# CD4+ T helper cells use CD154–CD40 interactions to counteract T reg cell–mediated suppression of CD8+ T cell responses to influenza

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**CD4+ T cells promote CD8+ T cell priming by licensing dendritic cells (DCs) via CD40–CD154 interactions. However, the initial requirement for CD40 signaling may be replaced by the direct activation of DCs by pathogen-derived signals. Nevertheless, CD40–CD154 interactions are often required for optimal CD8+ T cell responses to pathogens for unknown reasons. Here we show that CD40 signaling is required to prevent the premature contraction of the influenza-specific CD8+ T cell response. CD40 is required on DCs but not on B cells or T cells, whereas CD154 is required on CD4+ T cells but not CD8+ T cells, NKT cells, or DCs. Paradoxically, even though CD154-expressing CD4+ T cells are required for robust CD8+ T cell responses, primary CD8+ T cell responses are apparently normal in the absence of CD4+ T cells. We resolved this paradox by showing that the interaction of CD40-bearing DCs with CD154-expressing CD4+ T cells precludes regulatory T cell (T reg cell)–mediated suppression and prevents premature contraction of the influenza-specific CD8+ T cell response. Thus, CD4+ T helper cells are not required for robust CD8+ T cell responses to influenza when T reg cells are absent.**

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Abbreviations used: DT, diphtheria toxin; DTR, DT receptor; GC, germinal center; mLN, mediastinal LN; NP, nucleoprotein.

Primary CD8+ T cell responses often require help from CD4+ T cells, which produce cytokines and provide co-stimulation, including the engagement of CD40 by its ligand CD154 (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). In one model, CD4+ T cells engage CD40 on DCs and license them to become efficient antigen-presenting cells for naive CD8+ T cells (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998). However, other models suggest that CD4+ T cells provide help to CD8+ T cells by activating B cells and promoting CD40-dependent antibody responses (Bachmann et al., 2004) or that they engage CD40 on CD8+ T cells (Bourgeois et al., 2002) and directly promote CD8+ T cell activation or survival.

Interestingly, CD4+ T cell help is not required to prime all CD8+ T cells responses. Whereas CD8+ T cell responses to noninflammatory antigens are impaired in the absence of CD4+ T cells or CD40 signaling (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Feau et al., 2011), primary responses to some pathogens occur independently of CD4+ T cells or CD40 signaling (Whitmire et al., 1996, 1999; Shedlock and Shen, 2003; Shedlock et al., 2003; Sun and Bevan, 2003), possibly because of the direct activation of DCs through pathogen recognition receptors (Hamilton et al., 2001). Curiously, primary CD8+ T cell responses to influenza virus require CD40 signaling (Lee et al., 2003a) but not CD4+ T cells (Belz et al., 2002), suggesting that other cell types may express CD154 and license CD40-expressing targets in the absence of CD4+ T cells. Consistent with this view, activated CD8+ T cells (Hernandez et al., 2007; Wong et al., 2008) and natural killer T cells (NKT) express CD154 (Tomura et al., 1999) and may license DCs (Hernandez et al., 2007, 2008; Wong et al., 2008) and help B cells (Chang et al., 2012) in the absence of CD4+ T cells. In addition, CD154 is

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expressed on activated DCs (Johnson et al., 2009) and may directly activate CD40-expressing CD8+ T cells. However, the actual role of CD40 signaling and the cellular basis of CD40-mediated help to CD8+ T cells help are not fully understood.

Whereas helper CD4+ T cells promote T and B cell responses, FoxP3-expressing CD4+ regulatory T cells (T reg cells) suppress them (Kim et al., 2007; Campbell and Koch, 2011; Chung et al., 2011; Dietze et al., 2011; Linterman et al., 2011). Although the potent suppressive activity of T reg cells is neutralized during infection to allow robust immune responses to pathogens, T reg cells are also involved in the late stages of immune responses to resolve inflammation and curtail immunopathology (Suvas et al., 2003; Fulton et al., 2010; McNally et al., 2011). However, the relationship between CD40-mediated CD4+ T cell help and the immunosuppressive activity of T reg cells in CD8+ T cell responses to pathogens remains unexplored.

Here we determined what cells use CD40–CD154 interactions and how CD40 signaling promotes CD8+ T cell responses to influenza. We found that CD4+ T cells were the only cells to functionally express CD154 and that DCs were the only cells that required CD40 for optimal CD8+ T cell responses to influenza. However, rather than licensing DCs to prime naive CD8+ T cells, CD40 signaling was required to prevent the early contraction of the CD8+ T cell response. Despite the necessity for CD154 on CD4+ T cells, we also observed apparently normal CD8+ T cell responses in the absence of CD4+ T cells. Finally, we showed that CD8+ T cell responses were normal or even enhanced when T reg cells were depleted and that additional CD40 blockade did not change the CD8+ T cell response. Thus, our data demonstrate that CD154-expressing CD4+ T cells stimulate DCs through CD40 to counteract T reg cell–mediated suppression of the CD8+ T cell response during the contraction phase of the immune response.

## RESULTS

### Co-stimulation through CD40 prevents premature contraction of the CD8+ T cell response

To test whether CD40–CD154 interactions played a role in the initiation and expansion of the CD8+ T cell response to influenza, we infected C57BL/6 (B6) and *Cd40*$^{-/-}$ mice with influenza virus and followed the accumulation of influenza nucleoprotein (NP)-specific CD8+ T cells in the mediastinal LN (mLN). We found that the frequencies (Fig. 1 A) and numbers (Fig. 1 B) of NP-specific CD8+ T cells were similar in B6 and *Cd40*$^{-/-}$ mice 7 d after infection. However, NP-specific CD8+ T cells failed to accumulate in *Cd40*$^{-/-}$ mice compared with B6 mice on day 10 after infection (Fig. 1, A and B). Similar results were obtained in the lungs (Fig. 1, C and D) and in *Cd154*$^{-/-}$ mice (not depicted), as well as in B6 mice treated with a blocking anti-CD154 antibody (Fig. 1, E–H). These results indicated that CD40 signaling was not required for the initial expansion of influenza-specific CD8+ T cells, but instead prevented their early contraction.

### CD40 expression is required on DCs for influenza-specific CD8+ T cell responses

CD40 signaling on B cells is required for T-dependent B cell activation and antibody production (Han et al., 1995; Lee et al.,

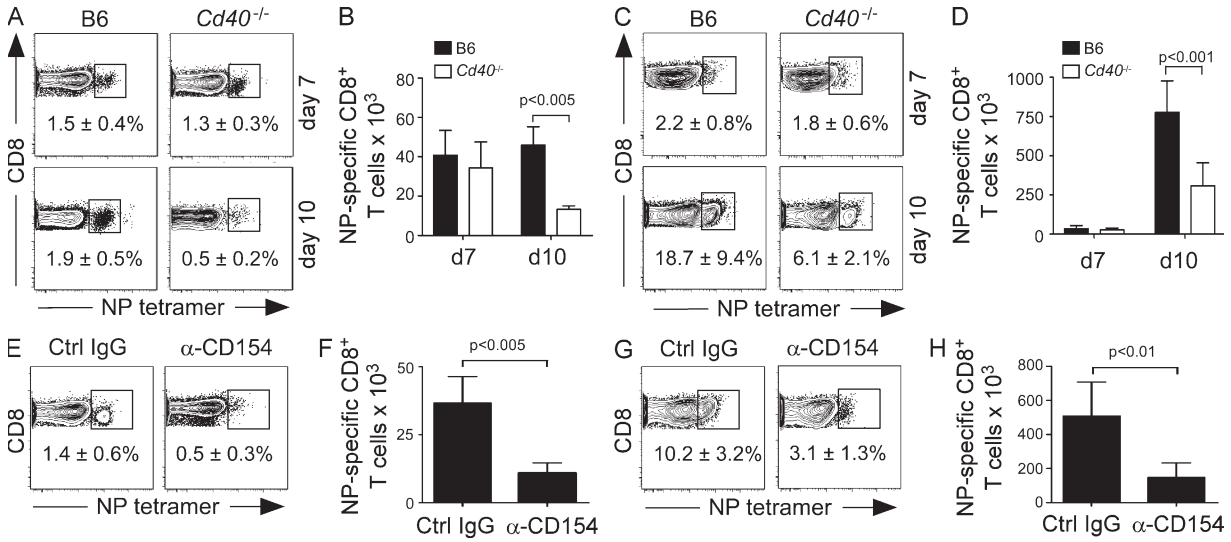


**Figure 1. CD40 signaling prevents premature contraction of influenza-specific CD8+ T cells.** (A–D) B6 and *Cd40*$^{-/-}$ mice were infected with influenza, and the frequency (A and C) and number (B and D) of NP-specific CD8+ T cells in the mLNs (A and B) and lungs (C and D) were determined by flow cytometry at the indicated times. A–D are representative of three independent experiments (mean ± SD of five mice per time point). (E–H) B6 mice were infected with influenza and treated with either 250 µg of the CD154-blocking antibody MR1 or control antibody, and the frequency (E and G) and number (F and H) of NP-specific CD8+ T cells in the mLNs (E and F) and lungs (G and H) were determined by flow cytometry on day 10 after infection. Plots were gated on CD8+ lymphocytes. E–H are representative of five independent experiments (mean ± SD of three to five mice per group). P-values were determined using a two-tailed Student's *t* test.

2003b; Goodnow et al., 2010; Vinuesa et al., 2010) but the role of B cells in CD8$^+$ T cell responses is relatively unexplored (Voeten et al., 2001). To test whether CD40 expression by B cells was required for the accumulation of NP-specific CD8$^+$ T cells, we generated BM chimeras that expressed CD40 normally (B-WT chimeras) or that were selectively CD40 deficient in the B lineage (B-*Cd40*$^{-/-}$ chimeras), infected them with influenza, and enumerated CD19$^+$PNA$^{hi}$FAS$^+$ germinal center (GC) B cells and NP-specific CD8$^+$ T cells in the mLNs on day 12 after infection. As expected (Lee et al., 2003b), the frequencies (Fig. 2 A) and numbers (Fig. 2 B) of GC B cells were reduced in B-*Cd40*$^{-/-}$ chimeras compared with those in B-WT chimeras. However, the frequencies (Fig. 2 C) and numbers (Fig. 2 D) of NP-specific CD8$^+$ T cells in B-*Cd40*$^{-/-}$ chimeras were equivalent to those in B-WT chimeras. Similar results were obtained when the NP-specific CD8$^+$ T cell response was evaluated in the lungs (not depicted). These results indicated that CD40-activated B cells were not required for NP-specific CD8$^+$ T cell expansion after influenza infection.

CD8$^+$ T cells may express CD40 and receive CD40 signals directly from CD4$^+$ T cells (Bourgeois et al., 2002). To determine whether CD40 acted directly on CD8$^+$ T cells, we reconstituted irradiated B6 mice with a 50:50 mix of B6.CD45.1 BM and *Cd40*$^{-/-}$ BM (CD45.2). We allowed the chimeric mice to reconstitute for 2 mo, infected them with influenza virus, and evaluated the NP-specific CD8$^+$ T cell response 12 d later. We found that total CD8$^+$ T cells were derived equally from both donors (Fig. 2 E) and that NP-specific CD8$^+$ T cells were also derived equally from both donors (Fig. 2, F and G). Similar results were obtained in the lung (not depicted). These results suggested that CD40 did not act directly on CD8$^+$ T cells and instead suggested that CD40 signaling on non-CD8$^+$ T cells indirectly promoted CD8$^+$ T cell responses to influenza.

To determine whether robust NP-specific CD8$^+$ T cell responses required sustained interactions with DCs beyond initial priming, we generated BM chimeras in which B6 mice were reconstituted with BM from mice that express the diphtheria toxin (DT) receptor (DTR) under the control of the

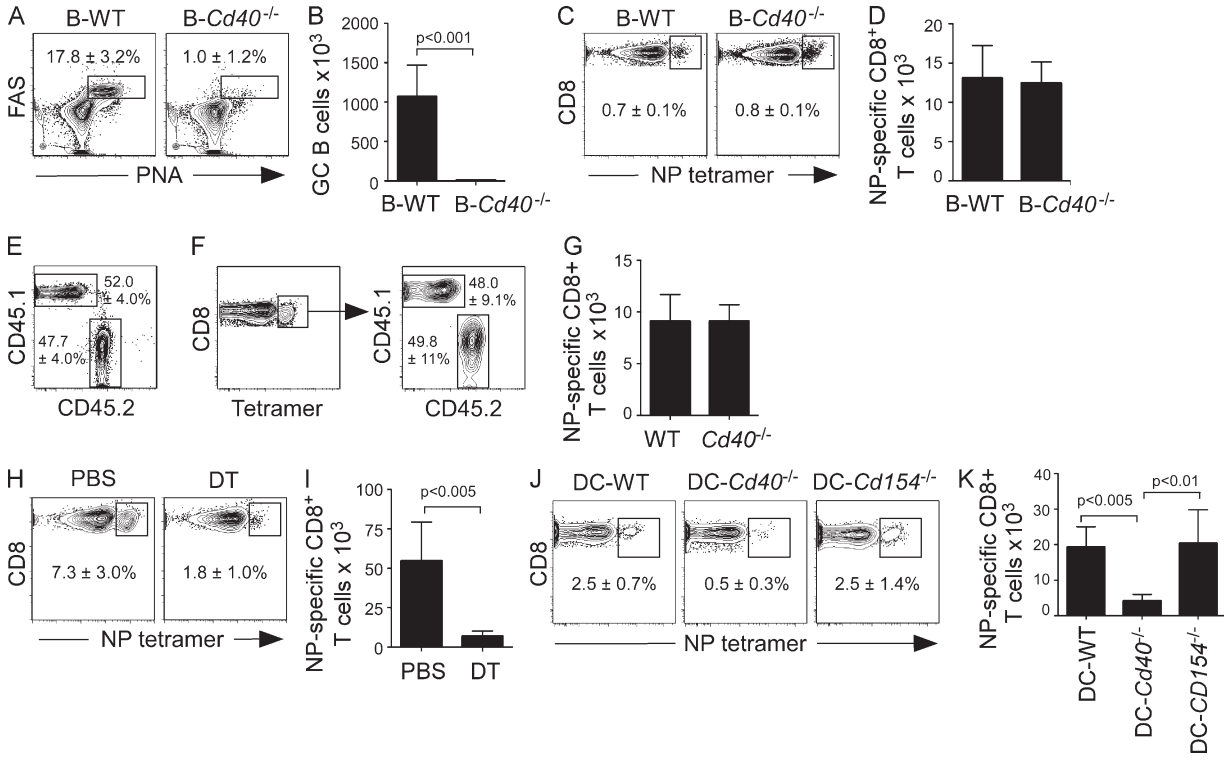


**Figure 2. CD40 is required on DCs but not T cells or B cells for efficient CD8$^+$ T cell responses to influenza.** (A–D) B-WT and B-*Cd40*$^{-/-}$ chimeras were infected with influenza, and the frequency (A) and number (B) of GC B cells as well as the frequency (C) and number (D) of NP-specific CD8$^+$ T cells were determined 12 d later. Plots were gated on CD19$^+$ lymphocytes (A) or CD8$^+$ lymphocytes (C). (E–G) B6:*Cd40*$^{-/-}$ mixed BM chimeras were infected with influenza, and the frequency of CD45.1$^+$ and CD45.2$^+$ cells in the total CD8$^+$ T cell population (E) and in the NP-specific CD8$^+$ T cell population (F), as well the number of CD45.1$^+$ and CD45.2$^+$ NP-specific CD8$^+$ T cells (G) were determined by flow cytometry on day 12 after infection. A–G are representative of two (A–D) or three (E–G) independent experiments (mean ± SD of five mice per group). (H and I) Irradiated B6 mice were reconstituted with CD11c-DTR BM, infected with influenza 8 wk later, and treated with either PBS or 60 ng DT on day 6 after infection, and the frequency (H) and number (I) of NP-specific CD8$^+$ T cells in mLNs were determined by flow cytometry on day 12. (J and K) DC-WT, DC-*Cd40*$^{-/-}$, and DC-*Cd154*$^{-/-}$ BM chimeras were infected with influenza and treated with DT on days 0, 3, 5, and 7 after infection, and the frequency (J) and number (K) of NP-specific CD8$^+$ T cells were determined by flow cytometry on day 10. H–K are representative of four (H and I) or two (J and K) independent experiments (mean ± SD of four to five mice per group). P-values were determined using a two-tailed Student's *t* test.

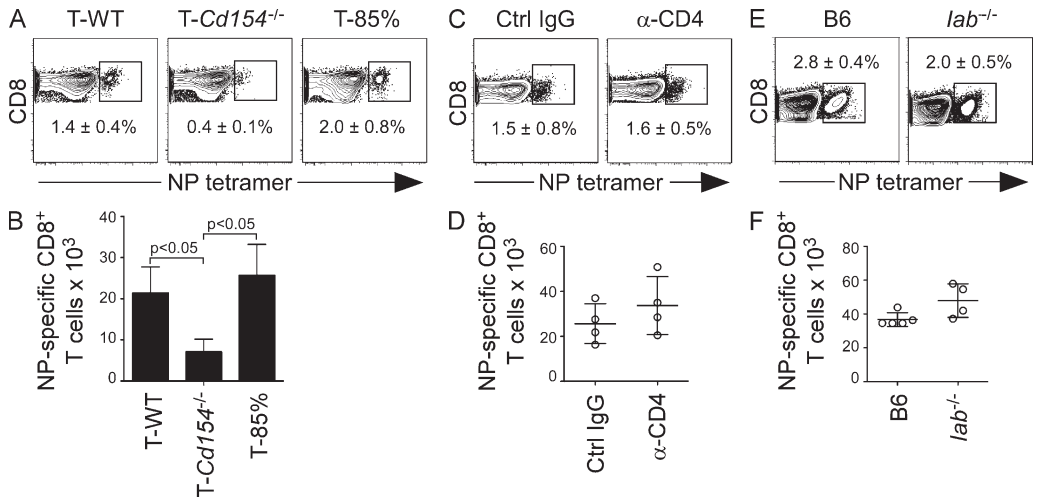


**Figure 3. The NP-specific CD8⁺ T cell response requires CD154 expression on T cells but is normal in the absence of CD4⁺ T cells.** (A and B) T-WT, T-*Cd154*$^{-/-}$, and T-85% chimeric mice were infected with influenza, and the frequency (A) and number (B) of NP-specific CD8⁺ T cells were determined on day 12. (C and D) B6 mice were infected with influenza and treated with 250 µg anti-CD4 or control IgG, and the frequency (C) and number (D) of NP-specific CD8⁺ T cells were calculated by flow cytometry on day 10. (E and F) B6 and *Iab*$^{-/-}$ mice were infected with influenza, and the frequency (E) and numbers (F) of NP-specific CD8⁺ T cells were calculated by flow cytometry on day 10. A–F are representative of three (A, B, E, and F) or four (C and D) independent experiments (mean ± SD of four to five mice per group). P-values were determined using a two-tailed Student's *t* test.

CD11c promoter (CD11c-DTR mice; Jung et al., 2002). 2 mo after reconstitution, we infected the chimeras with influenza, depleted CD11c-expressing cells with DT on days 6 and 10 after infection, and analyzed the NP-specific CD8⁺ T cell response on day 12. We found that after late DC depletion, both the frequencies (Fig. 2 H) and numbers (Fig. 2 I) of NP-specific CD8⁺ T cells were decreased in DT-treated mice. Similar results were obtained in the lungs (not depicted). These results confirmed that expansion of NP-specific CD8⁺ T cells required sustained interactions with DCs in the late phase of the primary response.

Prolonged interactions with CD154-expressing T cells may continue to license DCs that maintain NP-specific CD8⁺ T cell responses, or alternatively, CD154 expression on DCs themselves may be important for CD8⁺ T cell responses (Johnson et al., 2009). To distinguish between these possibilities, we generated mixed BM chimeras in which DCs selectively lacked CD40 (DC-*Cd40*$^{-/-}$ mice) or CD154 (DC-*Cd154*$^{-/-}$ mice) as well as control chimeras (DC-WT mice). 2 mo after reconstitution, we infected the chimeras with influenza, treated them with DT to ablate CD11c⁺ cells derived from the CD11c-DTR BM (León et al., 2012), and enumerated NP-specific CD8⁺ T cells in the mLN. We found that the frequencies (Fig. 2 J) and numbers (Fig. 2 K) of NP-specific CD8⁺ T cells were reduced in the DC-*Cd40*$^{-/-}$ chimeras compared with those in the DC-WT chimeras and the DC-*Cd154*$^{-/-}$ chimeras. Thus, CD40 but not CD154 is required on CD11c⁺ cells to generate optimal NP-specific CD8⁺ T cell responses.

### Optimal NP-specific CD8⁺ T cell responses require CD154 expression on CD4⁺ T cells

To determine whether CD154 expression on T cells was required for normal NP-specific CD8⁺ T cell responses, we generated BM chimeras in which CD154 deficiency was restricted to the T lineage (T-*Cd154*$^{-/-}$ mice) as well as control chimeras with normal expression of CD154 (T-WT mice) and a partial CD154 deficiency (T-85% mice). We infected the chimeras 2 mo after reconstitution and evaluated the NP-specific CD8⁺ T cell response 12 d later. We found that the frequencies (Fig. 3 A) and the numbers (Fig. 3 B) of NP-specific CD8⁺ T cells were similar in the T-WT and T-85% chimeras but were greatly diminished in the T-*Cd154*$^{-/-}$ chimeras. Similar results were obtained when the NP-specific CD8⁺ T cell response was analyzed in the lungs (not depicted). These results indicated that expression of CD154 on T cells was required to license normal NP-specific CD8⁺ T cell response to influenza.

Because CD4⁺ T cells are well known to express CD154 (Lee et al., 2002) and to promote CD8⁺ T cell responses in various experimental models (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998), we next tested whether the depletion of CD4⁺ T cells impaired NP-specific CD8⁺ T cell responses to influenza. We found that influenza-infected B6 mice treated with control antibody or with a depleting anti-CD4 antibody had nearly identical NP-specific CD8⁺ T cell responses at day 10 after infection (Fig. 3 C), even though the anti-CD4 treatment depleted >98% of CD4⁺ T cells (not depicted). To independently confirm these results, we analyzed the CD8⁺ T cell response in MHC class II–deficient (*Iab*$^{-/-}$) mice, which entirely lack CD4⁺ T cells. Consistent with our previous observation (Fig. 3, C and D), the frequencies (Fig. 3 E) and numbers (Fig. 3 F) of NP-specific CD8⁺ T cells in the mLN of *Iab*$^{-/-}$ mice were no different than those in B6 mice. These results indicated that NP-specific CD8⁺ T cells accumulate normally in the absence of CD4⁺ T cells.

CD154 can be expressed by multiple T cell subsets, including CD8+ T cells (Hernandez et al., 2007; Wong et al., 2008) and NKT cells (Tomura et al., 1999). Thus, we next evaluated the CD8+ T cell response in CD1d-deficient (*Cd1d*$^{-/-}$) mice, which selectively lack CD1d-restricted NKT cells (Sonoda et al., 1999; Smiley et al., 2005). We found that NP-specific CD8+ T cells equally accumulated in the mLNs (Fig. 4, A and B) and lungs (not depicted) of *Cd1d*$^{-/-}$ and B6 mice. To test whether CD154 expression on CD8+ T cells was required to maximize influenza-specific CD8+ T cell responses, we purified CD4+ and CD8+ T cells from the spleens of either B6 or *Cd154*$^{-/-}$ mice and transferred mixed populations of these cells to *Tcrβδ*$^{-/-}$ recipients. We infected the recipient mice with influenza 2 d after T cell transfer and enumerated NP-specific CD8+ T cells in the mLN at day 14. We found that the frequencies (Fig. 4 C) and numbers (Fig. 4 D) of NP-specific CD8+ T cells were similar when they were mixed with B6 CD4+ T cells, regardless of whether the CD8+ T cells were derived from B6 or *Cd154*$^{-/-}$ donors. However, NP-specific CD8+ T cells from B6 mice failed to accumulate when mixed with *Cd154*$^{-/-}$ CD4+ T cells (Fig. 4, C and D). As a control, we confirmed that the number of total CD8+ T cells were similar in all groups (Fig. 4 E). These results indicated that the expression of CD154 on CD4+ T cells, but not on CD8+ T cells, was essential for normal primary CD8+ T cell responses to influenza.

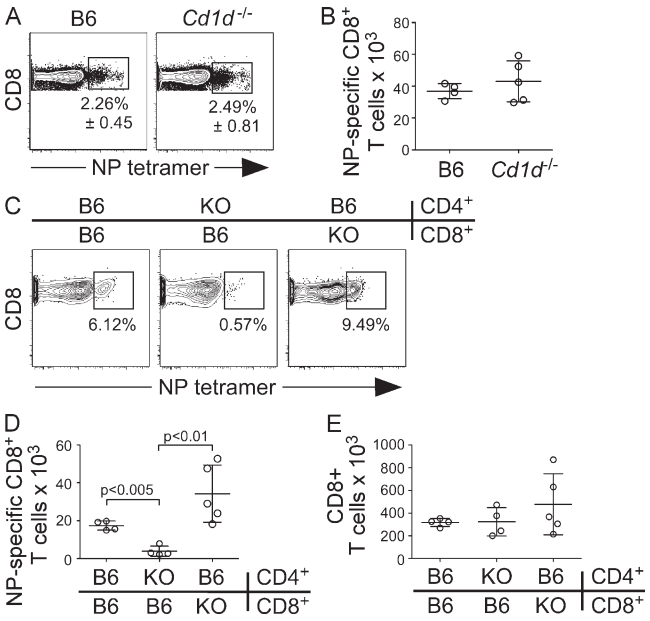


**Figure 4. NP-specific CD8+ T cell response requires CD154 expression on CD4+ T cells but not on NKT or CD8+ T cells.** (A and B) B6 and *Cd1d*$^{-/-}$ mice were infected with influenza, and the frequency (A) and number (B) of NP-specific CD8+ T cells were calculated by on day 10. (C–E) CD4+ and CD8+ T cells purified from spleens of naive B6 and *Cd154*$^{-/-}$ mice were adoptively transferred to *Tcrβδ*$^{-/-}$ recipients, which were infected with influenza the next day, and the frequency (C) and number (D) of NP-specific CD8+ T cells and total CD8+ T cells (E) were determined by flow cytometry 14 d later. A–E are representative of three independent experiments (mean ± SD of four to five mice per group). P-values were determined using a two-tailed Student's *t* test.

### NP-specific CD8+ T cell response does not require CD40 in the absence of CD4+ T cells

Our results paradoxically suggested that the interaction of CD40 on DCs with CD154 on CD4+ T cells helped the late CD8+ T cell response to influenza, even though the CD8+ T cell response was normal in the absence of CD4+ T cells. Based on these results, we hypothesized that CD40 signaling might be dispensable in the absence of CD4+ T cells. To test this possibility, we treated B6 mice with control or depleting anti-CD4 antibodies on days −2 and 4 as well as with control or blocking anti-CD154 antibodies on days 0 and 4 and evaluated the NP-specific CD8+ T cell response on day 10. We observed that the frequencies (Fig. 5, A and C) and numbers (Fig. 5, B and D) of NP-specific CD8+ T cells were reduced in the mLNs (Fig. 5, A and B) and lungs (Fig. 5, C and D) of mice that received anti-CD154 alone compared with mice that received control antibodies. However, NP-specific CD8+ T cells accumulated normally in mice that received anti-CD4 or a combination of anti-CD154 and anti-CD4 (Fig. 5, A–D). Similarly, we found that the blockade of CD154 compromised the NP-specific CD8+ T cell response in the mLNs and lungs of B6 mice but not in the mLNs and lungs of *Iab*$^{-/-}$ mice (Fig. 5, E–H). Moreover, in the reciprocal experiment, we observed that NP-specific CD8+ T cells failed to accumulate in *Cd154*$^{-/-}$ mice when compared with B6 mice (Fig. 5, I–L) but accumulated normally in *Cd154*$^{-/-}$ mice after CD4 depletion (Fig. 5, I–L). Collectively, these results demonstrated that the CD8+ T cell response to influenza does not require CD40 signaling when CD4+ T cells are absent.

### CD40 signaling prevents T reg cell–mediated suppression of NP-specific CD8+ T cell responses

The CD4+ T cell compartment is heterogenous and includes populations of effector T cells that provide help, as well as populations of T reg cells that are immunosuppressive. Thus, we hypothesized that, whereas CD154-expressing CD4+ T cells promoted CD8+ T cell responses, T reg cells suppressed them. To test this possibility, we infected DT-treated B6 and FoxP3-DTR mice (Kim et al., 2007) with influenza and evaluated the frequencies and numbers of FoxP3+CD4+ T reg cells and NP-specific CD8+ T cell on day 10 after infection. As expected, T reg cells were efficiently depleted in DT-treated FoxP3-DTR mice but not in B6 mice (Fig. 6 A). In addition, the frequencies (Fig. 6 B) and numbers (Fig. 6 C) of NP-specific CD8+ T cell were increased in T reg cell–depleted mice. We next administered DT to B6 and FoxP3-DTR mice, infected them with influenza virus, and treated them with either anti-CD154 or control antibody. As expected, CD154 blockade decreased the frequencies (Fig. 6 D) and numbers (Fig. 6 E) of NP-specific CD8+ T cells compared in B6 mice. However, CD154 blockade did not impair the accumulation of NP-specific CD8+ T cells in T reg cell–depleted FoxP3-DTR mice (Fig. 6, D and E).

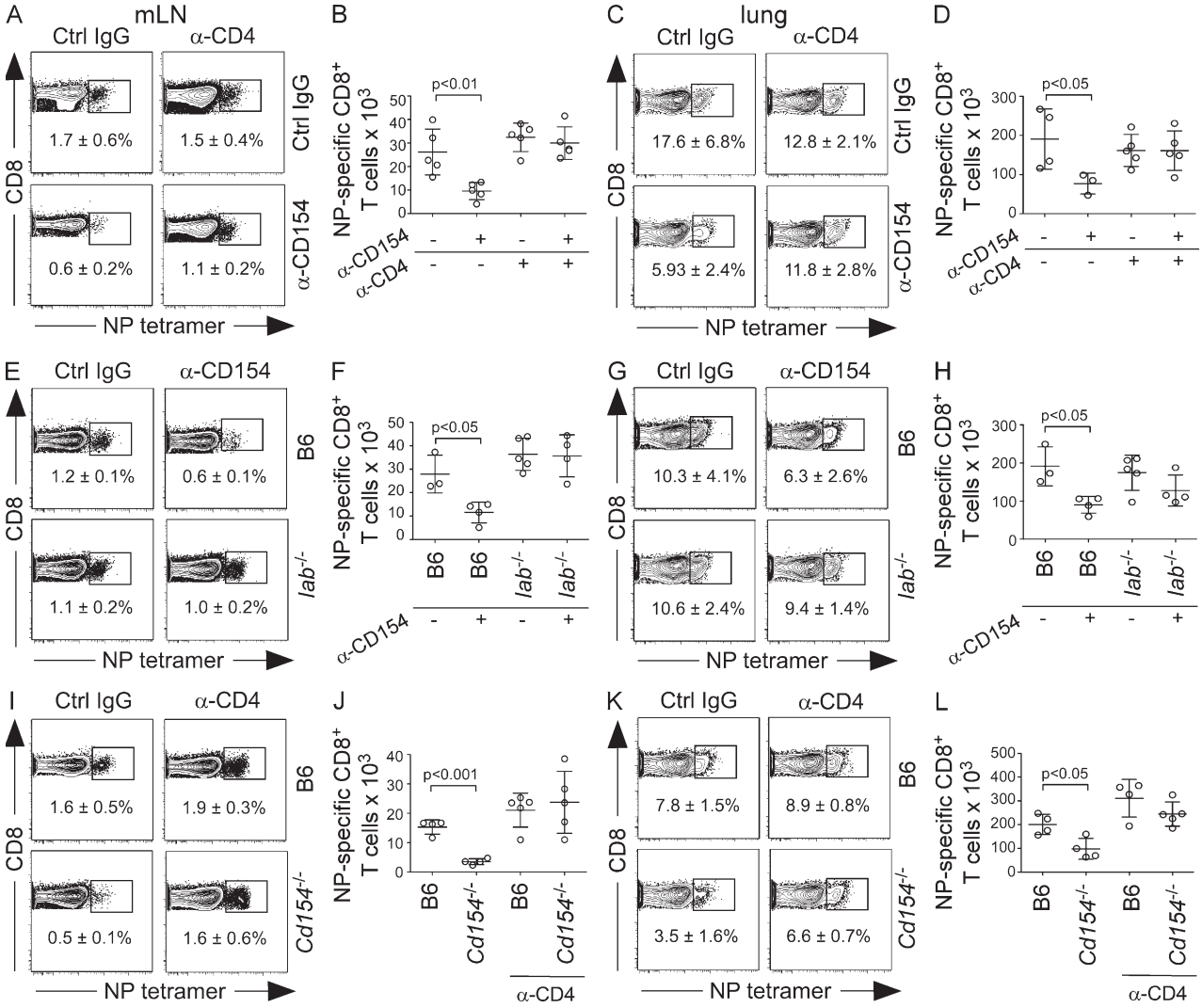


**Figure 5. The NP-specific CD8⁺ T cell response does not require CD40 signaling in the absence of CD4⁺ T cells.** (A–D) B6 mice were treated with combinations of control IgG, anti-CD4, and anti-CD154 and infected with influenza, and the frequency (A and C) and number (B and D) of NP-specific CD8⁺ T cells in the mLNs (A and B) or lung (C and D) were determined by flow cytometry on day 10. (E–H) B6 and *Iab*$^{-/-}$ mice were treated with anti-CD154 or control IgG and infected with influenza, and the frequency (E and G) and absolute number (F and H) of NP-specific CD8⁺ T cells in the mLNs (E and F) and lung (G and H) were calculated on day 10. (I–L) B6 and *Cd154*$^{-/-}$ mice were treated with anti-CD4 or control IgG and infected with influenza, and the frequency (I and K) and number (J and L) of NP-specific CD8⁺ T cells in the mLNs (I and J) and lung (K and L) were calculated by flow cytometry on day 10. A–L are representative of three independent experiments (mean ± SD of four to five mice per group). All plots were gated on CD8⁺ lymphocytes. P-values were determined using a two-tailed Student's *t* test.

To study whether T reg cells controlled NP-specific CD8⁺ T cell expansion late after infection, B6 and FoxP3-DTR mice were infected with influenza and treated with DT at days 6 and 9, and the frequencies and numbers of NP-specific CD8⁺ T cell were evaluated on day 12 after infection. We found that the frequencies (Fig. 6 F) and numbers (Fig. 6 G) of NP-specific CD8⁺ T cell were increased when T reg cells were depleted at late times after infection. Next, to determine whether CD40 signaling was required in the late stage of the influenza immune response to prevent premature contraction of the NP-specific CD8⁺ T cell population, we infected B6 and FoxP3-DTR mice with influenza virus, treated them with either control or anti-CD154 antibody on day 6 as well as with DT on days 6 and 9, and analyzed the NP-specific CD8⁺ T cell response in the mLN at day 13 after infection. We found that late CD154 blockade decreased the frequencies (Fig. 6 H) and numbers (Fig. 6 I) of NP-specific CD8⁺ T cells in B6 mice. However, late CD154 blockade did not impair the accumulation of NP-specific CD8⁺ T cells in FoxP3-DTR mice treated with DT at days 6 and 9 after infection (Fig. 6, H and I).

Because CD40 signaling may have a different impact on CD8⁺ T cell contraction in the lung, we infected B6 and FoxP3-DTR mice with influenza virus, treated them with either

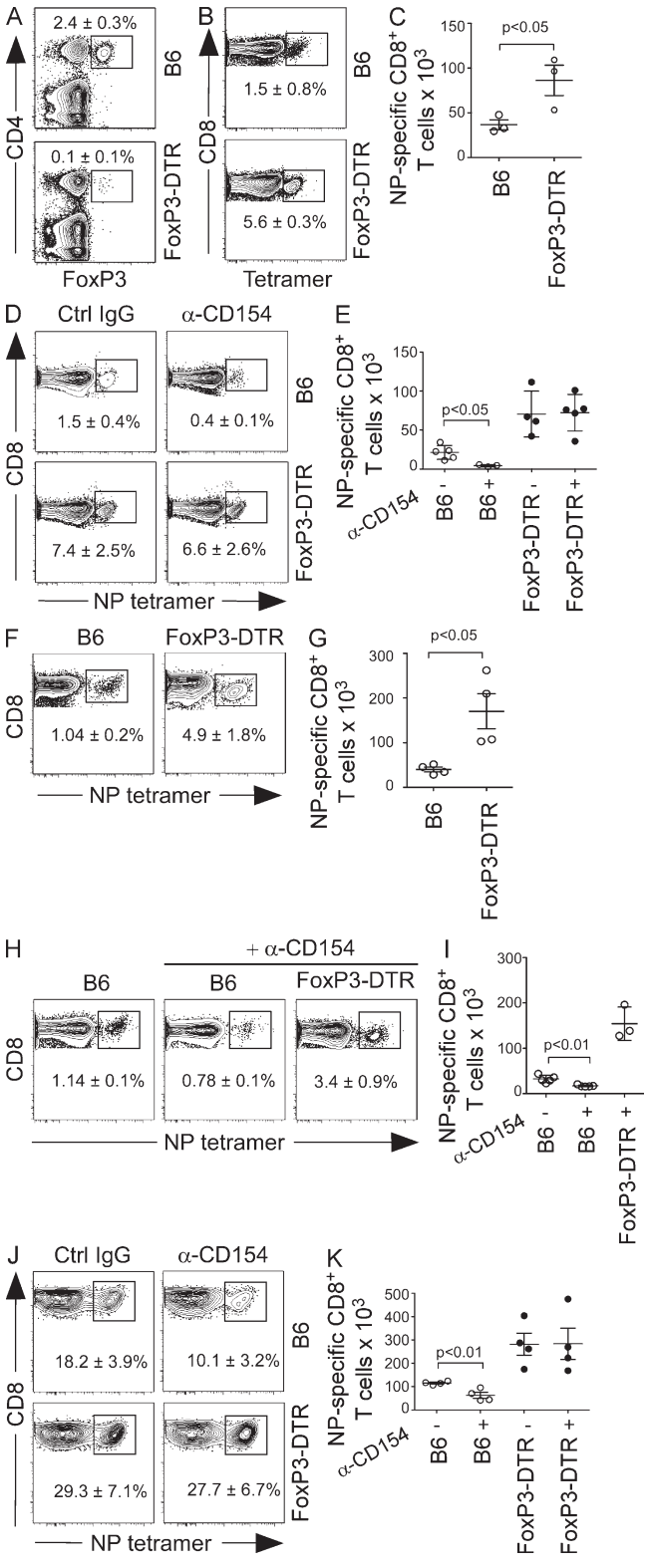


**Figure 6. The NP-specific CD8+ T cell response does not require CD40 signaling in the absence of T reg cells.** (A–C) B6 and FoxP3-DTR mice were infected with influenza and treated with DT on days 0, 4, and 7, and the frequency of FoxP3+CD4+ T cells (A) as well as the frequency (B) and number (C) of NP-specific CD8+ T cells were determined in the mLN by flow cytometry on day 10. (D and E) B6 and FoxP3-DTR mice were infected with influenza, treated with anti-CD154 or control IgG on days 0 and 4, and treated with DT on days 0, 4, and 7, and the frequency (D) and number (E) of NP-specific CD8+ T cells were determined in the mLNs by flow cytometry on day 10. (F and G) B6 and FoxP3-DTR mice were infected with influenza and treated with DT on days 6 and 9, and the frequency (F) and absolute number (G) of NP-specific CD8+ T cells were determined in the mLN by flow cytometry on day 12. F and G are representative of two independent experiments (mean ± SD of four to five mice per group). (H and I) B6 and FoxP3-DTR mice were infected with influenza, treated with anti-CD154 or control IgG on days 6 and 9, and treated with DT on days 6 and 9, and the frequency (H) and number (I) of NP-specific CD8+ T cells were determined in the mLN by flow cytometry on day 12. (J and K) B6 and FoxP3-DTR mice were infected with influenza, treated with anti-CD154 or control IgG on days 6 and 9, and treated with DT on days 6 and 9, and the frequency (J) and number (K) of NP-specific CD8+ T cells were determined in the lung by flow cytometry on day 12. A–E and H–K are representative of four (A–C), three (D and E), or two (H–K) independent experiments (mean ± SD of three to five mice per group). Plots are gated on CD8+ lymphocytes. P-values were determined using a two-tailed Student's *t* test.

control or anti-CD154 antibody 6 d later, and analyzed the NP-specific CD8+ T cell response in the lungs at day 13 after infection. We found that late CD154 blockade decreased the frequencies (Fig. 6 J) and numbers (Fig. 6 K) of NP-specific CD8+ T cells in B6 mice. However, the late blockade of CD154 did not impair the accumulation of NP-specific CD8+ T cells in FoxP3-DTR mice treated with DT at days 6 and 9 after infection (Fig. 6, J and K). Collectively, our results indicated that CD154-expressing CD4+ T cells were essential to prevent T reg cell–mediated suppression of the influenza-specific CD8+ T cell response in the late stages of the primary immune response.

## DISCUSSION

Here we show that CD40–CD154 interactions are required to promote robust influenza-specific CD8+ T cell responses. However, rather than being important for initial DC licensing and CD8+ T cell priming (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Feau et al., 2011), CD40 signaling appears to be important to prevent the premature contraction of the CD8+ T cell response. Consistent with conventional models (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Feau et al., 2011), we find that CD40–CD154 interactions occur between CD40-bearing DCs and CD154-expressing CD4+ T cells during influenza infection, despite apparently normal primary CD8+ T cell responses to influenza in the complete absence of CD4+ T cells (Belz et al., 2002). However, CD4+ T cells are not a homogenous population, and our data also suggest that CD4+ effector T cells express CD154 and maintain DC activation during the late phases of the CD8+ T cell response by counteracting the suppressive effects of FoxP3-expressing CD4+ T reg cells. Thus, CD40–CD154 interactions prevent the CD8+ T cell response from waning prematurely.

CD40 is a co-stimulatory molecule on B cells that, upon engagement with its ligand CD154, promotes $CD4^+$ T cell–dependent B cell activation and proliferation (Elgueta et al., 2009). CD154-expressing $CD4^+$ T cells also help other CD40-expressing APCs like DCs and monocytes (Bennett et al., 1998; Fujii et al., 2004; Munroe, 2009; Kurts et al., 2010). However, reports that CD40 expression is not restricted to APCs and can occur on fibroblasts (Fries et al., 1995; Brouty-Boyé et al., 2000), endothelial cells (Hollenbaugh et al., 1995; Maisch et al., 2002), and even T cells (Bourgeois et al., 2002) complicate the model, as do reports that CD154 can be expressed on NKT cells (Tomura et al., 1999), $CD8^+$ T cells (Hernandez et al., 2007, 2008), platelets (Inwald et al., 2003), and even DCs (Johnson et al., 2009). Thus, the partner cells that mediate CD40–CD154 interactions are not always clear. Nevertheless, we find that the expression of CD154 by $CD4^+$ T cells is both necessary and sufficient for robust $CD8^+$ T cell responses to influenza. Conversely, we also show that CD40 must be expressed on DCs for sustained $CD8^+$ T cell responses. Moreover, we find no evidence for a functional role of either CD154-expressing DCs or CD40-bearing $CD8^+$ T cells. Thus, our results are consistent with the conventional model in which CD154-expressing $CD4^+$ T cells interact with CD40-bearing DCs to promote their activation and antigen presentation.

Conventional models of primary $CD8^+$ T cell responses suggest that $CD8^+$ T cells responding to soluble, inert antigens, such as those in vaccines, require CD154-mediated $CD4^+$ T cell help to license DCs so that they can efficiently cross-present antigens. However later studies suggest that primary $CD8^+$ T cell responses to pathogens (Shedlock and Shen, 2003; Shedlock et al., 2003; Sun and Bevan, 2003), including those to influenza (Belz et al., 2002), do not require $CD4^+$ T cell help because infection triggers an innate inflammatory response that efficiently activates DCs in the absence of CD40 signaling (Hamilton et al., 2001). Nevertheless, $CD8^+$ T cell responses to influenza still require CD40–CD154 interactions (Lee et al., 2003a). Our new data show that CD40–CD154 interactions are not required for the initial licensing of DCs or $CD8^+$ T cell priming but are required during the late phase of the response to prevent premature contraction of $CD8^+$ T cells. Consistent with previous models, CD40 is required on DCs and CD154 is required on $CD4^+$ T cells, suggesting that DC licensing, and by extension antigen presentation, is still important during the late phase of the immune response. This idea is consistent with data showing that antigen-presenting DCs are important to maintain influenza-specific $CD8^+$ T cells in the lung (McGill et al., 2008).

Our data also show that CD40 signaling is required during the late phase of the immune response to counteract the suppressive activity of T reg cells. T reg cells are known to suppress $CD8^+$ T cell responses, in part, by suppressing antigen presentation by DCs (Boissonnas et al., 2010; Gorbachev and Fairchild, 2010). During influenza infection, T reg cell activity is likely minimized during the initial priming phase of the $CD8^+$ T cell response because of the direct activation of DCs by virus-derived molecules and by the innate production of inflammatory cytokines like IL-6 (Pasare and Medzhitov, 2003). However, during the late phase of the immune response, virus is being cleared, inflammation is being resolved, and the $CD8^+$ T cell response is being maintained by newly recruited DCs that are likely susceptible to the suppressive activities of T reg cells (Campbell and Koch, 2011). Thus, we posit that CD154-expressing $CD4^+$ effector cells are required at late times after infection to maintain DC activation and antigen presentation that will prevent the early contraction of the $CD8^+$ T cell response.

The maintenance of antigen-experienced effector $CD8^+$ T cells during the late phase of the immune response may also require co-stimulatory signals that are dependent on CD40 engagement. For example, the engagement of CD27 (Hendriks et al., 2000; Dolfi et al., 2008; Ballesteros-Tato et al., 2010), signaling through the β transmembrane domain of the TCR (Teixeiro et al., 2009), and signals from cytokines such as IL-2 (Williams et al., 2006; Feau et al., 2011), IL-7 (Kaech et al., 2003; Ma et al., 2006), and IL-15 (McGill et al., 2010) are required during the contraction phase of the immune response. Thus, FoxP3-expressing $CD4^+$ T reg cells may limit access to these signals and promote early $CD8^+$ T cell contraction, whereas CD154-expressing helper $CD4^+$ T cells may amplify these signals and promote prolonged $CD8^+$ T cell expansion. For example, T reg cells may suppress $CD8^+$ T cell responses by consuming IL-2 (Pandiyan et al., 2007). Conversely, $CD4^+$ helper T cells may enable autocrine production of IL-2 by $CD8^+$ T cells (Feau et al., 2011), perhaps by promoting CD70 expression on DCs (Feau et al., 2012). Similarly, T reg cells may consume IL-7 (Bayer et al., 2008), whereas $CD4^+$ helper T cells may promote IL-7 production (Carreno et al., 2009). Thus, the balance between $CD4^+$ T cell–dependent, CD154-mediated DC activation and T reg cell–mediated cytokine deprivation may determine the ultimate fate of effector $CD8^+$ T cells during the contraction phase of the immune response.

In summary, by showing that $CD4^+$ T helper cells use CD40–CD154 interactions to counteract the suppressive effects of T reg cells, our results reconcile the conflicting reports in the literature that CD40–CD154 interactions are required (Lee et al., 2003a) but that $CD4^+$ T cells are dispensable (Belz et al., 2002) for efficient primary $CD8^+$ T cell responses to influenza. Moreover, our results support the conventional model, in which CD154-expressing $CD4^+$ T cells interact with CD40-bearing DCs (Bennett et al., 1998; Ridge et al., 1998; Schoenberger et al., 1998; Feau et al., 2011), and exclude the potential contributions of direct CD40 signaling on $CD8^+$ T cells (Bourgeois et al., 2002) and CD154 expression by DCs (Johnson et al., 2009). However, unlike the conventional model, in which CD40 signaling is important for DC licensing and $CD8^+$ T cell priming, our results suggest that CD154–CD40 interactions are important to counteract the suppressive activity of T reg cells during the late phase of the immune response and prevent the premature contraction of influenza-specific $CD8^+$ T cells. Thus, our study reveals an unexpected complexity in the provision of $CD4^+$ T cell help via CD40–CD154 interactions.

## MATERIALS AND METHODS

**Mice.** B6, B6.129P2-Tnfrsf5$^{tm1kik}$ (*Cd40*$^{-/-}$), B6.129S2-Tnfsf5$^{tm1Imx}$ (*Cd154*$^{-/-}$), B6.129S2-IgH-6$^{tm1Cgn}$/J (μMT), B6.FVB-Tg(Itgax-DTR/EGFP)$^{57Lan}$/J (CD11c-DTR), B6.129P2-*Tcrb*$^{tm1Mom}$*Tcrδ*$^{tm1Mom}$ (*Tcrbd*$^{-/-}$), B6.129S-H2dIAb1-Ea (*Iab*$^{-/-}$), and B6.IgH$^{a}$.Thy-1$^{a}$.Ptrpc$^{a}$ (CD45.1) mice were originally obtained from the Jackson Laboratory. B6.CD1d-deficient (*Cd1d*$^{-/-}$) mice were originally obtained from S. Smiley (Trudeau Institute, Saranac Lake, NY). B6.129S6-*Foxp3*$^{tm1}$DTR (FoxP3-DTR) mice were obtained from A. Rudensky (Memorial Sloan-Kettering Cancer Center, New York, NY). All gene-targeted animals were on the B6 background and were bred within the University of Rochester Animal Facilities. The University of Rochester Animal Care and Use Committee approved all procedures.

**BM chimeras.** Chimeric mice were generated by irradiating recipient mice with 950 Rad from a $^{137}$Cs source delivered in two equal doses 4–5 h apart. After irradiation, lethally irradiated mice were intravenously injected with $5 \times 10^6$ total BM cells from the indicated donor mice and allowed to reconstitute for at least 8 wk before infection. To generate mice that lacked CD40 in the B cell compartment, we irradiated B cell–deficient μMT mice and reconstituted them with an 85:15 mix of BM from μMT and B6 donors (B-WT chimeras) or with an 85:15 mix of BM from μMT and *Cd40*$^{-/-}$ mice (B-*Cd40*$^{-/-}$ chimeras). To generate mice in which half of the cells were derived from B6 BM and half of the cells were derived from Cd154$^{-/-}$ BM, we reconstituted irradiated B6 mice with a 50:50 mix of BM from B6.CD45.1 mice and BM from Cd154$^{-/-}$ mice (CD45.2). To generate mice in which the DCs specifically lacked either CD40 or CD154, we reconstituted irradiated mice with an 85:15 mix of CD11c-DTR BM and B6 BM (DC-WT mice), an 85:15 mix of CD11c-DTR BM and CD40$^{-/-}$ BM (DC-CD40$^{-/-}$ mice), or an 85:15 mix of CD11c-DTR BM and Cd154$^{-/-}$ BM (DC-Cd154$^{-/-}$ mice). Mice were treated with DT before infection to eliminate the WT DCs. To generate mice that lacked CD154 in the T cell compartment, we reconstituted irradiated *Tcrβδ*$^{-/-}$ with an 85:15 mix of *Tcrβδ*$^{-/-}$ BM and *Cd154*$^{-/-}$ BM (T-*Cd154*$^{-/-}$ mice) or with an 85:15 mix of *Tcrβδ*$^{-/-}$ BM and B6 BM (T-WT mice). As a final control, we reconstituted irradiated *Tcrβδ*$^{-/-}$ mice with an 85:15 mix of B6 BM and Cd154$^{-/-}$ BM (T-85% mice).

**Infections and in vivo treatments.** Infections were performed intranasally with 500 egg infectious units of influenza A/PR8/34 in 100 μl PBS. To block CD40–CD154 interactions, mice were injected intraperitoneally with 250 μg anti-CD154 (MR-1; Bio X Cell) or 250 μg hamster IgG isotype-matched control antibody (Bio X Cell). To deplete CD4$^+$ cells, mice were treated intraperitoneally with 250 μg anti-CD4 (GK1.5; Bio X Cell) or 250 μg rat IgG isotype-matched control antibody (LTF-2; Bio X Cell). In indicated experiments, B6 and FoxP3-DTR received an intraperitoneal injection of 50 μg/kg DT (Sigma-Aldrich) on days 0, 4, and 7 after infection. To deplete CD11c$^+$ cells, CD11c-DTR BM chimeras were treated with 60 ng DT.

**Cell preparation and flow cytometry.** Cell suspensions from mLNs were centrifuged and resuspended in 150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM EDTA for 10 min to lyse red cells. Cells were then filtered through a 70-μm nylon cell strainer (BD), washed and resuspended in PBS with 5% donor calf serum, and 10 μg/ml FcBlock (2.4G2; Trudeau Institute) for 10 min on ice followed by staining with fluorochrome-conjugated antibodies or tetramer reagents. To obtain single-cell suspensions from lungs, lungs were cut into small fragments and digested for 45 min at 37°C with 0.6 mg/ml collagenase A (Sigma-Aldrich) and 30 μg/ml DNase I (Sigma-Aldrich) in RPMI-1640 medium (Gibco). Digested tissues were mechanically disrupted by passage through a wire mesh. Live mononuclear leukocytes were enriched from lung suspensions by density gradient centrifugation with 1-Step Polymorphs (Accurate Chemical). Cell were then centrifuged and resuspended in 150 mM $NH_4Cl$, 10 mM $KHCO_3$, and 0.1 mM EDTA for 10 min to lyse red cells. Cell suspension was filtered through a 70-μm nylon cell strainer (BD), washed, and resuspended in PBS with 5% donor calf serum and 10 μg/ml FcBlock (2.4G2) for 10 min before staining with fluorochrome-conjugated antibodies or tetramer reagents. Fluorochrome-labeled anti-FoxP3 (FJK-16s), anti-CD19 (1D3), and anti-B220 (RA3-6B2) were obtained from eBioscience. Fluorochrome-labeled anti-CD4 (RM4-5), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD8α (53-6.7), and anti-CD95 (RM4-5) were obtained from BD. Peanut agglutinin (PNA; L7381) was obtained from Sigma-Aldrich and conjugated with FITC. The H-2D$^b$NP$_{366–374}$ tetramer was obtained from the National Institutes of Health Tetramer Core Facility. Intracellular staining was performed using the mouse T reg cell staining kit (eBioscience) according to the manufacturer's instructions. Flow cytometry was performed using a FACSCanto II (BD) or a C6 Flow Cytometer (Accuri), available through the Flow Cytometry Core Facility at the University of Rochester.

**T cell purifications and adoptive T cell transfers.** CD4$^+$ and CD8$^+$ T cells were purified from spleens of naive mice using LS columns and anti-CD4 or andi-CD8 MACs beads (Miltenyi Biotec) according to the manufacturer's instructions. Cells were then stained with fluorochrome-labeled anti-CD4 (RM4-5) and anti-CD8α (53-6.7; BD). Single T cell preparations were >95% pure as determined by flow cytometry. CD4$^+$ ($15 \times 10^6$/mouse) and CD8$^+$ T cells ($10 \times 10^6$/mouse) from the indicated mice were transferred intravenously into naive *Tcrbd*$^{-/-}$ recipient mice.

**Statistical analysis.** The statistical significance of differences in mean values was analyzed using a two-tailed Student's *t* test. P-values of <0.05 were considered statistically significant.

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