Memory CD4 T Cells That Express CXCR5 Provide Accelerated Help to B Cells

Megan K. L. MacLeod, Alexandria David, Amy S. McKee, Frances Crawford, John W. Kappler and Philippa Marrack

J Immunol 2011; 186:2889-2896; Prepublished online 26 January 2011;
doi: 10.4049/jimmunol.1002955
http://www.jimmunol.org/content/186/5/2889

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/01/26/jimmunol.1002955.DC1

References
This article cites 46 articles, 19 of which you can access for free at:
http://www.jimmunol.org/content/186/5/2889.full#ref-list-1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2011 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
CD4 T cell help for B cells is critical for effective Ab responses. Although many of the molecules involved in helper functions of naive CD4 T cells have been characterized, much less is known about the helper capabilities of memory CD4 T cells, an important consideration for the design of vaccines that aim to prime protective memory CD4 T cells. In this study, we demonstrate that memory CD4 T cells enable B cells to expand more rapidly and class switch earlier than do primary responding CD4 T cells. This accelerated response does not require large numbers of memory cells, and similar numbers of primary responding cells provide less effective help than do memory cells. However, only memory CD4 T cells that express the B cell follicle homing molecule, CXCR5, are able to accelerate the response, suggesting that the rapidity of the Ab response depends on the ability of CD4 memory T cells to migrate quickly toward B cells. The Journal of Immunology, 2011, 186: 2889–2896.

The basis for immunological memory is that Ag-experienced lymphocytes respond better than their naive counterparts. Although this phenomenon is widely accepted, there is still a paucity of understanding of the mechanisms involved in enhanced memory responses. Memory cells are generated following the initial primary response in which Ag-specific cells first proliferate, differentiate, and then most, but not all, of these cells undergo apoptosis (1). The surviving memory cells differ from naive cells in two main ways. First, there are more Ag-specific cells in the memory as compared with the naive pool (2–4). Second, memory cells can make an effector response more rapidly after stimulation (1, 5). Which of these two factors is important for the improved responses observed upon reactivation is not clearly understood but is an important issue when considering the design of T cell-mediated vaccines.

Memory CD4 T cells could provide a protective response to pathogens by helping B cells make a more rapid Ab response (6, 7). That CD4 help is required for primary responding B cells to form germinal centers and produce high-affinity class-switched Ab is well established (8). These signals are supplied via cell surface molecules such as CD40L and ICOS, and by means of soluble molecules such as the cytokines IL-4 and IL-21 (9). The Abs generated by such a response protect the host against invading microorganisms by, for example, neutralizing the invader or improving uptake by phagocytic cells (10). The faster the Ab response, the more quickly the invading organisms can be controlled. Therefore, the rapid generation of class-switched Ab is an important consideration for vaccine design (6, 7).

There is some evidence that CD4 memory T cells can provide accelerated help for Ab responses (11–14); however, this may be limited to B cell responses directed toward haptens rather than more relevant Ags (15). More importantly, there is currently no mechanistic information to explain how CD4 memory T cells could provide an enhanced helper response.

T follicular helper (Tfh) cells, the CD4 T cells that provide help to B cells, are defined by their expression of the transcription factor, Bcl-6, and the cell surface markers ICOS, CXCR5, and programmed cell death 1 (PD-1) (16–20). Tfh memory cells have been defined in humans by these markers, in particular by the expression of CXCR5, the chemokine receptor that allows cells to migrate toward the B cell area of lymphoid organs (16). Moreover, these cells are able to provide effective help to B cells in vitro (20). Likewise, CD4 memory cells with similar surface properties have been found in the lymph nodes of mice that harbor persistent Ag (21); however, an enhanced helper activity by these cells has not been demonstrated.

In this study, we compared directly the functions and effectiveness of resting endogenous memory and naive CD4 T cells to help primary responding B cells to produce class-switched Ab to a protein Ag. We found that Ag-specific memory CD4 T cells do indeed stimulate primary B cell responses better than do naive T cells. This is not just a consequence of the increase in Ag-specific cell precursor frequency in the memory as compared with the naive T cell pool. Instead, this effect is contained within a subpopulation of memory cells that express CXCR5 at high levels, suggesting that their better function is due to their ability to migrate more rapidly to B cell follicles.

Materials and Methods
Mice, immunizations, and infections
Female B6 and B6.PL-Thy1a/CyJ (Thy1.1+) mice were obtained from The Jackson Laboratory. 508 TCR transgenic (Tg) mice expressing a TCR specific for I-A\(^{b}\)/3K (22) were bred at National Jewish Health. All mice were maintained in a specific pathogen-free environment in accordance
with institutional guidelines in the Animal Care Facility at National Jewish Health. Mice were age-matched within experiments. Mice were immunized with 10 μg 3K (FEAQKAKANKAK) supplied by The Molecular Resources Center at National Jewish Health or by JPT Peptide Technologies (Berlin, Germany) and 7 μg LPS (Escherichia coli; Difco) i.v. or, after the transfer of larger numbers of TCR Tg cells, recipients were immunized with 20 μg 3K plus 10 μg LPS. For 3K or GFp66-68 OVA conjugations, 3K or GFp61 peptide (GLNPDPDYKGYVKFKSFED) were conjugated to maleimide-activated OVA as described by the manufacturer (Pierce). Peptide/protein conjugates were tumbled with alum (Alhydrogel; Brentnagl Biosector) for 2 h at room temperature, and each mouse received 1 μg peptide/protein with 0.2 mg alum i.p. Mice were infected with a single dose of 2 × 10^7 PFU LCMV (Armstrong) i.p.

**Cell transfer**

Spleens and lymph nodes (brachial, axillary, inguinal, popliteal, lumbar) were taken from naive (Thermo Fisher Scientific) were coated with OVA protein at 100 μg/ml and an unknown number of cells that are specific for the CD4 class-switched anti-OVA Ab but did not increase the peak response. The most obvious is that the 3K-memory mice contain larger numbers of memory CD4 T cells than do naive animals. Naive CD4 T cells provide less effective help than do similar class-switched anti-OVA Ab but did not increase the peak response. To test the idea that precursor frequency alone was responsible for the efficiency of B cell help in 3K-memory mice, we examined the B cell response in animals that contained an equivalent number of naive and memory Ag-specific cells. We first counted the number of 3K-specific memory cells in a cohort of 3K-memory mice and then transferred 10-fold this number of naive T cells bearing an I-A^b/3K-specific Tg TCR into naive B6 mice. The 10-fold increase was used based on the well-established finding that only ~10% of T cells survive the transfer (4, 25, 26).

Statistical analysis

Data are presented as indicated in the figure legends, and statistical significance was determined using either a Student two-tailed t test or Mann–Whitney U test with GraphPad Prism software version 4.

**Results**

**Memory CD4 T cells accelerate primary Ab responses**

We first asked whether memory CD4 T cells could enhance a primary B cell response to a protein Ag. To determine this we needed to prime CD4 T cells to an Ag that did not prime a B cell response. To achieve this, we primed C57BL/6 (B6) mice with the I-A^b-binding peptide, 3K, delivered with the TLR agonist, LPS, as an adjuvant. The 3K peptide was used because T cells specific for it can be tracked with I-A^b MHC class II tetramers containing 3K. Moreover, we wanted to use a soluble Ag and adjuvant combination so that there would be little, if any, persistent Ag. This combination clearly primed CD4 T cells that could be stained with I-A^b/3K tetramer at early and late time points (Fig. 1A).

Ten to 14 wk later, the 3K-primed mice (hereafter 3K-memory mice) and naive age-matched mice were immunized with 3K peptide that had been chemically conjugated to OVA protein (3K-OVA). In the 3K-memory mice, we would initiate a memory T cell response to the 3K peptide but a primary T and B cell response to OVA protein, whereas there would be a primary T and B cell response to both Ags in the naive mice. The peptide/protein conjugate was delivered with alum, as this adjuvant induces a strong Ab response. The anti-OVA Ab response was analyzed over time in the sera of the immunized animals, with day 0 indicating the time point at which the mice were immunized with 3K-OVA plus alum. Immunized 3K-memory mice had levels of anti-OVA IgG above those in control mice at days 6 and 8 (Fig. 1B). However, by day 15 the levels of anti-OVA Ab were the same in the two groups. Therefore, Ag-specific memory CD4 T cells accelerated the production of class-switched anti-OVA Ab but did not increase the peak response.

**Naive CD4 T cells provide less effective help than do similar numbers of memory CD4 T cells**

There are a number of explanations for this accelerated response. The most obvious is that the 3K-memory mice contain larger numbers of CD4 T cells specific for 3K than do naive animals. Naive B6 mice contain approximately seventy 3K-specific cells (4) and an unknown number of cells that are specific for the CD4 epitopes found within OVA. The 3K memory mice contain ~1–5 × 10^5 3K-specific memory cells (data not shown) and presumably the same number of OVA-specific T cells as do naive mice.

**Flow cytometry**

PE-labeled I-A^b/3K was produced as described (23). PE-labeled I-A^b/GFp66-77 tetramer (referred to as I-A^b/GP66 in the text) was supplied by the National Institutes of Health Tetramer Core Facility. Single-cell suspensions from the spleens of immunized or infected mice were stained with tetramer at 37˚C for 2 h. Abs to surface markers were added and the cells incubated for a further 20 min at 4˚C. Tetramer^+ cells were defined by gating on live CD4^+CD44^− cells that were CD8, B220, F4/80, MHC class II negative. For Bc6 staining, cells were fixed with 4% paraformaldehyde after surface staining. OVA-Alexa 488 (Invitrogen) was incubated with spleen cells for 2 h at 37˚C. These cells were washed and then incubated with 2G42 (made at National Jewish Health) for 20 min before staining with cell surface Abs. Abs were either from BD Biosciences, eBioscience, or BioLegend, and anti-Bc6 was from Santa Cruz Biotechnology and peanut agglutinin (PNA) was from Vector Laboratories. Two to 5 million events were collected on a CyAn ADP (Beckman Coulter) and data analyzed using FlowJo version 8.8 (Tree Star).

**Intracellular cytokine staining**

For analysis of cytokine production by intracellular staining, splenocytes were activated ex vivo with 10 μg/ml GP66 and 1 μg/ml of GolgiPlug (BD Biosciences) for 6 h. Cells were stained with surface Abs and then fixed and permeabilized using the BD Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer’s instructions. Ag-specific cytokine was defined as staining above the level of staining from splenocytes cultured in the absence of peptide. Cells were gated on live CD4^+ cells that were CD8, B220, and MHC class II negative.

**OVA-specific ELISAs**

ELISAs were performed as described (24). Briefly, 96-well Immulon plates were coated with OVA protein at 100 μg/ml in PBS overnight at 4˚C. Following washing, the plates were blocked with 10% FCS/PBS before serum samples were added and titrated down the plate. To determine relative units, we used a positive control serum sample and an unknown number of cells that are specific for the CD4 class-switched anti-OVA Ab but did not increase the peak response. To test the idea that precursor frequency alone was responsible for the efficiency of B cell help in 3K-memory mice, we examined the B cell response in animals that contained an equivalent number of naive and memory Ag-specific cells. We first counted the number of 3K-specific memory cells in a cohort of 3K-memory mice and then transferred 10-fold this number of naive T cells bearing an I-A^b/3K-specific Tg TCR into naive B6 mice. The 10-fold increase was used based on the well-established finding that only ~10% of T cells survive the transfer (4, 25, 26).
One day following the transfer, the TCR Tg recipients, naive nontransferred mice, and the remaining 3K-memory mice were immunized with 3K-OVA plus alum. We have previously reported that primary responding T cells undergo more rounds of proliferation than do reactivated memory cells (4). Therefore, as expected, there were more I-\(\alpha\)/3K–specific cells present in the mice that had received naive TCR Tg cells than in the memory mice 8 d after immunization (Fig. 1C). Given this greater number of T cells, it may have been expected that the Ab response would be highest in these mice. However, at early time points, the primary anti-OVA response was greatest in the 3K-memory mice (Fig. 1D). The presence of the TCR Tg cells did increase the anti-OVA response compared with the nontransferred primary responding mice at day 8 (Fig. 1D). Importantly, memory TCR Tg cells could also provide accelerated help to primary responding B cells (Supplemental Fig. 1), demonstrating that the TCR Tg memory cells could act in an equivalent fashion to endogenous memory cells. Interestingly, the peak Ab response was equivalent in all groups, suggesting that the limiting factor for the total Ab response was the number of B cells that can differentiate into plasma cells rather than any limitation on T cell help.

### OVA-specific, class-switched germinal center B cells are increased in CD4 memory mice

To examine the B cell response more closely, we counted the number of OVA-specific B cells that had class-switched in the different groups of mice after immunization. Spleen B cells were stained with fluorescently labeled OVA, anti-IgG1, and PNA to identify OVA-specific, class-switched germinal center cells (Fig. 2A). The previously naive mice that had not been given naive TCR Tg cells contained a small number of class-switched OVA-specific B cells 8 d after immunization. The mice that had received TCR Tg cells had more of such B cells. However, the greatest percentage and number (Fig. 2B) of class-switched B cells was found in the 3K-memory mice. These data suggest that the memory T cells could induce B cells to enter the germinal center response more quickly than naive T cells could. Thus, the accelerated help afforded by CD4 memory T cells is not just a matter of increased numbers of T cells; that is, CD4 memory T cells have some intrinsic property that renders them more effective at early stages in the B cell response.

#### Activated naive and memory cells upregulate markers associated with Tfh cells

Reactivated memory cells are known to differentiate into effector cells more rapidly than do primary responding cells (5). To find out whether reactivated memory cells expressed proteins associated with Tfh cells more rapidly than did naive T cells, we tracked the expression of CXCR5, ICOS, and PD-1 on the activated cells. Many of the specific cells increased expression of these markers, and the kinetics of expression were similar in either primary (endogenous or TCR Tg) or secondary responding cells (Fig. 3A–C). It was possible that there were differences in Tfh cell marker expression at a time point earlier than day 6. There were not enough primary responding endogenous I-\(\alpha\)/3K tetramer\(^+\) cells to examine their expression of Tfh cell markers before day 6. However, the expression of CXCR5 and ICOS was equivalent in primary responding TCR Tg cells and endogenous memory cells at day 4 (Fig. 3D). The secondary responding memory cells did express slightly more PD-1 than did the primary responding TCR Tg cells at day 4 (Fig. 3D). However, there seems to be little difference associated with Tfh cells more rapidly than did naive T cells, we tracked the expression of CXCR5, ICOS, and PD-1 on the activated cells. Many of the specific cells increased expression of these markers, and the kinetics of expression were similar in either primary (endogenous or TCR Tg) or secondary responding cells (Fig. 3A–C). It was possible that there were differences in Tfh cell marker expression at a time point earlier than day 6. There were not enough primary responding endogenous I-\(\alpha\)/3K tetramer\(^+\) cells to examine their expression of Tfh cell markers before day 6. However, the expression of CXCR5 and ICOS was equivalent in primary responding TCR Tg cells and endogenous memory cells at day 4 (Fig. 3D). The secondary responding memory cells did express slightly more PD-1 than did the primary responding TCR Tg cells at day 4 (Fig. 3D). However, there seems to be little requirement for PD-1–PDL1 interactions at this early time point (27), suggesting that this small difference is unlikely to contribute to the accelerated OVA-specific Ab response in mice containing the memory CD4 T cells. Taken together, these data suggested that the memory and primary responding cells differentiate into Tfh cells at a similar rate following activation.

To confirm that the activated primary and memory cells expressed higher levels of CXCR5, ICOS and PD-1 and were not just binding more of the Abs nonspecifically because they were larger cells, we compared the level of binding of these Abs to the I-\(\alpha\)/3K tetramer\(^+\) cells to that on CD44\(^+\) CD4 T cells (naive) and
CD4+CD44hi CD4 T cells (memory phenotype). Although memory phenotype cells were of a similar size to the activated I-Ab/3K tetramer+ cells, they bound less of the Tfh cell marker Abs, and naive CD4 T cells bound less of the Abs and were also of a smaller size (Supplemental Fig. 2A). Moreover, to exclude the possibility that staining with the tetramer affected the expression of the markers, we examined the level of staining on in vivo-activated TCR Tg cells identified using either I-Ab/3K tetramer or a congenic marker (Thy1.2). There were no differences in the expression level of any of the makers on TCR Tg cells identified by the two different markers (Supplemental Fig. 2B). Therefore, we conclude that the I-Ab/3K tetramer+ cells do indeed express increased levels of CXCR5, ICOS, and PD-1.

The higher expression of CXCR5 by the memory cells should position these cells close to B cell follicles. To examine the location of the memory cells, we transferred naive TCR Tg cells into congenic hosts to increase the frequency of Ag-specific cells, allowing us to find the cells by immunofluorescence. Once these cells had developed into memory cells, we examined the location of the memory cells in relationship to B cell follicles and compared the location of these cells to that of transferred naive TCR Tg cells (Fig. 3E). The memory cells were, in general, found to be in closer proximity to B cell follicles than were naive T cells (Fig. 3F). This location and the higher expression of the Tfh cell markers suggest that memory CD4 T cells are poised ready to help B cells prior to reactivation.

LCMV-specific memory cells express higher levels of Tfh cell markers than do naive T cells

The best way to test whether the increased expression of the Tfh cell markers were sufficient for the better helper activity of CD4 memory T cells would be to purify various populations of T cells,
transfer them into naive animals, and evaluate their ability to provide enhanced help. This experiment would also allow evaluation of the functions of the transferred cells in the absence of any other factors that may have been induced in previously immunized animals.

This experiment was not feasible using 3K-activated memory cells, as too few memory cells are generated to allow isolation of sufficient cells for transfer. To increase the number of available endogenous Ag-specific memory cells we used mice that had previously been infected with LCMV. In such animals ~1–2% of CD4 T cells are specific for the immunodominant LCMV peptide (3), GP31–71 (referred to as GP66). Eight weeks postinfection, we stained splenocytes with I-Ab/GP66 MHC tetramer and Abs to PD-1, ICOS, and CXCR5 and also CCR7. The memory cells expressed higher levels of all Tfh cell markers compared with naive CD4 T cells (Fig. 4A). We could not, however, detect a population of cells that expressed two or three of these markers at a high level that would be equivalent to the Tfh cells found during the primary response (19, 28–30). This is demonstrated in Fig. 4B in which the expression of ICOS or PD-1 on cells that express CXCR5 at a high or low level was examined: these two populations expressed identical levels of ICOS or PD-1. Similarly, regardless of CXCR5 expression, the cells expressed equivalent levels of CCR7.

These data suggested that the memory cells that expressed CXCR5 may not be a memory equivalent of the Tfh cells described in primary responses (19, 28–30). To investigate this further, we examined the levels of the transcription factor Bcl6 in the memory cells. Memory and activated Ag-specific cells did express higher levels of Bcl6 than did naive CD4 T cells, with the recently activated cells expressing slightly more (Fig. 4C). The highest level of Bcl6 is thought to be in germinal center B cells (31), and IgG1+ PNA+CD19+ cells from recently immunized mice did express more Bcl6 than did any of the T cell populations. When we compared expression of Bcl6 in CXCR5hi and CXCR5lo tetramer+ memory cells there were no differences between the groups as illustrated by the mean fluorescence intensity (MFI) of Bcl6 in these populations (Fig. 4D). Interestingly, in primary responding cells, there appeared to be slightly more Bcl6 in the CXCR5lo tetramer+ cells than in the CXCR5hi cells. Whether this small difference has a biological significance remains to be determined. Regardless, it is difficult to conclude whether the CXCR5hi memory cells are a Tfh memory cell equivalent.

The accelerated helper activity of the memory CD4 T cells is contained within the CXCR5lo subset

Even though we could not clearly identify a population of Tfh Ag-specific memory cells in the LCMV memory mice, we noted a broad expression level of CXCR5 on these cells. The first step that CD4 T cells must take to provide help to B cells is to migrate toward the follicle. We therefore decided to find out whether expression of CXCR5, the protein that guides T cells to follicles, was correlated with the accelerated helper activity.

This experiment could not be done with prior sorting of the Ag-specific T cells, since staining with MHC class II tetramers requires incubation with tetramer at 37°C for at least 1 h; such conditions may induce TCR signaling and/or affect the phenotype of the cells. Instead, CXCR5lo and CXCR5hi CD4+CD44+ cells were sorted from the spleens and lymph nodes of mice infected 8 wk previously with LCMV without regard to their Ag specificity. A small fraction of the sorted populations were stained with I-Ab/GP66 MHC class II tetramers to count the number of Ag-specific cells in each population (Fig. 5A). Of note, both populations bound to the tetramer at an equivalent level, suggesting that they were of similar affinities (Fig. 5B). The tetramer staining allowed us to estimate the percentage of CXCR5hi and CXCR5lo cells that were I-Ab/GP66-specific, allowing us to transfer the same numbers of Ag-specific T cells into naive hosts. Because the memory cells expressed a range of CXCR5, we were unable to separate the
CXCR5 high and low cells cleanly, instead enriching for either high or low expression (Fig. 5B).

Memory cell recipients, as well as age-matched mice that had not received any cells, were immunized with OVA that had been chemically conjugated to the GP66 peptide plus alum 1 d after transfer. As a positive control, we also immunized a cohort of the LCMV-infected mice (LCMV memory mice) that contained a greater frequency of GP66-specific cells: LCMV memory mice contained $1 \times 10^5$ I-Ab/GP66 tetramer$^+$ cells while the adoptive transfer recipients contained $\sim 100–400$ I-Ab/GP66 tetramer$^+$ cells after the transfer (data not shown).

Using the congenic marker to identify the transferred cells, we found that the number of Ag-specific transferred cells in the recipients 8 d after immunization was low but similar in mice that received CXCR5$^{hi}$ or CXCR5$^{lo}$ cells (Fig. 5C). The percentages of I-Ab/GP66 tetramer$^+$ cells out of total transferred cells increased dramatically following immunization, from 1–2% on the day of transfer to 10–30% at day 8, indicating that both populations had reactivated and responded to the immunization (Fig. 5A, SD).

When we examined the phenotype of the reactivated memory cells, a similar percentage expressed PD-1. Many of the CXCR5$^{hi}$ cells had upregulated CXCR5, and slightly more of them expressed ICOS than the CXCR5$^{hi}$-enriched cells (Fig. 5E). Transfer of the memory cells did not inhibit the host response, as similar numbers of I-Ab/GP66 tetramer$^+$ host cells were found in all the primary responding mice (Supplemental Fig. 3).

To find out whether the transferred memory subsets could provide accelerated help to the host primary responding B cells, we examined the anti-OVA Ab response 8 d after immunization. The LCMV-immunized mice made a mixed IgG1/IgG2c response that was greater than the response in primary responding animals, supporting our findings in the 3K-OVA experiments (Fig. 6). The magnitude of the Ab response was similar in mice that received CXCR5$^{hi}$ memory cells and mice that had not received any transferred T cells. In contrast, mice that received CXCR5$^{hi}$ memory cells had greater levels of OVA-specific IgG1, at levels that were similar to those in mice previously infected with LCMV (Fig. 6A). This improved response was not evident for IgG2c anti-OVA Abs (Fig. 6B), even though the CXCR5$^{hi}$ cells could make IFN-γ (Supplemental Fig. 4). Perhaps this reflects the influence of the numerically larger recipient T cell response that is primed in the type 2 environment following alum immunization.

**Discussion**

Memory responses are considered to be better than primary responses, but what is meant by “better” for CD4 memory T cells is not well defined. In this study we conclusively show that memory CD4 T cells primed either by Ag and adjuvant or by a viral infection provide accelerated help for B cell responses. We demonstrate that this acceleration of the B cell response is due to an intrinsic difference between naive and memory cells and that this differences correlates with high expression of the B cell follicle homing molecule, CXCR5, on the memory CD4 T cells.

Adaptive immune responses to infections and vaccines lead to the generation of memory cells that can protect the host from subsequent infections. The advantages of memory CD8 T cells and B cells are obvious: faster responses that generate effector cells or molecules that directly clear or neutralize the pathogen or its toxins (10, 32). Understandably, these cell types have been found to be long-lived, although human memory CD8 T cells may have relatively shorter lifespans than do those in mice (33–35). CD4 memory T cells, on the other hand, have a shorter lifespan both in

**FIGURE 5.** Both CXCR5$^{hi}$ and CXCR5$^{lo}$ cells respond following transfer and reactivation in vivo. A, Eight weeks after LCMV infection, spleens and lymph nodes were taken and CD4$^+$CD44$^{hi}$ T cells expressing high or low levels of CXCR5 were sorted as described in Materials and Methods. Some of the sorted cells were stained with I-Ab/GP66 tetramer. Cells are gated as in Fig. 4A, with the numbers indicating the percentages of cells in the I-Ab/GP66 tetramer$^+$CD44$^{hi}$ gate. B, Intensity of tetramer binding and the expression of CXCR5 on the I-Ab/GP66 tetramer$^+$CD44$^{hi}$ cells after the sort. C, The sorted cells were transferred into congenically marked mice, with each recipient receiving between 1 and 4 $\times 10^5$ cells that were I-Ab/GP66 tetramer$^+$CD44$^{hi}$. These mice, age-matched mice, and a cohort of LCMV memory mice were immunized with GP66-OVA plus alum 1 d after the transfer (day 0). Eight days later, the numbers of I-Ab/GP66 tetramer$^+$ cells that were donor derived were examined. Cells were gated as in Fig. 4A and on the donor cells. Each point represents one mouse, and the line shows the means of the group. The data are combined from three experiments with three to five mice per group. D, Representative FACS plots of the cells in C. Cells are gated on donor$^+$CD4 cells. The number indicates the percentage in the gate: I-Ab/GP66$^+$CD44$^{hi}$. E, ICOS, PD-1, and CXCR5 expression on the donor I-Ab/GP66 tetramer$^+$CD44$^{hi}$ on day 8. The data are from one to three experiments, with each symbol representing a mouse, and the line shows the mean of the group.
humans and in mice when compared with CD8 T cells or B cells (3, 4, 33, 36–38). This may reflect the critical role that CD4 memory cells play as catalysts for both CD8 T cell and B cell responses in the primary responses, but that, once developed, the CD8 T cells and B cells probably do not require the presence of many specific CD4 memory T cells to be reactivated (39, 40). However, there are many infectious diseases that cause significant mortality or morbidity against which there are no current vaccines or no effective vaccines; in particular, intracellular organisms that are adept at hiding from Ab or CD8 T cells or pathogens that alter their immune targets (7, 41). For such infections, vaccines are required that create cells that can mobilize a multifaceted attack on the pathogen. To make progress in the design of such vaccines, a greater appreciation of the in vivo functions of memory CD4 T cells is required.

With this in mind, several studies have investigated the contribution of CD4 memory T cells to B cell responses (11–15, 42). These studies have proved inconclusive, providing conflicting results perhaps because they have examined T cell responses in artificial environments and have not addressed a mechanism for any enhanced help that was observed. Bone marrow–resident memory CD4 T cells have been shown to improve the affinity maturation of an Ab response compared with similar memory cells that reside in the spleen (43). Again, however, no mechanism was described for this effect, and any help for primary responding B cells, which reside in secondary lymphoid organs, would be delayed as the memory cells migrate from the bone marrow to the priming site. Our studies shed new light on this issue enabled, perhaps, by the more physiological numbers and sources of cells used in our studies.

Our results show that naive B cells do indeed respond and switch to the production of Ig isotypes other than IgM more rapidly if they are helped by CD4 memory, rather than primary, T cells. This result was not simply a numbers issue, as the same numbers of primary responding T cells were unable to induce early IgG from the responding B cells. The data suggest that the superior effects of the CD4 memory T cells were due to a subset of cells that bore CXCR5, the receptor for CXCL13, the chemokine that attracts cells to B cell follicles (44). These CD4 memory T cells also expressed the chemokine receptor that attracts cells to the T cell zone of lymphoid organs, CCR7, which recognizes CCL19 and CCL21 (44). Although we do not directly show that CXCR5 expression is sufficient for the enhanced helper function, we found that the memory cells were more likely to be located close to B cell follicles compared with naive CD4 T cells. This proximity may account for their superior helper function, since other, apparently similar CD4 memory T cells, that bear low levels of CXCR5 prior to reactivation were not able to deliver the same degree of help.

The CD4 memory T cells only affected early Ab production to the Ag. Later, Ab production was the same regardless of the presence of the memory T cells. This suggests that other factors may limit the differentiation of activated B cells to plasma cells. Such a relatively small shift in timing may nevertheless be significant, especially in cases in which the immune response is dealing with an infection that rapidly changes its B cell, but not its CD4 epitopes, as is the case for influenza (6).

Recently, the T cells that migrate to B cell follicles to provide help have been identified as a separate subset of helper T cells, Thf cells (9). The CD4 memory T cells produced in the experiments described in this study all bore or contained higher levels of markers characteristic of Thf cells (ICOS, PD-1, CXCR5, and Bcl-6) compared with naive cells (9, 31). However, we could not separate out a population of Ag-specific memory cells that expressed high levels of all the markers. Only those cells that bore high levels of CXCR5, regardless of their levels of expression of other Thf cell markers, provided superior help. These results suggest that it is an oversimplification to assign T cells to a particular subset based simply on their gene expression. In the end, the important criterion may be function.

Thf cells can make either a Th1 or Th2 response (45). The experiments described in this article involved CD4 memory T cells that were primed in vivo in the presence of the infectious virus, LCMV, or LPS, conditions that should generate Th1-like cells, although to a different degree: all GP66-reactive T cells make IFN-γ following LCMV infection, whereas LPS-primed memory cells are less committed to the Th1 lineage, as a smaller percentage of them make IFN-γ (3, 4). In the experiments reported in this article, the only animals that made a type 1 Ab response (indicated by a switch to IgG2c) were those that had been infected with LCMV before immunization with Ag and the Th2-inducing adjuvant, alum, suggesting that LCMV-primed cells are fully committed to their Th1 phenotype. The switch to a type 2 (IgG1) rather than a type 1 Ab response in the LPS-primed mice probably reflects a lesser commitment of these cells to IFN-γ production and the presence of IL-4 producing primary responding T cells to epitopes within the OVA protein. In the case of the transferred LCMV memory cells, the small number of IFN-γ-committed memory cells are reactivated in the presence of a numerically larger primary host response that develops in a Th2 environment. This suggests that the IL-4 from these latter cells overrides the IFN-γ made by the reactivated memory cells, inducing a type 2 Ab response from the responding B cells (46).

In summary, we show that some memory CD4 T cells have an intrinsic ability to provide accelerated help to primary responding B cells. Important questions remain as to how best to prime or maintain CXCR5-memory cells, a critical consideration for the design of vaccines against pathogens that are adept at altering their Ab targets to escape immunodetection.

Acknowledgments
We thank Dr. Dirk Homann for supplying LCMV, the National Institutes of Health Tetramer Core Facility for I-Aβ/GP66,77 tetramer, Josh Loomis and Shirley Sobus for cell sorting and assistance with microscopy, Catherine Haluszczak for assistance with immunofluorescence, and all members of the Kappler Marrack laboratory for intellectual contributions to this project.

Disclosures
The authors have no financial conflicts of interest.

FIGURE 6. CXCR5+–enriched Ag-specific memory cells provide accelerated help for IgG1 but not IgG2c responses. The experiment was performed as described in Fig. 5, but sera was prepared from the blood of the GP-OVA plus alum-immunized mice and the amount of OVA-specific IgG1 (A) or IgG2c (B) was examined 8 d after immunization. Each point represents one mouse and the line shows the means of the group. The data are combined from three experiments with three to five mice per group.
References


Supplemental Figures:

**Supplemental Figure 1: TCR Tg memory cells also provide accelerated help to primary responding B cells**

5x10^4 3K specific TCR Tg cells were transferred i.v. into wild-type mice. These mice (TCR Tg recall) and non-transferred mice (endogenous recall) were immunized with 3K+LPS then rested for 7 weeks. At this time point, age-matched naïve mice received 10^5 3K specific TCR Tg cells (TCR Tg primary) then all these animals and naïve age-matched mice (endogenous primary) were all immunized with 3K-OVA+alum i.p. The mice were bled 7 days after this immunization and the amount of anti-OVA IgG1 antibodies in the serum examined. The data are from one experiment with 12 mice in each group at the timepoint shown.

**Supplemental Figure 2: Activated IA^b/3K tetramer+ cells express higher levels of the Tfh markers than naïve or memory phenotype CD4 T cells and MHC tetramer staining does not alter expression of Tfh markers**

A: 3K memory mice, naïve mice, or naïve mice that had received 3K specific TCR Tg cells were immunized with 3K-OVA+alum i.p. Eight days later, either the size of or the expression of CXCR5, ICOS and PD1 on the IA^b/3K tetramer+ cells, or memory phenotype cells (CD4+CD44^hi) were compared to that of naïve CD4 T cells (CD4+CD44^lo, shaded histogram). Representative histograms from one mouse per condition are shown from 3 experiments with 3-4 mice per group. Numbers in the top right of the histogram indicate the MFI of the indicated marker on the named cell type represented by the black line.

B: 5x10^4 3K specific TCR were transferred i.v. into congenic (B6PL) recipients that were immunized the following day with 3K-OVA+alum i.p. On day 8, the TCR Tg cells were identified by staining with either IA^b/3K tetramer or anti-Thy1.2 and the expression of CXCR5, PD1 or ICOS examined on the cells. Cells are gated on CD4+ IA^b/3K tetramer+ cells negative for B220, F4/80, CD8 and MHCII (dashed blue line), CD4+Thy1.2+ cells negative for B220, F4/80, CD8 and MHCII (red line) or on naïve CD4 T cells (grey shaded histogram). The data are from one individual mouse that is representative of a total of 6 mice from two experiments.
Supplemental Figure 3: The transfer of sorted memory cells does not affect the host primary response to LCMV
CXCR5\textsuperscript{hi} or CXCR5\textsuperscript{lo} CD44\textsuperscript{hi} CD4 cells were transferred or not into congenic mice that were immunized the following day with GP66-OVA+alum. 8 days later, the number of host IA\textsuperscript{b}/GP66 CD44\textsuperscript{hi} cells in the spleen that were CD4+ but negative for CD8, B220, F4/80 and MHC class II was examined by flow cytometry. Each symbol represents a mouse and the line shows the mean of the group, the data are representative of three independent experiments.

Supplemental Figure 4: CXCR5\textsuperscript{hi} IA\textsuperscript{b}/GP66 specific cells make IFN\gamma
Spleen cells from mice infected with LCMV 8 weeks previously were stimulated with GP66 peptide in the presence of golgi plug for 6 hours. In the first panel, the percent of CD44\textsuperscript{hi} IFN\gamma\textsuperscript{+} positive cells out of CD4+B220-CD8-MHC class II- cells. The second panel is gated on the CD44\textsuperscript{hi}IFN\gamma\textsuperscript{+} cells and shows the separation of this population into CXCR5\textsuperscript{hi} and CXCR5\textsuperscript{lo} cells. The third panel is gated on the CD44\textsuperscript{hi}IFN\gamma\textsuperscript{+} CXCR5\textsuperscript{hi} or CXCR5\textsuperscript{lo} cells and shows that both populations make similar levels of IFN\gamma.
Supplemental Figure 1, MacLeod et al
Supplemental Figure 2, MacLeod et al

A

Cell Type

CD4+ CD44 hi

IAβ/tetramer+ TCR Tg
  Primary

IAβ/tetramer+ B6 Primary

IAβ/tetramer+ 3K memory/ secondary

% of max

B

indicated cell type
naive CD4 T cells

TCR Tg cells gated using IAβ/3K tetramer
TCR Tg cells gated using Thy1.2
Naive CD4 T cells