

CD4 memory T cells survive and proliferate but fail to differentiate in the absence of CD40

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Secondary T cell responses are enhanced because of an expansion in numbers of antigen-specific (memory) cells. Using major histocompatibility complex class II tetramers we have tracked peptide-specific endogenous (non-T cell receptor transgenic) CD4 memory T cells in normal and in costimulation-deficient mice. 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.

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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 bone marrow-derived DCs. Class II tetramers provide an elegant and physiological means to investigate CD4 T cell memory, enabling the *ex vivo* examination of endogenously generated antigen-specific T cells for periods in excess of 200 d after immunization. CD40–CD154 interactions were required for both the priming of naive cells and the formation of memory cells. However, once a population of antigen-specific memory cells had formed, CD40 signals were not required for either their maintenance or their

The online version of this article contains supplemental material.

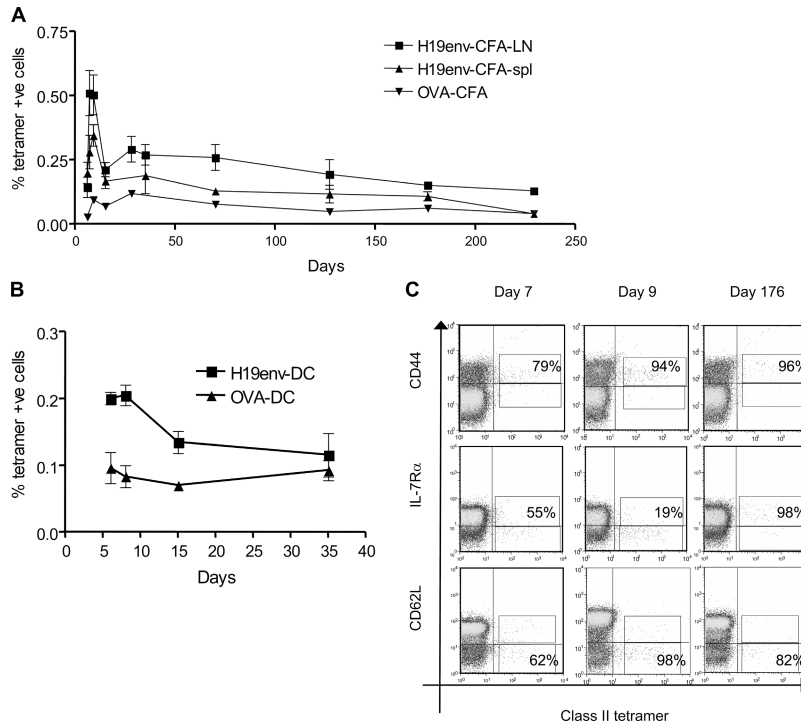


Figure 1. Class II tetramers can be used to enumerate antigen-specific cells. (A) H19-Env-CFA-immunized mice, three to four per group, were killed at various times after immunization, and cells from the draining LNs (■) or spleens (▲) were stained with class II tetramers. Control mice were immunized with OVA-CFA and stained with the tetramers (▼). The mean percentage of tetramer⁺ cells out of total CD4 cells is shown, and error bars show the SEM. (B) Bone marrow-derived DCs were activated with LPS, pulsed with H19-Env or OVA peptide, and 10⁶ cells were injected i.v. into C57BL/6 mice. At various days after transfer, spleens were removed and cells were stained with class II tetramers and

labeled antibodies (■). The mean percentage of tetramer⁺ cells out of total CD4 cells of four mice per group is shown, and error bars show the SEM. Background staining was set by staining cells from mice immunized with OVA-pulsed DCs (▲). (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}.

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 in mice immunized with an irrelevant peptide, OVA_{323–339}, in CFA, also shown in Fig. 1 A. Typically, this level of staining was below 0.1% of total CD4⁺ cells. After day 8, a contraction phase followed that left \sim 0.15% tetramer-binding cells, corresponding to \sim 10⁴ total cells, in the spleen. The percentage and number of specific cells remained above background for at least 229 d after immunization in the draining LN. In the spleen, tetramer⁺ cells were visible on day 176, but tetramer staining had fallen to background levels by day 229 (Fig. 1 A). In contrast, after transfer of peptide-pulsed DCs, the response peaked at around 0.2% of CD4 T cells and had dropped to background levels (mice immunized with OVA peptide-pulsed DCs) by day 35 (Fig. 1 B).

We examined the phenotype of activated and memory tetramer⁺ cells over this period. During activation, the antigen-specific CD4 T cells up-regulated expression of CD44 and down-regulated CD62L (Fig. 1 C, Day 7). By day 9, all

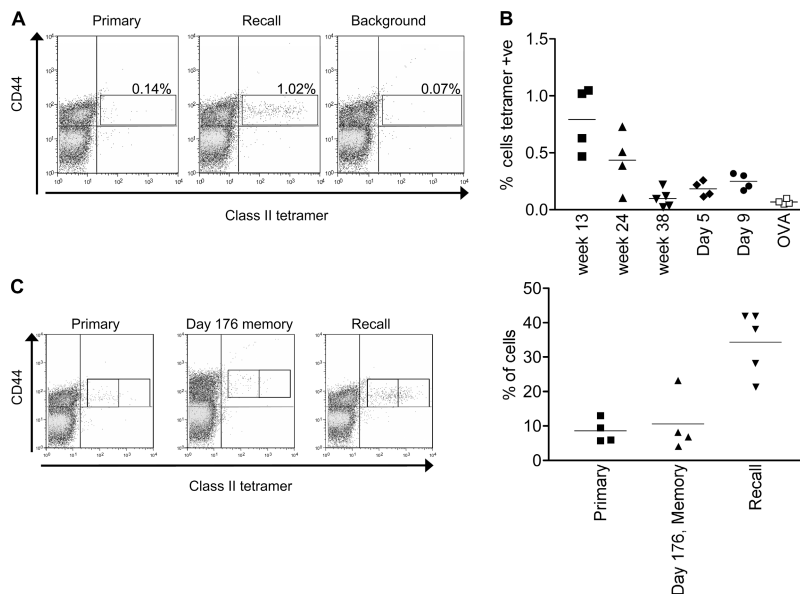


Figure 2. The recall response is faster and larger than the primary response. C57BL/6 mice were injected with H19-Env-pulsed DCs, rested for 13, 24, or 38 wk, and boosted with H19-Env-CFA. Cells from the spleens and draining LNs were stained with class II tetramers and analyzed as in Fig. 1. (A) Example staining of LN cells from the recall response after 13 wk, the primary response, and background staining. The number shows the percentage of CD44^{hi} 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.

the tetramer⁺ cells were CD44^{hi} and CD62L^{lo} and they maintained this phenotype throughout the memory phase of the response.

As IL-7R α 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 α 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 α (Fig. 1 C, middle). By day 9, although the majority of cells were IL-7R α ^{lo}, a substantial proportion was still IL-7R α ⁺. By day 14, the majority of tetramer-binding cells in the spleen were IL-7R α ⁺. However, in the draining LN, even by day 32 only ~60% 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 α ⁺, 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.

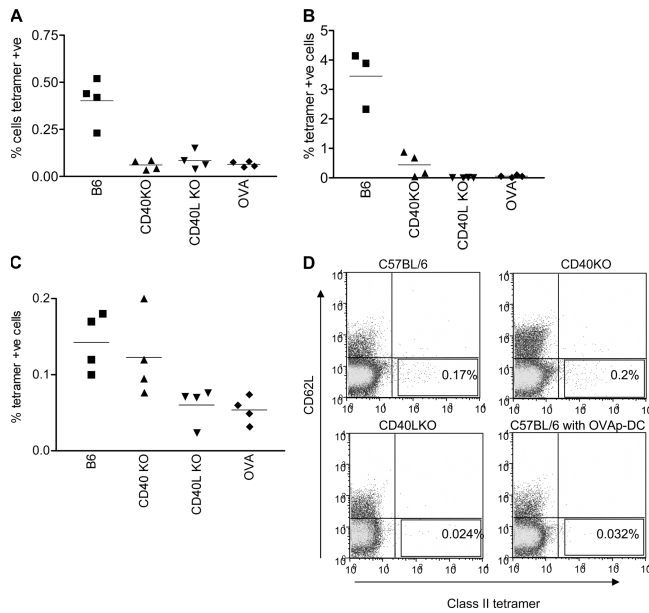


Figure 3. CD40–CD40L signals are required for T cell priming.

(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 μ g/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

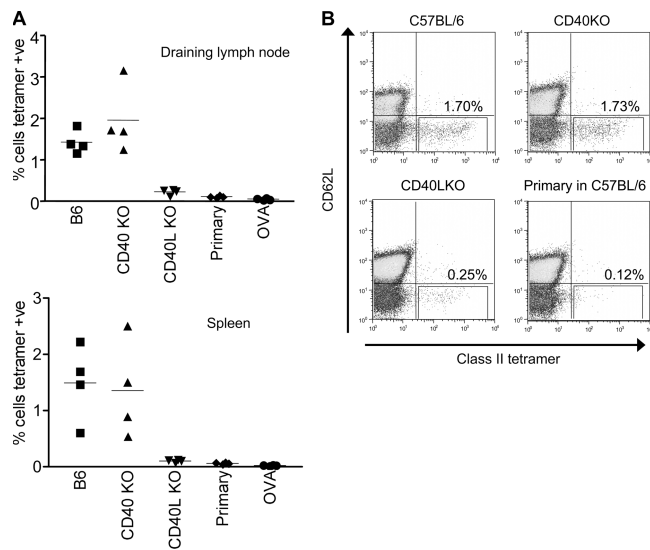


Figure 4. CD4 memory T cells survive and proliferate after boosting in CD40-deficient mice. (A) WT B6, CD40KO, and CD154KO mice were immunized s.c. with peptide-pulsed, LPS-matured WT DCs and boosted with peptide-CFA after 5 wk. Mice were killed on day 6 after boost, and draining LNs and spleens were taken for tetramer staining. Each point represents the percentage of tetramer⁺ cells out of CD4 cells in one mouse, and the line shows the mean of each group. One representative of five experiments is shown. (B) Representative FACS plots shown from the draining LNs of boosted mice, the primary response (mice immunized with only peptide-CFA) from the experiment summarized in A. Cells gated as in Fig. 1. The number of activated (CD62L^{lo}) tetramer⁺ cells as a percentage of CD4 cells is indicated. One representative of five experiments is shown.

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- γ 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 peptide-CFA or cells from naive mice. In primed and boosted CD40KO mice, the percentage of IFN- γ -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- γ effector response. Representative FACS dot plots of the IFN- γ 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- γ (in WT mice) is in line with similar recently published data (25, 26).

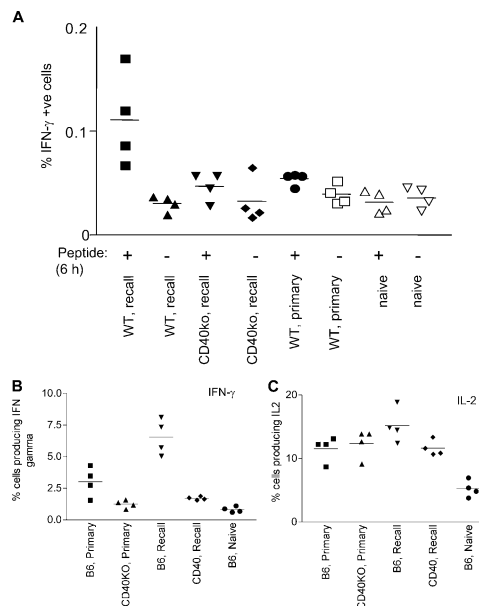


Figure 5. Effector cytokine production is reduced in CD40KO mice. Splenocytes from C57BL/6 and CD40KO mice primed with WT DCs and boosted with peptide-CFA were activated *in vitro* with peptide (A) or PMA/ionomycin (B and C). In A, the IFN- γ -producing cells were detected by cell surface cytokine capture assay and in B and C by intracellular cytokine staining. The percentages of total CD4 cells producing either IFN- γ (A and C) or IL-2 (B) are shown. Each point represents one mouse, and the line shows the mean of each group. Data are representative of three (A) or four (B and C) experiments.

We also tested IFN- γ , 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- γ response described above, only WT mice (and not CD40KO mice) exhibited a significant increase in IFN- γ -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- γ 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- γ 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

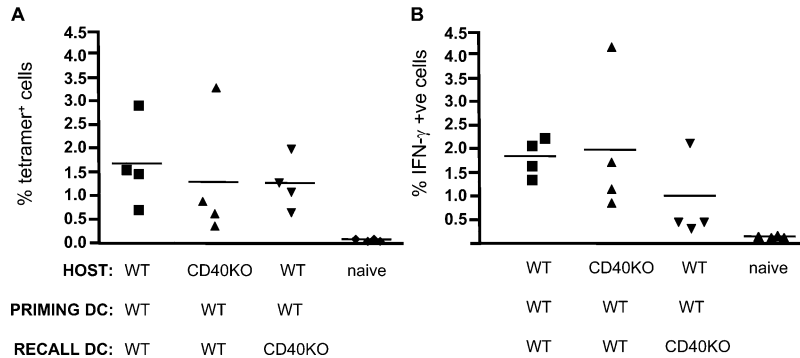


Figure 6. Recall IFN- γ secretion is dependent on CD40. WT or CD40KO mice were immunized with H19-Env peptide-pulsed, LPS-matured WT DCs. 4 wk later, the mice were boosted with peptide-pulsed, LPS-matured DCs from WT or CD40KO mice as indicated. Antigen-specific cells were detected by tetramer staining (A), and the

frequency of peptide-induced IFN- γ -secreting cells was measured by the cell surface cytokine capture assay (B). All mice were boosted with peptide in CFA at 2 wk. Each point represents one mouse, and the line shows the mean of each group. Data are representative of two such experiments.

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. Moodycliffe 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).

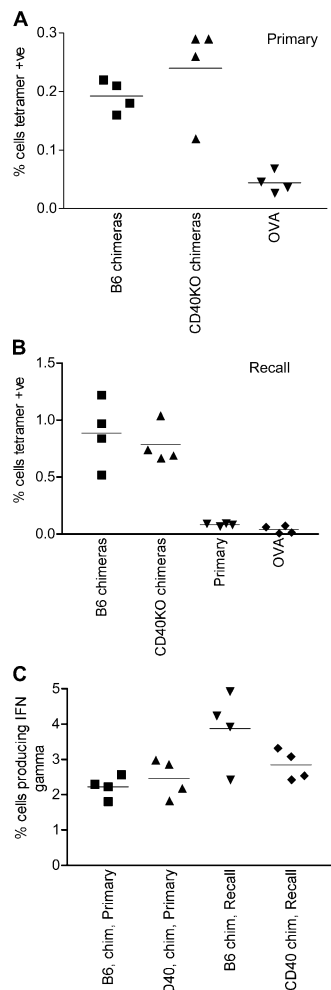


Figure 7. CD40-bearing B cells are not essential in the recall cytokine response. Mixed bone marrow chimeras were made as described in Materials and methods, such that the B cell compartment lacked CD40, whereas the majority of the DCs were CD40 sufficient. 8 wk after reconstitution, WT and B cell CD40KO chimeras were immunized s.c. with peptide-pulsed DCs. (A) Percentage of tetramer⁺ cells out of CD4 cells in C57BL/6 and CD40KO B cell chimeras primed with WT DCs s.c. Spleens and draining LNs were taken on day 7 and stained with class II tetramers. (B) Percentage of tetramer⁺ cells out of CD4 cells after priming with DCs and boosted with H19-Env-CFA after 10 wk. (C) Percentage of CD4 cells producing IFN- γ in the primary and secondary response after incubation in vitro with PMA/ionomycin as in Fig. 5 (B and C). Each point represents one mouse, and the line shows the mean of each group. Results are representative of three experiments.

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

long-term memory cells, but no effectors, to develop, representing a divergent pathway of differentiation (43).

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., CD62L^{lo}, CD44^{hi}). 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-7R α) 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-7R α (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-7R α (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 μ g H19-Env (EPLTSL-TPRCNTAWNRLKL) or OVA_{323–339} peptide (ISQAVHAAHAEINEA-GR) that binds to H2-A^b (both 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 DC 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 μ g/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 \times 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. Phycoerythrin-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-7R α (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 \times 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 U/ml penicillin, and 100 μ g/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 μ g/ml LPS (Sigma-Aldrich) for 12–13 h, and then reharvested. The DCs were incubated for 90 min with 50 μ g/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 10⁶ cells per well and incubated for 3 d at 37°C and 5% CO₂ with 1 μ g/ml H19-Env peptide in IMDM supplemented with 5% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (all from Invitrogen), and 50 μ M 2-mercaptoethanol (BDH Laboratories).

Detection of antigen-specific IFN- γ . Single cell suspensions were prepared as described above, and splenocytes were cultured with 8 μ g/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 μ g/ml streptomycin. The production of IFN- γ was then assessed using an IFN- γ capture and detection kit (Miltenyi Biotec) according to the manufacturer's instructions. In brief, the cells were harvested,

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 MACSmix 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 $4\text{--}5 \times 10^6$ cells per well in 24-well plates and incubated for 4 h with 10 ng/ml PMA and 1 $\mu\text{g}/\text{ml}$ ionomycin (both from Sigma-Aldrich) in the presence of Golgi Stop (Becton Dickinson). Cells were transferred into V-shaped 96-well plates and washed with FACS buffer before staining at 4°C with APC-labeled anti-CD4 (BD Biosciences) for 15 min. After washing, cells were fixed for 20 min with Cytofix and then washed in Cytoperm (Becton Dickinson). Anti-cytokine antibodies (anti-IL-2-PE and anti-IFN- γ -FITC; BD Biosciences) diluted in Cytoperm were added for 45 min. The cells were washed in Cytoperm three times, resuspended in PBS, and acquired on a FACSCalibur.

Bone marrow chimeras. Recipient mice were lethally irradiated with 1150c Gy 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 magnetic 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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REFERENCES

- Kroczek, R.A., H.W. Mages, and A. Hutloff. 2004. Emerging paradigms of T-cell co-stimulation. *Curr. Opin. Immunol.* 16:321–327.
- Grewal, I.S., and R.A. Flavell. 1998. CD40 and CD154 in cell-mediated immunity. *Annu. Rev. Immunol.* 16:111–135.
- Banchereau, J., F. Bazan, D. Blanchard, F. Briere, J.P. Galizzi, C. van Kooten, Y.J. Liu, F. Rousset, and S. Saeland. 1994. The CD40 antigen and its ligand. *Annu. Rev. Immunol.* 12:881–922.
- Cella, M., D. Scheidegger, K. Palmer-Lehmann, P. Lane, A. Lanzavecchia, and G. Alber. 1996. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T–T help via APC activation. *J. Exp. Med.* 184:747–752.
- Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heuffer, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184:741–746.
- Howland, K.C., L.J. Ausubel, C.A. London, and A.K. Abbas. 2000. The roles of CD28 and CD40 ligand in T cell activation and tolerance. *J. Immunol.* 164:4465–4470.
- Yang, Y., and J.M. Wilson. 1996. CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. *Science*. 273:1862–1864.
- Whitmire, J.K., M.K. Slifka, I.S. Grewal, R.A. Flavell, and R. Ahmed. 1996. CD40 ligand-deficient mice generate a normal primary cytotoxic T-lymphocyte response but a defective humoral response to a viral infection. *J. Virol.* 70:8375–8381.
- Oxenius, A., K.A. Campbell, C.R. Maliszewski, T. Kishimoto, H. Kikutani, H. Hengartner, R.M. Zinkernagel, and M.F. Bachmann. 1996. CD40–CD40 ligand interactions are critical in T–B cooperation but not for other anti-viral CD4⁺ T cell functions. *J. Exp. Med.* 183:2209–2218.
- Miga, A.J., S.R. Masters, B.G. Durell, M. Gonzalez, M.K. Jenkins, C. Maliszewski, H. Kikutani, W.F. Wade, and R.J. Noelle. 2001. Dendritic cell longevity and T cell persistence is controlled by CD154–CD40 interactions. *Eur. J. Immunol.* 31:959–965.
- Hochweller, K., and S.M. Anderton. 2004. Systemic administration of antigen-loaded CD40-deficient dendritic cells mimics soluble antigen administration. *Eur. J. Immunol.* 34:990–998.
- Schepers, K., M. Toebes, G. Sotthwes, F.A. Vyth-Dreese, T.A. Dellemijn, C.J. Melief, F. Ossendorp, and T.N. Schumacher. 2002. Differential kinetics of antigen-specific CD4⁺ and CD8⁺ T cell responses in the regression of retrovirus-induced sarcomas. *J. Immunol.* 169:3191–3199.
- Iwashiro, M., T. Kondo, T. Shimizu, H. Yamagishi, K. Takahashi, Y. Matsubayashi, T. Masuda, A. Otaka, N. Fujii, and A. Ishimoto. 1993. Multiplicity of virus-encoded helper T-cell epitopes expressed on FBL-3 tumor cells. *J. Virol.* 67:4533–4542.
- Kaech, S.M., J.T. Tan, E.J. Wherry, B.T. Konieczny, C.D. Surh, and R. Ahmed. 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat. Immunol.* 4:1191–1198.
- Kondrack, R.M., J. Harbertson, J.T. Tan, M.E. McBreen, C.D. Surh, and L.M. Bradley. 2003. Interleukin 7 regulates the survival and generation of memory CD4 cells. *J. Exp. Med.* 198:1797–1806.
- Li, J., G. Huston, and S.L. Swain. 2003. IL-7 promotes the transition of CD4 effectors to persistent memory cells. *J. Exp. Med.* 198:1807–1815.
- Crawford, F., H. Kozono, J. White, P. Marrack, and J. Kappler. 1998. Detection of antigen-specific T cells with multivalent soluble class II MHC covalent peptide complexes. *Immunity*. 8:675–682.
- Rees, W., J. Bender, T.K. Teague, R.M. Kedl, F. Crawford, P. Marrack, and J. Kappler. 1999. An inverse relationship between T cell receptor affinity and antigen dose during CD4(+) T cell responses in vivo and in vitro. *Proc. Natl. Acad. Sci. USA*. 96:9781–9786.
- Savage, P.A., J.J. Boniface, and M.M. Davis. 1999. A kinetic basis for T cell receptor repertoire selection during an immune response. *Immunity*. 10:485–492.
- Busch, D.H., and E.G.P. Am. 1999. T cell affinity maturation by selective expansion during infection. *J. Exp. Med.* 189:701–710.
- Slifka, M.K., and J.L. Whitton. 2001. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat. Immunol.* 2:711–717.
- London, C.A., M.P. Lodge, and A.K. Abbas. 2000. Functional responses and costimulator dependence of memory CD4⁺ T cells. *J. Immunol.* 164:265–272.
- Croft, M., L.M. Bradley, and S.L. Swain. 1994. Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* 152:2675–2685.

24. Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha. 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat. Immunol.* 1:47–53.
25. Foulds, K.E., L.A. Zenewicz, D.J. Shedlock, J. Jiang, A.E. Troy, and H. Shen. 2002. Cutting edge: CD4 and CD8 T cells are intrinsically different in their proliferative responses. *J. Immunol.* 168:1528–1532.
26. Smith, K.M., J.M. Brewer, C.M. Rush, J. Riley, and P. Garside. 2004. In vivo generated Th1 cells can migrate to B cell follicles to support B cell responses. *J. Immunol.* 173:1640–1646.
27. van Essen, D., P. Dullforce, T. Brocker, and D. Gray. 2000. Cellular interactions involved in Th cell memory. *J. Immunol.* 165:3640–3646.
28. Linton, P.J., J. Harbertson, and L.M. Bradley. 2000. A critical role for B cells in the development of memory CD4 cells. *J. Immunol.* 165:5558–5565.
29. Fillatreau, S., and D. Gray. 2003. T cell accumulation in B cell follicles is regulated by dendritic cells and is independent of B cell activation. *J. Exp. Med.* 197:195–206.
30. Gupta, M., E. Satyaraj, J.M. Durdik, S. Rath, and V. Bal. 1997. Differential regulation of T cell activation for primary versus secondary proliferative responses. *J. Immunol.* 158:4113–4121.
31. Moodycliffe, A.M., V. Shreedhar, S.E. Ullrich, J. Walterscheid, C. Bucana, M.L. Kripke, and L. Flores-Romo. 2000. CD40–CD40 ligand interactions in vivo regulate migration of antigen-bearing dendritic cells from the skin to draining lymph nodes. *J. Exp. Med.* 191:2011–2020.
32. Gudmundsdottir, H., A.D. Wells, and L.A. Turka. 1999. Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *J. Immunol.* 162:5212–5223.
33. Itano, A.A., S.J. McSorley, R.L. Reinhardt, B.D. Ehst, E. Ingulli, A.Y. Rudensky, and M.K. Jenkins. 2003. Distinct dendritic cell populations sequentially present antigen to CD4 T cells and stimulate different aspects of cell-mediated immunity. *Immunity.* 19:47–57.
34. Schulz, O., D.A. Edwards, M. Schito, J. Aliberti, S. Manickasingham, A. Sher, and C. Reis e Sousa. 2000. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity.* 13:453–462.
35. Gramaglia, I., A. Jember, S.D. Pippig, A.D. Weinberg, N. Killeen, and M. Croft. 2000. The OX40 costimulatory receptor determines the development of CD4 memory by regulating primary clonal expansion. *J. Immunol.* 165:3043–3050.
36. Rogers, P.R., J. Song, I. Gramaglia, N. Killeen, and M. Croft. 2001. Ox40 promotes bcl-xl and bcl-2 expression and is essential for long-term survival of cd4 T cells. *Immunity.* 15:445–455.
37. Zammit, D.J., L.S. Cauley, Q.M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity.* 22:561–570.
38. Filatenkov, A.A., E.L. Jacovetty, U.B. Fischer, J.M. Curtsinger, M.F. Mescher, and E. Ingulli. 2005. CD4 T cell-dependent conditioning of dendritic cells to produce IL-12 results in CD8-mediated graft rejection and avoidance of tolerance. *J. Immunol.* 174:6909–6917.
39. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science.* 297:2060–2063.
40. Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. Von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat. Immunol.* 4:225–234.
41. Opferman, J.T., B.T. Ober, and P.G. Ashton-Rickardt. 1999. Linear differentiation of cytotoxic effectors into memory T lymphocytes. *Science.* 283:1745–1748.
42. Hu, H., G. Huston, D. Duso, N. Lepak, E. Roman, and S.L. Swain. 2001. CD4+ T cell effectors can become memory cells with high efficiency and without further division. *Nat. Immunol.* 2:705–710.
43. Lanzavecchia, A., and F. Sallusto. 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science.* 290:92–97.
44. Roman, E., E. Miller, A. Harmsen, J. Wiley, U.H. Von Andrian, G. Huston, and S.L. Swain. 2002. CD4 effector T cell subsets in the response to influenza: heterogeneity, migration, and function. *J. Exp. Med.* 196:957–968.
45. Ahmadzadeh, M., S.F. Hussain, and D.L. Farber. 2001. Heterogeneity of the memory CD4 T cell response: persisting effectors and resting memory T cells. *J. Immunol.* 166:926–935.
46. Marzo, A.L., K.D. Klonowski, A. Le Bon, P. Borrow, D.F. Tough, and L. Lefrancois. 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat. Immunol.* 6:793–799.
47. Lacombe, M.H., M.P. Hardy, J. Rooney, and N. Labrecque. 2005. IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization. *J. Immunol.* 175:4400–4407.
48. Merica, R., A. Khoruts, K.A. Pape, R.L. Reinhardt, and M.K. Jenkins. 2000. Antigen-experienced CD4 T cells display a reduced capacity for clonal expansion in vivo that is imposed by factors present in the immune host. *J. Immunol.* 164:4551–4557.
49. Homann, D., L. Teyton, and M.B. Oldstone. 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. *Nat. Med.* 7:913–919.
50. London, C.A., V.L. Perez, and A.K. Abbas. 1999. Functional characteristics and survival requirements of memory CD4+ T lymphocytes in vivo. *J. Immunol.* 162:766–773.
51. Varga, S.M., and R.M. Welsh. 1998. Stability of virus-specific CD4+ T cell frequencies from acute infection into long term memory. *J. Immunol.* 161:367–374.
52. Gray, D. 2002. A role for antigen in the maintenance of immunological memory. *Nat. Rev. Immunol.* 2:60–65.
53. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1:167–178.
54. Xu, J., T.M. Foy, J.D. Laman, E.A. Elliott, J.J. Dunn, T.J. Waldschmidt, J. Elsemore, R.J. Noelle, and R.A. Flavell. 1994. Mice deficient for the CD40 ligand. *Immunity.* 1:423–431.
55. Inaba, K., M. Inaba, N. Romani, H. Aya, M. Deguchi, S. Ikehara, S. Muramatsu, and R.M. Steinman. 1992. Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* 176:1693–1702.
56. Stockinger, B., T. Zal, A. Zal, and D. Gray. 1996. B cells solicit their own help from T cells. *J. Exp. Med.* 183:891–899.