During an immune response T cells proliferate, differentiate, and give rise to memory cells. The signals driving the former two processes are reasonably well characterized; however, the identity of the costimulatory molecules involved in generating memory cells is still not clear. CD40 is a member of the TNFR family and is expressed on activated DCs and B cells (1). It binds to CD154 on activated T cells and is an initiator of the cross-talk between T cells and APCs (2, 3). CD40 acts as a master regulator of APC function, inducing DC maturation and cytokine production (4–6), and consequently the interaction between CD154 (CD40-ligand) and CD40 is critical for the development of a mature immune response.

Studies in CD40KO and CD154KO mice have provided insights into the role of CD40–CD154 interactions in T cell priming. There have been reports of reduced CD4 and CD8 T cell priming (7); however, others have found a defect only in the CD4 response (8) or no defect at all in the T cell response (9). These variations perhaps reflect the different immunization strategies used and inherent differences between viral infection and protein-based immunizations. Moreover, the CD4 T cell response has primarily been examined indirectly using in vitro proliferation assays. Studies involving the adoptive transfer of CD154KO TCR transgenic cells showed that, at this high precursor frequency, the KO cells can proliferate in vivo; however, the response was aborted at an early stage (6, 10). Similarly, exogenous CD40-deficient DCs failed to sustain T cell activation after transfer to WT mice (10, 11). The importance of CD40 in the reactivation of memory T cell responses has not so far been defined.

In this study, class II tetramers were used to track antigen-specific responses generated by immunization with peptide emulsified in CFA or by peptide-pulsed wild-type dendritic cells. CD4 memory T cells were detectable after immunization for more than 200 days, although decay was apparent. Memory cells generated in CD40 knockout mice by immunization with peptide-pulsed wild-type dendritic cells survived in the absence of CD40 and proliferated when boosted with peptide (plus adjuvant) in a CD40-independent fashion. However, differentiation of the memory cells into cytokine-producing effector cells did not occur in the absence of CD40. The data indicate that memory cells can be generated without passing through the effector cell stage.
proliferation. The CD40–CD154 costimulatory signals were required, however, for the differentiation of IFN-γ-producing effector T cells. These data suggest that CD40–CD154 interactions regulate primary and memory T cell responses in different ways. Specifically, although primary CD4 T cells require CD40–CD154 interactions for expansion, memory T cells can survive and expand in a CD40–CD154-independent fashion but do require CD40–CD154 interactions for full effector cell differentiation.

RESULTS

Phenotypic characterization of peptide-induced antigen-specific CD4 T cells

We used MHC class II tetramers to track antigen-specific T cells through expansion and contraction phases of the response and into the subsequent memory phase. The I-A^b class II tetramers (12) contain the peptide from the immunodominant epitope, H19-Env, from the envelope protein of murine Moloney leukemia virus (13). C57BL/6 mice were immunized with H19-Env delivered either in CFA or on peptide-pulsed bone marrow–derived DCs.

After immunization with H19-Env-CFA, the peak of the H19-Env–specific CD4^+ T cell response was between days 8 and 10 at around 0.5% of CD4 cells (Fig. 1 A). Background staining was set by the level of tetramer binding cells from mice immunized with an irrelevant peptide, OVA peptide–pulsed DCs (Fig. 1 A). (C) Phenotype of tetramer^+ cells after CFA immunization. Cells gated on CD4^+ lymphocytes, excluding propidium iodide^+ and F4/80^+ cells. First column shows day 7 (primary response), second column shows day 9 (peak of the primary response), and the third column shows cells from day 176 after immunization. The number shows the percentage of tetramer^+ cells that are also CD44^hi, IL-7R^α^+, or CD62L^lo^.
the tetramer$^+$ cells were CD44$^+$ and CD62L$^-$ and they maintained this phenotype throughout the memory phase of the response.

As IL-7R$\alpha$ is thought to be a functional marker for the precursors of CD8 (14) and CD4 (15, 16) memory cells, the kinetics of expression of IL-7R$\alpha$ was investigated. At day 7, before the peak of the response, about half of the tetramer$^+$ cells in the draining LN had down-regulated IL-7R$\alpha$ (Fig. 1 C, middle). By day 9, although the majority of cells were IL-7R$\alpha^{lo}$, a substantial proportion was still IL-7R$\alpha^+$. By day 14, the majority of tetramer-binding cells in the spleen were IL-7R$\alpha^+$. However, in the draining LN, even by day 32 only $\approx60\%$ of antigen-specific cells stained positive, presumably reflecting continuing activation (from the peptide-CFA depot) at this site but not in the spleen (not depicted). All of the long-term memory cells (at day 176) at both sites were IL-7R$\alpha^+$, perhaps indicating the importance of this cytokine in long-term memory cell survival.

The recall response is faster and larger than the primary response

To investigate whether antigen-specific CD4 T cells were still present in mice that had been injected with peptide-pulsed DCs, these mice were challenged with peptide-CFA. A classical rapid and large recall response was measured up to 24 wk after primary immunization, demonstrating that antigen-specific memory CD4 T cells were present in these mice (Fig. 2 A). However, the size of the recall response was reduced at 24 wk compared with 13 wk after priming, and by 38 wk only two out of six mice made a small recall response to the peptide-CFA challenge (Fig. 2 B). This suggests that the memory cells declined over time as they had in the peptide-CFA–immunized mice. In long-term memory experiments, age-matched naive control mice were also immunized (not depicted). These mice gave similar primary responses to young mice, indicating that the loss of memory was not simply an age-related impairment of immune function/cell division.

The intensity of tetramer staining is thought to correlate with the avidity of the TCR for peptide-MHC (17–19). This staining intensity has been found to increase in the recall response (18, 20). We also found an increase in the intensity of tetramer staining of the responding cells after secondary stimulation (Fig. 2 C). This was not observed in memory cells that had not been boosted, suggesting that the maturation was a result of selective expansion of higher avidity cells during the secondary response. However, this increase in the intensity of tetramer staining has also been shown to be due to the localization of TCR in rafts rather than an actual increase in the avidity of the interaction between TCR and peptide-MHC (21). Regardless, the change in intensity of staining indicates a change in the cells involved in the recall response compared with those in the primary response, a change that may partly explain the increased speed of the recall response.

Figure 2. The recall response is faster and larger than the primary response. C57BL/6 mice were injected with H19-Env–pulsed DCs, rested for 13, 24, or 38 wk, and boosted with H19-Env-CFA. Cells from the spleens and draining LNs were stained with class II tetramers and analyzed as in Fig. 1. (A) Example staining of LN cells from the recall response after 13 wk, the primary response, and background staining. The number shows the percentage of CD44$^+$ tetramer$^+$ cells out of CD4 cells. (B) Percentage of tetramer$^+$ cells out of CD4 cells in the LNs of boosted C57BL/6 mice immunized with DCs, rested for the indicated time, and then boosted with H19-Env-CFA and killed on day 5. Day 5, primary response to H19-Env-CFA; Day 9, peak of the primary response to H19-Env-CFA; OVA, background staining from mice primed with OVA-pulsed DCs and boosted with OVA-CFA. Each point represents one mouse, and the line shows the mean of each group. (C) The intensity of tetramer staining was analyzed by defining high and low staining as shown, and the percentage of tetramer$^+$ cells in the high staining box was analyzed.
Figure 3. CD40–CD40L signals are required for T cell priming. (A) C57BL/6, CD40KO, and CD154KO mice were immunized with H19-Env in CFA. 9 d later, spleens and draining LNs were taken and cells were stained with class II tetramers. Data from draining LNs are shown with each point representing the percentage of CD4 cells that were tetramer + in an individual mouse, and the mean of the group is shown. (B) Splenocytes from immunized mice were activated with 1 μg/ml H19-Env peptide. After 4 d of culture, the percentage of tetramer + cells in the CD4 + population was measured by FACS. (C) WT DC primed T cells in CD40KO mice. C57BL/6, CD40KO, and CD154KO mice were immunized with H19-Env peptide-pulsed, LPS-matured DCs from WT mice. The percentage of tetramer + cells in the CD4 + population was measured by FACS in the draining LNs 6 d later. (A–C) Each point represents one mouse, and the line shows the mean of each group. Results are representative of three independent experiments. (D) Representative FACS dot plots from the experiment shown in C. Control mice were immunized with OVA-CFA (A and B) or OVA-pulsed DCs (C and D) and stained with the H19-Env tetramers.

Antigen-specific CD4 T cell priming in CD40KO mice is restored by WT DCs

To evaluate the role of the costimulatory interaction between CD154 and CD40 in CD4 T cell activation, CD40KO and CD154KO and WT C57BL/6 mice were primed with H19-Env peptide in CFA. As expected, CD4 T cells from WT mice bound the tetramer; however, tetramer staining of cells from CD40KO or CD154KO mice did not rise above background at any of the time points examined (Fig. 3 A and not depicted for days 15 and 28).

To examine whether priming had occurred at a level below detection by the tetramers ex vivo, splenocytes from the mice were activated in vitro with peptide for 4 d. Expansion of antigen-specific cells was detected in splenocytes from C57BL/6 mice; however, tetramer staining of the cells from CD40KO and CD154KO mice remained at background levels (Fig. 3 B).

We found that the priming could be restored in CD40KO mice if they were injected with a cohort of peptide-pulsed LPS-activated WT DCs. The primary response in both the spleen and draining LN was similar in C57BL/6 and CD40KO mice (Fig. 3, C and D). However, in CD154KO mice, in which WT DCs cannot restore the genetic defect, tetramer staining remained at background levels. The peptide-pulsed WT DCs used to immunize CD40KO mice were matured with LPS; however, it is clear that this does not bypass any requirement for the CD40 signal. In Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050711/DC1, we show that LPS-activated CD40KO DCs could not prime T cells in CD40KO mice and, in addition, these mice did not respond to antigen delivered with LPS in incomplete Freund’s adjuvant.

CD4 memory T cells survive and proliferate after boosting in CD40-deficient mice

Previous studies have suggested that the activation of memory cells does not require the same level of costimulation as naive cells (22–24). By using WT DCs to restore priming in CD40KO mice, a role for CD40–CD154 costimulation in the survival and recall response of T cells could be examined. Thus, CD40KO mice were immunized with WT LPS-activated, peptide-pulsed DCs and after 4–18 wk were boosted with peptide in CFA. The spleen and draining LN were taken on day 5 after the boost. The secondary expansion of tetramer + T cells in the draining LN (Fig. 4, A and B) and the spleen (Fig. 4 C) of C57BL/6 and CD40KO mice was absolutely equivalent. Furthermore, in both groups the peak of the response at day 5 was dramatically larger than that in the primary response in C57BL/6 mice immunized with peptide in CFA alone, demonstrating that this was a bona fide memory response.

We next investigated if the memory T cells generated in CD40KO mice differed qualitatively from those formed in WT mice. We found them to be equivalent in their expression of CD44 and CD62L, and they also exhibited the avidity increase (increased intensity of tetramer staining) seen in WT T cells (shown in Fig. 2 C). They are also detectable (by staining after boost) for a similar length of time after priming in vivo. These comparisons of the CD4 memory T cells generated in CD40KO and WT mice are shown in Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20050711/DC1.

Tetramer staining slightly above background levels was detected in the draining LNs of CD154KO mice that had first been primed with WT DCs and then boosted with peptide–CFA. However, this response was deficient compared with that in WT or similarly treated CD40KO mice. Thus, a small population of memory T cells may be generated in the absence of CD40–CD154 interactions.

Differentiation of CD4 memory T cells to produce IFN-γ requires CD40

T cell proliferation is only one component of the immune response to antigen. To establish whether the restored T cell proliferation in the CD40KO mice was accompanied by an equivalent cytokine response, the antigen-specific IFN-γ
response was measured. Because TCR levels fall after peptide stimulation in vitro, it was not possible to carry out tetramer and intracellular cytokine staining simultaneously. Instead, antigen-specific cytokine production from long-term primed cells was measured after a 6-h in vitro stimulation with peptide using a cell surface cytokine capture assay (see Materials and methods). The percentage of CD4 cells making IFN-\(\gamma\) in C57BL/6 mice primed with WT DCs and boosted with peptide-CFA was significantly greater than that in similarly treated CD40KO mice (Fig. 5 A; \(P = 0.01\)). Furthermore, this response was greater than that in C57BL/6 mice immunized with only peptide-CFA or cells from naive mice. In primed and boosted CD40KO mice, the percentage of IFN-\(\gamma\)-producing CD4 cells did not rise above basal levels (cells incubated without peptide) and did not exceed the response of cells from naive C57BL/6 mice. Thus, although antigen-specific memory cells proliferated in the absence of CD40, these cells were unable to make an IFN-\(\gamma\) effector response. Representative FACS dot plots of the IFN-\(\gamma\) secretion data shown in Fig. 5 are provided in Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20050711/DC1. The frequency of tetramer\(^+\) CD4 T cells producing IFN-\(\gamma\) (in WT mice) is in line with similar recently published data (25, 26).

We also tested IFN-\(\gamma\), IL-2, and IL-4 production after in vitro restimulation of spleen or LN cells from immunized CD40\(^{−/−}\) or WT mice with PMA/ionomycin for 4 h ex vivo. The production of IL-2 by total CD4 cells was equivalent in C57BL/6 and CD40KO mice after priming with DCs and boosting with CFA (Fig. 5 C). In agreement with the antigen-specific IFN-\(\gamma\) response described above, only WT mice (and not CD40KO mice) exhibited a significant increase in IFN-\(\gamma\)-producing CD4 T cells after boosting with peptide-CFA (Fig. 5 B; \(P = 0.008\)). In summary, the percentage of CD4 T cells producing IFN-\(\gamma\) was lower in CD40KO mice compared with that in C57BL/6 mice in both primary and recall responses (Fig. 5 B). No IL-4-producing cells were detected in the response of either C57BL/6 or CD40KO mice (not depicted).

The lack of IFN-\(\gamma\) secretion in the absence of CD40 during boosting might be due to a dependence of secondary effector differentiation on CD40 or, alternatively, it could be the result of an impairment in the capacity of the memory cells generated in the CD40KO mice to differentiate. To test this, we primed CD40KO and WT mice with peptide-pulsed WT DCs and then rechallenged them 4 wk later with CD40KO (or WT) DCs. This experiment, shown in Fig. 6, confirmed that memory cells from either WT or CD40KO mice required the presence of CD40 on APCs for their differentiation to effectors. Thus, the memory cells generated in
CD40KO mice differentiated into IFN-γ producers when restimulated with WT DCs, whereas the memory cells formed in WT mice differentiated very poorly when boosted with peptide-pulsed CD40KO DCs.

**DCs but not B cells are required for effector T cell differentiation recall responses**

We wished to confirm the identity of the CD40-bearing APCs in vivo necessary for effector T cell differentiation. We (27) and others (28) have proposed that B cells are particularly important in maintaining or supporting memory CD4 T cell responses, and we set up bone marrow chimeric mice in which the CD40 mutation was restricted to B cells (29). In these chimeras, activated T cells could interact via CD40 with DCs and macrophages, but not with B cells. The percentage of tetramer+ T cells activated after the transfer of peptide-pulsed WT DCs was the same in B cell CD40KO chimeras as in WT chimeras (Fig. 7 A). As expected, the recall response was also similar in the two sets of chimeras after boosting with H19-Env-CFA (Fig. 7 B).

The IFN-γ response after priming with WT DCs was equivalent in CD40KO B cell chimeras and WT chimeras (Fig. 7 C). Similarly, in CD40KO B cell and WT chimeras, which had been immunized with DCs and boosted with H19-Env-CFA after 10 wk, the number of IFN-γ-secreting CD4 T cells was similar (Fig. 7 C). Thus, interactions with DCs are sufficient to restore the defect in the secondary effector cell response.

**DISCUSSION**

Tracking memory cells using MHC class II tetramers led us to investigate the requirements for costimulatory signals in T cell responses in vivo. There have been reports that memory cells require less costimulation than naive cells; however, the majority of these experiments have been performed in vitro (22, 23, 30). The findings reported here validate these studies, and advance them as the current data is derived entirely ex vivo.

Normal immunization of CD40-deficient mice fails to evoke clonal expansion of naive CD4 T cells; however, by transferring WT DCs into CD40KO mice, primary and recall responses were restored. This demonstrates several points: (a) The T cells activated in CD40KO mice in response to WT DCs developed into memory cells; (b) these memory cells survived in a CD40-deficient environment; (c) the memory T cells were able to proliferate in response to antigen rechallenge in the absence of CD40, demonstrating a qualitative difference between naive and memory CD4 T cells; and (d) the memory cells could not, however, differentiate to produce effector cytokines without CD40-CD154 signals.

That antigen-specific cells were not detected after immunization of CD40KO and CD154KO mice with peptide-CFA could be due to several factors. Moodycliff e et al. (31) have reported a requirement for a CD154 signal to DCs in the skin to induce their migration to draining LNs. This seems an improbable explanation for our data, as we were able to see presentation of antigen in the recall response in CD40-deficient mice. Indeed, free antigen is likely to reach lymphoid organs by draining from the immunization site (32, 33).

The most likely explanation for the defect of priming in CD40KO mice is that the T cell–DC interaction may be curtailed in the absence of this maturation signal to the APCs. The adoptive transfer and immunization of CD154-deficient TCR transgenic T cells into WT mice, or the transfer of antigen-pulsed CD40-deficient DCs to WT mice, results in a brief proliferative response followed by a crash of the transgenic T cells (6, 10, 11). Miga et al. (10) argue that the CD40 signal to the DCs is required for the full activation of the DCs. For example, CD40–CD154 signals induce increased expression of costimulatory molecules such as CD80 and CD86 and, importantly, also greatly enhance DC IL-12 production (4, 5, 34). It is also likely that T cell activation in vivo in the absence of CD40 is impaired because of a failure of OX40L signals (11, 29, 35, 36).
Immunization with WT DCs circumvented this defect. Antigen-specific CD4 T cells in the CD40KO mice proliferated to the same level as in WT mice. These primed T cells were indistinguishable in phenotype from memory cells generated in WT mice and survived in the CD40-deficient environment for at least 18 wk (Fig. S2). When reimmunized with peptide plus CFA they responded by proliferating just as well as memory cells in WT mice (Fig. 4). However, they were incapable of differentiating into IFN-γ-producing effector T cells. So the costimulation requirements of memory cells for cell division are less stringent than naive cells, but differentiation still requires some, if not all, of the same costimuli. The importance of B cells in supporting this differentiation was investigated using bone marrow chimeras given our previous data suggesting a dependence of CD4 T cell memory on B cells (27). The observation that the effector (IFN-γ) response is restored if CD40+ DCs are present rules out any obligatory CD40-dependent role for B cells. Moreover, WT bone marrow-derived DCs were sufficient to restore the IFN-γ response in DC-primed CD40KO mice. This demonstrates, using two immunization protocols, that DCs play an important role as APCs in the CD4 recall response, as they do in CD8 recall responses (37).

The requirement for CD40–CD154 interactions for CD4 memory T cell differentiation observed here may be explained in three ways. First, continued signaling via CD40 on host APCs may be required during the primary response. In such a model, this continued signaling would then imprint the capacity of the expanding CD4 T cell population to differentiate into effector cells during the recall phase. Second, CD40–CD154 interactions may be required during the memory phase to maintain the capacity of memory T cells to differentiate upon renewed antigen encounter. Finally, and perhaps most likely, memory CD4 T cells may proceed through cell division independently of CD40–CD154 interactions but would still require this interaction for functional maturation. In this model, DCs would have the capacity to control memory T cell responses not through regulation of memory T cell expansion, but by licensing memory helper T cells for effector cell differentiation. This final interpretation is strongly supported by the observation that memory cells generated in WT mice differentiated only poorly when boosted with peptide-pulsed CD40KO DCs and, conversely, memory cells formed in CD40KO mice could differentiate into IFN-γ producers perfectly well when restimulated with WT DCs (Fig. S3). The licensing or programming may be mediated by inflammatory cytokines, such as IL-12 (38). Although we have presumed in these explanations that it is CD40 activation of APCs that is critical, we cannot rule out a role for CD40 that may be expressed by the CD4 T cells themselves (39).

The immunization of CD40KO mice with peptide-pulsed DCs gives rise to negligible IFN-γ production in the primary response (Fig. 5 B), even though clonal expansion is instigated and the cells survive into the memory phase and can be reactivated many weeks later. This is a clear indication that memory cells can be generated without passage through the effector cell stage. This linear development of memory cells from effector cells has been suggested by several recent publications (40–42). As most lineage studies so far published refer to CD8 T cells, this may simply represent a difference between CD4s and CD8s. On the other hand, it is more attractive to suppose that there are several ways to generate the different memory cell subsets. In the circumstance presented here, subnormal quality or quantity of stimulus allows...
The tetramer-binding memory cells in this study are homogeneous and stable in their expression of molecules that would mark them out as effector memory cells (e.g., CD62Llo, CD44hi). This homogeneity in marker expression is contrary to studies by Roman et al. (44) and Ahmadzadeh et al. (45), who have described heterogeneity within an antigen-specific memory pool based on CD62L staining. There are various differences between these studies and ours, including the use of TCR transgenic T cells as well as the presence of CFA-peptide depots to provide a potential for continued activation of cells. However, it is of interest in this context that Marzo et al. (46) have recently found that at low precursor frequency (e.g., in endogenous T cell populations) the effector-memory CD8 population is predominant and stable over long periods.

The expression of the α-chain of the IL-7R (IL-7Rα) is also stable on our long-term tetramer-binding CD4 memory cells (98%; Fig. 1 C). It is interesting that at the peak of the primary expansion (day 9) only a minority (19%; Fig. 1 C) of the tetramer+ memory cells bear IL-7Rα (down from 55% 2 d earlier). On the face of it, this fits with the notion that reactivity to IL-7 marks out the memory precursors that will survive the contraction phase and move into the memory pool (14–16). However, it takes more than 30 d for the majority of the tetramer+ memory cells to express IL-7Rα (61% at day 32; not depicted), an indication that entry into the CD4 memory pool is not absolutely dependent on IL-7R, a point recently made by Lacombe et al. (47).

Using the MHC class II peptide tetramers, antigen-specific CD4 memory T cells were visible ex vivo more than 200 d after immunization with peptide plus CFA, and recall responses were detectable for more than 150 d after immunization with peptide-pulsed DCs. After this time, these polyclonal CD4 memory T cells would undergo significant secondary clonal expansions upon antigen restimulation, in contrast to the relatively poor secondary expansion observed with transferred TCR transgenic CD4 memory T cells (48). Our longitudinal studies tracking antigen-specific memory cells in mice immunized with either peptide in a persistent form (with CFA) or with a transient exposure (peptide-pulsed DCs) also demonstrated a slow decline in CD4 memory T cell survival over time. A similar decrease in CD4 memory cells was described by Homann et al. (49), although there have been reports of stable CD4 memory pools (50, 51). It is no surprise to us that under competitive conditions memory cells decay and do not survive indefinitely (52). Despite this, the basis of the homeostatic loss under these normal competitive conditions still needs resolution.

MATERIALS AND METHODS

Mice. C57BL/6, CD40KO (53), and CD154KO (54) were bred and maintained in specific pathogen-free conditions at the School of Biological Sciences, Animal Facility at the University of Edinburgh. 6–10 wk old mice were age and sex matched in experiments. All experiments were covered by a Project License granted by the Home Office under the Animal (Scientific Procedures) Act of 1986. Locally, this license was approved by the University of Edinburgh Ethical Review Committee.

Immunizations. Mice were immunized with 100 μg H19-Env (EPLTSL-TPR.CNTAWNRLKL) or OVA223-259 peptide (SQAVHAHAHAEINEAGR) that binds to H2-A for both mice were made and supplied by Advanced Biotechnology Centre, Imperial College, London). The peptides were emulsified in CFA (Sigma-Aldrich) and injected s.c. in both hind legs. For bone marrow–derived CD4 immunizations, mice were injected with 106 DCs either i.v. in the tail vein or s.c. in the hind legs. In recall experiments, mice were first primed with peptide-pulsed DCs s.c., boosted with peptide-CFA when indicated, and killed on day 5. All injections were in the hind leg. In one experiment, LPS (from Salmonella typhimurium; Sigma-Aldrich) was used as adjuvant. It was delivered (at 30 μg/mouse) together with peptide in incomplete Freund’s adjuvant.

Tetramer and antibody staining. Single cell suspensions were prepared from spleens and inguinal LNs, and erythrocytes were lysed using Red Blood Cell Lysis Solution (Sigma-Aldrich). Approximately 1–2 × 106 cells were plated in round-bottom 96-well plates and washed in IMDM (Sigma-Aldrich) containing 10% FCS before the addition of PE-labeled class II tetramers diluted in IMDM plus 10% FCS. Tetramers were made as described previously (17). The cells were incubated at 37°C for 3 h with gentle agitation every 20–30 min to prevent clumping of cells. Allophycocyanin (APC)-labeled anti-CD4 (BD Biosciences), R. Phycocerythrin-Cy5-labeled anti-F4/80 (Serotec), and FITC-labeled anti-CD44 (142.5; in-house) or FITC-labeled anti-CD62L (MEL-14; in-house) or FITC-labeled anti-IL-7Rα (CD127; eBioscience) were added and incubated for 10–15 min at room temperature. Cells were washed three times in FACS buffer (PBS with 2% FCS and 0.05% sodium azide). Propidium iodide (BD Biosciences) was added before acquisition. 200,000 live events were collected on a FACSCalibur flow cytometer (Becton Dickinson) and analyzed using FlowJo software (TreeStar). Viable lymphocytes were defined using FSC/SSC parameters and propidium iodide exclusion, and tetramer+ cells were identified as CD4+ F4/80+ live lymphocytes.

Bone marrow–derived DCs. DCs were derived from bone marrow cells according to the procedure developed by Inaba et al. (55). A single cell suspension of the bone marrow from the femurs and tibias of 6–10 wk-old WT C57BL/6 or CD40KO mice was prepared and red blood cells were lysed. Cells were plated in 24-well plates at 3.75 × 105 in 1 ml in 10% FCS, 5% GM-CSF (supernatant from a GM-CSF-producing cell line; reference 56), and RPMI medium (Sigma-Aldrich) supplemented with 2 mM l-glutamine, 100 μM penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Cells were grown for 7 d and washed on days 3 and 6, with the FCS in the medium being replaced with 0.5% mouse serum (Harlan UK).

DCs were harvested on day 7, incubated at 37°C together with 0.1 μg/ml LPS (Sigma-Aldrich) for 12–13 h, and then reharvested. The DCs were incubated for 90 min with 50 μg/ml peptide and washed extensively with PBS before injection into mice.

In vitro activation. Single cell suspensions, prepared as described above, were plated in 96-well plates at 104 cells per well and incubated for 3 d at 37°C and 5% CO2 with 1 μg/ml H19-Env peptide in IMDM supplemented with 5% FCS, 2 mM l-glutamine,100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen), and 50 μM 2-mercaptoethanol (BDH Laboratories).

Detection of antigen-specific IFN-γ. Single cell suspensions were prepared as described above, and splenocytes were cultured with 8 μg/ml H19-Env peptide for 6 h (or without peptide) at 37°C and 5% CO2 in RPMI containing 1% mouse serum supplemented with 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The production of IFN-γ was then assessed using an IFN-γ ELISA (Becton Dickinson). Histology was performed on 5 μm sections of paraffin-embedded tissues (H&E) and was read by the terminal pathology service of the University of Edinburgh.

Statistical analysis. Data were analyzed using GraphPad Prism (version 5). Comparisons were made using Student’s t test (unpaired, 2-tailed) or analysis of variance (ANOVA) test followed by Bonferroni’s and Tukey’s multiple comparison tests as appropriate. Significance was indicated by *p < 0.05, **p < 0.01, ***p < 0.001.
washed, and incubated with the bi-specific capture antibody (anti-CD45- anti-IFN-γ). Extra medium was added to the cells that were then incubated for an additional 45 min in the MACSxms to rotate the cells throughout the incubation period. After additional washes, the cells were stained with PE-labeled detection anti-IFN-γ and anti-B220-PerCP (BD Biosciences), and anti-CD4-APC and anti-CD44-FITC. Propidium iodide (BD Biosciences) was added before acquisition. 500,000 live events were collected on a FACSCalibur flow cytometer and analyzed using FlowJo software. IFN-γ-producing CD4 cells were identified by gating out B cells and dead cells (all FL3− cells), and gating on CD4+ cells.

### Intracellular cytokine staining.

Cells were plated at 4–5 × 10^6 effector cells in WT and CD40KO mice. Figs. S1–S3 are available at http://

#### Bone marrow chimeras.

Recipient mice were lethally irradiated with 1150cGy from a Caesium γ source 24 h before bone marrow reconstruction as described previously (29). Bone marrow was removed from donor mice and depleted of T cells using anti-Thy1 microbeads on a MACS column (both from Miltenyi Biotec) according to the manufacturer’s instructions. Between 3 and 5 × 10^6 cells were injected into the irradiated recipients.

Mixed chimeras were made by reconstituting with 80% μMT (B cell-deficient) bone marrow and 20% CD40KO bone marrow. B cells can only develop from the CD40KO bone marrow and were therefore CD40 deficient. Control WT chimeras were made with 80% μMT bone marrow and 20% C57BL/6 bone marrow. The chimeric hosts were then left for 8 wk to allow full reconstruction from the donor cells. Reconstitution was confirmed by FACS analysis of splenocytes when the chimeras were killed.

### Statistics.

Data are expressed, when appropriate, as mean and SEM. Significance was assessed using unpaired t tests, and p-values <0.05 were considered significant.

### Online supplemental material.

Fig. S1 shows that the priming of CD4 T cells in CD40KO mice cannot be bypassed by LPS signals alone. Fig. S2 shows a comparison of phenotype, avidity, and longevity between memory CD4 T cells generated in WT and CD40KO mice. Fig. S3 shows representative flow cytometry dot plots of cytokine (IFN-γ) secretion by secondary effector cells in WT and CD40KO mice. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20050711/DC1.

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