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CD40 on APCs Is Needed for Optimal Programming, Maintenance, and Recall of CD8+ T Cell Memory Even in the Absence of CD4+ T Cell Help

Maria Genevive H. Hernandez,*† Lianjun Shen,* and Kenneth L. Rock2*

CD40 stimulation is one of the many signals that can activate APCs and we have recently shown it to have a unique function in generating maximum primary CD8+ T cell responses. However, whether CD40 signaling plays a role in memory CD8+ T cell responses is still not completely understood. In this study, we show that in the absence of CD40 on all APCs or specifically on dendritic cells, memory CD8+ T cells are generated but at significantly reduced levels. This reduction is due to a contribution of CD40 at several different steps in the generation of CD8+ memory. In the initial T cell response, CD40 contributes to maximizing not only the number of effector cells that are generated but also the programming of ones that will differentiate into memory. Subsequently, CD40 is needed to maintain maximal numbers of the committed memory cells in a manner that is independent of the immunizing Ag. Finally, when memory CD8+ T cells are reactivated there is a variable requirement for CD40 depending on whether CD40 or CD4+ Th cells were present during the primary response. Therefore, CD40 signaling on APCs plays an important role in all phases of a memory CD8+ T cell response. The Journal of Immunology, 2008, 180: 4382–4390.

Following exposure to Ag, naive CD8+ T cells undergo a proliferation and differentiation pathway that culminates in the establishment of long-lived memory. Full memory differentiation is a gradual process that is accompanied by stable changes in gene expression and takes several weeks to complete (1). The initial encounter of naive CD8+ T cells with Ag has been demonstrated to induce an instructional program for memory development (2–4). However, the signals that are required for the development and function of memory CD8+ T cells are still not completely defined.

Various cytokines have been implicated in generation and maintenance of memory CD8+ T cells. For example, inflammatory cytokines such as type I IFNs and IL-12 have been shown to increase the expansion of effector CD8+ T cells and also enhance their differentiation into memory cells (5–9). Three members of the γ-chain family of cytokines—IL-2, IL-7, and IL-15—are also important for CD8+ T cell memory. IL-2 signals, during both priming and reactivation, are required for secondary expansion of memory CD8+ T cells (10, 11). Meanwhile, IL-15 is critical in the generation as well as homeostasis of memory CD8+ T cells (12). IL-7 is also important in the maintenance of memory CD8+ T cells (12, 13). In connection with this, it has been shown that IL-7R is expressed in memory CD8+ T cell precursors as well as long-lived memory CD8+ T cells (14–16).

CD40 is a member of the TNFR superfamily that is expressed constitutively on all APCs while its ligand, CD40L/CD154 is expressed mainly on activated CD4+ T cells (17–19). Binding of CD40L to CD40 results in potent APC activation and is considered to be the mechanism by which CD4+ T cells provide help for the induction of primary CD8+ T cell responses (20–23). Several studies have shown that CD40-CD40L interactions are important for antiviral CD8+ T cell memory. Although CD40L−/− mice mount strong primary CTL responses to viruses such as lymphocytic choriomeningitis virus (LCMV), Pichinde, and vesicular stomatitis virus, they are unable to permanently control virus replication. This has been demonstrated to be largely due to the generation of lower numbers of memory CD8+ T cells and not due to a defect in their maintenance (24–29). In addition, it has also been found that for the noninflammatory male HY Ag, CD40 expression on CD8+ T cells plays a role in memory generation (30). However, a number of reports have also demonstrated that CD40 is dispensable for the development of functional CD8+ T cell memory upon immunization with virus-like particles or infection with other viruses or bacteria (31–34).

It has long been known that in many situations, CD4+ T cell help is required to generate effective CD8+ T cell memory (35, 36). The absence of memory CD8+ T cell activity in mice lacking functional CD4+ T cells was initially attributed to inefficient priming of naive CD8+ T cells. However, several studies have demonstrated that CD4+ T cell help is required for the long-term maintenance and secondary expansion of memory CD8+ T cells (37–42). The exact mechanism by which CD4+ T cells promote memory generation and maintenance is still unknown. Aside from APC licensing, it is thought that it involves direct CD40-CD40L interactions between CD4+ T cells and CD8+ T cells (30). Additionally, in viral infections, CD40-dependent Ab responses may limit the viral load and prevent clonal

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exhaustion (43). However, the requirement for CD4+ T cells in long-lasting CD8+ T cell memory is not absolute, as fully functional memory CD8+ T cells can be detected in some systems despite the absence of CD4+ T cells or B cells (44, 45).

We have recently shown that CD40-CD40L interactions between dendritic cells (DCs) and Ag-specific CD8+ T cells are important in inducing maximal primary responses independent of a requirement for CD24+ T cell help (46). In this study, we investigate whether CD40 signaling also plays a role in the generation, maintenance, and function of memory CD8+ T cells. Using two different systems, LCMV infection and DC-based immunization, we show that the absence of CD40 results in impaired CD8+ T cell memory whether CD4+ T cell help is present during priming or not. CD40 contributes at many stages during the memory response including influencing the initial clonal expansion of activated cells, the commitment to differentiation into memory cells, and their subsequent maintenance. Moreover, depending on the signals that were received during these various steps, CD40 can also sometimes play a role in the reactivation of established memory cells.

**Materials and Methods**

**Mice**

C57BL/6J (wild-type (WT)) and B6.129P2-Cd40tm1Kik (CD40+/-) mice were purchased from The Jackson Laboratory. CD40+/- breeders were also obtained from The Jackson Laboratory as well as D. Greiner (University of Massachusetts Medical School, Worcester, MA) and used as a source of mice. Mice used as hosts were from 5 to 10 wk of age. P-14 TCR-transgenic (Tg) breeders were originally obtained from R. Welsh (University of Massachusetts Medical School, Worcester, MA) and then bred with B6.SJL-Ptprca Pep3b/Boy mice to yield CD45.1 congenic marker. One day later, we infected the hosts with Cd40+/- mice expressing the CD45.1 T cell marker. All mice were bred and housed in specific pathogen-free conditions at the University of Massachusetts Medical School animal facility.

**Cells**

Bone marrow-derived DCs (BMDC) were generated by flushing cells from femurs and tibias of mice and culturing them in complete medium, with 10 ng/ml GM-CSF and 5 ng/ml IL-4 (Corixa) added on days 1 and 4. On day 7, both adherent and nonadherent cells were harvested and incubated for 2–4 h at 37°C with the SIINFEKL peptide from chicken OVA (OVA peptide) or the KAVYNFATC peptide from LCMV glycoprotein (gp33 peptide) at a concentration of 1 μg of peptide per 5 x 10^6 cells. The cells were washed once with complete medium and twice with HBSS (Invitrogen Life Technologies) before immunization.

**Adoptive transfer and virus infection**

Spleen and lymph node cells from P-14/CD45.1+ mice were depleted of RBC, labeled with CFSE, and injected i.v. into new hosts containing P-14 cells on day 4 postchallenge. The P-14 cells proliferated robustly in both WT and CD40-/- hosts, with the peak of expansion occurring at days 5–6 postinfection. However, even though the P-14 cells expanded considerably in the CD40-/- hosts, in the majority of experiments (five of seven) there was a small, but statistically significant, increase in the number of P-14 cells in the WT hosts at the peak of the response (Fig. 1A). For the cells that had responded in the WT vs CD40-/- hosts, there was initially no difference in the percentage of IFN-γ-producing cells (Fig. 1B). This indicates that the cells that proliferated were able to differentiate into functional effectors whether CD40 was present on host APCs or not.

WT mice containing memory P-14 cells were also challenged i.v. with WT or CD40-/- DCs pulsed with the gp33 peptide. Memory P-14 expansion and IFN-γ production were likewise analyzed on day 4 postchallenge.

**DC immunization and assessment of endogenous CD8+ T cell memory**

WT mice were immunized i.v. with 1 x 10^6 WT or CD40-/- DCs pulsed with OVA peptide. In some cases, the hosts were depleted of CD4+ T cells using the GK1.5 Ab before immunization. After at least 6 wk, the mice were challenged i.v. with 1 x 10^6 OVA peptide-pulsed WT or CD40-/- DCs. Memory responses were evaluated on day 4 postchallenge through in vivo CTL assay and intracellular IFN-γ staining.

**Abs and FACS analysis**

Samples were depleted of RBC and then stained with combinations of the following Abs: PerCP-anti-CD8 (53-6.7; BD Pharmingen), PE- or allophycocyanin-anti-CD45.1 (A20; eBioscience), Annexin V FL (BD Pharmingen), allophycocyanin-anti-CD127 (A7R34; eBioscience), and PE-anti-CD62L (MEL-14; eBioscience). Flow cytometry was done with a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

**In vivo CTL assay**

The in vivo CTL assay was performed as described previously (47). Briefly, splenocyte targets were pulsed with OVA peptide or control peptide and labeled with different concentrations of CFSE. The targets were then mixed at a 1:1 ratio and injected i.v. into immunized and unimmunized control mice. After 12–20 h, spleen or blood was collected and the percentage of target cell killing was calculated using the formula: 100 – ((percentage of relevant peptide pulsed in immunized/percentage of irrelevant peptide pulsed in control)/(percentage of relevant peptide pulsed in control/percentage of irrelevant peptide pulsed in control)) x 100.

**Intracellular IFN-γ staining**

Spleen and blood samples were depleted of RBC and incubated with the indicated peptide in the presence of brefeldin A (Golgi Plug; BD Pharmingen) and rIL-2 for 5 h at 37°C. The cells were then stained with anti-CD8 and -CD45.1 Ab, fixed, and permeabilized using cytotox/Cytoperm buffer (BD Pharmingen), and stained with anti-IFN-γ (XMG1.2; BD Pharmingen). The samples were washed twice with perm/wash buffer and analyzed by flow cytometry.

**Statistics**

Data were analyzed for statistical significance with a two-tailed Student t test using Microsoft Excel software. Differences in T cell responses were considered significant when a probability value of p < 0.05 was obtained.

**Results**

**Decreased numbers of memory cells in CD40-/- hosts**

We sought to determine whether CD40 plays a role in memory CD8+ T cell generation, programming, maintenance, and reactivation. To have a better understanding of what role CD40 plays in each of these processes, we first used an adoptive transfer system wherein we injected WT and CD40-/- mice with CFSE-labeled P-14 (LCMV gp33-specific TCR Tg) T cells expressing the CD45.1 congenic marker. One day later, we infected the hosts with LCMV (Armstrong strain) to stimulate strong primary responses. We then monitored the P-14 response by staining for CD8+ T cells. All P-14 cells were depleted of RBC, labeled with CFSE, and injected i.v. into new hosts containing P-14 cells on day 4 postchallenge. The P-14 cells proliferated robustly in both WT and CD40-/- hosts, with the peak of expansion occurring at days 5–6 postinfection. However, even though the P-14 cells expanded considerably in the CD40-/- hosts, in the majority of experiments (five of seven) there was a small, but statistically significant, increase in the number of P-14 cells in the WT hosts at the peak of the response (Fig. 1A). For the cells that had responded in the WT vs CD40-/- hosts, there was initially no difference in the percentage of IFN-γ-producing cells (Fig. 1B). This indicates that the cells that proliferated were able to differentiate into functional effectors whether CD40 was present on host APCs or not.
When we followed the fate of the T cells that had responded in the primary response, we found that the P-14 cells in the CD40-/- animals underwent a greater contraction compared with those in the WT hosts. The degree of contraction was calculated by dividing the P-14 frequency at the indicated time point by the P-14 frequency at the peak of the response (day 6). Thus, while ~50% of the peak P-14 population remained in the WT hosts 2 wk postinfection, only ~30% remained in the CD40-/- hosts (Fig. 1C). By 4 wk postinfection, the P-14 numbers had gone down to 34 and 16% of the respective peak responses (Fig. 1C). These differences in contraction between WT and CD40-/- hosts were statistically significant. Nevertheless, the P-14 cells that remained in the WT and CD40-/- hosts had the same phenotype: they were CFSE-negative, CD44high, and contained similar proportions of both CD62Lhigh and CD62Llow cells (data not shown).

In some experiments (two of seven), the initial P14 T cell response was similar in magnitude between WT and CD40-deficient hosts. The basis for this variation between experiments is not clear but it is useful because it allowed us to follow cohorts of cells that were initially equal in number at the peak of the primary response. Even when the initial response was identical in the CD40-positive and -negative hosts, this parity was not maintained and at later time points the number of P-14 cells remaining in the CD40-/- hosts was ~2-fold less compared with the WT hosts (Fig. 1D). Examination of earlier time points revealed that whereas the P-14 population in the WT hosts was still at peak levels 1 wk after infection, the P-14 population in the CD40-/- hosts had already started to decline (data not shown). Not only was the rate of P-14 contraction faster in the CD40-/- hosts, but the magnitude of contraction was also greater.

Overall, these results indicate that CD40 signaling is not necessary to generate memory CD8+ T cells. However, in the absence of CD40 there is a roughly 2-fold reduction in the number of functional memory cells. Because this reduction is seen even in experiments where the initial T cell expansion is equal in CD40-positive and -negative hosts, CD40 must play a role in memory generation beyond just influencing the magnitude of the primary response.

Reduced memory CD8+ T cells numbers in CD40-/- hosts is not due to differences in apoptosis

We investigated whether the reduced numbers of memory CD8+ T cell in CD40-/- hosts was due to increased apoptosis of CD8+ T cells. On day 4 postinfection, the majority of P-14 cells in both the WT and CD40-/- hosts were annexin V+, while the host CD8+ T cells exhibited very little annexin V binding (Fig. 2A and data not shown). This is consistent with a previous study of LCMV infection showing similarly high levels of annexin V staining in virus-specific CD8+ T cells (48). In comparing the T cells in WT and CD40-/- hosts, we found a small but statistically greater increase in the percentage of annexin V+ cells in the CD40-deficient environment (Fig. 2A). By day 6 postinfection, the percentage as well as the mean fluorescence intensity (MFI) of annexin V+ P-14 cells had started to decrease (Fig. 2). By day 12 postinfection, the percentage as well as MFI of annexin V+ P-14 cells had returned to similar levels as that of the host CD8+ T cells (Fig. 2) and we detected no further changes in annexin V staining at later time points (data not shown). Because the proportion of P-14 cells that bind annexin V as well as the intensity of annexin V binding was the same at all but one of the time points examined, the difference in memory CD8+ T cell numbers between WT and CD40-/- hosts is most likely not due to an increased rate of apoptosis. However, we cannot exclude the possibility that differences in the rate of apoptosis that are too small to reliably measure could still contribute to the reduction in memory CD8+ T cells that we observed.

Reduced memory CD8+ T cell numbers in CD40-/- hosts correlate with IL-7Ra expression

There have been recent reports that IL-7Ra expression serves as a marker for identifying effector cells that will develop into memory cells (14–16). We therefore decided to examine IL-7Ra expression by the P-14 cells. At the peak of the response (day 6), the percentage of IL-7Ra+ P-14 cells was similarly low in the WT and
CD40−/− hosts (25 vs 29%; Fig. 3). This finding is consistent with the down-regulation of IL-7Rα expression by effector cells. By day 12, however, while the percentage of IL-7Rα+ P-14 cells in the WT hosts had started to increase, the percentage of IL-7Rα+ P-14 cells in the CD40−/− hosts remained low (44 vs 27%; Fig. 3).

Furthermore, whereas ~70% of the P-14 cells in the WT hosts had reacquired IL-7Rα expression by day 25, only ~33% of the P-14 cells in the CD40−/− hosts had done so (Fig. 3). Interestingly, this 2-fold difference in IL-7Rα expression correlates with the roughly 2-fold difference in memory CD8+ T cell numbers between WT and CD40−/− hosts.

**CD40 signals both during and after priming influence memory CD8+ T cell differentiation and survival**

We next investigated whether CD40 signaling is still important once the naive CD8+ T cells have clonally expanded and are undergoing differentiation into memory cells. To examine this issue, we transferred P-14 cells from LCMV-infected mice into new, uninfected WT and CD40−/− hosts. We harvested spleens 11–13 days postinfection to ensure that the virus has been cleared. We also normalized the number of splenocytes that we injected such that they contained equal numbers of P-14 cells. At the indicated time points, the mice were bled and the survival of transferred P-14 T cells was determined. A, Survival of P-14 T cells primed in WT or CD40−/− hosts and transferred into WT hosts. B, Survival of P-14 T cells primed in WT hosts and transferred into WT or CD40−/− hosts. The data are presented as the percentage of P-14 T cells of the total CD8+ T cells in the blood. The results shown are representative of three independent experiments with three to five mice per group. ***, p < 0.005; **, p < 0.01; *, p < 0.001.
greater cell loss when we transferred the P-14 effector cells that have been primed in CD40<sup>−/−</sup> hosts into new WT hosts (Fig. 4A). By 1 wk posttransfer, there was an ~3-fold difference in the number of P-14 cells that initially came from the WT compared with the CD40<sup>−/−</sup> hosts. At later time points, there was a further decrease in the number of P-14 cells that came from CD40<sup>−/−</sup> hosts. Therefore, during the primary response CD40 in the host environment appears to be necessary to program CD8<sup>+</sup> T cells for optimal differentiation into long-lived memory cells.

Interestingly, when P-14 effector cells that were activated in WT mice were transferred into CD40-deficient hosts, there was also a marked reduction in their cell number (Fig. 4B). This indicates that after T cells have been activated and have gone through their rounds of proliferation, there continues to be a role for CD40 signaling in the continued development and/or maintenance of memory CD8<sup>+</sup> T cells.

**Memory cells generated in the absence of CD40 are functional**

The above data indicate that in the absence of CD40, memory cells are generated, albeit at lower levels than in the presence of CD40. We investigated whether the memory cells generated in the absence of CD40 were fully functional. Upon stimulation ex vivo with gp33 peptide 10 wk after infection, the percentage of IFN-γ-producing P-14 cells in WT and CD40<sup>−/−</sup> hosts was similar (Fig. 5A).

We next investigated whether the memory cells generated in the presence or absence of CD40 were as functional upon a secondary antigenic challenge in vivo. To address this issue, we challenged the WT hosts that received effector P-14 T cells from either WT or CD40<sup>−/−</sup> mice with a vaccinia virus construct that expresses the LCMV glycoprotein (Vac-gp). We performed the challenge 10 wk posttransplant, when the effector cells had already differentiated into memory cells, and we monitored memory responses by looking at the expansion of CD45.1<sup>+</sup> P-14 T cells in the blood. Memory P-14 T cells that were initially primed in CD40<sup>−/−</sup> hosts were able to expand considerably upon challenge. However, when compared with the memory cells that were initially primed in WT hosts, their expansion was slightly reduced, although the difference was not statistically significant (Fig. 5B). In terms of effector function, the percentage of IFN-γ-producing memory P-14 T cells was also similar (Fig. 5C).

To examine whether CD40 is required for optimal reactivation of memory CD8<sup>+</sup> T cells, we challenged WT mice that were infected at least 3 mo previously with LCMV, and which contained memory P-14 cells, with WT or CD40<sup>−/−</sup> DCs that have been pulsed with gp33 peptide. We detected an increase in memory P-14 cell numbers 4 days after challenge with peptide-pulsed DCs. However, WT and CD40<sup>−/−</sup> DCs induced similar levels of memory P-14 expansion (Fig. 5D). When we looked at memory effector function, we found that the percentage of IFN-γ-producing P-14 cells was the same regardless of whether WT or CD40<sup>−/−</sup> DCs were used for challenge (Fig. 5E).

Taken together, these results show that the memory cells that develop in the absence of CD40 are functional and that CD40 signaling on DCs is not required during the recall of memory CD8<sup>+</sup> T cell responses; an exception to this conclusion will be described below.

**Priming with CD40<sup>−/−</sup> DCs leads to a weaker memory response**

The experiments described above indicate that CD40 expression on host APCs can affect the generation of CD8<sup>+</sup> T cell memory but do not define the specific APC involved in this process. We previously showed that CD40 signaling on APCs, specifically on DCs, is involved in maximizing primary CD8<sup>+</sup> T cell responses. Therefore, we examined the importance of CD40 signaling on DCs in the above processes. Furthermore, we sought to extend our findings to an endogenous system that contains normal frequencies of polyclonal Ag-specific CD8<sup>+</sup> T cells.

To determine whether CD40 signaling on DCs plays a role in CD8<sup>+</sup> T cell memory, we first evaluated memory responses in mice that were immunized with WT or CD40<sup>−/−</sup> DCs. We injected WT mice i.v. with WT or CD40<sup>−/−</sup> DCs pulsed with OVA peptide and examined in vivo CTL responses 1 wk later to make sure that priming occurred (data not shown). We then waited at least 6 wk after immunization and challenged the mice i.v. with OVA peptide-pulsed WT DCs. Endogenous memory responses were evaluated by looking at in vivo CTL activity against OVA peptide-pulsed targets as well as IFN-γ production. Mice immunized with...
WT DCs showed strong memory CTL responses, exhibiting as much as 90% target cell killing by day 4 postchallenge (Fig. 6A). In contrast, mice immunized with CD40<sup>−/−</sup> DCs mounted significantly weaker memory CTL responses, with only ~43% target cell killing (Fig. 6A). In control naive mice that were injected with peptide-pulsed DCs for the first time, CTL activity was not observed until day 7 postchallenge (data not shown); therefore, the responses we are measuring at day 4 are due to the recall of memory cells. Analysis of cytokine production by spleen cells directly ex vivo also revealed that mice immunized with CD40<sup>−/−</sup> DCs have less IFN-γ-producing cells upon challenge compared with mice immunized with WT DCs (Fig. 6B). We obtained similar data when we performed the challenge 3 mo after immunization (data not shown). These results imply that naive CD8<sup>+</sup> T cells primed in the absence of CD40 can develop into memory cells; therefore, CD40 stimulation of APCs during priming is not absolutely required to induce a memory CD8<sup>+</sup> T cell response. However, there is an ~2-fold reduction in memory responses in mice immunized with CD40<sup>−/−</sup> DCs, which is similar to what we have previously observed for primary responses.

Memory CD8<sup>+</sup> T cell responses in the absence of CD4<sup>+</sup> T cell help

Several groups have reported that the presence of CD4<sup>+</sup> Th cells only during the priming of CD8<sup>+</sup> T cells is essential in the development of functional CD8<sup>+</sup> T cell memory (37, 38, 40). These findings were obtained using immunization with tumor cells or infection with vaccinia virus or *Listeria monocytogenes* to induce primary responses. We examined whether the CD8<sup>+</sup> T cell memory induced by DC immunization had a similar dependence on CD4<sup>+</sup> T cell help. Moreover, because it is generally assumed that CD40 stimulation is provided by Th cells, we also determined whether the reduction in memory responses that we observed in CD40<sup>−/−</sup> DC-immunized mice was due to the involvement of CD4<sup>+</sup> T cells. We immunized host mice that were acutely depleted of CD4<sup>+</sup> T cells with OVA peptide-pulsed WT and CD40<sup>−/−</sup> DCs and challenged them 6 wk later with WT DCs. Treatment of host mice with the anti-CD4 Ab GK1.5 for 2 consecutive days before immunization resulted in loss of CD4<sup>+</sup> T cells lasting up to 2 wk. Surprisingly, we detected memory CD8<sup>+</sup> T cell responses even when we depleted the host mice of CD4<sup>+</sup> T cells during priming. In fact, there was no difference in target cell killing between CD4-sufficient and CD4-deficient mice (Fig. 6A). More interestingly, in mice that were depleted of CD4<sup>+</sup> T cells, the memory CTL activity was still weaker in the mice that were immunized with CD40<sup>−/−</sup> DCs compared with the mice that were immunized with WT DCs (Fig. 6A). This result is reminiscent of the data we have previously obtained for primary responses. It suggests that the effect of CD40 on memory CD8<sup>+</sup> T cell responses can likewise be independent of CD4<sup>+</sup> T cell help.

Variable requirement for CD40 during endogenous memory CD8<sup>+</sup> T cell responses

Finally, we investigated whether CD40 signaling on DCs is involved in the recall response of memory CD8<sup>+</sup> T cells. We challenged mice immunized with WT or CD40<sup>−/−</sup> peptide-pulsed with
either WT or CD40−/− DCs pulsed with the same peptide. Consistent with our results described above, we observed equally strong memory CTL responses when mice that were immunized with WT DCs were challenged with either WT or CD40−/− DCs (Fig. 7A). Surprisingly, however, mice that were immunized with CD40−/− DCs showed much lower memory CTL activity when challenged with CD40−/− DCs compared with WT DCs (Fig. 7A). In other words, memory cells that were generated in the absence of CD40 still require CD40-sufficient APCs for an optimal response.

Interestingly, depletion of CD4+ T cells at the time of immunization also resulted in reduced memory CTL activity in mice immunized with WT DCs and challenged with CD40−/− DCs (Fig. 7B). Moreover, CD4-depleted mice that were both immunized and challenged with CD40−/− DCs had the most striking reduction in memory CTL responses (Fig. 7B). Altogether, these results indicate that for memory cells the signals received during their generation can influence their subsequent need for CD40 on APCs. Moreover, the results suggest that these “programming signals” can be provided by either CD40 or other signals from CD4+ T cells.

Discussion

There is some previous evidence that CD40-CD40L interactions contribute to the generation of optimal CD8+ T cell memory (24–29). Our present results confirm these findings and extend them by showing that the role of the CD40 pathway in this process is more multifaceted than previously appreciated. There are a number of important differences between our studies and ours previously reported. First, ours is the first study that has systematically followed the fate of Ag-specific cells during priming, contraction, and recall in the presence or absence of CD40 signals and to define the contributions of this molecule at these different stages. Second, we have looked more broadly at two different kinds of antigenic challenge (viral infection and cell-associated immunization) and analyzed responses of both TCR-Tg and endogenous CD8+ T cells. Third, the experiments in which we confined CD40 deficiency to DCs definitively show the importance of CD40 on the ability of these specific APCs to stimulate both primary and memory CD8+ T cell responses, even in the absence of CD4+ T cell help. Fourth, we used CD40-deficient mice whereas most of the previous studies that have looked at the role of CD40 signals in memory CD8+ T cell responses used CD40L-deficient animals. These two situations may not be equivalent because it has been suggested that there may be some alternate ligands/receptors for these molecules (49, 50).

Fifth, while we have previously demonstrated that CD40 and CD4+ T cell help have nonredundant functions during naive CD8+ T cell priming, we now show that they also have unique roles for CD8+ T cell memory.

The generation of a memory T cell response can be divided into at least four steps and we show that although it is not absolutely required, the CD40 pathway contributes at all of these stages. The first step in the generation of memory cells is the activation and clonal expansion of naïve T cells. It has been suggested that the magnitude of the primary response is one of the factors that influences the number of memory cells that are generated (51). The idea here is that a certain percentage of responding cells will differentiate into the memory state so that there is a causal link between the strength of the initial response and the number of memory cells generated. We and others have shown that CD40 plays a role in the optimal expansion of CD8+ T cells (46). Therefore, by increasing the number of cells generated in the primary response, CD40 may correspondingly increase the number of memory cells. However, we clearly find that this is not the only effect of CD40 in memory generation.

The second step is the differentiation of activated effector cells into memory cells. It has previously been demonstrated that CD4+ T cells promote the commitment of CD8+ T cells into memory. But whether CD4+ T cells mediate this effect through CD40 and/or some other mediator has not been directly shown. We found that CD8+ T cells primed in CD40−/− hosts did not optimally differentiate into long-lived memory cells even when the number of cells at the peak of the response was equivalent to that WT hosts. Moreover, memory cell numbers were reduced whether they were kept in the original hosts or transferred into new CD40-sufficient hosts. Because the P-14 T cells come from a WT (CD40-sufficient) background, it was formally possible that the memory cells were at reduced numbers because they were being rejected in CD40−/− hosts. However, examination of the transferred non-CD8+ cells revealed that these CD40+ cells survived equally well in WT and CD40−/− hosts (data not shown). Therefore, the reduction in the number of memory P-14 T cells in CD40−/− hosts is due to the role of CD40 in memory differentiation and not rejection.

There is data showing that CD40 expression on CD8+ T cells, and not APCs, is important in the generation of memory CD8+ T cells (30). Again, the adoptively transferred T cells in our study come from a WT background and CD40 deficiency was confined only to the hosts. Hence, our results indicate that CD40 signaling on CD8+ T cells is of little or no significance to the development of memory in our system. The discrepancy between the results could be explained by the nature of the Ags used—HY-expressing male cells which are noninflammatory, in the previous study vs LCMV, a strongly inflammatory virus, in our case. However, our findings that memory CTL activity is impaired in mice immunized with CD40-deficient DCs makes this less probable and further strengthens the view that the role of CD40 in the commitment process is most probably on APCs, similar to what we have shown for primary responses.

It is likely that CD4+ T cells are responsible for most of the CD40 stimulation on APCs. However, this has not been directly shown and in addition, there is at least one study that provides evidence for a CD4+ T cell-dependent, but CD40/CD40L-independent CD8+ T cell memory (31). Remarkably, our data reveal that immunization with peptide-pulsed DCs, a noninflammatory Ag, can induce the development of functional memory CD8+ T cells in a manner that is dependent on CD40 but independent of CD4+ T cell help. Therefore, even for the development of CD8+ T cell memory, the function of CD4+ T cell help and CD40 are not always equivalent. Our data that normal memory can be generated in CD4-depleted hosts indicate that the CD40 stimulation can come from other sources. Based on our earlier work, the source of CD40L is likely to be the Ag-specific CD8+ T cells.

The third step is the maintenance of CD8+ T cells that have committed to the memory state. Our serial transfer experiments clearly demonstrate that CD40 signals both during and after priming promote the survival of memory CD8+ T cells. Because the transfer is occurring into hosts that have not been exposed to the virus, this maintenance step is independent of the presence of a specific Ag. This suggests that CD40 is controlling the ability of the host environment to support the survival of memory CD8+ T cells, presumably through effects on APCs that are not driven by the immunizing (cognate) Ag. This role of CD40 was not appreciated in previous studies using CD40L−/− mice infected with LCMV that only found a defect in the number of memory cells generated in the absence of CD40L but not a defect in maintenance (28). Furthermore, while some studies have shown impaired CD8+ T cell memory, we demonstrate that priming in the absence of CD40 generates functionally competent memory cells, albeit at

The generation of memory T cells is a process that is crucial for the immune system’s ability to respond to future infections. CD40, a member of the tumor necrosis factor receptor superfamily, plays a pivotal role in this process. CD40-CD40L interactions are essential for the generation of optimal CD8+ T cell memory, as demonstrated by the findings that CD8+ T cells primed in CD40−/− hosts did not optimally differentiate into long-lived memory cells even when the number of cells at the peak of the response was equivalent to that in WT hosts. Moreover, memory cell numbers were reduced whether they were kept in the original hosts or transferred into new CD40-sufficient hosts. The results suggest that CD40 signaling on CD8+ T cells is of little or no significance to the development of memory in the system. The discrepancy between the results could be explained by the nature of the Ags used—HY-expressing male cells which are noninflammatory, in the previous study vs LCMV, a strongly inflammatory virus, in the case of CD40's effect on memory T cells. However, our findings that memory CTL activity is impaired in mice immunized with CD40-deficient DCs makes this less probable and further strengthens the view that the role of CD40 in the commitment process is most probably on APCs, similar to what we have shown for primary responses.

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CTL activity is more severe when CD4+/H11001 IL-7, a cleared in both hosts as assayed by RT-PCR (data not shown). Early as day 12 postinfection, a time point at which virus has been reduced numbers. Because these earlier studies were mostly done using CD40L-deficient mice, the poor recall abilities of the memory cells could be due to the combined effects of CD40 on the different phases of the response (24, 25, 29). Another explanation is that perhaps, CD40+/H11001 and CD40L+/H11001 are not necessarily equivalent, as alternative ligands/receptors for these molecules have been reported (49, 50). By doing the serial transfer experiments, we show that there is no absolute requirement for CD40 on host APCs in memory CD8+ T cell programming.

The fourth step is the reactivation of memory cells upon re-exposure to Ag. Surprisingly, we found that the role for CD40 interactions in the fourth step can vary depending on the previous experience of the memory T cell. When memory CD8+ T cells were generated in a CD40-sufficient environment or using CD40-sufficient DCS, their subsequent reactivation during challenge did not require CD40 on APCs. In contrast, recall responses were reduced when CD40 was absent during both the priming and reactivation of CD8+ T cells. Furthermore, the reduction in memory CTL activity is more severe when CD4+ T cells were depleted during priming. There are at least two explanations, which are not necessarily mutually exclusive, that could account for these results. First, because we and others have shown that CD8+ T cell responses can occur independently of CD40 or CD4+ T cell help when the frequency of responders is high enough, it is possible that the requirement for CD40 or CD4+ T cells during memory is a direct consequence of the lower numbers of memory cells that are formed in the absence of CD40. Depletion of CD4+ T cells during priming might further reduce the number of memory cells that are formed and thereby increase the CD40 dependence at recall, although we have not seen evidence for such an effect. Second, it is possible that CD4+ T cell help and CD40 are each delivering unique signals, such that memory cells generated in an environment containing a complete set of “helper” signals are in fact qualitatively different from the ones that were primed in the absence of one or more of those signals. For CD4+ T cell help, it has been shown that its presence only during but not after priming is what is essential for the ability of memory CD8+ T cells to undergo secondary expansion. In the case of CD40, it seems that the CD40-dependent APC signal to the T cell needs to have been received, but it can be either during priming or at challenge. Furthermore, the requirement for CD40 during reactivation is also affected by whether CD4+ T cell help was present during priming. This implies that the fate of Ag-specific CD8+ T cells primed in the absence of CD40 is not fixed or irreversible. If this is true, it would be reminiscent of the ability of exogenous IL-2 to rescue the proliferative and functional defects of “unhelped” memory CD8+ T cells (40).

As a consequence of the role of CD40 in these various steps, T cells primed in a CD40-deficient environment generate fewer memory cells. More effector cells are lost and fewer memory cells are maintained. At any time, the number of T cells is determined by a balance between proliferation, survival, apoptosis, and homeostatic turnover and it is not yet clear which of these various steps is impacted by CD40. We only found a small difference in the percentage of apoptotic P-14 T cells between WT and CD40+/H11001−/H11001 hosts at an early stage after infection and not thereafter. However, it is possible that annexin V assays could miss very small differences in rates of apoptosis which could lead to differences in the number of memory cells present over time. What was more interesting is that we observed a correlation between CD40 expression in the host and IL-7Rα expression as early as day 12 postinfection, a time point at which virus has been cleared in both hosts as assayed by RT-PCR (data not shown). IL-7, a y-chain cytokine, has been implicated in the survival of both naive and memory CD8+ T cells, owing to its ability to induce the expression of the antiapoptotic molecules Bcl-2 and Bcl-xL in IL-7R-expressing cells (12–16, 52). Thus, it is possible that CD40 effects on IL-7R expression could be causally related to the increased numbers of memory cells in the CD40-sufficient environment. However, it is not clear at this point how CD40 affects the ability of the responding CD8+ T cells to reacquire IL-7R expression.

In summary, our results show that CD40 can contribute to optimal memory responses at all four phases of the memory response: the primary clonal expansion, the commitment of responding cells to memory, maintenance of committed cells, and, under some conditions, the reactivation of memory cells. CD40 is playing these roles on APCs of the host. Our experiments transferring CD40-positive and -negative APCs show that one of the key APCs in which CD40 is operating is the DC. Our findings suggest that manipulating CD40 stimulation might be a useful strategy for improving CD8+ T cell memory in vaccinated individuals or immunocompromised patients.

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Disclosures
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References


