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# Epitope-Specific Regulation of Memory Programming by Differential Duration of Antigen Presentation to Influenza-Specific CD8+ T Cells

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## SUMMARY

**Memory CD8+ T cells are programmed during the primary response for robust secondary responsiveness. Here we show that CD8+ T cells responding to different epitopes of influenza virus received qualitatively different signals during the primary response that altered their secondary responsiveness. Nucleoprotein (NP)-specific CD8+ T cells encountered antigen on CD40-licensed, CD70-expressing, CD103−CD11b$^{hi}$ dendritic cells (DCs) at later times in the primary response. As a consequence, they maintained CD25 expression and responded to interleukin-2 (IL-2) and CD27, which together programmed their robust secondary proliferative capacity and interferon-γ (IFN-γ)-producing ability. In contrast, polymerase (PA)-specific CD8+ T cells did not encounter antigen-bearing, CD40-activated DCs at later times in the primary response, did not receive CD27 and CD25 signals, and were not programmed to become memory CD8+ T cells with strong proliferative and cytokine-producing ability. As a result, CD8+ T cells responding to abundant antigens, like NP, dominated the secondary response.**

## INTRODUCTION

The generation of memory CD8+ T cells that rapidly expand after secondary challenge is essential for sustained antiviral immunity. Dendritic cells (DCs) prime naive T cell responses, and early studies suggest that a brief encounter between naive T cells and antigen-bearing DCs is sufficient to trigger their differentiation into effector and memory CD8+ T cells without additional stimulation (Kaech and Ahmed, 2001; van Stipdonk et al., 2001). Later studies, however, show that repeated encounters with antigen-bearing DCs are important for optimal primary CD8+ T cell responses (McGill et al., 2008; Zammit et al., 2006) and that responding CD8+ T cells are conditioned to become functional memory cells during the contraction phase of the primary immune response, a phenomenon termed memory programming (Kaech and Wherry, 2007; Teixeiro et al., 2009; Williams et al., 2006).

The cellular and molecular basis of memory programming is not entirely understood, but is thought to require CD4+ T cell help (Shedlock and Shen, 2003; Sun and Bevan, 2003), IL-2 signaling through CD25 (Williams et al., 2006), engagement of CD27 by its ligand, CD70 (Hendriks et al., 2000) and, in some cases, interactions between CD40 and its ligand, CD154 (Borrow et al., 1996; Lee et al., 2003). In fact, the licensing of CD40-expressing DCs by CD154-expressing CD4+ T cells can be a major component of help for primary CD8+ T cell responses against some pathogens, as well as nonreplicating antigens due to the ability of CD40 to activate DCs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998), and due to its ability to prevent regulatory T (Treg) cell-mediated suppression (Ballesteros-Tato et al., 2013). However, primary responses to some pathogens appear to bypass the requirement for CD4 and CD40 help (Borrow et al., 1998; Hamilton et al., 2001; Whitmire et al., 1996), possibly due to direct activation of DCs through pathogen-recognition receptors. Nevertheless, even when primary CD8+ T cell responses do not require CD40 signaling, memory CD8+ T cell responses are often severely impaired in *Cd40*$^{-/-}$ or *Cd154*$^{-/-}$ mice (Borrow et al., 1998), in part because of CD40-dependent expression of CD70, which engages CD27 on T cells and promotes memory CD8+ T cell programming (Feau et al., 2012; Hendriks et al., 2000).

Here we show that influenza nucleoprotein (NP)-specific and polymerase (PA)-specific memory CD8+ T cells differentially utilize the IL-2:CD25, CD70:CD27, and CD40:CD154 signaling pathways. NP-specific memory T cells have prolonged interactions with CD40-licensed, antigen-bearing DCs, maintain CD25 expression for up to 10 days after infection and utilize CD70:CD27 interactions for programming. In contrast, PA-specific CD8+ T cells concluded their interactions with antigen-bearing DCs and downregulate CD25 expression prior to day 6 after infection. As a result, PA-specific CD8+ T cells do not engage CD40-licensed, CD70-expressing DCs during the late phase of the primary response and fail to differentiate into fully programmed memory cells with robust secondary proliferative capacity. Thus, CD8+ T cells of different specificities, even during the same infection, receive qualitatively distinct sets of signals during the late phase of the primary response resulting in differential memory programming. These differences strongly impact the immunodominance hierarchy of the secondary response and

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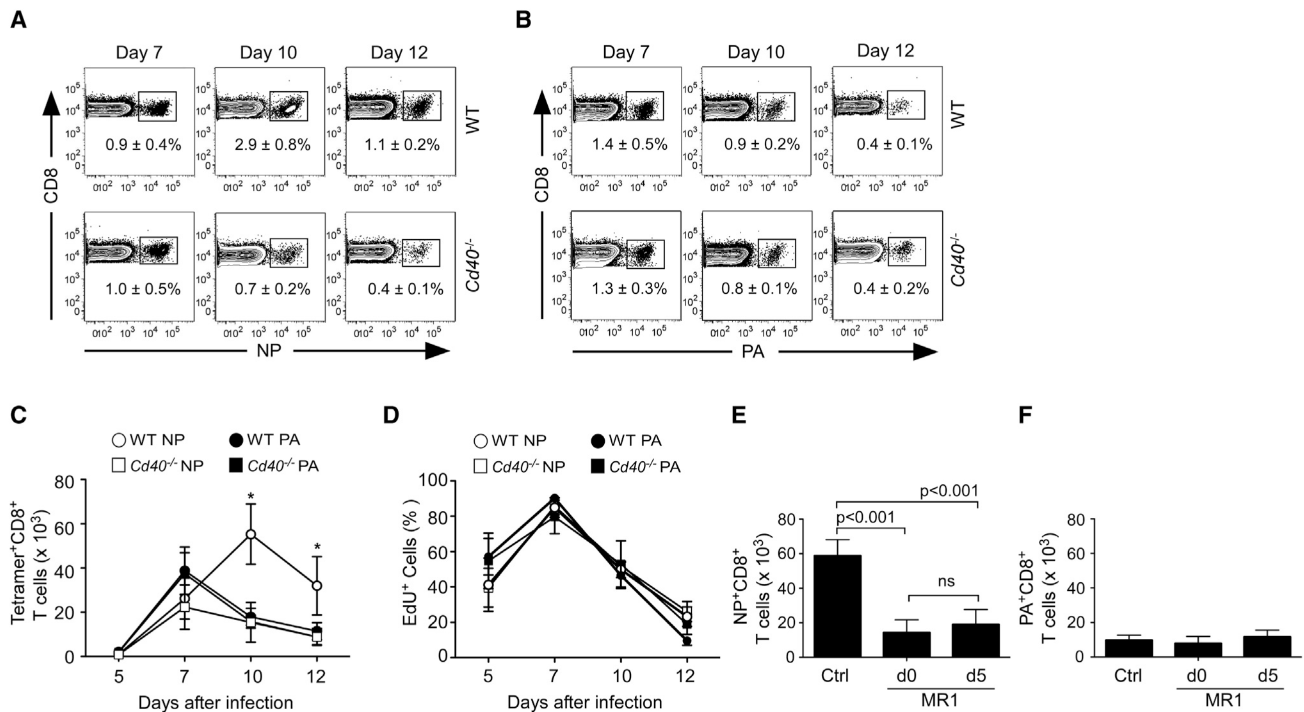



**Figure 1. CD40 Signaling Delays the Contraction of NP-Specific CD8+ T Cells**

WT and *Cd40*$^{-/-}$ were infected with PR8 and the frequencies of NP-specific (A) and PA-specific (B) CD8+ T cells, as well as the numbers of NP-specific and PA-specific CD8+ T cells (C) in the mLNs are shown. Data are representative of three experiments (mean ± SD of five mice per group; *p < 0.005).

(D) WT and *Cd40*$^{-/-}$ mice were injected with 0.5 mg of 5-ethynyl-2′-deoxyuridine every 6 hr starting 24 hr before sacrifice and the frequency of EdU+ cells among NP and PA-specific CD8+ T cells in the mLNs are shown. Data are representative of three experiments (mean ± SD of four to five mice).

(E and F) C57BL/6 mice were infected with PR8 and treated with 250 μg of the CD154-blocking antibody, MR1, or control antibody on day 0 or day 5 after infection. NP-specific (E) and PA-specific (F) CD8+ T cells were enumerated on day 10 in mLNs. Data are representative of three experiments (mean ± SD of five mice per group). p values were determined with a two-tailed Student's t test.

might represent a mechanism to enhance the fitness of the memory T cell responses.

## RESULTS

### NP-Specific, but Not PA-Specific, CD8+ T Cell Expansion Requires CD40 Signaling

To determine the role of CD40 signaling in primary CD8+ T cell responses to influenza, we infected WT and *Cd40*$^{-/-}$ mice with A/PR8/34 (PR8) influenza virus and followed the kinetics of NP- and PA-specific CD8+ T cell accumulation in the mediastinal lymph nodes (mLNs). We found that the initial (day 7) NP-specific CD8+ T cell response was similar in WT and *Cd40*$^{-/-}$ mice (Figure 1A). However, NP-specific CD8+ T cells continued to expand through day 10 in WT mice, whereas they contracted in *Cd40*$^{-/-}$ mice (Figure 1A). In contrast, PA-specific CD8+ T cells expanded equivalently in WT and *Cd40*$^{-/-}$ mice through day 7 and thereafter contracted equivalently in both groups (Figure 1B). Thus, CD40 deficiency altered the kinetics of the primary CD8+ T cell response to NP, but not that of PA (Figure 1C). Importantly, the differences in T cell accumulation did not appear to be due to altered proliferation, as NP-specific and PA-specific CD8+ T cells incorporated 5-ethenyl-2′-deoxyuridine (EdU) at similar rates in WT and *Cd40*$^{-/-}$ at all times tested (Figure 1D).

To directly test whether CD40 signaling was important for the initial priming of NP-specific CD8+ T cells or to delay the contraction phase, we treated WT mice with control antibody or MR1 (anti-CD154) at the time of infection or 5 days later and measured CD8+ T cell responses on day 10. We found that MR1 treatment starting on day 0 or day 5 resulted in equivalent reductions in NP-specific CD8+ T cells (Figure 1E). In contrast, we observed no differences in the accumulation of PA-specific CD8+ T cells (Figure 1F). These results showed that the late expansion of NP-specific CD8+ T cell response was compromised in the absence of CD40 signaling, regardless of whether initial priming occurred in a CD40 sufficient environment and further demonstrated that NP and PA-specific CD8+ T cell responses have differential requirements for CD40 signaling.

### CD40 Signaling Programs NP-Specific Memory CD8+ T Cells

To determine whether the altered primary response in *Cd40*$^{-/-}$ mice impacted the differentiation of influenza-specific memory CD8+ T cells, we first enumerated NP- and PA-specific memory cells in WT and *Cd40*$^{-/-}$ mice 8 weeks after infection. We found that despite the differences in the primary response, the number (Figure 2A) and phenotype (Figure 2B) of NP-specific memory CD8+ T cells were similar in WT and *Cd40*$^{-/-}$ mice prior to

secondary infection and were indistinguishable from the number and phenotype of the PA-specific memory $CD8^+$ T cells (Figures 2A and 2B).

To test whether there were functional differences in the populations of memory cells, we infected WT and *Cd40*$^{-/-}$ mice with PR8, allowed memory cells to develop for 8 weeks, and challenged the memory mice with influenza A/HK-X31 (X31). Because PR8 and X31 viruses express different hemagglutinin (HA) and neuraminidase (NA) subtypes (H1N1 in PR8, H3N2 in X31), antibodies generated to PR8 do not neutralize X31. However, the genome segments encoding NP and PA are identical in PR8 and X31 (Baez et al., 1980). Thus, memory T cells generated following infection with one virus will response to challenge infection with the other virus. We found that the secondary expansion of NP-specific $CD8^+$ T cells was impaired in *Cd40*$^{-/-}$ mice, whereas the secondary expansion of PA-specific $CD8^+$ T cells was similar in WT and *Cd40*$^{-/-}$ mice (Figures 2C–2E). Interestingly, the impaired NP-specific $CD8^+$ T cell response in *Cd40*$^{-/-}$ mice was similar to the "normal" PA-specific $CD8^+$ T cell response in WT mice when expressed as total numbers (Figure 2D) or as fold-expansion from resting memory cells (Figure 2E). Similar results were obtained when memory cells were allowed to develop for 100 days prior to X31 rechallenge (Figure 2F–2I) or when NP and PA-specific memory $CD8^+$ T expansion was evaluated in the mLN (see Figure S1A and S1B available online).

Given that the ability to produce interferon-$\gamma$ (IFN-$\gamma$) is another hallmark of properly programmed memory $CD8^+$ T cells (Williams et al., 2006), we next analyzed the capacity of NP-specific and PA-specific memory $CD8^+$ T cells from WT and *Cd40*$^{-/-}$ mice to produce IFN-$\gamma$. Cells from the lungs of WT and *Cd40*$^{-/-}$ mice were stimulated with $NP_{366–374}$ or $PA_{224–233}$ peptides 6 days after secondary challenge and the frequency of IFN-$\gamma$-producing, NP- and PA-specific $CD8^+$ T cell populations was determined by combining tetramer and intracellular cytokine staining (Dimopoulos et al., 2009). We found that more than 40% of the NP-specific memory $CD8^+$ T cells from WT mice made IFN-$\gamma$, but only 21% of the NP-specific $CD8^+$ T cells from *Cd40*$^{-/-}$ mice made IFN-$\gamma$ (Figure 2J). In contrast, only 27% of the PA-specific memory $CD8^+$ T cells from WT mice made IFN-$\gamma$, similar to the frequency in cells from *Cd40*$^{-/-}$ mice. (Figure 2K) These differences were magnified when calculated as total numbers (Figure 2L). Thus, the lack of CD40 signaling impaired both the secondary proliferative capacity and the IFN-$\gamma$-producing ability of NP-specific memory $CD8^+$ T cells, whereas these characteristics were already impaired in PA-specific memory $CD8^+$ T cells from WT mice and, as a result, the loss of CD40 had little impact.

To determine whether CD40-deficiency affected the ability of $CD8^+$ T cells to protect against a lethal challenge, we infected WT and *Cd40*$^{-/-}$ mice with a sublethal dose of X31, allowed them to recover for 8 weeks, and then challenged the memory mice as well as naive WT mice with a normally lethal dose of PR8. As expected, naive WT mice rapidly lost weight (Figure 2M) and 60% of the animals succumbed to infection (Figure 2N). By contrast, all WT and *Cd40*$^{-/-}$ memory mice were protected from lethal challenge (Figure 2N). However, WT memory mice lost almost no weight after challenge infection (Figure 2M), whereas *Cd40*$^{-/-}$ memory mice lost nearly 12% body weight over the first 5 days after infection and did not recover until after day 8. Consistent with the severity of the weight loss, we found high viral titers in the lungs of all naive WT mice, very low titers in memory WT mice and slightly increased titers in memory *Cd40*$^{-/-}$ mice on day 6 after challenge (Figure 2O).

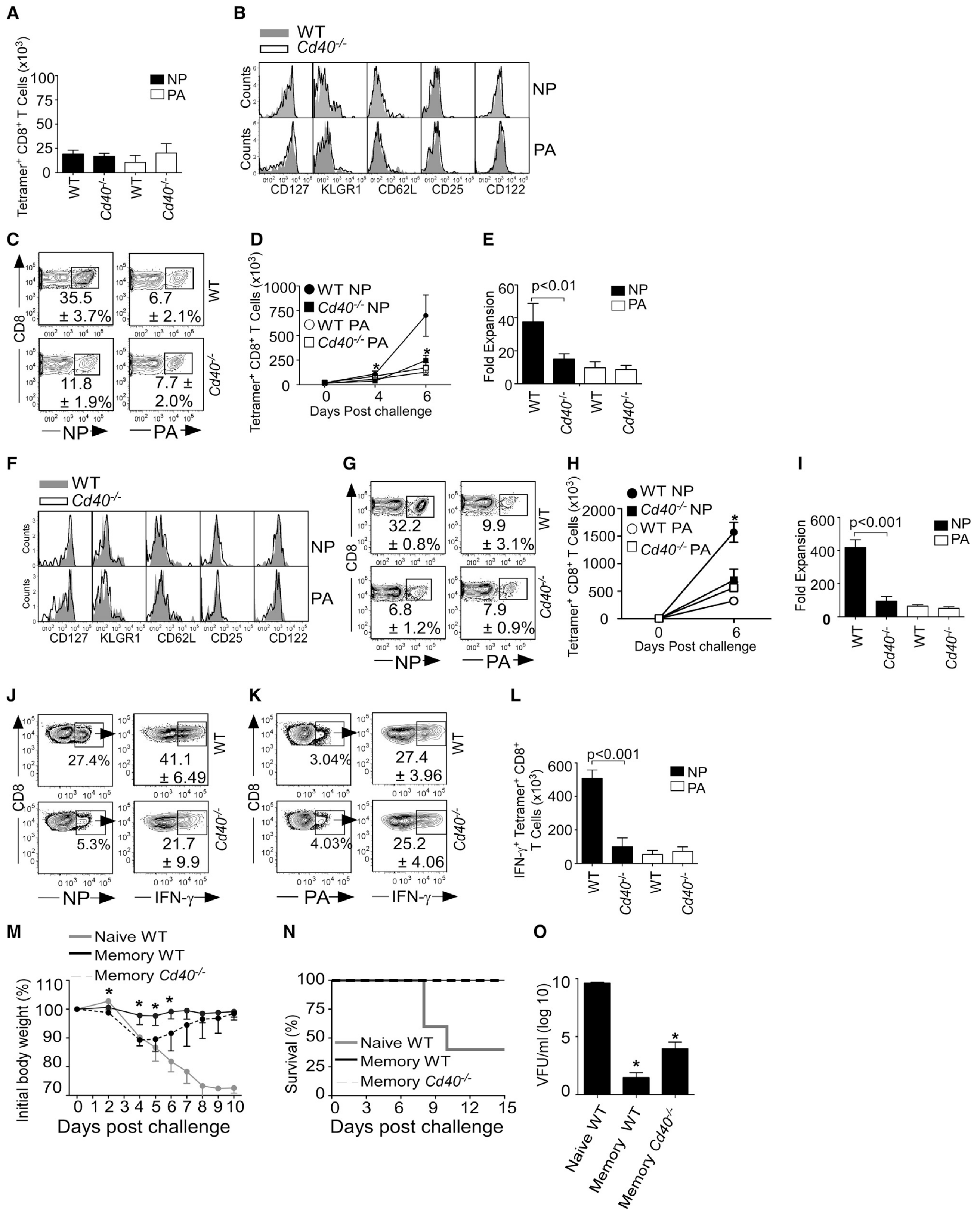
We next determined whether CD40 signaling during the primary response was required to program functional memory NP-specific $CD8^+$ T cells. Therefore, we sorted total $CD44^{hi}$ $CD8^+$ memory T cells from WT (Figure 3A) and *Cd40*$^{-/-}$ mice (Figure 3B) 8 weeks after primary PR8 infection and adoptively transferred equivalent numbers of NP-specific $CD8^+$ T cells (CD45.2) into naive CD45.1 recipient mice. We challenged recipients 24 hr after transfer with X31 and assessed the host ($CD45.1^+$) and donor ($CD45.2^+$) NP-specific $CD8^+$ T cell responses in the lungs on day 6 after challenge. We found that the frequencies (Figures 3A and 3B) and numbers (Figure 3C) of host NP-specific $CD8^+$ T cells were similar in the two groups. However, the frequencies (Figures 3A and 3B) and numbers (Figure 3D) of donor NP-specific $CD8^+$ T cells were reduced in recipients of *Cd40*$^{-/-}$ cells compared to recipients of WT cells.

We also transferred equal numbers of PA-specific $CD8^+$ memory T cells from WT (Figure 3E) and *Cd40*$^{-/-}$ mice (Figure 3F) to CD45.1 recipients, challenged them with X31 and assayed memory T cell expansion 6 days after rechallenge. In this case, we found no differences in the expansion of host PA-specific $CD8^+$ T cells (Figure 3G) or the donor PA-specific $CD8^+$ T cells from WT and *Cd40*$^{-/-}$ mice in the lungs of recipients (Figure 3H). These data suggested that CD40 signaling during the primary response was necessary for programming NP-specific, but not PA-specific $CD8^+$ memory T cells.

To determine whether CD40 signaling played any role in the secondary expansion of NP-specific memory $CD8^+$ T cells, we sorted memory $CD8^+$ T cells from WT mice that were treated with control or CD154-blocking antibody (MR1) during the primary infection (Figure 3I). We then adoptively transferred equal numbers of NP-specific memory $CD8^+$ T cells to naive CD45.1 mice, treated recipient mice with control antibody or MR1, and then challenged all groups with X31. We found that the secondary expansion of donor NP-specific $CD8^+$ T cells was not impaired by treatment with MR1 during the challenge, but that treatment with MR1 during the primary response did impair the secondary expansion of NP-specific $CD8^+$ T cells in WT recipients (Figure 3J). Similar results were obtained when WT donor NP-specific memory $CD8^+$ T cells were transferred into *Cd40*$^{-/-}$ mice (data not shown). Taken together, our results confirmed that absence of CD40 signaling during primary response compromised optimal NP-specific memory T cell expansion regardless of whether rechallenge occurred in a CD40-sufficient environment.

### *Cd40*$^{-/-}$ DCs Poorly Present Influenza NP to $CD8^+$ T Cells

Given the role of CD40 signaling in DC licensing (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998), we next tested whether *Cd40*$^{-/-}$ DCs could present influenza-derived epitopes at late times during the primary immune response. To do that, we purified $CD11c^+$ DCs from the mLNs of day 7 infected WT or *Cd40*$^{-/-}$ mice and cocultured them for 3 days with

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carboxyfluorescein succinimidyl ester (CFSE)-labeled $CD8^+$ T cells that were sorted from the mLN of influenza-infected WT mice. We found that WT DCs expanded $CD8^+$ T cells more efficiently than did *Cd40*$^{-/-}$ DCs (Figure 4A). We next analyzed the expansion of NP-specific and PA-specific $CD8^+$ T cells in the cultures. We found that WT DCs expanded NP-specific $CD8^+$ T cells much more efficiently than did *Cd40*$^{-/-}$ DCs (Figures 4B and 4C). In contrast, although only a few PA-specific $CD8^+$ T cells were expanded in either culture, they expanded to the same extent in cultures with WT or *Cd40*$^{-/-}$ DCs (Figures 4B and 4C). Thus, these results suggested that lack of CD40 signaling compromised the ability of *Cd40*$^{-/-}$ DCs to expand NP-specific, but not PA-specific, $CD8^+$ T cells.

Given studies showing that DCs program $CD8^+$ T cells during the early stages of priming (Kaech and Ahmed, 2001; van Stipdonk et al., 2001), we next performed depletion studies to address whether DCs also acted later in the primary response to program NP-specific memory $CD8^+$ T cells. In the first experiment, we reconstituted irradiated B6 recipients with bone marrow (BM) from CD11c-diptheria toxin receptor (DTR) mice, allowed them to recover for 8 weeks and infected them with PR8. We then depleted CD11c-expressing cells with DT on day 6 after infection and enumerated NP and PA-specific $CD8^+$ T cells in the mLN on day 12. We found that the frequency (Figure 4D) and number (Figure 4E) of NP-specific $CD8^+$ T cells were dramatically decreased in the mLNs of DT-treated mice, confirming that the late expansion of NP-specific $CD8^+$ T cell required antigen presentation by DCs. In contrast, DC depletion on day 6 did not affect the accumulation of PA-specific $CD8^+$ T cells (Figures 4D and 4E).

In a second experiment, CD11c-DTR BM chimeras were treated with DT every 3 days between day 6 and day 40 after infection. Mice were then allowed to recover for two weeks, which was sufficient time for normal numbers of DCs to return (data not shown), and we enumerated NP-specific and PA-specific memory $CD8^+$ T cells in control and DT-treated mice on day 55. We found that the numbers of NP-specific and PA-specific memory $CD8^+$ T cells were similar in the lungs (Figure 4F) and mLNs (Figure 4G) of both groups, suggesting that late DC depletion did not alter the number of memory NP and PA-specific $CD8^+$ T cells generated after the primary infection. Mice were then rechallenged with X31 and the accumulation of NP and PA-specific $CD8^+$ T cells in the lungs was assessed 6 days later. We found that the frequency (Figure 4H) and total number (Figure 4I) of responding NP-specific memory $CD8^+$ T cells was compromised in the lungs of DT-treated mice. However, the frequency and number of PA-specific $CD8^+$ T cells was not affected by DT treatment (Figures 4H and 4I). Similar results were obtained in the mLN and spleen (Figures S2A and S2B). These results suggested that CD40-licensed DCs presented antigen to NP-specific, but not PA-specific, $CD8^+$ T cells at late times after infection and that sustained antigen presentation programmed NP-specific $CD8^+$ memory T cells to optimally expand after rechallenge.

## CD40 Signaling Controls Cross-Presentation by $CD103^-CD11b^+$ DCs

To better understand how CD40 signaling controls DC function in response to influenza, we next enumerated DC subsets at various times after infection in WT and *Cd40*$^{-/-}$ mice. Although WT and *Cd40*$^{-/-}$ mice contained similar numbers of most DC subsets in the mLNs, there were more $CD103^-CD11b^{hi}$ DCs in the mLNs of WT mice than in *Cd40*$^{-/-}$ mice on day 10 (Figures S3A–S3C). However, there were no differences in the numbers of DCs in the lungs of WT and *Cd40*$^{-/-}$ mice at any time (Figures S3D and S3E). We also examined the expression of the costimulatory molecules CD80, CD86, and CD70 and found slightly higher expression of CD80 and CD86 on $CD103^-CD11b^{hi}$ and $CD103^+CD11b^{lo}$ tDCs in both the mLN and lungs of *Cd40*$^{-/-}$ mice (Figures S3F and S3G). In contrast, there was no difference in the expression of CD70 on these cells at any time (Figures S3H and S3I). Thus, the numbers, subset distribution and maturation of DCs in both the lungs and mLNs appeared relatively normal in *Cd40*$^{-/-}$ mice.

Given the apparently normal maturation of *Cd40*$^{-/-}$ DCs, we next tested whether they were functional antigen-presenting cells. To do this, we purified total $CD11c^+$ DCs from the mLNs of WT and *Cd40*$^{-/-}$ mice that had been infected with influenza 7 days earlier, pulsed them in vitro with $NP_{366–374}$ peptide, and cultured them with CFSE-labeled $CD8^+$ T cells from the mLNs of day 7 influenza infected mice. We found that NP-specific $CD8^+$ T cells proliferated similarly in response to both WT and *Cd40*$^{-/-}$ DCs pulsed with a wide range of peptide concentrations (Figure 5A). Next, to test the capacity of *Cd40*$^{-/-}$ DCs to cross-present exogenous protein antigens, we purified total $CD11c^+$ DCs from the mLNs of WT or *Cd40*$^{-/-}$ mice infected

**Figure 2. NP-Specific Memory $CD8^+$ T Cell Responses Require CD40**

(A and B) WT and *Cd40*$^{-/-}$ mice were infected with PR8 and the number (A) and phenotype (B) of NP-specific and PA-specific $CD8^+$ T cells in the lungs are shown at 8 weeks. Data are representative of three experiments (mean ± SD of 5 mice per group).

(C–E) WT and *Cd40*$^{-/-}$ mice were infected with PR8 and challenged with X31 8 weeks later. The frequencies (C) and total numbers (D) of NP-specific and PA-specific $CD8^+$ T cells in the lungs are shown. Data are representative of three experiments (mean ± SD of five mice per group; *$p < 0.005$).

(E) The relative expansion of NP- and PA-specific $CD8^+$ T cells in the lungs of C57BL/6 and *Cd40*$^{-/-}$ mice was calculated on day 7. Data are representative of three experiments (mean ± SD of five mice per group).

(F) WT and *Cd40*$^{-/-}$ mice were infected with PR8 and the phenotype of NP-specific and PA-specific $CD8^+$ T cells in the lungs was determined 100 days later.

(G–I) WT and *Cd40*$^{-/-}$ mice were infected with PR8 and challenged with X31 100 days later, and the frequencies (G) and total numbers (H) of NP-specific and PA-specific $CD8^+$ T cells in the lungs are shown (*$p < 0.005$). (I) The fold expansion of NP- and PA-specific $CD8^+$ T cells in the lungs of C57BL/6 and *Cd40*$^{-/-}$ mice was calculated on day 6. The frequency (J and K) and number (L) of IFN-$\gamma$ producing cells among either WT or *Cd40*$^{-/-}$ NP and PA-specific $CD8^+$ T cells were determined by intracellular staining and tetramer costaining after restimulation with $NP_{366–374}$ peptide (J and L) or $PA_{224–233}$ peptides (K and L) 6 days after challenge. Data are representative of two experiments (mean ± SD of four to five mice per group).

(M–O) WT and *Cd40*$^{-/-}$ mice were infected with 500 EIU of X31 and challenged with 5000 EIU PR8 8 weeks later. As a control, naive WT mice were infected with 5000 EIU PR8. Weight loss (M) and survival (N) are shown. Viral titers in the lungs were determined at day 6 (O). Data are representative of two experiments (mean ± SD of five to ten mice per group; *$p < 0.05$). p values were determined using a two-tailed Student's t test.

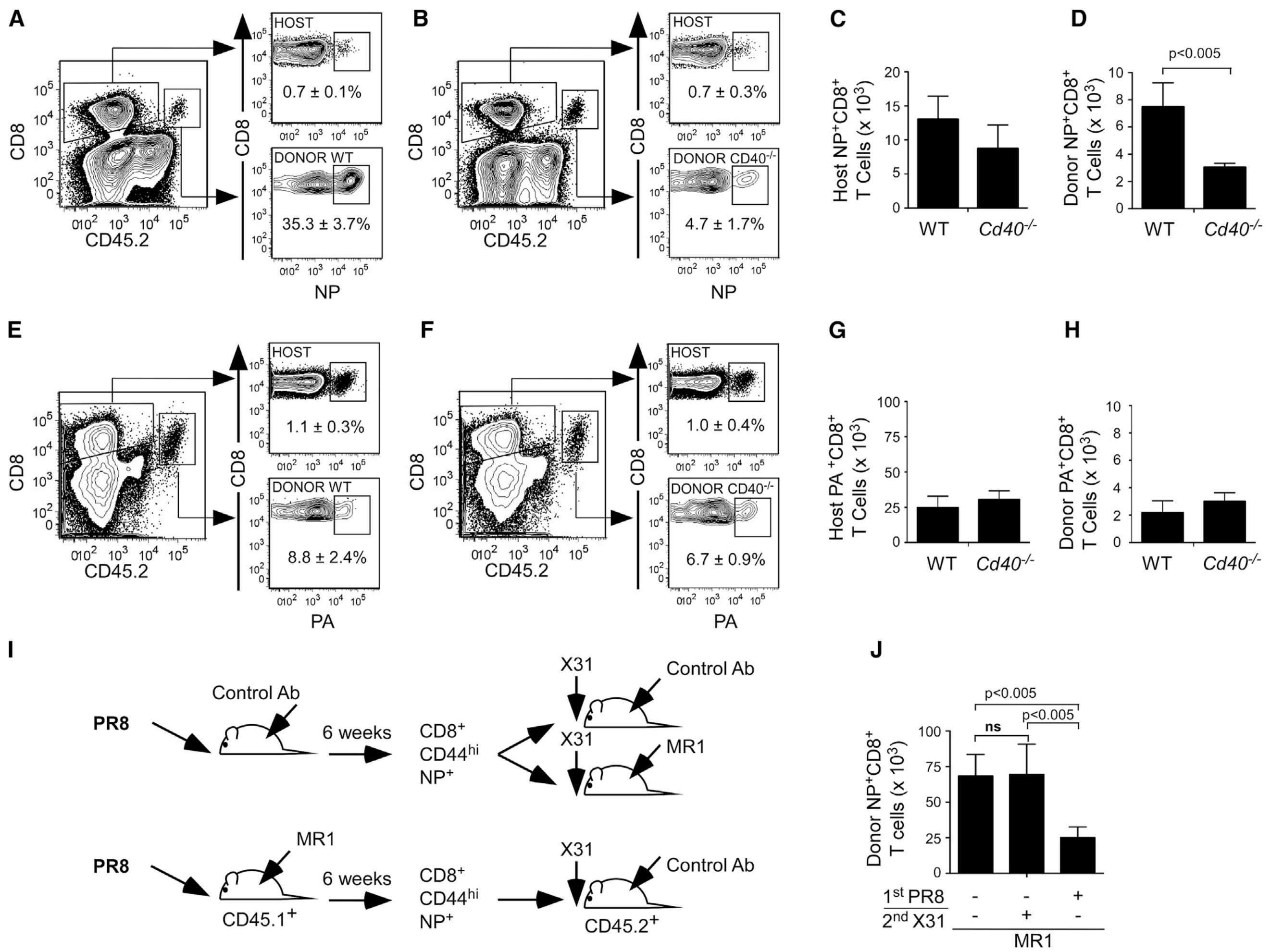


**Figure 3. CD40 Signaling during Priming Programs NP-Specific Memory CD8⁺ T Cells**

(A–D) WT and *Cd40*$^{-/-}$ mice (both CD45.2) were infected with PR8 and 8 weeks later, memory CD8$^+$CD44$^{hi}$ T cells were sorted from the spleens and populations containing 4 × $10^3$ WT or *Cd40*$^{-/-}$ NP-specific CD8$^+$CD44$^{hi}$ T cells (A–D) or populations containing 4 × $10^3$ WT or *Cd40*$^{-/-}$ PA-specific CD8$^+$CD44$^{hi}$ T cells (E–H) were transferred into naive CD45.1$^+$ mice, which were infected with X31 24 hr later. The numbers of host (c) and donor (D) NP-specific CD8$^+$ T cells, as well as the number of host (G) and donor (H) PA-specific CD8$^+$ T cells in the lungs of recipient mice are shown. Data are representative of three experiments (mean ± SD of four to five mice per group).

(I–J) C57BL/6 mice were treated with 250 μg of MR1 or control IgG and infected with PR8. Six weeks later, CD8$^+$CD44$^{hi}$ cells were sorted from the donor mice and populations containing 4 × $10^3$ NP-specific CD8$^+$ T cells were transferred into CD45.2 recipient mice. The recipients were infected with X31 the next day and treated with MR1 or control IgG. The number of donor NP-specific CD8$^+$ T cells in lungs of recipient mice were determined with flow cytometry 7 days later (I). Data are representative of two experiments (mean ± SD of four to five mice per group). p values were calculated with a two-tailed Student's t test.

with influenza 7 days earlier, pulsed them with soluble OVA, and cultured them with CFSE-labeled OT-I cells. We found that *Cd40*$^{-/-}$ DCs poorly cross-presented soluble OVA compared to WT DCs (Figure 5B–D). Importantly, the failure of *Cd40*$^{-/-}$ DCs to expand OT-I T cells was reversed when we pulsed DCs with $OVA_{257–264}$ peptide (Figure 5D).

Given that CD103$^-$CD11b$^{hi}$ DCs are the dominant population of DCs in the mLN and lung after influenza infection (Ballesteros-Tato et al., 2010; GeurtsvanKessel et al., 2008) and that these cells are the major subset that presents NP to CD8$^+$ T cells at the peak of infection (Ballesteros-Tato et al., 2010), we next tested the ability of CD103$^-$CD11b$^{hi}$ DCs from WT and *Cd40*$^{-/-}$ mice to induce the proliferation of effector NP-specific CD8$^+$ T cells. We found that WT CD103$^-$CD11b$^{hi}$ DCs efficiently expanded NP-specific CD8$^+$ T cells, whereas *Cd40*$^{-/-}$ CD103$^-$CD11b$^{hi}$ DCs did not (Figure 5E). In contrast, although only a few PA-specific CD8$^+$ T cells expanded in culture, they expanded equivalently in cultures with WT and *Cd40*$^{-/-}$ DCs (Figure 5F). These results demonstrate that CD40 controls the ability of CD103$^-$CD11b$^{hi}$ DCs to present NP, but not PA, late after infection.

To determine whether CD103$^-$CD11b$^{hi}$ DCs were impaired in their ability to cross-present soluble antigens, we sorted CD103$^-$CD11b$^{hi}$ DCs from infected WT or *Cd40*$^{-/-}$ mice, pulsed

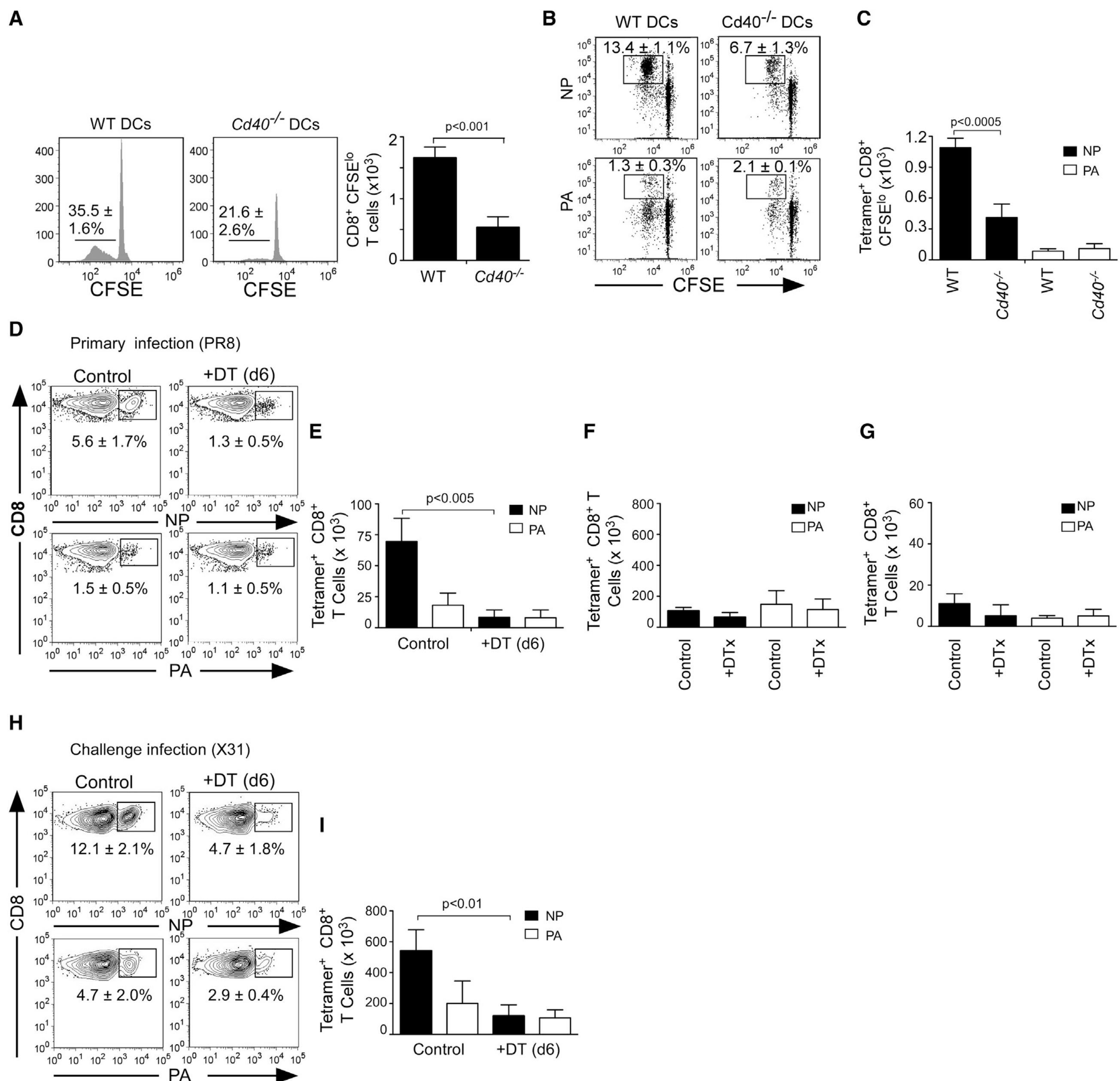


**Figure 4. Limited Presentation of NP by DCs in the Absence of CD40**

$CD8^+$ T cells were purified from day 7 PR8-infected WT mice, labeled with CFSE, and cultured for 3 days with $CD11c^+$ DCs purified from the mLNs of day 7 PR8-infected WT or *Cd40*$^{-/-}$ mice. The frequency and number of $CFSE^{lo}CD8^+$ T cells are shown (A). The frequency (B) and number (C) of NP-specific and PA-specific $CFSE^{lo}CD8^+$ T cells are shown. Data are representative of three experiments (mean ± SD of four samples per group).

(D and E) C57BL/6 mice were irradiated and reconstituted with CD11c-DTR-EGFP bone marrow. Reconstituted mice were infected with PR8 and injected i.p. with PBS or 60 ng DTX on day 6 after infection and then analyzed on day 12. The frequency (D) and numbers (E) of NP-specific and PA-specific $CD8^+$ T cells in the mLNs are shown. Data are representative of four experiments (mean ± SD of four to five mice per group).

(F and G) CD11c-DTR-EGFP BM chimeras were infected with PR8 and injected i.p. with PBS or 60 ng DTX every 3 days between day 6 and 40. The numbers of resting NP-specific and PA-specific memory $CD8^+$ T cells in lungs (F) and mLNs (G) are shown at 2 weeks. Data are representative of three experiments (mean ± SD of four to five mice per group).

(H and I) CD11c-DTR-EGFP BM chimeras were infected with PR8 and injected i.p. with PBS or 60 ng DTX every 3 days between day 6 and 40. Two weeks later, mice were challenged with X31 and the frequency (H) and numbers (I) of NP-specific and PA-specific $CD8^+$ T cells in the lungs are shown at day 6. Data are representative of two experiments (mean ± SD of four to five mice per group). All p values were calculated with a two-tailed Student's t test.

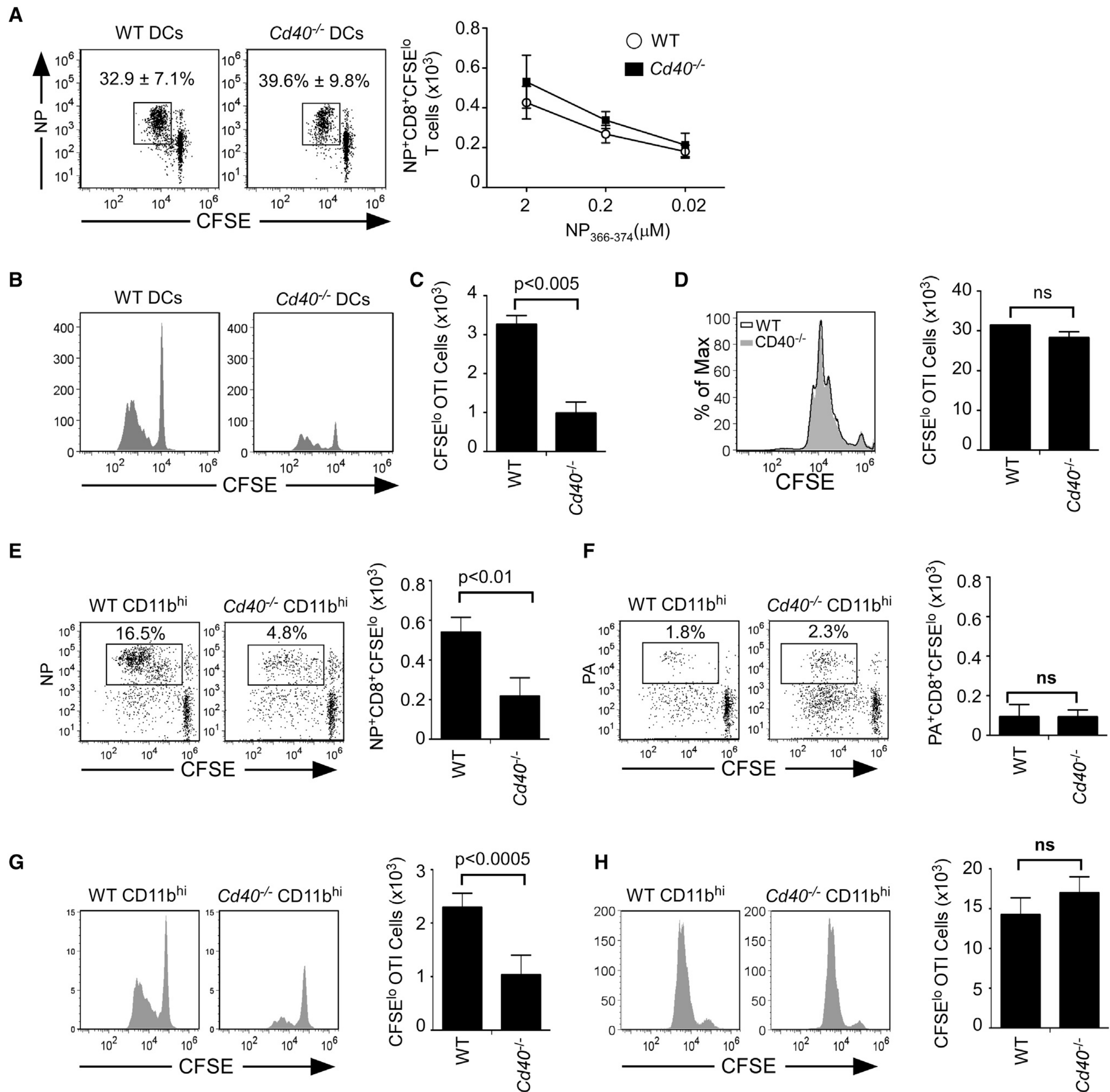


**Figure 5. Cross-Presentation by CD103⁻CD11b⁺ tDC Is Compromised in *Cd40*$^{-/-}$ Mice**

(A) CFSE-labeled CD8$^+$ T cells from day 7 influenza infected mLNs were cultured for 72 hr with WT or *Cd40*$^{-/-}$ CD11c$^+$ cells pulsed with $NP_{366–374}$ peptide, and the frequency (left) and number (right) of divided NP-specific CD8$^+$ T cells is shown. Data are representative of three experiments with four samples per group.

(B–D) CD11c$^+$ cells from mLNs of day 7 influenza-infected C57BL/6 or *Cd40*$^{-/-}$ mice were pulsed with 5 μg/ml OVA (B and C) or 0.5 μg/ml $OVA_{257–264}$ (D) and cultured for 72 hr with CFSE-labeled OT-I cells. Results are representative of three experiments with four samples per group.

(E and F) CFSE-labeled CD8$^+$ T cells from mLNs of day 7 infected mice were cultured with CD103$^-$CD11b$^+$ tDCs from the mLN of day 7 infected C57BL/6 or *Cd40*$^{-/-}$ mice and the frequency and number of divided NP-specific (E) or PA-specific CD8$^+$ T cells (F) are shown. Data are representative of three experiments with four samples per group.

(G and H) CFSE-labeled OT-I cells were cultured for 72 hr with CD103$^-$CD11b$^+$ tDCs cells from mLNs of day 7 C57BL/6 or *Cd40*$^{-/-}$ that were pulsed with 5 μg/ml OVA (G) or 0.5 μg/ml $OVA_{257–264}$ (H). Data are representative of three experiments with four samples per group. All p values were calculated with a two-tailed Student's t test.

them with either soluble OVA protein or OVA peptide, and tested their ability to prime naive OTI cells. We found that *Cd40*$^{-/-}$ CD103$^-$CD11b$^{hi}$ DCs poorly cross-presented soluble OVA protein to CD8 T cells (Figure 5G) but that those same DCs pulsed with peptide-primed naive CD8 T cells normally (Figure 5H). Taken together, our results suggest that CD40 signaling controls

antigen processing and cross-presentation rather than the accumulation and maturation of DCs.

### Control of Memory Programming by CD70-Expressing CD103⁻CD11b⁺ DCs

CD103⁻CD11b$^{hi}$ DCs express CD70, the ligand for CD27, which is a costimulatory molecule that facilitates the late expansion of CD8⁺ T cell responses and might be involved in memory programming (Ballesteros-Tato et al., 2010; Hendriks et al., 2000). To test whether CD27 engagement by CD70-expressing CD103⁻CD11b$^{hi}$ DCs was important for NP-specific or PA-specific CD8⁺ T cell responses, we treated WT mice with anti-CD70 blocking antibody 4 days after primary infection and enumerated NP and PA-specific CD8⁺ T cells on day 10. We found that anti-CD70 treatment impaired NP-specific CD8⁺ T cell expansion without affecting the PA-specific CD8⁺ T cell response (Figures 6A and 6B). Similarly, we found that the in vitro expansion of NP-specific CD8⁺ T cells by CD103⁻CD11b⁺ DCs was also markedly inhibited by CD70 blockade, whereas the expansion of PA-specific CD8 T cells was not affected (Figures 6C and 6D).

To determine whether CD27 signaling played a role in memory CD8⁺ T cell programming, we next treated WT mice with anti-CD70 antibody 4 days after primary infection, waited 8 weeks for memory to develop, and enumerated NP-specific memory CD8⁺ T cells. We found that the frequencies and numbers of NP-specific memory T cells were similar in mice treated with anti-CD70 or control antibodies (Figure 6E). We next challenged memory mice with X31. We found that NP-specific memory CD8⁺ T cell expansion was compromised in the lungs of mice that received anti-CD70 during primary infection but that the expansion of PA-specific memory CD8⁺ T cells was unaffected (Figures 6F and 6G). Thus, both CD40 and CD27 are required at late times during the primary response to elicit fully functional NP-specific memory CD8⁺ T cells, whereas PA-specific memory CD8⁺ T cells develop normally in the absence of these costimulatory signals.

### Costimulation through CD40 and CD27 Maintain IL-2-Responsive T Cells

IL-2 signaling through CD25 is required for T cell expansion and memory formation (Williams et al., 2006) and might be dependent on CD40 (Wolkers et al., 2011) and CD27 costimulation (Huang et al., 2006). Therefore, we next determined whether NP and PA-specific CD8⁺ T cells expressed CD25 after influenza infection. We found that NP and PA-specific CD8⁺ T cells expressed similar amounts of CD25 early after infection (Figure 7A). However, whereas NP-specific CD8⁺ T cells maintained CD25 expression on day 10, PA-specific CD8⁺ T cells downregulated CD25 (Figure 7B).

To test whether CD25 was important for the accumulation of NP-specific CD8⁺ T cells, we made mixed BM chimeras in which irradiated WT mice (CD45.1⁺) were reconstituted with 50% WT CD45.1⁺ BM and 50% CD45.2⁺*Cd25*$^{-/-}$ BM. Chimeric mice were infected with PR8 and the expansion of WT (CD45.1) and *Cd25*$^{-/-}$ (CD45.2) NP-specific and PA-specific CD8⁺ T cells was assessed. We found that although equivalent numbers of NP-specific CD8⁺ T cells were generated from WT and CD25-deficient CD8⁺ T cells 7 days after infection (data not shown), many more WT NP-specific CD8⁺ T cells than *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells had accumulated by day 12 (Figures 7C and 7D). In contrast, similar numbers of PA-specific CD8⁺ T cells were generated from WT and *Cd25*$^{-/-}$ precursors (Figures 7C and 7D). Similar results were obtained in the lungs (data not shown).

We next challenged the WT:*Cd25*$^{-/-}$ chimeras with X31 8 weeks after the initial infection and measured the expansion of WT and *Cd25*$^{-/-}$ CD8⁺ T cells in the lung 6 days later. We found that the secondary expansion of *Cd25*$^{-/-}$ NP-specific memory CD8⁺ T cell was compromised compared to their WT counterparts (Figure 7E). Thus, CD25 expression was important for both the primary and secondary expansion of NP-specific CD8⁺ T cells.

To connect CD40 signaling and CD25 expression, we analyzed whether NP- and PA-specific CD8⁺ T cells expressed CD25 in WT and *Cd40*$^{-/-}$ mice. We found that both NP- and PA-specific CD8⁺ T cells expressed similar amounts of CD25 in WT and *Cd40*$^{-/-}$ mice on day 7 (Figure 7F). In contrast, although NP-specific CD8⁺ T cells continued to express CD25 on day 10 in WT mice, they had decreased CD25 expression in *Cd40*$^{-/-}$ mice (Figure 7G). Unlike NP-specific CD8⁺ T cells, PA-specific CD8⁺ T cells had already downregulated CD25 expression on day 10 after infection in WT mice and the amount of CD25 was not affected by the loss of CD40 (Figures 7F and 7G). Similar results were obtained in mice treated with MR1 5 days after infection (data not shown).

To directly confirm that CD40 signaling was important for the accumulation of CD25⁺ NP-specific CD8⁺ T cells, WT:*Cd25*$^{-/-}$ chimeras were treated with MR1 to block CD40 signaling and WT and *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells were enumerated 12 days after infection. We found that WT NP-specific CD8⁺ T cells accumulated to a greater extent than *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells in isotype control-treated mice, whereas WT and *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells accumulated similarly in MR1-treated mice (Figure 7H). These results suggested that CD40 signaling helps to maintain CD25 expression, and thus IL-2 responsiveness by NP-specific, but not PA-specific, CD8⁺ T cells.

In order to connect CD25 and CD27, we next gated the NP-specific CD8⁺ T cells into CD25$^{hi}$ and CD25$^{lo}$ subsets and measured CD27 expression with flow cytometry. We found that CD25$^{hi}$ cells expressed more CD27 compared to CD25$^{lo}$ cells (Figure 7I), suggesting that CD27 and CD25 expression are also functionally linked. To test this possibility, we treated WT:*Cd25*$^{-/-}$ chimeric mice with control or anti-CD70 blocking antibody 4 days after infection and enumerated WT and *Cd25*$^{-/-}$ NP -specific CD8⁺ T cells on day 12. We found that the accumulation of *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells was severely impaired in chimeras treated with control antibody, whereas the accumulation of both WT and *Cd25*$^{-/-}$ NP-specific CD8⁺ T cells was impaired to the same extent in anti-CD70 treated mice (Figure 7J). Thus, the loss of CD70 and CD25 appear to impact the same process of late T cell expansion, which is when memory programming occurs.

Taken together, these results suggest that CD8 T cells are programmed to become fully functional memory cells by prolonged antigen presentation and interactions between IL-2:IL2R, CD40:CD40L, and CD70:CD27. In the absence of extended antigen presentation, CD8⁺ T cells do not receive the appropriate

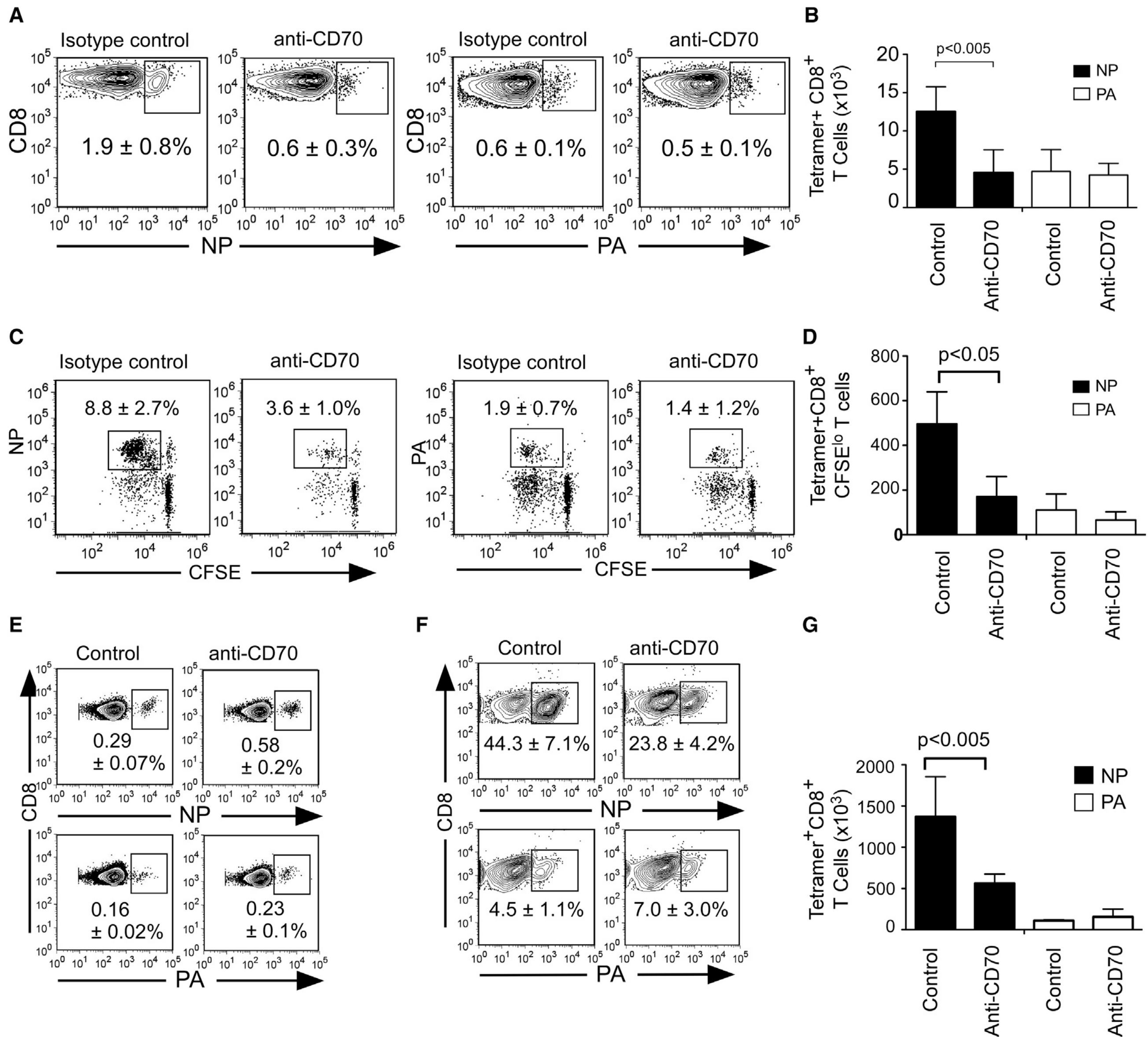


**Figure 6. CD70-Expressing CD103⁻CD11b⁺ DCs Program NP-Specific CD8⁺ T Cells**

(A and B) C57BL/6 mice were infected with PR8 and treated with 500 μg anti-CD70 or control IgG 4 days after infection, and the frequency (A) and number (B) of NP and PA-specific CD8⁺ T cells in the mLNs on day 10 are shown. Data are representative of three experiments (mean ± SD of four to five mice per group).
(C and D) CD8⁺ T cells from mLNs of day 7 infected C57BL/6 mice were cultured for 72 hr with CD103⁻CD11b⁺ tDCs and either anti-CD70 or control IgG and the frequency (C) and number (D) of divided NP and PA-specific CD8⁺ T cells is shown. Results are representative of three experiments (mean ± SD of four samples).
(E) C57BL/6 were infected with PR8 and treated with 500 μg anti-CD70 or control IgG 4 days after infection and the frequencies of NP and PA-specific CD8⁺ T cells in the lungs at 8 weeks are shown. Data are representative of two experiments (mean ± SD of four to five mice per group).
(F) C57BL/6 were infected with PR8 and treated with 500 μg anti-CD70 or control IgG 4 days after infection, challenged 8 weeks later with X31, and the frequencies (left) and numbers (right) of NP and PA-specific CD8⁺ T cells in the lungs are shown. Data are representative of two experiments (mean ± SD of four to five mice per group). p value was calculated with a two-tailed Student's t test

costimulatory signals and are not programmed to become fully functional memory cells.

## DISCUSSION

Our results demonstrate that CD8⁺ T cells responding to different epitopes in influenza have different requirements for the CD40, CD27, and CD25 signaling pathways. Primary and secondary CD8⁺ T cell responses to NP require these pathways, whereas CD8⁺ T cell responses to PA are unchanged by their absence. This observation is contrary to the current paradigm, which suggests that CD8⁺ T cell responses to some types of antigens, such as purified proteins in subunit vaccines, are dependent on CD40 signaling to properly licensed DCs, whereas CD8 T cell

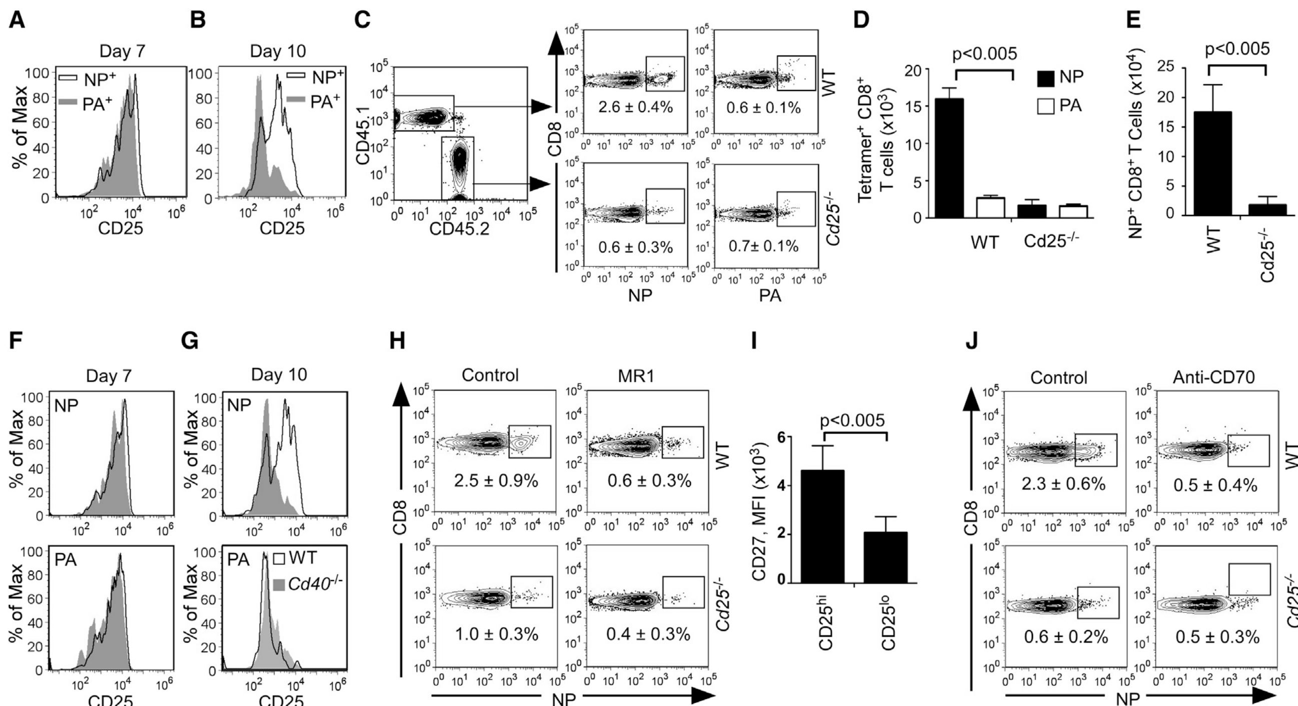


**Figure 7. CD40 Promotes Survival of $CD25^+$ $CD8^+$ T Cells**

(A and B) Expression of CD25 on NP and PA-specific $CD8^+$ T cells in the mLN. Data are representative of three experiments of four to five mice per group.

(C and D) WT:*Cd25*$^{-/-}$ chimeras were infected with PR8 and the frequency (C) and numbers (D) of WT and *Cd25*$^{-/-}$ NP-specific and PA-specific $CD8^+$ T cells in the mLNs on day 12 are shown. Data are representative of three experiments (mean ± SD of four to five mice per group).

(E) WT:*Cd25*$^{-/-}$ chimeras were infected with PR8 and challenged with X31 8 weeks later, and the numbers of WT and *Cd25*$^{-/-}$ NP-specific $CD8^+$ T cells on day 6 are shown. Data are representative of two experiments (mean ± SD of five mice per group).

(F and G) Expression of CD25 on NP and PA-specific $CD8^+$ T cells in the mLN of influenza-infected C57BL/6 and *Cd40*$^{-/-}$ mice. Data are representative of three experiments (four to five mice per group).

(H) WT:*Cd25*$^{-/-}$ chimeras were infected with PR8 and treated with 250 μg of MR1 or control IgG and the frequency of NP-specific $CD8^+$ T cells from WT or *Cd25*$^{-/-}$ donors in the mLN on day 12 is shown. Data are representative of two experiments (mean ± SD of four to five mice).

(I) Expression of CD27 on $CD25^{hi}$ or $CD25^{lo}$ NP-specific $CD8^+$ T cells in the mLN of day 10 influenza-infected mice (MFI; mean fluorescence intensity). Data are representative of three experiments (mean ± SD of four to five mice).

(J) WT:*Cd25*$^{-/-}$ chimeras were infected with PR8 and treated with anti-CD70 or control antibody, and the frequency of NP-specific $CD8^+$ T cells from WT or *Cd25*$^{-/-}$ donors is shown. Data are representative of two experiments (mean ± SD of four to five mice). p values were calculated with a two-tailed Student's t test.

responses to virulent pathogens, like influenza, might not rely on CD40:CD154 interactions because DCs are fully activated by pathogen-sensing molecules (Hamilton et al., 2001). If this paradigm is correct, then one would expect that $CD8^+$ T cells responding to any epitope of a particular antigen or pathogen would be consistent in their requirements for CD40-mediated DC licensing. In contrast, our data demonstrate that there can be dramatic differences between $CD8^+$ T cell responses to different epitopes from the same pathogen. Thus, factors other than initial DC activation must control the requirement for CD40 signaling for $CD8^+$ T cell responses to some antigens.

For example, our data demonstrate that $CD11c^+$ cells (presumably DCs) are required for the continued expansion of NP-specific $CD8^+$ T cells beyond day 5, whereas these cells are not at all required for normal $CD8^+$ T cell responses to PA. Thus, we conclude that the duration of antigen presentation for these two antigens is very different. Consistent with this idea, T cells responding to PA expand for the first 7 days after influenza infection and subsequently contract. In contrast, T cells responding to NP continue to accumulate through days 10–12. Given that $CD103^-CD11b^{hi}$ DCs, which are the only cells to express CD70, the ligand for CD27, dominate the late phase of the primary response to influenza (Ballesteros-Tato et al., 2010), it makes sense that $CD8^+$ T cells responding to epitopes that are presented during this period are exposed to qualitatively distinct DCs and utilize very different pathways of costimulation.

Each of the signaling pathways required in the late phase of the primary response (CD40, CD25, CD27) appears to control different aspects of late primary expansion and memory programming. For example, CD40 signaling appears to be important for successful cross-priming during this period. Thus, in the absence of CD40, cross-priming is inefficient, NP is poorly presented, and NP-specific $CD8^+$ T cell expansion and memory programming are ineffective. Blockade of either CD70 or CD25 has no additional effect, because in the absence of antigen, costimulation is irrelevant. Antigen-receptor signaling is also likely to be important for IL-2 production, which reinforces the expression of CD25. Thus, in the absence of CD40, NP is not presented and CD25 expression is not maintained.

The functions of CD25 and CD27 are also likely to be interrelated. For example, CD27 engagement by CD70-expressing DCs is probably important for preventing the apoptosis of $CD8^+$ T cells responding to IL-2 (Dolfi et al., 2008; Peperzak et al., 2010). Thus, although IL-2 signaling is important for late primary expansion and memory programming, it does not work unless the T cells encounter CD70-expressing DCs that prevent their apoptosis and promote their survival. Importantly, none of these mechanisms apply to PA-specific $CD8^+$ T cells, because PA is poorly presented during the late phase of the immune response. Thus the genetic ablation or pharmacological blockade of CD40, CD25, or CD27 pathways has no effect on PA-specific $CD8^+$ T cell responses.

In light of these data, we propose an alternative model, in which CD40-licensed, CD70-expressing, $CD103^-CD11b^{hi}$ DCs cross-present abundant antigens during the late phase of the primary response. T cells recognizing antigens on these DCs express CD25, respond to IL-2, and receive survival signals through CD27, which together program T cells to become memory T cells with robust secondary proliferative capacity and cytokine-producing ability. In contrast, T cells responding to antigens like PA, which are poorly presented during the late phase of the primary response, do not receive these signals and are not programmed be become highly proliferative memory $CD8^+$ T cells. This model is consistent with previous data showing that NP-specific memory $CD8^+$ T cells, but not PA-specific memory $CD8^+$ T cells, dominate the secondary response to influenza and promote beneficial outcomes (Belz et al., 2000; Crowe et al., 2003; La Gruta et al., 2010).

The differences in the presentation of NP and PA by DCs during the primary response may be explained by the nature of antigens themselves. For example, the amount of NP and PA contained in mature influenza virions is widely different—with 560 NP molecules per virion and only 8 PA molecules (one per RNA strand) per virion. Thus, one could envision a scenario in which both NP and PA are directly presented to CD8 T cells early after infection by influenza-infected DCs that are activated by pathogen-recognition receptors. However, at later times after infection, when the number of virally infected cells is low and the majority of antigen is in the form of cellular debris and neutralized virions, then DCs must acquire antigens exogenously and stimulate CD8 T cells by cross-priming. Given that cross-presentation is much more efficient at high doses rather than low doses of antigen (Kurts et al., 1998), then the processing and presentation of NP would be dramatically favored over PA. This conclusion is also consistent with data showing that subdominant antigens are often poorly cross-presented (Otahal et al., 2005) and that the immunodominance hierarchy can be a function of antigen dose (Jenkins et al., 2006; La Gruta et al., 2006). Importantly, previous studies show that the recall response to PA can be enhanced by engineering the PA epitope into the influenza neuraminidase protein, which is much more abundant than polymerase (La Gruta et al., 2006). Although the previous studies did not specifically examine memory programming or a requirement for CD40, they are consistent with our model in which epitopes in more abundant proteins are preferentially cross-presented at late times in the primary response and, as a result, preferentially receive memory programming signals.

In this model, only T cells recognizing epitopes in abundant antigens would be programmed appropriately by CD70-expressing $CD103^-CD11b^{hi}$ DCs. This model also represents a mechanism for the immune system to enhance the efficiency of memory T cell responses. Differential cross-presentation by $CD103^-CD11b^{hi}$ DCs would lead to a selection process that favors the expansion of T cells recognizing more abundant antigens and skews memory responses toward those antigens. As a consequence, the responding memory $CD8^+$ T cells would more likely encounter antigen on DCs, as well as nonprofessional APCs such as lung epithelial cells, and more effectively eliminate the pathogen. Thus, the fitness of the memory response would be improved. This view is consistent with studies showing that NP, but not PA-derived epitopes, are strongly expressed by lung-epithelial cells (Crowe et al., 2003), the primary target of influenza virus, and that PA-specific memory $CD8^+$ T cells are ineffective or even detrimental in controlling influenza infection when compared to NP-specific memory CD8 T cells (Crowe et al., 2003).

In summary, our data provide insights into the mechanisms regulating memory $CD8^+$ T cell programming, as well as the role of extended antigen presentation by DCs. Collectively, this information will be useful in the rational design of vaccines and development of immunotherapies that target $CD8^+$ T cell responses.

## EXPERIMENTAL PROCEDURES

### Mice, Infections, Chimeras, EdU, and Antibody Treatment

C57BL/6 (WT), B6.129P2-Tnfrsf5$^{tm1kik}$ (*Cd40*$^{-/-}$), B6.129S2-Tnfsf5$^{tm1Imx}$ (*Cd154*$^{-/-}$), B6.Tgn(TcrOVA)$^{1100Mjb}$ (OT-I), B6.129S2-IgH-6$^{tm1Cgn}$/J (μMT), B6.129S4-IL2ra$^{tm1Dw}$/J, (*Cd25*$^{-/-}$) B6.FVB-Tg(Itgax-DTR/EGFP)$^{57Lan}$/J (CD11c-DTR), and B6.IgH$^a$.Thy-1$^a$.Ptrpc$^a$ (CD45.1) mice were obtained from Trudeau Institute and were bred in the University of Rochester (UR) or University of Alabama at Birmingham (UAB) animal facilities. Infections were performed intranasally in 100 μl with 500 egg infectious units (EIU) of PR8 or X31 (primary infection) and 5000 EIU of PR8 or X31 (secondary infection). Viral titers were quantified with a viral foci assay (Rangel-Moreno et al., 2008). In some experiments, mice were injected intraperitoneally with 500 μg anti-CD70 (FR70), 500 μg rat immunoglobulin G2b (IgG2b) (LTF-2), 250 μg anti-CD154 (MR-1), or 250 μg hamster IgG (all from Bioxcell). Proliferating cells were labeled by intravenously injecting 0.5 mg of EdU (Invitrogen) three times every 6 hr starting 24 hr before sacrifice. BM chimeric mice were generated by lethally irradiating recipients with 950 Rads from a $^{137}Cs$ source delivered in a split dose and reconstituting them with $10^7$ total BM cells. Mice were allowed to reconstitute for 8–12 weeks before infection. In some cases, B6 CD11c-DTR BM chimeras received an intraperitoneal injection of 60 ng DT (Sigma) on days 6 and 10 after infection. All experimental procedures involving animals were approved by the appropriate UR or UAB animal welfare committees.

### Cell Preparation and Flow Cytometry

Cells were prepared from lungs cut into small fragments and digested for 45 min at 37°C with 0.6 mg/ml collagenase A (Sigma) and 30 μg/ml DNase I (Sigma) in RPMI-1640 medium (GIBCO). Digested lungs were dispersed by passage through a wire mesh. Live cells were obtained by density-gradient centrifugation with 1-Step Polymorphs (Accurate Chemical). Cells were obtained from mLNs and spleens by disruption through 70 μm nylon cell strainer (BD Biosciences). Red blood cells were lysed with 150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM EDTA. Fc receptors were blocked with antibody 2.4G2 (10 μg/ml; Trudeau Institute), followed by staining with MHC class I tetramers or fluorochrome-conjugated antibodies. The H-2D$^b$ class I tetramers containing $NP_{366–374}$ peptide or $PA_{224–233}$ peptide were generated by NIH Tetramer Core Facility. Fluorochrome-labeled anti-CD8α (53-6.7), anti-CD4 (RM4-5), anti-CD27 (L6.3A10), anti-CD40 (3/23), anti-CD44 (IM7), anti-CD45.1 (A20),

anti-CD45.2 (104), anti-CD86 (GL1), anti-CD11b (MI/70), anti-Ly6C (AL-21), anti-B220 (RA3-6B2), anti-CD25 (7D4), anti-CD122 (TM-B1), anti-CD62L (MEL-14), and anti-MHC class II (AF6-120.1) were from BD Biosciences. Anti-CD11c (N418), anti-CD70 (FR70), and anti-CD103 (2E7), anti-CD80 (16-10AI), anti-CD127 (A7R34), and anti-KLGR1 (2F-1) were from eBioscience. For intracellular staining and tetramer costaining, cells were stimulated with $NP_{366–374}$ or $PA_{224–233}$ peptides (2 μg/ml) and 40U of rIL-2 in the presence of Brefeldin-A (10 μg/ml) for 3 hr. Cells were then surface stained, washed, and fixed, and intracellular staining for IFN-γ (clone XMG1.2, eBioscience) was performed with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions and adapted from (Dimopoulos et al., 2009). Flow cytometry was performed with a FACSCanto II (BD Biosciences) or a C6 Flow Cytometer (Accuri) and analyzed with Flowjo software.

### Cell Purification, CFSE Labeling, and Adoptive Transfer

$CD8^+$ T cells from influenza-infected C57BL/6 mice or OT-I mice were obtained by depletion of $CD11c^+$ cells with anti-CD11c MACS beads followed by positive selection with anti-CD8 MACS beads (Miltenyi Biotec). All T cell preparations were more than 95% pure. In some experiments, $CD8^+$ T cells were labeled for 10 min at 37°C with 5 μM CFSE (Molecular Probes).

$CD8^+CD44^{hi}$ memory T cells were sorted from spleens of C57BL/6 or *Cd40*$^{-/-}$ mice with a FACSAria (BD Biosciences) after positive selection with anti-CD8 MACS beads. Cell numbers were normalized to the concentration of antigen-specific T cells and 4 × $10^4$ $CD8^+CD44^{hi}$ $D^bNP^+$ or $CD8^+CD44^{hi}$ $D^bPA^+$ T cells were transferred intravenously into naive C57BL/6, *Cd40*$^{-/-}$, or CD45.1 recipient mice. DCs were enriched from pooled mLNs of C57BL/6, *Cd40*$^{-/-}$, or *Cd154*$^{-/-}$ with anti-CD11c MACS beads. In some experiments, DC subsets were sorted with a FACSAria. All sorted DC subsets were more than 95% pure.

### In Vitro Culture

Cells were cultured in RPMI-1640 supplemented with sodium pyruvate, HEPES, pH 7.4, nonessential amino acids, penicillin, streptomycin, 2-mercaptoethanol, and 10% heat-inactivated FCS (all from GIBCO). Sorted DCs (1 × $10^3$) and CFSE-labeled T cells (1 × $10^4$) were cultured for 72 hr at 37°C in 100 μl in round-bottomed 96-well plates. In some experiments, we added soluble OVA protein at 5 μg/ml or $OVA_{257–264}$, $NP_{366–374}$, or $PA_{224-233}$ peptides at 0.5 μg/ml. In some cases, anti-CD40 (10C8), or anti-CD70 (FR70; eBioscience) or rat IgG2b isotype-matched control antibody (KLH; Bioxcell) was added to the culture at a final concentration of 25 μg/ml.

### Statistical Analysis

The statistical significance of differences in mean values was analyzed with a two-tailed Student's t test. p values of less than 0.05 were considered statistically significant.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.06.007.

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