4⁺ T cells, we studied virus-specific CD4⁺ T cells throughout the primary, memory, and secondary effector phases of the immune response following acute lymphocytic choriomeningitis virus (LCMV) infection. We report here that a distinct CXCR5⁺ subset of antigen-specific CD4⁺ T cells preferentially recalled a Tfh cell secondary response following transfer and
Figure 1. Phenotypic Heterogeneity of Virus-Specific CD4+ T Cells Is Maintained during Effector and Memory Differentiation
We adoptively transferred $2 \times 10^6$ CD45.1+ LCMV-specific naive SMARTA transgenic CD4+ T cells into CD45.2+ naive recipients that were then infected with $2 \times 10^5$ PFU of LCMV Armstrong. FACS plots are gated on CD4+CD45.1+ SMARTA cells at the indicated time points relative to infection.

(A) Kinetics of splenic SMARTA CD4+ T cells.
(B) CXCR5, PD-1, ICOS, GL-7, Ly6c, and granzyme B analysis of naive, effector, and memory SMARTA CD4+ T cells.
(C) Analysis of T-bet and Bcl6 expression.
(D) Spleen and Blood analysis of effector and memory SMARTA CD4+ T cells.
(E) Mean fluorescent intensity (MFI) of CXCR5 in effector and memory SMARTA CD4+ T cells.
(F) Percent of SMARTA cells in different organs on Day 7 and Day 100-135.
(G) Analysis of Ly6c expression in SMARTA CD4+ T cells on Day 8, Day 46, and Day 105.

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challenge with virus, whereas CXCR5- memory cells generated a Th1 cell secondary response. Based on these findings, we propose a model in which Th1 and Thf cells differentiate to become Th1 and Thf memory cells, respectively, poised to preferentially recall their previously programmed lineage-associated gene expression patterns and effector functions upon antigen rechallenge. These findings have important implications for vaccine design, where adjuvants and strategies that promote a higher quantity and quality of memory Thf cells may enable enhanced humoral immunity following prime and boost vaccination.

RESULTS

Phenotypic Heterogeneity of Virus-Specific CD4+ T Cells Is Maintained during Effector and Memory Differentiation

To determine whether heterogeneity in the effector CD4+ T cell population persists during memory development, we performed a longitudinal analysis of Th1- and Thf-cell phenotypic marker expression on LCMV-specific CD4+ T cells following acute LCMV infection. Congenically-marked (CD45.1) naive SMARTA transgenic (Tg) CD4+ T cells specific for the LCMV GP66–77 epitope were adoptively transferred into recipient mice, and donor SMARTA cells (CD4+CD45.1+ gated) were analyzed at effector and memory time points following infection (Figure 1A). We observed that approximately 45% of virus-specific effector (day 7 postinfection) SMARTA cells expressed CXCR5 and high amounts of PD-1 and ICOS and contained a subpopulation of GL-7hi germinal center Tfh cells (Yusuf et al., 2010), consistent with a Thf phenotype (Figure 1B). Furthermore, the majority of CXCR5+ effector cells downregulated Ly6c expression and expressed low levels of granzyme B, while the CXCR5- effector cells displayed higher Ly6c and granzyme B expression (Figure 1B). In addition, CXCR5+ effector SMARTA cells expressed Bcl6 and low amounts of T-bet, whereas CXCR5- effector cells were Bcl6 negative and expressed high amounts of T-bet (Figure 1C). Similar to LCMV-specific SMARTA Tg cells, endogenous LCMV GP66–77 specific (tetramer+) effector CD4+ T cells in LCMV-infected B6 mice exhibited the same dichotomy of CXCR5+ and CXCR5- cells with similar expression patterns of PD-1, Ly6c, Bcl6, and T-bet (see Figure S1A and S1B available online). These data demonstrate the generation of both Thf and Th1 virus-specific effector cells during LCMV infection, which could be generally distinguished by Ly6c and CXCR5 expression. In agreement with this, Ly6c+ SMARTA effector cells localized predominantly within the B cell follicle and germinal centers, whereas Ly6c- effector cells were generally outside of B cell follicles and germinal centers (Figure S1F).

We observed that CXCR5+ Thf effector cells were lower for Psgl1 expression than CXCR5- Th1 effector cells (Figure S1C). This finding was consistent with a previous report that showed Bcl6-dependent downregulation of Psgl1 expression on Thf cells (Poholek et al., 2010). Another recent study also using the LCMV infection model used the markers Psgl1 and Ly6c in combination to identify effector and memory subsets of LCMV-specific CD4+ T cells (Marsh et al., 2011). Their study described the Psgl1hiLy6c+ (Tbet+) effector subset as Thf cells, the Psgl1hiLy6clo (Tbet-) effector subset as Th1 cells, and the Psgl1loLy6c+ effector cells as less terminally differentiated Th1 cells (Marsh et al., 2011). However, we observed that the Psgl1+Ly6c+ population did not account for all CXCR5+ Thf effector cells, and the Psgl1+hiLy6c+ effector population was composed of a similar proportion of both CXCR5+ Thf and CXCR5- Th1 effector cells (Figures S1D and S1E). Thus, Psgl1 used in combination with Ly6c does not clearly distinguish between Thf and Th1 lineage effector cells (Figures S1D and S1E). Furthermore, unlike at effector time points, the Psgl1+hiLy6c+ memory subset had higher CXCR5 expression than the Psgl1+loLy6c+ memory subset (Figures S1D and S1E), indicating that the combination of Psgl1 and Ly6c markers is not useful for subsetting cells with the most Thf-like qualities at both effector and memory time points.

Interestingly, whereas PD-1, ICOS, GL-7, and Bcl6 expression were absent on virus-specific memory cells (day 105), approximately 40% of SMARTA cells maintained CXCR5 expression, albeit at decreased surface expression relative to effector Thf cells (Figures 1B and 1C), a pattern which was also observed in endogenous GP66–77 tetramer+ memory cells (Figure S1B). Antigen-specific CXCR5+ memory cells were also observed in the blood but had reduced Bcl6 expression relative to their effector counterparts (Figures 1D and 1E). CXCR5+ effector cells were abundant in blood and secondary lymphoid organs; however, unlike CXCR5- Thf effector cells, CXCR5+ cells were almost entirely excluded from nonlymphoid tissues such as lung, liver, and IEL (Figure 1F). As expected, the total number of memory SMARTA CD4+ T cells in each tissue was reduced compared to the effector stage (Table S1), and coincided with a similar pattern in the tissue distribution of CXCR5+ and CXCR5- cells at the memory time point (Figure 1F). Thus, antigen-specific CXCR5+ cells are mostly found in lymphoid organs and excluded from nonlymphoid tissues at both effector and memory time points.

At memory time points, CXCR5+ memory cells fall into two subsets: Ly6c+ and Ly6c-. Following the contraction phase of antigen-specific Th1 and Thf effector cells, the CXCR5- Ly6chi, CXCR5+Ly6clo, and CXCR5+Ly6c+ CD4+ SMARTA subsets are maintained into the memory phase in stable numbers (Figure 1G). Together, these data show that distinct effector T helper subsets (Th1 and Thf) develop from a clonal population of virus-specific cells and suggest that these populations are maintained as distinct lineages within the heterogeneous memory pool.
Transcriptional Profiling Suggests Lineage Relationships between Tfh Effector and Memory Cells and between Th1 Effector and Memory Cells

To examine potential relationships between effector and memory cells of the Th and Th1 cell lineages, we performed gene expression profiling of sorted CXCR5+Ly6c^hi, CXCR5+Ly6c^int, and CXCR5+Ly6c^lo SMARTA subsets at effector and memory time points. These analyses revealed many genes that were commonly regulated between the Th1 effector and CXCR5+Ly6c^hi Th1-like memory cells and that distinguished them from Th1 effector and CXCR5+ Tfh-like memory cells (Figure 2A). Similarly, many genes that were expressed in Th1 effector cells were also expressed in the CXCR5+Ly6c^lo and CXCR5+Ly6c^int memory subsets, whereas expression of these same genes was downregulated in Th1 effector and memory cells (Figure 2A). We performed gene set enrichment analysis (GSEA) of the microarray data sets to further evaluate the degree that gene expression patterns were shared between these various subsets. These analyses revealed that gene expression patterns by CXCR5+Ly6c^hi Th1 memory cells were enriched in the effector Th1 cell expression profile (Enrichment Score: 0.78) (Figure S2A), whereas the upregulated gene set in CXCR5+Ly6c^lo memory cells was enriched in the Th1 effector profile (Enrichment score: 0.70) (Figure S2B).

Expression analysis of specific genes by CXCR5+Ly6c^hi memory cells revealed a similar pattern as the CXCR5+Ly6c^lo Th1 effector subset, showing higher levels of B7x21 (T-bet), Pdmd1 (Blimp-1), and Ly6c and no Cxcr5 expression compared to the CXCR5+Ly6c^lo memory subset (Figures 2B and 2C). In contrast, CXCR5+Ly6c^lo memory cells maintained Cxcr5 expression and intermediate levels of T-bet gene expression and did not express Pdmd1 (Blimp-1), a pattern similar to CXCR5+Ly6c^lo Th1 effector cells (Figures 2B and 2C). Furthermore, Bcl6 transcript levels remained slightly upregulated in the CXCR5+Ly6c^lo memory cells (relative to naive cells). Similar patterns of Bcl6 and Pdmd1 (Blimp-1) gene expression were confirmed by real-time PCR (Figure S2C). Together, these data indicate that the gene expression patterns of Cxcr5 and these transcription factors that define the Th1 and Tfh lineages in CD4^+ T cells are maintained (although at lower expression amounts compared to effector cells) in subsets of resting memory cells. In addition, we found that gene expression of the transcription factor Plagl1 (Abdollahi, 2007), a gene with no previously reported role in CD4^+ T cell differentiation, distinguished between the Th1 and Tfh cell populations at both the effector and memory phases of the immune response (Figure 2C).

We next examined the expression of genes that encode cytokine and chemokine receptors, costimulatory and inhibitory receptors, cytokines, and cytotoxic molecules. Expression of Th1-associated genes including Il-2ra, Ilng, Cc25, and Gzmb were even more highly expressed in the CXCR5^+Ly6c^lo compared to the CXCR5^+Ly6c^int and CXCR5^+Ly6c^lo memory populations (Figures 2D and 2E). In contrast, genes involved in Th function and differentiation (Crotty, 2011), including Cxcr5, Cxcr4, Il6ra, Pdcd1 (PD-1), Cd200, and Sh2d1a (SAP), were more highly expressed in memory CXCR5^+Ly6c^lo compared with memory CXCR5^+Ly6c^int cells (Figure 2B and 2D–2F; Figure S2D). Of note, several genes related to the cytotoxic potential of effector cells, including those that code for granzyme B and other granzymes, perforin, and Fas ligand were preferentially increased in the CXCR5^+Ly6c^lo SMARTA effector cells (Figure 2E). Interestingly, the CXCR5^+Ly6c^lo memory population in many cases displayed a gene expression pattern that was intermediate between that of the CXCR5^+Ly6c^lo and CXCR5^+Ly6c^lo memory populations (Cxcr5, Bcl6, Pdmd1, B7x21, Pdmg1, Ccr6, Ccl5, Prf1, and Ctd2a), suggesting more heterogeneity and/or lineage pluripotency within this population (Figures 2B–2F). Collectively, these data show that memory CXCR5^+Ly6c^lo and CXCR5^+Ly6c^lo CD4^+ T cell populations are enriched for Th1 and Tfh associated gene expression programs, respectively, and may thus be poised to preferentially recall distinct T helper effector responses upon antigen re-exposure.

Th1 and Tfh Memory CD4^+ T Cells Are Committed for Recall of Lineage-Specific Functions

To determine whether the generation of secondary Th1 versus Th1 effector CD4^+ T cells arise from these distinct memory populations, we sorted memory SMARTA cells into CXCR5^+Ly6c^hi, CXCR5^+Ly6c^lo, and CXCR5^+Ly6c^int subsets and transferred them into naive recipient mice, then challenged these mice with LCMV Armstrong (acute infection) (Figure 3A). The expansion of the CXCR5^+Ly6c^lo and CXCR5^+Ly6c^lo responding memory populations was relatively similar in the spleen 7 days postinfection (between 62- and 69-fold, assuming a 10% take in the spleen following adoptive transfer), whereas the CXCR5^+Ly6c^lo subset expanded more extensively (approximately 248-fold) (Figure 3B). Interestingly, CXCR5^+Ly6c^lo and CXCR5^+Ly6c^lo memory SMARTA cells preferentially recalled a Tfh phenotype following rechallenge, becoming mostly CXCR5^+ effector cells, whereas the majority of secondary effectors derived from the transferred CXCR5^+Ly6c^lo memory cells remained Ly6c^lo and CXCR5^− (Figure 3C). Furthermore, both the CXCR5^+Ly6c^lo and CXCR5^+Ly6c^lo memory cells gave rise to significantly increased frequencies of CXCR5^+ ICOS^hi Tfh effector cells and contained significantly more GL-7^+ germinal center Tfh cells relative to those derived from CXCR5^+ Ly6c^lo memory cells.
memory cells (Figures 3D and 3E; Figure S3A). Secondary effector cells derived from the CXCR5^Ly6c^lo and CXCR5^Ly6c^int memory populations displayed significantly higher Bcl6^+ and lower T-bet expression relative to those derived from CXCR5^Ly6c^hi memory CD4^+ T cells (Figures 3F and 3G; Figure S3D). Importantly, the transfer of CXCR5^memory

Figure 3. Th1 and Tfh Memory CD4^+ T Cells Are Committed for Recall of Lineage-Specific Functions

CD45.1 congenically marked CXCR5^Ly6c^hi (blue), CXCR5^Ly6c^lo (red), and CXCR5^Ly6c^int (green) memory CD4^+ subsets (between days 56 and 101 post-infection) were FACS purified to >97%. We adoptively transferred 8 x 10^3 sorted cells into naive CD45.2 recipients, and recipient mice were then infected with LCMV Armstrong 16–20 hr later. In (C)–(I), the phenotype and function of transferred SMARTA cells were analyzed 7 days postinfection.

(A) Representative postsort analysis of memory SMARTA subsets and cartoon of experimental setup.

(B) Absolute number of transferred CD4^+ CD45.1^ SMARTA splenocytes 7 days following rechallenge with LCMV. The relative fold increase of each population, assuming a 10% take of transferred SMARTA cells, is shown above each bar.

(C) Representative CXCR5 and Ly6c analysis.

(D) Chart shows the percent of transferred SMARTA cells that are CXCR5^ICOS^+ Tfh cells.

(E) The percent of SMARTA cells that are CXCR5^GL-7^+ germinal center Tfh cells.

(F) Bcl6 MFI of SMARTA cells.

(G) T-bet MFI of SMARTA cells.

(H) Chart shows the percent of B220^+ gated splenic B cells that are Fas^+PNA^+ germinal center B cells. Data were combined from two experiments.

(I) Chart shows the percent of transferred SMARTA cells that are CXCR5^GzmB^hi cells. Data in (B), (D), (E), and (I) are combined from four independent experiments for a total of n = 11–14 mice per group. Data in (F) and (G) were from a single experiment (n = 3 per group) and were representative of two to four independent experiments. Statistically significant p values are shown and were determined using a two-tailed unpaired Student’s t test. Error bars represent the SEM. See also Figure S3.
SMARTA cells promoted the rapid appearance (within 7 days postinfection) of Fas+PNA+ germinal center B cells compared to the transfer of CXCR5+Ly6cint memory cells (Figure 3H). Together, these data indicate that CXCR5+ memory cells are biased toward a Tfh cell recall response.

To further define the characteristics of CXCR5+Ly6cint, CXCR5+Ly6cint, and CXCR5+Ly6cint memory SMARTA cells, we evaluated their capacity to generate a Th1 cell secondary response. Effector cells derived from CXCR5+Ly6cint memory cells had significantly increased levels of T-bet expression (Figure 3G; Figure S3D). In addition, the majority of these cells was CXCR5+ and had an enhanced capacity for granzyme B expression in both the spleen (Figure 3I; Figure S3A) and in lung (Figure S3G). In contrast, although the majority of effector cells generated from CXCR5+Ly6cint and CXCR5+Ly6cint subsets were CXCR5+, those that became CXCR5+ cells had a diminished capacity to express granzyme B (Figure 3I; Figure S3A), even in the lung, a nonlymphoid tissue that enriches for Th1 effector cells (Figure S3G). Thus, CXCR5+Ly6cint Th1 memory cells efficiently produce Th1 effector cells following antigen challenge, whereas CXCR5+Ly6cint and CXCR5+Ly6cint Tfh memory cells have a cell-intrinsic restriction that limits their capacity to express granzyme B. In addition, significantly fewer effector cells derived from CXCR5+Ly6cint and CXCR5+Ly6cint Th1 subset (compared with those from CXCR5+Ly6cint memory cells) produced IFN-γ and had lower expression of this cytokine, being more consistent with the IFN-γ expression levels of Th1 cells (Figure S3H).

To determine whether the CXCR5+Ly6cint, CXCR5+Ly6cint, and CXCR5+Ly6cint memory subsets are committed to their respective Th1 and Tfh cell lineages, we compared their responses to the primary effector response generated from uncommitted (naive) SMARTA CD4+ T cells. CXCR5+Ly6cint memory cells exhibited Th1 lineage commitment, producing a significantly higher frequency of granzyme B+ and IFN-γ+ effector cells, whereas CXCR5+Ly6cint and CXCR5+Ly6cint memory cells generated a significantly higher frequency of Th1 cells and fewer Th1-like cells compared to the primary effector response (Figures S3B, S3F, and S3H). We thus conclude that the CXCR5+Ly6cint subset are Th1 memory cells, and that both CXCR5+Ly6cint and CXCR5+Ly6cint subsets are Tfh memory cells.

To determine whether Th1 and Tfh memory cells maintain their respective lineage biases in the absence of antigen, sorted Th1 memory and Tfh memory SMARTA cells were adaptively transferred into naive recipient mice and rested for 28 days before rechallenging these recipients with acute LCMV infection (Figure 4A). Consistent with our previous results (Figure 3), CXCR5+ Th1 memory cells preferentially generated Tfh effector cells (Figure 4B) and GL7+ GC Tfh cells (Figure 4C). Furthermore, these effector cells expressed significantly more Bcl6 and less T-bet (Figures 4D and 4E). The CXCR5+ effector cells that were generated from the CXCR5+ Th1 memory population also had significantly higher ICOS expression (Figure 4F) and exhibited an impairment for granzyme B expression compared with those generated from CXCR5+Th1 memory cells (Figures 4G and 4H). After challenge, memory Th1 cells generated Th1 effector cells with high T-bet and granzyme B expression (Figure 4). Thus, the capacity of CXCR5+Th1 and CXCR5+Tfh memory cells to recall their lineage-specific responses is not dependent on the continued presence of antigen during the maintenance phase of memory differentiation.

**Cells with a Central Memory Phenotype Exist within the Tfh and Th1 Memory Populations**

We next evaluated whether a central memory phenotype (by CD62L and CCR7 expression) was associated with CXCR5 expression by memory cells. As expected, within 5 days post-LCMV infection, SMARTA cells had downregulated CD62L, followed by the gradual re-expression by memory cells (Figure S4A). Compared to day 8 effector cells that were almost entirely CD62L+ and had downregulated CCR7, all memory SMARTA cells expressed some surface CD62L and CCR7, being either low or intermediate for each of these surface markers (Figure S4B). Further analysis showed that cells expressing high levels of surface CD62L and CCR7 existed within both CXCR5+ and CXCR5− memory populations (Figure S4C). Thus, cells of a central memory phenotype exist within both Th1 and Tfh memory populations (Figure S4C). To determine whether CD62L+ compared to CD62L− Th1 and Tfh memory cells exhibited different T helper lineage recall potential following rechallenge, we sorted CD62L− and CD62L+ subsets of CXCR5+ and CXCR5− memory cells, transferred them into congenic recipient mice, and challenged these mice with LCMV (Figure S4D). Both CD62L+ and CD62L− Th1 memory cells preferentially generated CXCR5− Th1 effector cells to similar degrees, whereas CXCR5+CD62L+ and CD62L− memory cells generated substantial Tfh effector cells (Figure S4E). These data suggest that whereas CD62L− central memory phenotype cells exist within both Th1 and Tfh memory populations, the CXCR5− Th1 memory and CXCR5− Tfh memory cells maintain and recall their relative lineage-associated phenotypes regardless of CD62L expression.

**Tfh Memory CD4+ T Cells Recall a Tfh-like Response Even in B Cell Deficient Recipient Mice**

Interaction with cognate B cells and continued ICOS signaling are required for stabilizing the Tfh phenotype and the maintenance of primary effector Tfh cells (Crotty, 2011). We next evaluated whether memory Tfh cells could recall aspects of the Tfh effector program when reactivated in the absence of B cells by transferring them into B cell deficient recipients prior to LCMV infection (Figure 5A). As expected, GL-7+ (germinal center phenotype) Tfh-like effector cells were nearly undetectable in B cell deficient recipient mice following infection (Figure S5). Similar to what has been previously reported (Johnston et al., 2009), naive SMARTA cells transferred into B cell deficient mice generated a significantly reduced frequency of CXCR5+ Tfh-like effector cells at day 7 postinfection, which continued to decrease to less than 10 percent by day 10 (Figures 5B and 5C). In contrast, approximately 30 percent of effectors derived from CXCR5+Ly6cint and CXCR5+Ly6cint memory cells were CXCR5+ Tfh-like cells at day 7, and surprisingly this frequency was maintained at approximately 35%–45% at day 10 in the absence of B cells (Figures 5B and 5C). Thus, compared to naive CD4+ T cells, CXCR5+Ly6cint and CXCR5+Ly6cint memory cells have a cell-intrinsic capacity to recall and maintain aspects of the Tfh phenotype such as CXCR5 expression, even in the absence of B cells. These results clearly demonstrate...
the persistence of memory CD4+ T cells with commitment to the Tfh lineage.

Epigenetic Modifications of the Granzyme B Locus Distinguish Tfh Memory from Th1 Memory CD4+ T Cells

We have shown that granzyme B expression is strikingly different between Th1 and Tfh cells at an effector time point (Figures 1 and 2; Figure S3), and is most efficiently re-expressed by CXCR5+/Ly6ch Th1 memory cells following viral rechallenge (Figure 3; Figure S3). We next tested whether the difference in granzyme B expression occurs very early in CD4+ lineage differentiation following activation of naive CD4+ T cells. Rapidly following activation, naive SMARTA cells had diverged into distinct granzyme B+ and granzyme B− populations, even in undivided cells, that persisted as separate populations while undergoing proliferation during the first 4 days of LCMV infection (Figure 6A). Granzyme B expression correlated with high T-bet expression as early as day 2 postinfection (Figure 6B), demonstrating that very early following activation (within only a few rounds of cell division), high granzyme B expression is restricted to Th1 cells. Day 5 CXCR5− Th1 cells expressed high levels of granzyme B, while expression was mostly diminished by day 9, and absent in CXCR5+/SMARTA memory cells (Figure 6C; Figure 1B).

The bimodal distribution of granzyme B expression during effector CD4+ T cell differentiation, in conjunction with our observation that granzyme B transcript is significantly upregulated only in CD4+ Th1 effector cells, suggests that CD4+ Tfh cells retain a transcriptional regulatory mechanism for repressing granzyme B expression. Epigenetic modifications serve as a mechanism for a dividing cell population to “remember” the transcriptional status of the parental cell population (Reik, 1996).
To determine whether the restriction of granzyme B expression is coupled to repressive epigenetic modifications in effector and memory Tfh cells, we analyzed the DNA methylation status in the Gzmb transcriptional regulatory region in naive, Th1 and Tfh effector, and Th1 and Tfh memory cells following LCMV infection. We found that the Gzmb locus becomes unmethylated exclusively in Th1 effector cells and remains unmethylated in Th1 memory cells, whereas CD4+ Tfh cells retain the naive DNA methylation program at the Gzmb locus throughout effector and memory differentiation (Figure 6D). Thus, our data show that methylation of the Gzmb locus can be used to distinguish CD4+ Th1 cells from Tfh cells and further confirm the lineage relationship between effector and memory Tfh cells. Furthermore, the repression of cytotoxic molecule expression such as granzyme B and perforin (Figure 2E) in Tfh cells may be essential for preventing the unwanted destruction of antigen-presenting B cells.

We also examined the methylation status of several other loci in Th1 and Tfh lineage cells. Surprisingly, the Il21 locus was demethylated in both Tfh and Th1 cells and remained unmethylated in both memory populations (Figure 6E). Although IL-21 is characterized as a cytokine critical for Tfh function, Th1 cells can also express it, albeit with reduced transcript levels (Figure 2) (Fahey et al., 2011; Spolski and Leonard, 2010). Similarly, the Ifng locus became demethylated in both Tfh and Th1 cells and remained unmethylated in memory cells (Figure 6E). These data suggest that both Th1 and Tfh memory CD4+ T cells transitioned through an effector stage of differentiation, and that the Ifng and Il21 loci may be similarly poised for potential
rapid reexpression in both subsets of memory cells following antigen reencounter. The *Pdcd1* (PD-1) locus was demethylated following infection in both Tfh and Th1 cells, although there may be slightly more methylation in Th1 compared to Tfh memory cells (Figure 6E).

**DISCUSSION**

There is considerable interest in understanding the generation of CD4+ Tfh memory cells. However, this topic remains controversial because several recent studies have reported different conclusions regarding the existence of a committed population of memory Tfh cells (Liu et al., 2012; Lüthje et al., 2012; MacLeod et al., 2011; Marshall et al., 2011; Pepper et al., 2011; Weber et al., 2012). In this study, by using the mouse model of acute LCMV infection, we show that there are distinct populations of virus-specific memory CD4+ T cells with commitment to either the Tfh or Th1 cell lineages. Our findings indicate that antigen-specific memory CD4+ T cells that maintain CXCR5 expression are biased toward the recall of a Tfh effector response, whereas CXCR5+Ly6Ch memory cells efficiently recall Th1 effector cells. These findings are consistent with a model in which the concomitant generation of a pool of resting memory Th1 or Tfh cells arise from the corresponding effector Th1 and Tfh cells, respectively. Commitment of memory CD4+ T cells to Th1 or Tfh lineages provides cells that are poised for the lineage-specific reexpression of effector molecules upon reexposure to antigen (see model in Figure S6).

In contrast to our findings, by using a *Listeria monocytogenes* infection model, Pepper et al. suggested that memory CD4+ T cells that express CXCR5 and CCR7 are a central memory population with a capacity to fully reconstitute Th1 and Tfh effector populations (Pepper et al., 2011). Whereas our study also shows that there is more pluripotency among the Tfh compared to Th1 memory population, there is still clear evidence of Tfh lineage commitment in CXCR5+ memory CD4+ T cells. The differing results seen in recall responses from CXCR5+ memory...
cells between our study and the report by Pepper et al. may result from the different infection systems used (LCMV versus *Listeria monocytogenes*). The report by Pepper et al. suggests that Tfh cells do not enter the memory pool based on the absence of CXCR5<sup>hi</sup>/PD-1<sup>hi</sup> cells at memory time points (Pepper et al., 2011). In our study, LCMV-specific CD4<sup>+</sup> T cells (both SMARTA and endogenous Gp66–77 tetramer<sup>+</sup>) at memory time points show decreased CXCR5 and relatively absent PD-1, ICOS, and Bcl6 expression levels by FACS staining. We interpret this observation (the lack of CXCR5<sup>PD-1</sup> memory cells) to signify that activation molecules such as PD-1 (and to some extent CXCR5) are downregulated on memory Tfh cells following antigen clearance, rather than indicate the disappearance of Tfh lineage cells altogether from cells entering the memory pool.

A recent study by Lüthje et al. using an *Il21* GFP reporter system showed that both GFP<sup>+</sup> and GFP<sup>−</sup> subsets of CXCR5<sup>+</sup> Tfh cells efficiently enter the memory pool of CD4<sup>+</sup> T cells following adoptive transfer. In contrast with our observations, they observed a higher frequency of CXCR5<sup>+</sup> effectors generated from Tfh-derived memory cells following influenza rechallenge (Lüthje et al., 2012). Clear interpretation of their study is limited by the lack of analysis of antigen-specific cells at effector versus memory and recall time points (either by MHC tetramer staining or by using TCR transgenic cells). Their study also showed that the majority of GFP<sup>+</sup> (*Il21<sup>+</sup>*) effector cells were CXCR5<sup>+</sup> Tfh effector cells (Lüthje et al., 2012). Our data show that both Tfh and Th1 effector cells express *Il21* following LCMV infection and that the *Il21* locus becomes demethylated in both Tfh and Th1 effector subsets. Therefore, IL-21 expression in itself is not a clear universal indicator of Tfh helper lineage. While our data indicate that some CXCR5<sup>Ly6c<sup>lo</sup></sup> and CXCR5<sup>Ly6c<sup>int</sup></sup> memory cells become CXCR5 negative following reactivation, we observed that such CXCR5<sup>−</sup> (Th1-like) effector cells do not express granzyme B to the same extent as secondary effector cells derived from Th1 memory cells. Our findings suggest that although there is some degree of heterogeneity and/or plasticity within the Tfh (CXCR5<sup>Ly6c<sup>lo</sup></sup> and CXCR5<sup>Ly6c<sup>int</sup></sup>) memory population, these cells do not yield secondary effector cells with the same capacity for Th1 cell function as memory Th1 (CXCR5<sup>Ly6c<sup>hi</sup></sup>) cells.

Several observations within our study clearly show that memory CD4<sup>+</sup> T cells with commitment to the Tfh lineage exist within the memory pool following acute LCMV infection. First, the Tfh and Th1 cell lineage dichotomy that is established very early following LCMV infection based on both CXCR5 surface expression and the restriction of the capacity for granzyme B expression can be tracked into the memory pool. Indeed, similar to their effector precursors that do not express granzyme B, CXCR5<sup>+</sup> memory cells carry repressive DNA methylation marks at the Gzmb locus, and the CXCR5<sup>−</sup> minority cell population that emerges from the CXCR5<sup>+</sup> memory cells following reexposure to antigen shows a delayed or impaired ability to express granzyme B. It has been shown that maintenance of repressive DNA methylation programs at the Il4 and Foxp3 loci are essential for preventing aberrant expression of these molecules in non-Th2 and non-Treg CD4<sup>+</sup> T cell lineages, respectively (Josefowicz et al., 2009; Makar et al., 2003). Thus, the maintenance of DNA methylation at the Gzmb locus (and other Th1-specific loci) in Tfh memory cells may reinforce their Tfh lineage commitment by repressing genes used by Th1 lineage cells (Wilson et al., 2009). Therefore, differential DNA methylation at the Gzmb locus can be used as an additional criterion for distinguishing CD4<sup>+</sup> Tfh memory from Th1 memory cells. Second, compared to the primary response generated from uncommitted naive CD4<sup>+</sup> T cells of the identical TCR specificity (SMARTA transgenic), CXCR5<sup>+</sup> Tfh memory cells are more biased toward generating Tfh cells. Third, transfer experiments to B cell deficient mice demonstrate that a large fraction of memory CXCR5<sup>+</sup> Tfh cells have acquired cell-intrinsic programs that allow them to recapitulate portions of the Tfh phenotype (including CXCR5 expression) following reactivation in the absence of bidirectional signals from B cells that are required for the generation and maintenance of primary effector Tfh cells.

In agreement with our findings, several recent reports provide evidence supporting the existence of memory Tfh cells. Marshall et al. observed that a subset of memory cells with similar phenotypic features as Tfh cells existed within the memory cell pool (Marshall et al., 2011). MacLeod et al. demonstrated that CXCR5<sup>+</sup> memory cells enhance the kinetics of B cell expansion and class switching, and suggested that such CXCR5 expression on memory cells promotes migration to areas where encounter with cognate B cells can occur following reintroduction of antigen (MacLeod et al., 2011). Similarly, findings by Liu et al. suggest that effector Tfh cells enter the memory T cell pool and retain their preference for recall of Tfh cells; however, only very early memory time points were examined (Liu et al., 2012). Another study suggested that rechallenge of memory cells derived from adoptively transferred Tfh effector cells exhibited a Tfh phenotype 2.5 days after reimmunization; however, this phenotype was diminished 6 days postreactivation (Weber et al., 2012). Morita et al. reported that CXCR5<sup>+</sup>CD4<sup>+</sup> T cells found in human blood are functional counterparts to Tfh cells found in lymphoid organs and suggest that these may be memory Tfh cells (Morita et al., 2011).

The repression of cytotoxic potential appears to be a hallmark of Tfh cells, and expression of key cytolytic molecules such as granzyme B and perforin was inhibited in Tfh but not Th1 effector cells. Together, these data suggest that Tfh differentiation is coupled to the restriction in cytotoxic potential by preventing expression of Th1 cell-related killing apparatus components. The epigenetic repression of cytotoxic molecules by Tfh cells may be essential to prevent the unwanted destruction of anti-CD4<sup>+</sup> Tfh cells for essential signals (Cannons et al., 2010; Crotty et al., 2003, Crotty, 2011; Qi et al., 2008).

Our findings demonstrate that acute LCMV infection induces a strikingly balanced response of both Tfh and Th1 effector CD4<sup>+</sup> T cells and robust long-lived memory populations for both of these lineages. This raises the question as to whether such Tfh and Th1 balance and memory generation is achieved by other viral infections, vaccine vectors, and protein immunization strategies. In addition, balancing the generation of Tfh memory and other lineages of CD4<sup>+</sup> memory T cells may be essential for providing secondary responses that provide both optimal CD4<sup>+</sup> T cell help for antibody responses, as well as CD4<sup>+</sup> T cells with effector function. Thus, future studies are needed to determine how different adjuvants, inflammatory cytokines,
costimulatory molecules, and interactions with different types of antigen-presenting cells can be used to specifically induce either Tfh or Th1 memory cells.

The majority of our successful vaccines rely on neutralizing antibody and long-lived humoral responses for protective immunity (Plotkin et al., 2013). Gaining a better understanding of the development, function, and contribution of Tfh memory cells within the context of prime and boost vaccination and pathogen challenge will provide avenues for rational vaccine design (D’Argenio and Wilson, 2010). Developing effective vaccines for pathogens such as HIV and malaria will likely require the generation of high-titer broadly neutralizing antibody responses for pathogens such as influenza vaccines by targeting conserved epitopes (Sette and Rappuoli, 2010) may possibly be improved by optimally inducing memory Tfh cells. It is likely that vaccine strategies that fail to induce the generation of primary Tfh cells will subsequently leave a gap in the pool of antigen-specific memory CD4+ T cells, resulting in suboptimal boosting of antibody responses. Conversely, immunogens, adjuvants, or strategies that promote robust Tfh responses and drive commitment and maintenance of high numbers of antigen-specific memory Tfh cells may enhance the quality and/or quantity of antibody production following antigen boost or pathogen encounter. Thus, it is critical to design rational prime and boost strategies for optimal generation of Tfh memory cells.

EXPERIMENTAL PROCEDURES

Mice and Adoptive Transfers

Congeneically marked (CD45.1) CD4+ T cell splenocytes specific to the GP66–77 epitope of LCMV obtained from naive SMARTA TCR transgenic mice (Oxenius et al., 1998) were intravenously transferred into naive C57BL/6 (CD45.2) mice (Jackson Laboratory, Bell Harbor, ME). In order to generate SMARTA chimeric mice with a high number of memory SMARTA cells, thus facilitating memory cell isolation for adoptive transfer studies, we transfected 2 × 10^6 naive SMARTA CD4+ T cells. Approximately 24 hr post-transfer, chimeric mice were infected intraperitoneally (i.p.) with 2 × 10^5 PFU of LCMV Armstrong. For adoptive transfer of memory SMARTA cells, CD4+ splenocytes from chimeric mice (day 56–101 postinfection) were enriched by using a MACS CD4+ T cell Isolation Kit II (Miltenyi). Enriched CD4+ T cells were stained and sorted to isolate CD45.1+ SMARTA cells to greater than 98% purity. For rechallenge experiments, 8 × 10^5 sorted memory (or naive) SMARTA cells were adoptively transferred into naive C57BL/6 mice (CD45.2) or B cell-deficient μMT mice that were subsequently infected 24 hr later with 2 × 10^5 PFU of LCMV Armstrong. Animal experiments were conducted in accordance with Emory University IACUC protocols.

FACS Analysis and Sorting

Cells were stained as described previously (Youngblood et al., 2011) with fluorochrome-conjugated antibodies (purchased from BD, eBiosciences, BioLegend, Vector Laboratories, and Invitrogen). A three-step CXC5R1 staining was performed as described by Johnston et al. (2009) by using purified rat anti-mouse CXCR5 (BD), a secondary Biox-Sp-conjugated AffiniPure F(Ab')2, Goat anti-rat IgG (Jackson ImmunoResearch) and finally with streptavidin-APC or streptavidin-PECy7 (Invitrogen). For Bcl8 and T-bet staining, cells were first stained for surface antigens, followed by permeabilization, fixation, and staining by using the FlowP3 Permeabilization/Fixation Kit and protocol (eBioscience). Intracellular cytokine staining was done by standard techniques following 5 hr stimulation with GP66–77 peptide (Murali-Krishna et al., 1998). Cell sorting was performed by using a FACS Aria II (BD), and flow cytometry data were collected on a FACS Canto II (BD). FACS data were analyzed by using FlowJo software (TreeStar).

Microscopy

Spleens were frozen in OCT compound (Fisher Scientific, Waltham, MA) and sectioned. Sections were fixed in cold acetone for 10 min, dried, and stained with the indicated antibodies. Images were acquired using a Zeiss Axioskop 2 Plus microscope with 10×/0.25 and 20×/0.50 (magnification/aperature) objectives and a Zeiss AxioCam MRc5 camera. Image overlays were performed with ImageJ software (National Institutes of Health [NIH], Bethesda, MD).

RNA Isolation and Microarray Analysis

RNA from sorted cells was purified (Qiagen), linearly amplified (NuGEN), and hybridized to Affymetrix mouse 430 2.0 arrays (Memorial Sloan-Kettering Cancer Center, Genomics Core Facility). Gene pattern 3.4 and the associated modules were used to analyze the microarray data. Gene set enrichment analysis (GSEA) was performed as described previously (Subramanian et al., 2005).

Genomic DNA Methylation Analysis

Bisulfite modification of genomic DNA from FACS purified cells was performed using the Zymo Research EZ DNA methylation kit. Bisulfite modified DNA was PCR amplified with locus-specific primers (Table S2) as previously described (Youngblood et al., 2010).

Statistical Analysis

All experiments were analyzed using Prism 4. Statistically significant p values of <0.05 are indicated and were determined by using a two-tailed unpaired Student’s t test.

ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE43863.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.02.020.

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