Prolonged antigen presentation by immune complex–binding dendritic cells programs the proliferative capacity of memory CD8 T cells

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The commitment of naive CD8 T cells to effector or memory cell fates can occur after a single day of antigenic stimulation even though virus-derived antigens (Ags) are still presented by DCs long after acute infection is resolved. However, the effects of extended Ag presentation on CD8 T cells are undefined and the mechanisms that regulate prolonged Ag presentation are unknown. We showed that the sustained presentation of two different epitopes from influenza virus by DCs prevented the premature contraction of the primary virus-specific CD8 T cell response. Although prolonged Ag presentation did not alter the number of memory CD8 T cells that developed, it was essential for programming the capacity of these cells to proliferate, produce cytokines, and protect the host after secondary challenge. Importantly, prolonged Ag presentation by DCs was dependent on virus-specific, isotype-switched antibodies (Abs) that facilitated the capture and cross-presentation of viral Ags by FcγR-expressing DCs. Collectively, our results demonstrate that B cells and Abs can regulate the quality and functionality of a subset of antiviral CD8 T cell memory responses and do so by promoting sustained Ag presentation by DCs during the contraction phase of the primary T cell response.

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Abbreviations used: Ag, antigen; AID, activation-induced deaminase; BCDT, B cell depletion therapy; DT, diphtheria toxin; DTR, DT receptor; FcR, Fc receptor; IC, immune complex; MedLN, mediastinal LN; NA, neuraminidase; NP, nucleoprotein; PA, polymerase.

Antigen (Ag) processing and presentation is essential for the activation and differentiation of T cells. Although many cell types can function as APCs for CD8 T cells, naive T cells are initially activated by DCs (Lanzavecchia and Sallusto, 2001). The fate of activated T cells is dictated, in part, by TCR signal strength (Zehn et al., 2012), which is regulated by the amount of available Ag (Leignadier and Labrecque, 2010), by the ability of DCs to process and present Ag (Prlic et al., 2006; Obst et al., 2007), and by the affinity of the TCR for its MHC-peptide ligand (Zehn et al., 2009). T cell fate is also controlled by co-stimulatory and inflammatory signals, which can be modulated by endogenous or pathogen-derived molecules that activate DCs (Guermonprez et al., 2002; Mescher et al., 2006). Despite the complexity of interactions between DCs and T cells, CD8 T cells can be sufficiently

activated within 24 h to differentiate into effector and memory cells (Kaech and Ahmed, 2001; van Stipdonk et al., 2001). However, CD8 T cells responding to natural infections, such as influenza, rarely encounter Ag for such a brief period. Instead, CD8 T cells experience numerous encounters with Ag-bearing cells, first in the draining LN (Henrickson et al., 2008) and later in infected or inflamed tissues where T cells may engage other Ag-bearing APCs, including DCs, macrophages, and nonhematopoietic cells (McGill et al., 2008; Hufford et al., 2011). In each case, APCs may provide T cells with a different array of signals. Thus, the ultimate fate of the responding T cell is influenced by the amount of available Ag, the magnitude of the initial inflammatory response, and the type of APC, all of which change throughout the course of infection.

Once pathogens are cleared, inflammation gradually subsides and Ag becomes limiting. This process leads to the contraction of the

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acute effector CD8 T cell response and the survival of a much smaller cohort of memory CD8 T cells (Harty and Badovinac, 2008). These memory CD8 T cells are poised to rapidly respond to secondary encounter with Ag, in part because they receive programming signals during the primary response which imprints the cells with the ability to rapidly proliferate and exert effector functions (Arens and Schoenberger, 2010). CD8 T cell memory programming requires encounter with Ag-presenting DCs, signals through the IL-2R (Williams et al., 2006; Feau et al., 2012), and co-stimulation via CD40–CD154 (Arens and Schoenberger, 2010) and CD27–CD70 pathways (Hendriks et al., 2000; Dolfi et al., 2011; Feau et al., 2012). CD8 memory programming is facilitated when inflammation is low, possibly because inflammatory signals bias CD8 T cell differentiation toward terminal effector differentiation (Pham et al., 2009; Pipkin et al., 2010). Although memory CD8 T cell programming can occur very early in the immune response when Ag is abundant (Prlic et al., 2006), Ag presentation by DCs occurs for weeks after pathogen clearance (Jelley-Gibbs et al., 2005; Zammit et al., 2006; Turner et al., 2007) and some studies suggest that memory CD8 T cells can be programmed during the contraction phase of the primary response when Ag is limiting (Hendriks et al., 2000). Consistent with this idea, Ag presentation during the contraction phase of the primary immune response can increase the magnitude of the primary effector CD8 T cell response and affect the distribution and function of the responding effectors (Zammit et al., 2005, 2006; McGill et al., 2008; Ballesteros-Tato et al., 2010). However, it is not clear whether sustained Ag presentation also affects the differentiation or programming of memory CD8 T cells.

In addition to CD8 T cells, Abs are instrumental for resolving acute viral infections. Virus-specific, isotype-switched Abs, which are produced within 5–6 d of infection (Lee et al., 2005; Mozdzanowska et al., 2005), control Ag availability by preventing continued infection and by binding Ag and targeting it to Fc receptor (FcR)–expressing phagocytic cells (Nimmerjahn and Ravetch, 2008). Ab-dependent targeting of Ag to FcγR-expressing DCs not only facilitates Ag capture by DCs but also promotes DC activation (Regnault et al., 1999) and cross-presentation of viral Ags to CD8 T cells (Amigorena and Bonnerot, 1999; Gil-Torregrosa et al., 2004). However, despite the importance of immune complexes (ICs) in regulating Ag availability and APC activation, the requirement for ICs in regulating primary antiviral CD8 T cell responses or the development of functional memory CD8 T cells has not been examined.

Here, we investigated the role of isotype-switched, influenza-specific Abs in the generation, maintenance, and recall responses of influenza-specific memory CD8 T cells. We found that primary CD8 T cell responses to epitopes present in influenza nucleoprotein (NP) and neuraminidase (NA) contracted more rapidly in mice that were transiently depleted of B cells at the time of infection. Despite the early contraction of the CD8 T cell response in B cell–depleted mice, the number of virus-specific resting memory CD8 T cells was similar to that of normal mice. However, these CD8 memory cells made defective cytokine responses after restimulation, expanded poorly

after challenge infection, and were less protective. Depletion of DCs after day 7 of infection also led to an early contraction of the CD8 T cell response and the development of functionally impaired memory CD8 T cells. The requirement for B cells could be overcome by treating mice with influenza–immune serum or influenza-specific mAbs during the primary response, which restored the CD8 T cell recall response. However, the addition of influenza-specific IgG only restored the CD8 T cell recall response when FcγR–expressing DCs were present after the first week of infection. Thus, prolonged Ag presentation by FcγR-expressing DCs to CD8 T cells during the primary infection was facilitated by nonneutralizing Abs that promoted the acquisition, processing, and presentation of Ag, which was required for the programming of fully functional CD8 memory cells. Collectively, these data reveal a novel Ab-dependent mechanism that improves the overall fitness of antiviral memory CD8 T cell responses.

RESULTS
Prolonged Ag presentation by DCs promotes NP–specific primary and secondary CD8 T cell responses

Previous studies show that sustained Ag presentation by DCs to CD8 T cells prolongs the primary influenza-specific CD8 T cell response (McGill et al., 2008; Ballesteros-Tato et al., 2010; Kim et al., 2010). To determine whether sustained Ag presentation by DCs also impacts the generation or maintenance of influenza-specific memory CD8 T cells, we reconstituted lethally irradiated C57BL/6J (B6) mice with BM from CD11c–diphtheria toxin receptor transgenic mice (CD11c-DTR; Jung et al., 2002) and allowed them to reconstitute for 8 wk. We subsequently infected the chimeric mice with influenza X31 virus (H3N2), treated them with DT every 3 d from days 7–50 after infection, and evaluated the NP-specific CD8 T cell response in the draining mediastinal LN (medLN) on days 15 and 60 (Fig. 1 A). We found that DC depletion beginning on day 7 significantly decreased the frequency and number of NP-specific CD8 T cells on day 15 (Fig. 1, B and C) but had no measurable impact on the frequency or number of NP-specific CD8 memory T cells on day 60 (Fig. 1, D and E). Importantly, this was not due to DTR–induced deletion of a CD11c-expressing subpopulation of CD8 T cells (Jung et al., 2002), as day 7 postinfection NP-specific CD8 T cells did not express the CD11c-driven DTR transgene and linked reporter gene (EGFP) and were not sensitive to DTR treatment (unpublished data). Thus, Ag presentation by DCs after day 7 increased the overall magnitude of the primary NP-specific CD8 T cell response. However, Ag presentation by DCs after day 7 was not required to generate memory CD8 T cells.

We next treated chimeric mice with DT every 3 d from day 7, 14, or 21 through day 50 (Fig. 1 F). We waited 10 d after the last DT administration (day 60) to allow DCs to repopulate the LN (Fig. 1 G), challenged the mice with influenza A/PR8/34 (PR8–H1N1), and evaluated the NP-specific CD8 T cell response 6 d later. We found that the frequencies and numbers of NP-specific CD8 T cells were significantly decreased in the medLNs of mice that were depleted of CD11c^{+}
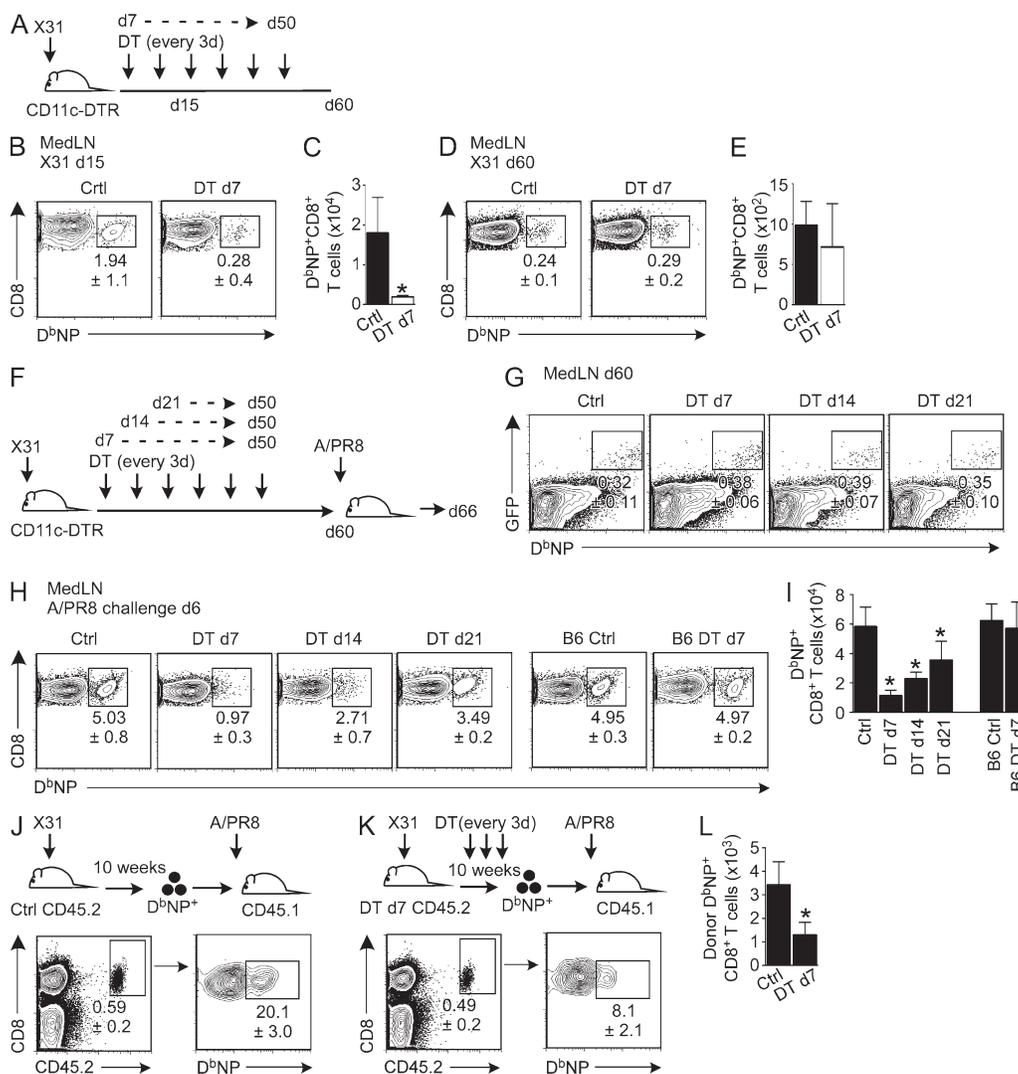
Figure 1. Prolonged Ag presentation by DCs during the primary response enhances NP–specific CD8 recall responses. (A–E) B6 mice were irradiated and reconstituted with CD11c-DTR-GFP BM. Reconstituted mice (A) were infected with X31 and injected with PBS or DT every 3 d, starting on day 7. The frequencies (B and D) and absolute numbers (C and E) of NP$^+$ CD8$^+$ T cells in the medLN on day 15 (B and C) and day 60 (D and E). (F–I) CD11c-DTR BM chimeras were infected with X31 and injected with PBS or DT every 3 d, starting on day 7, 14, or 21 and continuing until day 50. The mice were rested for 10 d to allow DCs to recover and the frequency of the DCs expressing the CD11c-DTR-GFPP transgene in the medLN on day 60 was assessed by flow cytometry (G). The remaining chimeras were challenged with PR8 virus and the frequencies (H) and absolute numbers (I) of NP$^+$ CD8$^+$ T cells in the medLN on day 6 after challenge were determined. (J–L) CD11c-DTR chimeras were infected with X31 virus and treated with PBS or DT beginning on day 7 and continuing until day 50. On day 70, memory CD8$^+$CD44^{hi} T cells were sorted from the spleens of both groups of mice and analyzed by flow. Equal numbers (5 × 10^3) of NP$^+$ CD8$^+$CD44^{hi} T cells were transferred into naive CD45.1$^+$ recipients that were infected with PR8 24 h later. The frequencies and numbers of medLN NP$^+$ CD8 T cells from control (J and L) or DT-treated (K and L) donors were determined 6 d after challenge. Data are representative of two or more independent experiments (mean ± SD of 4–5 mice per group; *, P < 0.01 vs. control).

cells beginning on days 7, 14, and, to a lesser extent, 21 relative to control mice (Fig. 1, H and I).

To ensure that the decreased NP$^+$ CD8 T response was not due to a nonspecific effect of the DT, we administered DT every 3 d to influenza–infected B6 mice (that do not express DTR) from days 7–50, challenged them on day 60, and measured the CD8 memory recall response 6 d later. Neither the frequencies nor the numbers of NP-specific CD8 T cells in the B6 mice were affected (Fig. 1, H and I), indicating that the defective CD8 recall response observed in DT-exposed

CD11c-DTR chimeras was most likely due to the depletion of DCs during the late phase of the primary response.

Finally, to ensure that the decreased NP-specific CD8 recall response observed in the DT-treated CD11c-DTR chimeras was not due to functional changes in the DCs that repopulate the host once DT treatment is stopped, we sorted CD44^{hi} CD8 T cells from CD45.2$^+$ control and DT-treated chimeras 10 wk after primary X31 infection and adoptively transferred equivalent numbers of NP-specific CD8 T cells into naive CD45.1$^+$ B6 recipients. We infected the recipients
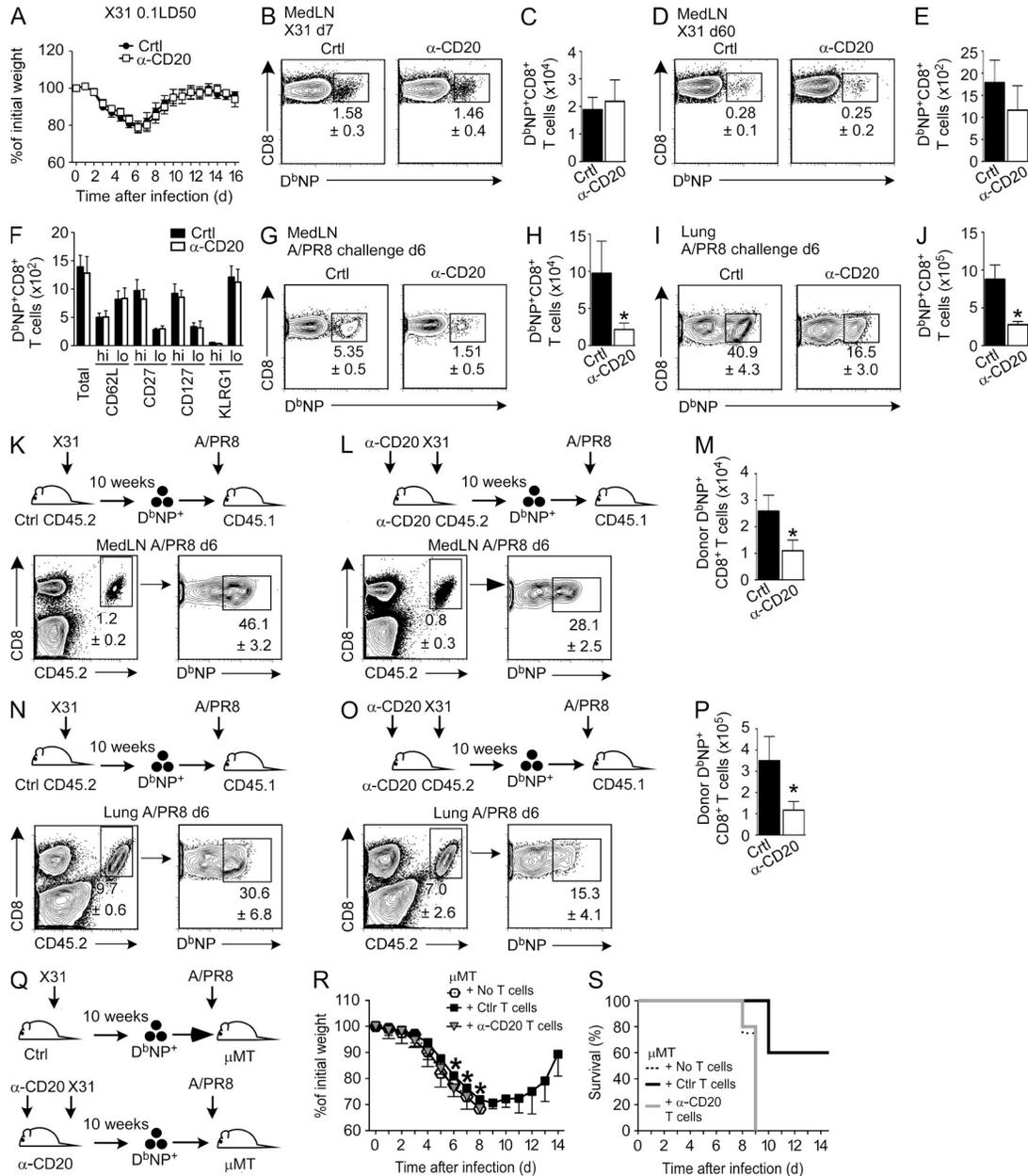
Figure 2. B cell depletion during the primary infection impairs NP–specific CD8 T cell recall responses. (A–F) Control and anti-CD20–treated B6 mice were infected with X31. Body weight changes were assessed for 16 d after virus inoculation (A). The frequencies and total numbers of NP+ CD8 T cells in the medLN were determined on days 7 (B and C) and 60 (D and E). Day 60 NP+ CD8 T cells were analyzed for expression of CD62L, CD27, CD127, and KLRG1. The frequencies (Fig. S1) and numbers (F) of the different populations were determined. (G–J) Control and anti-CD20–treated B6 mice were infected with X31, challenged with PR8 10 wk later, and analyzed on day 6 after challenge. The frequencies and total numbers of NP+ CD8 T cells in the medLN (G and H) and lung (I and J) were determined. (K–P) Memory CD8+CD44hi T cells were sorted from the spleens of B6 or anti-CD20–treated mice 10 wk after primary X31 infection. Equal numbers of NP+ CD8+CD44hi T cells (5 × 10^3/recipient) were transferred into naive CD45.1+ mice. Recipient mice were infected with PR8 24 h later and analyzed on day 6 after infection. The frequencies and numbers of NP+ CD8 T cells from B6 (K and N) or anti-CD20–treated (L and O) donors present in the recipient medLN (K–M) and lungs (N–P) were determined 6 d after infection. (Q–S) Memory CD8+ T cells were sorted from the spleens of B6 or anti-CD20–treated mice 10 wk after primary X31 infection (Q). Equal numbers of NP+ CD8+CD44hi T cells (5 × 10^4/recipient) were transferred into naive μMT mice. Recipient mice were infected with PR8 24 h later. Weight loss (R) and survival (S) of the recipients were monitored for 2 wk. Data are representative of two or more independent experiments (mean ± SD of 4–5 mice per group; *, P < 0.01 vs. control).

with PR8 and monitored the CD8 recall response 6 d later. We found that the frequencies and numbers of donor NP-specific CD8 T cells were significantly decreased in mice that received memory CD8 T cells from CD11c–depleted donors compared with mice that received memory CD8 T cells from control donors (Fig. 1, J–L). Thus, extended Ag presentation

by DCs during the later stages of primary influenza infection, although dispensable for the development of NP-specific CD8 memory cells, was critically important for the development of NP-specific memory CD8 T cells that could rapidly expand after challenge infection.

B cells regulate the quality of the NP–specific CD8 recall response

Given that sustained Ag presentation by DCs was needed for the development of functional NP+ CD8 memory cells, we hypothesized that Ab-containing ICs were required for sustained Ag presentation by DCs. To test this hypothesis, we administered anti-CD20 (Hamel et al., 2008) to deplete B cells 4 d before infection with X31 and then monitored the NP-specific CD8 T cell response in the medLN on days 7 and 60. Consistent with published data showing that B cell–deficient and B6 mice clear X31 virus infection with identical kinetics (Topham et al., 1996), we observed equivalent weight loss and recovery in B cell–depleted and control mice (Fig. 2 A). We also observed similar frequencies and numbers of NP-specific CD8 T cells in both groups of mice on days 7 (Fig. 2, B and C) and 60 (Fig. 2, D and E) in the medLN. Furthermore, we found that the expression of CD62L, CD27, CD127, and KLRG1 was similar on NP-specific CD8 T cells in the medLN of B cell–depleted and control mice on day 60 (Fig. 2 F and Fig. S1).

To test the effect of B cell depletion on the memory CD8 T cell recall response, we next infected B cell–depleted and control mice with X31, waited 10 wk, and challenged them with PR8. Interestingly, the magnitude of the NP-specific CD8 T cell recall response was significantly decreased in both the medLN (Fig. 2, G and H) and lungs (Fig. 2, I and J) of B cell–depleted mice. To ensure that the attenuated NP-specific CD8 T cell recall response observed in the B cell depleted mice was not due to the absence of B cells at the time of challenge, we sorted CD44^{hi} CD8 T cells from CD45.2+ control and B cell–depleted mice 10 wk after primary X31 infection, adoptively transferred equivalent numbers of NP-specific CD8 T cells into naive CD45.1+ B6 mice, and infected the recipients with PR8. 6 d after challenge, we found that the frequencies and numbers of donor NP+ CD8 T cells were significantly decreased in both the medLNs (Fig. 2, K–M) and lungs (Fig. 2, N–P) of mice that received CD8 memory T cells from B cell–depleted donors compared with mice that received memory CD8 T cells from control donors.

Finally, to determine whether NP-specific memory CD8 T cells generated in the presence of B cells were more protective after challenge infection, we infected B cell–depleted or control B6 mice with X31, waited 60 d, and transferred equal numbers of CD44^{hi} NP-specific CD8 memory T cells from these mice into naive μMT mice, which are highly susceptible to PR8 (Rangel-Moreno et al., 2008). We then infected the recipients with PR8 and monitored survival and weight loss (Fig. 2 Q). As expected, μMT mice that did not receive memory T cells rapidly lost weight (Fig. 2 R) and succumbed to infection by day 9 (Fig. 2 S). In contrast, μMT mice that received CD8 memory T cells from B cell–sufficient donors regained body weight (Fig. 2 R) and more than half the animals fully recovered from infection (Fig. 2 S). However, μMT mice that received CD8 memory T cells from B cell–depleted donors all succumbed to infection by day 9 and were indistinguishable from the mice that did not receive memory CD8 T cells (Fig. 2, R and S). Collectively, these data showed that, although B cells were not required for the initial expansion of NP-specific CD8 effector T cells or for the development of NP-specific memory CD8 T cells, the presence of B cells during the primary infection was necessary to generate NP-specific memory CD8 T cells that could undergo rapid secondary expansion and provide protection after challenge infection.

B cells facilitate prolonged Ag presentation by DCs and extend the duration of the primary NP-specific CD8 T cell response

Given that the NP-specific CD8 T cell recall response to influenza was highly dependent on the presence of B cells during the primary infection, we next evaluated the kinetics of the primary NP-specific CD8 T cell response in B cell–depleted and control mice. We found that the magnitude of the NP-specific CD8 T cell response in B cell–depleted mice and control mice was equivalent on day 7 (Fig. 3, A–C). However, the NP-specific CD8 T cell response contracted more rapidly between days 10 and 20 in B cell–depleted mice compared with control mice (Fig. 3, A–C). Thus, B cell depletion appeared to accelerate contraction of NP-specific CD8 effectors.

The development of both primary and memory CD8 T cell responses to influenza is critically dependent on Ag-presenting DCs during the first week of infection (Belz et al., 2004). Because the development of optimal CD8 memory responses to influenza required B cells as well as DCs after the first week of infection, we hypothesized that B cells might modulate the DCs by enhancing the Ag-presenting capacity of the DCs. To test this hypothesis, we infected control and B cell–depleted mice with X31 and enumerated CD11c+ DCs on day 12 after infection. We did not observe significant differences in the numbers of total CD11c+, CD103+, or CD103− DCs in the medLN (Fig. 3 D and Fig. S2) or lung (not depicted) between WT and B cell–depleted mice. We also purified CD11c+ DCs from the medLNs of B cell–depleted and control mice on day 12 and co-cultured them with CFSE-labeled CD8 TCR transgenic OT-I cells in vitro in the presence of specific Ag (OVA protein and OVA[257-264] peptide). We found that DCs from B cell–depleted and control mice were similarly able to induce proliferation of naive OT-I CD8 T cells when provided with either whole protein or peptide (Fig. 3, E and F), indicating that DCs from B cell–depleted and control mice were similarly able to acquire, process, and present Ags that were provided ex vivo.

To test whether DCs could present influenza Ags obtained in vivo, we infected B cell–depleted mice and controls with X31, purified CD11c+ DCs from the medLNs or lungs on day 12, and co-cultured them for 3 d with CFSE-labeled CD8 T cells sorted from the medLNs of day 7 X31-infected B6 mice. We found that the lung DCs, regardless of whether they were isolated from B cell–depleted or control mice, did
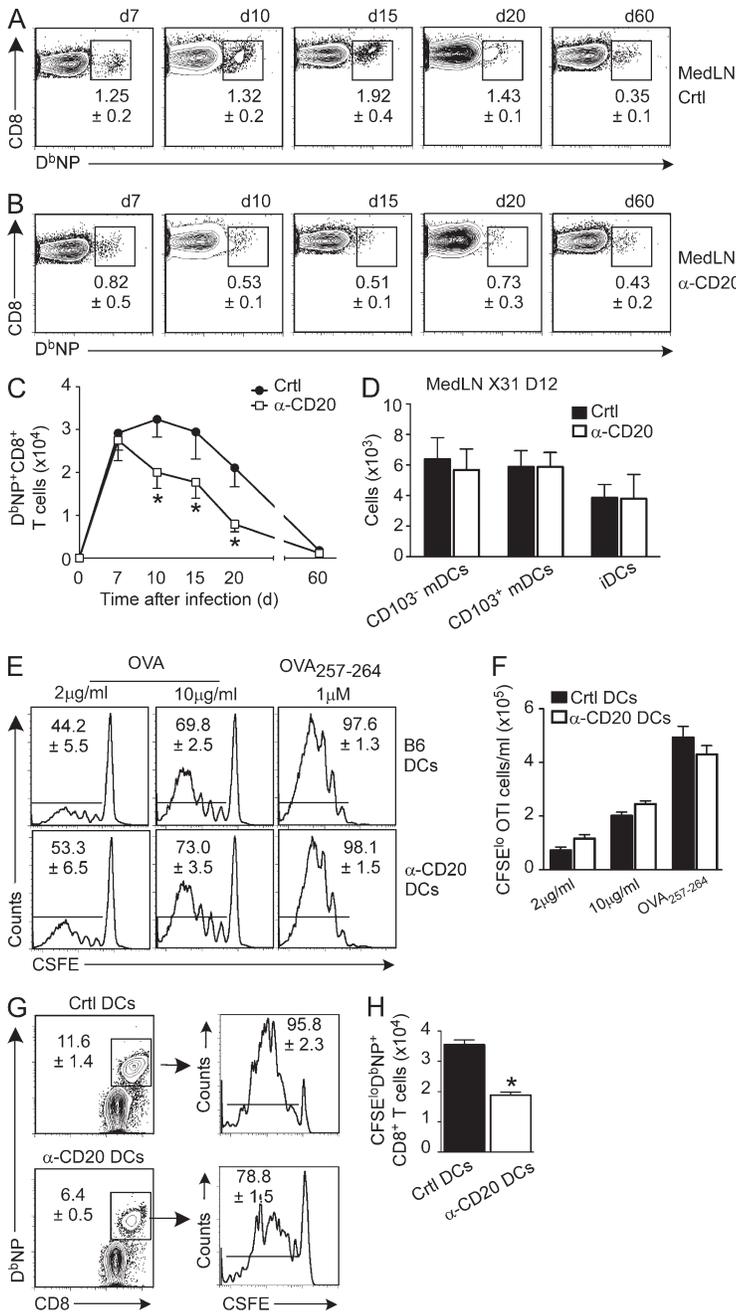
Figure 3. B cell depletion induces accelerated contraction of NP–specific primary CD8 T cell responses and impairs late DC Ag presentation to NP+ CD8 T cells. Control and anti-CD20–treated B6 mice were infected with X31. Frequencies (A and B) and absolute numbers (C) of NP+ CD8 T cells in the medLNs were determined at indicated time points using flow cytometry. The numbers of mature and immature DC subpopulations (mDCs and iDCs) in medLN were determined on day 12 (D) according to gating strategy in Fig. S2. Day 12 CD11c+ medLN DCs from X31-infected control or anti-CD20–treated mice were purified and co-cultured for 3 d with purified CFSE-labeled OT-I cells in the presence of OVA protein or OVA$_{257-264}$ peptide (E and F) or with CFSE-labeled CD8 T cells isolated from medLNs of day 7 X31-infected B6 mice (G and H). The frequencies (E and G) and total numbers (F and H) of CFSE^{lo}CD8+ T cells were determined by flow cytometry. Data are representative of three independent experiments (mean ± SD of 5 mice per time point; *, P < 0.01 vs. control).

not expand NP-specific CD8 T cells (unpublished data). In contrast, DCs from the medLNs of control mice efficiently expanded NP-specific CD8 T cells, whereas DCs from B cell–depleted mice poorly expanded NP-specific CD8 T cells (Fig. 3, G-H). Thus, although B cell depletion had no obvious effect on the number of DCs in the LN or lung on day 12 after infection, it impaired the capacity of DCs in the medLN to present NP to CD8 T cells during the late phase of the primary response.

NP-specific Abs sustain Ag presentation by LN DCs and delay the contraction of the primary NP-specific CD8 T cell response

To distinguish whether the premature contraction of the NP-specific CD8 T cell response in B cell–depleted mice was due to the loss of isotype-switched Ab or to the loss of the B cells themselves, we compared the NP-specific CD8 T cell response in B cell–depleted mice and *Aicda*$^{-/-}$ mice that lack activation-induced deaminase (AID), an enzyme required for isotype switching and affinity maturation (Muramatsu et al., 2000). Interestingly, the kinetics of the NP-specific CD8 response in *Aicda*$^{-/-}$ mice mirrored that observed in B cell–depleted mice (Fig. 4, A–C). Thus, AID-expressing, isotype-switched, and/or affinity-matured B cells were required to prevent premature contraction of the primary NP-specific CD8 T cell response.

To test whether influenza-specific, isotype-switched Abs facilitated prolonged Ag presentation by DCs and thereby

extended the primary CD8 T cell response, we purified CD11c^{+} DCs on day 12 after X31 infection from control or B cell–depleted mice that had received influenza-immune serum from B6 mice (immune serum) or from μMT mice (Ab-deficient control serum) and co-cultured them for 3 d with CFSE-labeled CD8 T cells from the medLNs of day 7 X31-infected B6 mice. We found that DCs from B cell–depleted mice treated with control serum poorly induced NP-specific CD8 T cell expansion, whereas DCs from B cell–depleted mice treated with immune serum were as effective as DCs from control mice in expanding the NP-specific CD8 T cells (Fig. 5, A and C). Importantly, the failure of DCs from B cell–depleted mice treated with control serum to expand NP-specific CD8 T cells was not due to an inability to stimulate CD8 T cells because addition of exogenous NP$_{366-374}$ peptide to the co-cultures induced comparable expansion of NP-specific CD8 T cells, regardless of the origin of the DCs (Fig. 5, B and C). Thus, influenza-specific serum Abs enhanced the capacity of DCs to capture NP Ag that could then be processed and presented to CD8 T cells.

To determine whether the accelerated contraction of the NP-specific CD8 response observed in B cell–depleted mice was also due to the loss of influenza-specific Abs, we infected control and B cell–depleted mice with X31, transferred control or immune serum on days 10 and 14, and enumerated NP-specific CD8 T cells in the medLN on day 20. As expected, the frequencies and numbers of NP-specific CD8 T cells were lower in the B cell–depleted mice that received control serum compared with intact mice (Fig. 5, D and E). However, the NP-specific CD8 T cell response was restored to normal levels when immune serum was transferred to the B cell–depleted animals (Fig. 5, D and E).

Given these results, we hypothesized that NP-containing ICs must target Ag to DCs, which then process and present NP peptides to NP-specific CD8 T cells. To test this hypothesis, we infected B cell–depleted mice with X31, treated them on day 10 with a mixture of purified anti-NP mAbs (LaMere et al., 2011a) or isotype control mAbs, purified CD11c^{+} DCs from the medLN on day 12, and co-cultured these DCs with CFSE-labeled T cells from day 7 infected medLNs. As expected, the NP-specific CD8 T cells did not proliferate when cultured without DCs (Fig. 5 F). However, DCs from B cell–depleted mice that received anti-NP mAbs expanded NP-specific CD8 T cells more efficiently than DCs from B cell–depleted mice that received isotype control mAbs, and were as effective as DCs from the B cell–sufficient mice (Fig. 5, F and H). Importantly, DCs from all groups were similarly able to stimulate T cells when supplied with exogenous NP$_{366-374}$ peptide (Fig. 5, G and H). Finally, transfer of purified anti-NP mAbs to infected B cell–depleted animals on days 10 and 14 restored the primary NP-specific CD8 T cell response to the levels observed in control mice on day 20 after infection (Fig. 5, I and J). These data suggested that anti-NP mAbs supported extended Ag presentation by DCs and prevented premature contraction of the primary NP-specific CD8 T cell response.

NP-specific Abs regulate the quality of the NP-specific CD8 T cell memory response

To address whether NP-specific Abs affected the quality of NP-specific CD8 memory cells, we restimulated memory CD8 T cells from B cell–depleted animals or control mice that had been administered NP-specific or control mAbs during the primary infection, and evaluated their ability to coproduce IL-2 and IFN-γ, as multi-cytokine production is an attribute of functional memory CD8 T cells (Kristensen et al., 2002). We observed that the frequency and number of IL-2– and IFN-γ–coproducing NP-specific CD8 T cells were decreased in B cell–depleted mice compared with control mice (Fig. 6, A and B). However, treatment of B cell–depleted mice with NP-specific mAbs during the primary infection rescued the capacity of the memory NP-specific CD8 T cells to coproduce IL2 and IFN-γ (Fig. 6, A and B).

To assess whether the presence of influenza-specific Abs during the primary response also enhanced the virus-specific recall response, we infected control and B cell–depleted mice with X31 and transferred control serum, immune serum, purified anti-NP mAbs, or isotype control Abs to the infected animals on days 10, 14, and 21 after infection. We then waited
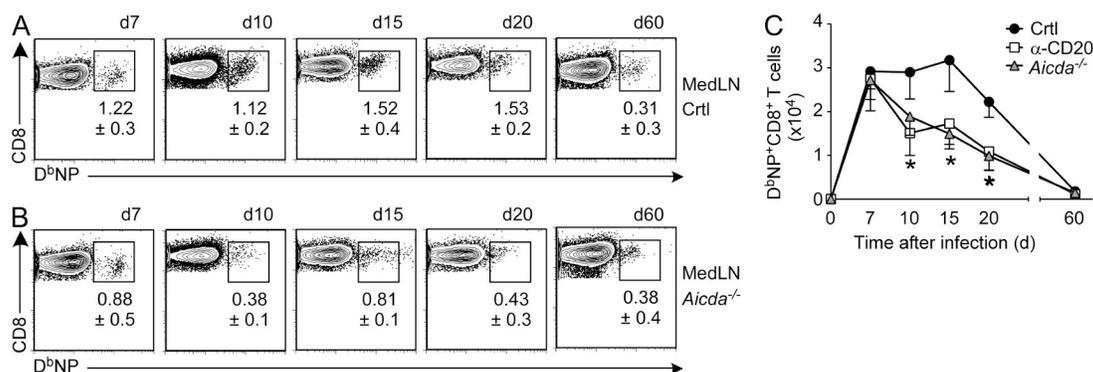
Figure 4.   AID deficiency causes accelerated contraction of NP-specific primary CD8 T cell responses. *Aicda*$^{-/-}$ and B6 mice or anti-CD20–treated B6 mice were infected with X31. Frequencies (A and B) and absolute numbers (C) of NP[+] CD8 T cells in the medLNs were determined at the indicated time points using flow cytometry. Data are representative of three independent experiments (mean ± SD of 5 mice per group per time point; *, P < 0.01 vs. B6).
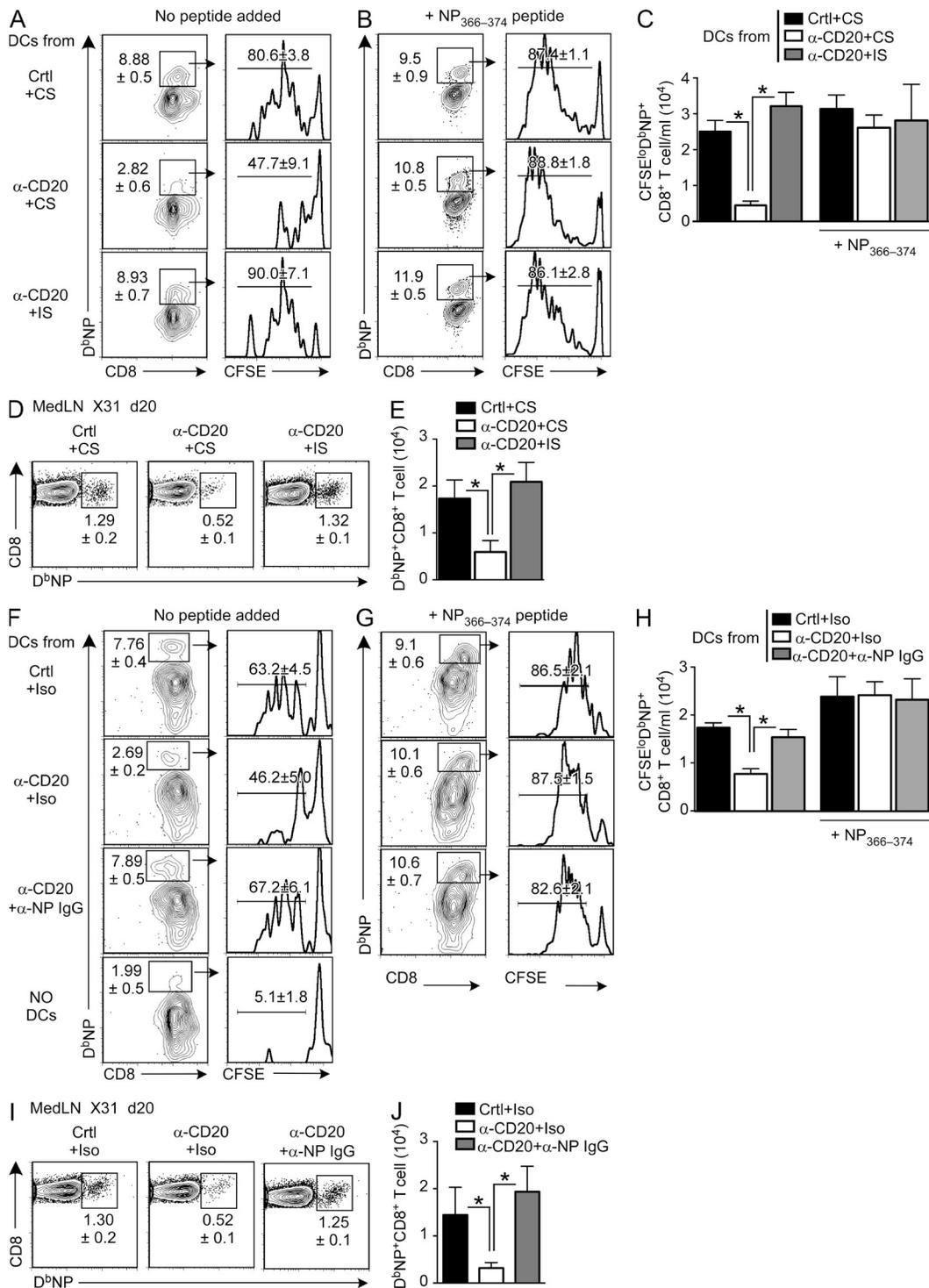
Figure 5. NP–specific Abs delay the contraction of NP⁺ primary CD8 T cell responses and rescue late DC Ag presentation to NP⁺ CD8 T cells. Control and anti-CD20–treated B6 mice were infected with X31 and treated on days 10, 14, and 21 with Ab⁺ flu immune serum (IS) or Ab^neg control serum (CS; A–E) or with a mixture of flu NP-specific IgG1, IgG2a, and IgG2b mAbs or isotype control Abs (Iso; F–J). DCs from the medLN of day 12 control or anti-CD20–treated mice that received CS or IS on day 10 (A–C), or that received anti-NP mAbs or isotype control Abs on day 10 (F–H) were co-cultured for 3 d with CFSE-labeled CD8 T cells purified from medLN of day 7 X31-infected B6 mice. Exogenous NP$_{366–374}$ peptide was added to some of the cultures (B and G). The frequencies (A, B, F, and G) and numbers (C and H) of CFSE^lo NP⁺ CD8 T cells were determined by flow cytometry. On day 20 after infection, the frequencies (D and I) and numbers (E and J) of NP⁺ CD8 T cells in the medLN of the B cell–depleted and control mice, treated on days 10 and 14 with flu-specific or control Abs, were determined using flow cytometry. Data are representative of three independent experiments (mean ± SD of 5 mice per group; *, P < 0.01).
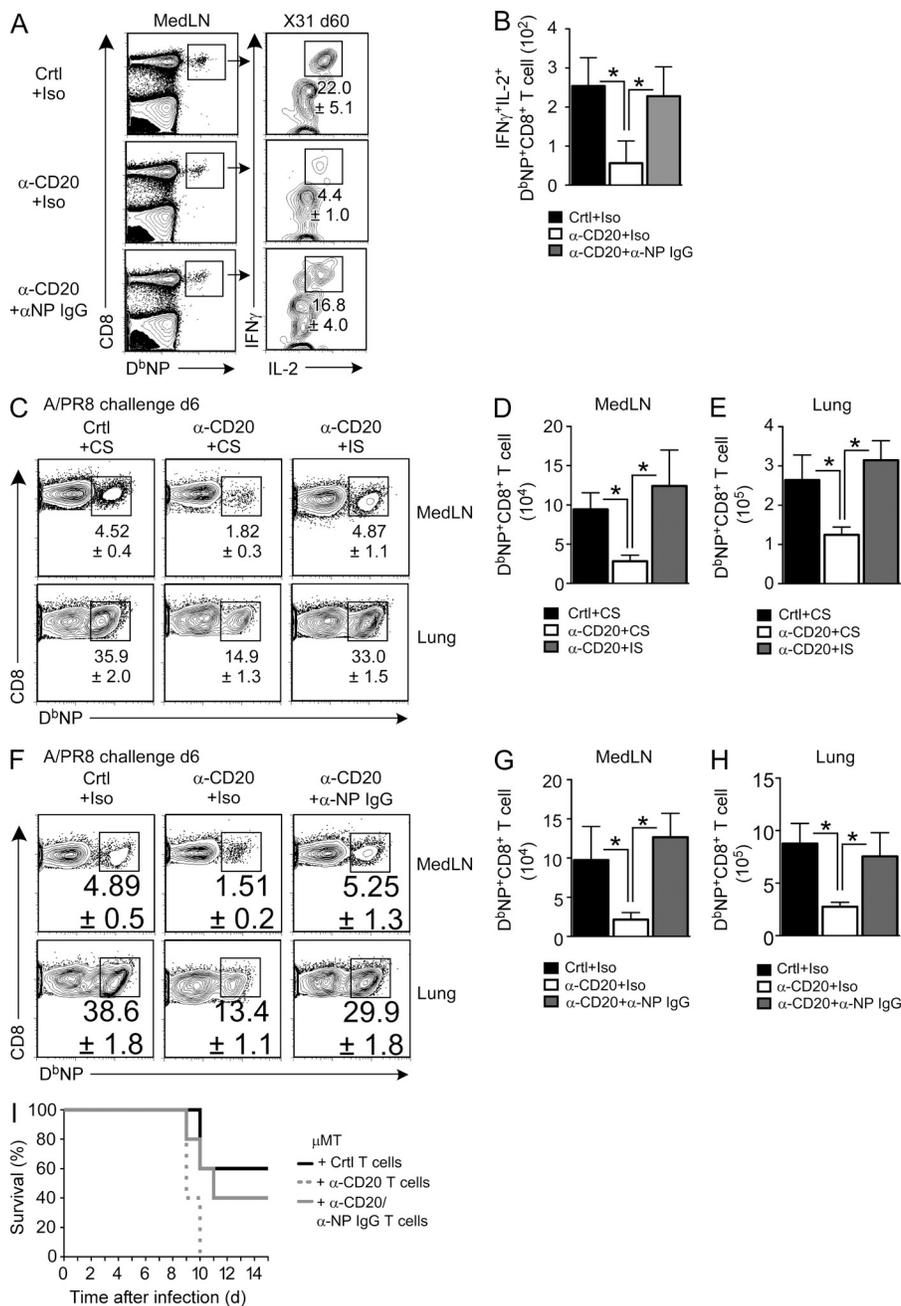
Figure 6. NP–specific Abs rescue NP+ recall responses in B cell–depleted mice. (A and B) Control and anti-CD20–treated B6 mice were infected with X31 and treated on days 10, 14, and 21 with Ab+ flu immune serum (IS) or Ab^neg control serum (CS) or with a mixture of flu NP-specific IgG1, IgG2a, and IgG2b mAbs or isotype control Abs (Iso). On day 60, the medLN cells were restimulated in vitro with NP$_{366-374}$ peptide and the frequencies (A) and numbers (B) of IFN-γ+IL-2+ NP-specific CD8 T cells were determined by ICCS. (C–H) The same groups of mice were challenged with PR8 virus on day 70 after primary infection, and the CD8 recall response in the medLN (C, D, F, and G) and lung (C, E, F, and H) was measured 6 d later. The frequencies (C and F) and numbers (D, E, G, and H) of NP+ CD8 T cells were determined using flow cytometry. (I) B6 or anti-CD20–treated B6 mice were infected with X31 and treated or not with anti-NP mAbs on days 10, 14, and 21. On day 70, CD44^hi memory CD8 T cells were sorted from the spleens and equal numbers of NP+ CD8 T cells (5 × 10^4/recipient) were transferred into naive μMT mice. Recipient mice were infected with a lethal dose of PR8 virus 24 h later. Survival of the recipients was monitored for 2 wk. Data are representative of three independent experiments (mean ± SD of 5 mice per group; *, P < 0.01).

10 wk and challenged the mice with PR8. As expected, the NP-specific CD8 T cell recall responses in both LNs and lungs were attenuated in B cell–depleted mice that received control serum (Fig. 6, C–E) or isotype control Ab (Fig. 6, F–H). However, the NP-specific CD8 T cell recall responses in LNs and lungs of B cell–depleted mice that received immune serum (Fig. 6, C–E) or NP-specific mAbs (Fig. 6, F–H) were restored to the levels observed in control mice.

Finally, to test whether NP-specific memory CD8 T cells that develop in the presence of NP-specific mAbs more effectively mediate protection from infection, we purified memory CD8 T cells from X31-immune control or B cell–depleted

mice that had been treated with control or NP-specific Ab, transferred equal numbers of NP-specific memory CD8 T cells into naive μMT recipients, and infected them with PR8. We observed that all of the μMT mice that received memory CD8 T cells from B cell–depleted donors that were treated with control Abs succumbed to infection by day 9 (Fig. 6 I). In contrast, 50% of the recipients receiving CD8 memory T cells from B cell–depleted donors that were treated with NP-specific Abs during the primary infection recovered from infection and were as protected from challenge infection as mice receiving memory CD8 T cells from normal donors (Fig. 6 I). Thus, the presence of NP-specific mAbs during the primary infection
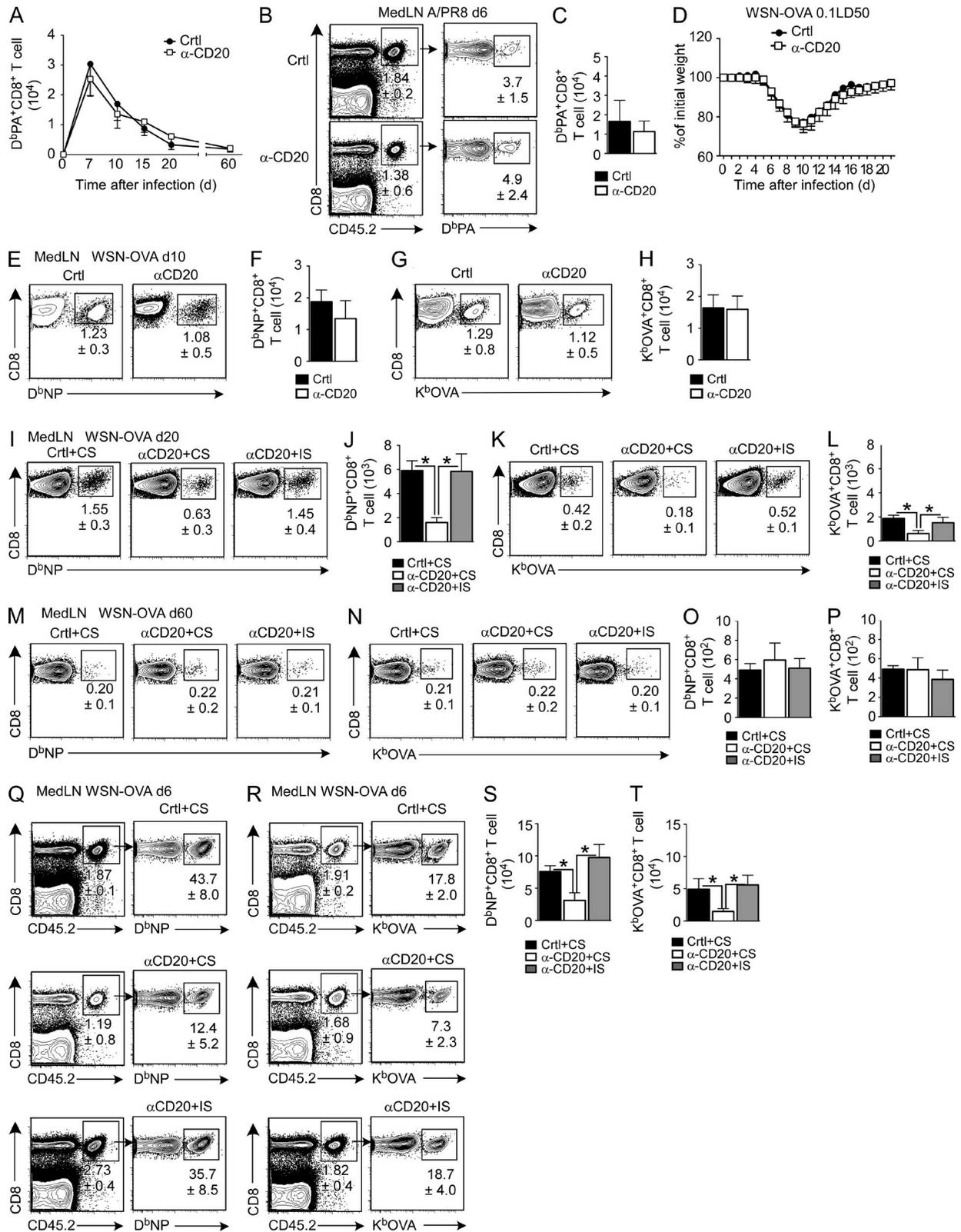
Figure 7. B cell depletion differentially impacts the response of individual influenza CD8 T cell specificities. Control and anti-CD20–treated B6 mice were infected with X31. (A) The numbers of PA^{+} CD8 T cells in the medLNs were determined at indicated time points using flow cytometry. (B and C) Memory CD8^{+}CD44^{hi} T cells were sorted from the spleens of B6 or anti-CD20–treated mice 10 wk after primary X31 infection. 5×10^3 PA^{+} CD8^{+}CD44^{hi} T cells were transferred into naive CD45.1^{+} mice. Recipient mice were infected with PR8 24 h later. The frequencies (B) and numbers (C) of

facilitated the generation of NP-specific memory CD8 T cells that were able to produce cytokines, expand, and protect after reexposure to Ag.

CD8^{+} T cells responding to distinct influenza Ags are differentially reliant on B cells

To evaluate whether the development of highly functional CD8 T cell memory is always reliant on Ab and B cells, we examined CD8 responses to two additional influenza epitopes. First, we examined the primary and secondary CD8 T cell response to influenza polymerase (PA) in B cell–depleted mice. We found that PA-specific CD8 T cells expanded equivalently in control and B cell–depleted mice through day 7 and then contracted at the same rate in both groups (Fig. 7 A). Moreover, we observed that adoptively transferred CD44^{hi} PA-specific memory CD8 T cells from control or B cell–depleted mice expanded equivalently in PR8-challenged congenic recipients (Fig. 7, B and C). Likewise, DC depletion after day 7 of the primary infection did not affect the primary or secondary PA-specific CD8 T cell response (unpublished data). Thus, B cells and extended Ag presentation by DCs are dispensable for the development and recall potential of PA-specific CD8 memory cells.

Next, we tested whether B cells or Ab were required for CD8 T cell responses to the influenza NA protein. To follow the CD8 response to NA, we infected control and B cell–depleted mice with recombinant influenza WSN-OVA virus that expresses the ovalbumin SIINFEKL CD8 epitope in the stalk of the influenza NA protein (NA-OVA; Topham et al., 2001) and monitored the NA-OVA–specific CD8 T cell response using OVA-specific (Kb/SIINFEKL) MHC Class I tetramers and the NP response using NP$_{366-374}$ tetramers. We observed equivalent weight loss and recovery in B cell–depleted and control mice infected with WSN-OVA (Fig. 7 D). Moreover, we found that NP-specific (Fig. 7, E and F) and NA-OVA–specific (Fig. 7, G and H) CD8 T cells expanded equivalently in the medLN of both groups at the peak of the primary response on day 10. By day 20 after infection, the NP-specific CD8 T cell response had contracted more in B cell–depleted mice compared with nondepleted controls and this more rapid contraction was reversed when immune serum was transferred to the B cell–depleted animals on day 15 after infection (Fig. 7, I and J). Interestingly, the day 20 NA-OVA–specific CD8 T cell response was also greatly decreased in B cell–depleted mice that received control serum compared with B cell–depleted mice that received immune serum (Fig. 7, K and L). Thus, B cell depletion accelerated the contraction of both
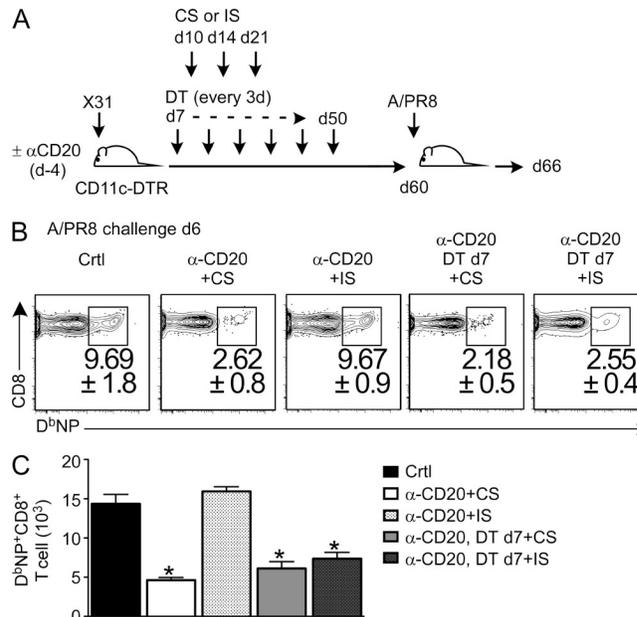
Figure 8. Prolonged presentation of NP Ag by DCs during the primary response requires Ab. B6 mice were irradiated and reconstituted with CD11c-DTR BM. Reconstituted mice (A) were treated with anti-CD20 or control Ab, infected with X31 4 d later and injected with PBS or DT every 3 d, starting on day 7 after infection and continuing until day 50. Mice were also treated with flu Ab[+] immune serum (IS) or Ab[neg] control serum (CS) on days 10, 14, and 21 after infection. Mice were challenged with PR8 10 d after the last DT exposure. The frequencies (B) and absolute numbers (C) of NP[+] CD8 T cells in medLN on day 6 after challenge were determined using flow cytometry. Data are representative of three independent experiments (mean ± SD of 4–5 mice per group; *, P < 0.001 vs. control).

NP-specific and NA-OVA–specific CD8 effectors after primary infection.

Although the contraction of the NA-OVA–specific CD8 response was accelerated in mice lacking influenza-immune serum, the frequencies and numbers of NP-specific (Fig. 7, M and O) and NA-OVA–specific (Fig. 7, N and P) CD8 memory cells were equivalent in all groups of mice. To test whether B cell depletion during primary infection affected the NA-OVA–specific CD8 recall response, we adoptively transferred equivalent numbers of day 70 memory NA-OVA–specific or NP-specific CD8 T cells isolated from CD45.2[+] control and immune serum–treated B cell–depleted mice into naive congenic B6 mice. We infected the recipients with WSN-OVA virus and monitored the response of the donor CD8 T cells 6 d later. We found that the frequencies and numbers of both donor

donor PA[+] CD8 T cells in the medLN were determined 6 d later. (D) Control and anti-CD20–treated B6 mice were infected with WSN-OVA. Infected mice were monitored for body weight changes over 3 wk. (E–P) B6 and anti-CD20–treated B6 mice were infected with WSN-OVA and then treated with Ab[+] WSN-OVA immune serum (IS) or Ab[neg] control serum (CS) on days 15 and 21 after infection. The frequencies and total numbers of NP^{+} and NA-OVA^{+} CD8 T cells in the medLN were determined on day 10 (E–H), day 20 (I–L), and day 60 (M–P). (Q–T) CD8^{+} T cells were sorted from the same groups of mice 10 wk after primary infection with WSN-OVA. Equal numbers (5 × 10³) of NP^{+} (Q and S) or NA-OVA^{+} (R and T) CD8^{+} T cells were transferred into naive CD45.1^{+} mice. Recipient mice were infected with WSN-OVA 24 h later. The frequencies (Q and R) and numbers (S and T) of donor CD8 T cells in the medLN were determined 6 d later. Data are representative of two independent experiments (mean ± SD of 5 mice per group; *, P < 0.01).

NP-specific (Fig. 7, Q and S) and NA-OVA–specific CD8 T cells (Fig. 7, R and T) were significantly decreased in the medLNs of mice receiving cells from B cell–depleted mice that were treated with control serum compared with recipients of intact control mice or B cell–depleted mice that received immune serum. Thus, the absence of B cells during the primary infection led to intrinsic defects in the secondary expansion ability of NP-specific memory CD8 T cells and NA-OVA–specific memory CD8 T cells, but not PA-specific CD8 memory T cells.
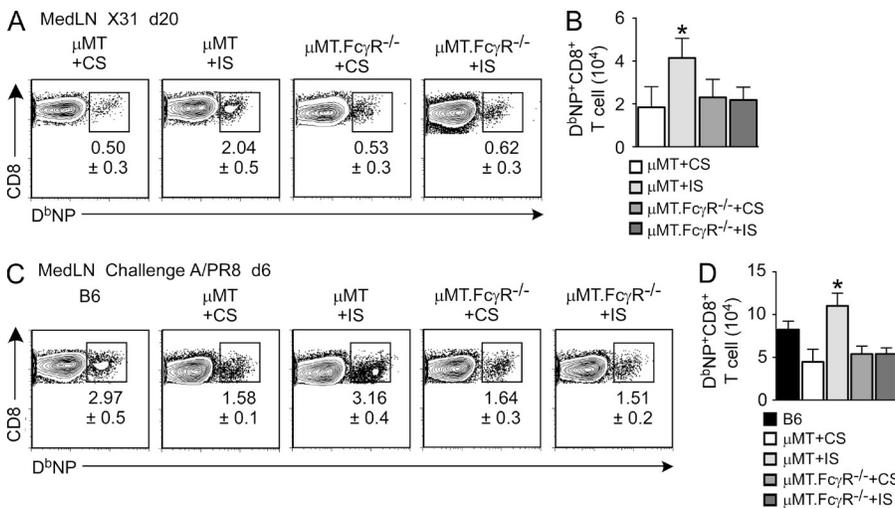
The NP–specific CD8 T cell recall response requires extended Ab–dependent Ag presentation by DCs during the primary infection

To test whether influenza-specific Abs induced the development of fully functional NP-specific memory CD8 T cells via a DC-dependent mechanism, we generated CD11c-DTR BM chimeras, waited 8 wk for reconstitution, and then depleted B cells with anti-CD20 in a cohort of the animals. We infected both groups with X31, waited 7 d, and then depleted DCs in a cohort of the B cell–depleted mice by treating the mice with DT every 3 d between days 7 and 50 after infection. We also transferred control serum or immune serum on days 10, 14, and 21 after infection. On day 50, we rested the mice for 10 d to allow for DC repopulation and then challenged the mice with PR8. 6 d after the challenge infection, we monitored the NP-specific CD8 T cell response in the medLN (Fig. 8 A). As expected, control mice made a robust NP-specific CD8 T cell recall response after the challenge infection (Fig. 8, B and C). However, the NP-specific CD8 T cell response was attenuated in mice that were B cell depleted before the primary infection and in mice that were depleted of B cells before infection and depleted of DCs starting on day 7 (Fig. 8, B and C). The NP-specific CD8 T cell recall response was restored to control levels in the B cell–depleted mice that received immune serum during the primary infection (Fig. 8, B and C), but immune serum did not restore the response in mice that were B cell depleted and depleted of CD11c+ cells starting

on day 7 (Fig. 8, B and C). Thus, specific Abs enhanced the development of functional memory CD8 T cells via a DC-dependent mechanism.

FcγR modulates primary and recall NP-specific CD8 T cell responses

Given that Ab-dependent Ag presentation by DCs was required for the development of functional NP-specific memory CD8 T cells, we hypothesized that NP-specific Abs likely targeted the NP Ag to FcR-expressing DCs during the primary response. To test this hypothesis, we tested whether the stimulatory FcγRs (FcγRI, FcγRIII, and FcγRIV) were necessary for the primary or secondary NP-specific CD8 T cell response to influenza. We crossed Fc common γ chain–deficient mice (Takai et al., 1994) to B cell–deficient μMT mice to generate mice (μMT.FcγR$^{-/-}$) that lacked B cells, Ab, and all three stimulatory FcγRs (LaMere et al., 2011a). We infected B6 control, μMT, and the μMT.FcγR$^{-/-}$ mice with X31, transferred either immune serum or control serum on days 10, 14, and 21 after infection, and monitored the NP-specific CD8 response on day 20. We then rested the remaining animals for 8 wk, challenged the mice with PR8, and assessed the NP-specific CD8 T cell response on day 6 after challenge. Similar to our previous results using B cell–depleted mice, we observed that the contraction of the primary NP-specific CD8 T cell response was accelerated in μMT mice that received control serum (Fig. 9, A and B) and that the recall response was also attenuated in these mice (Fig. 9, C and D). Identical results were observed in the μMT.FcγR$^{-/-}$ mice treated with control serum (Fig. 9, A–D). Although immune serum restored the primary (Fig. 9, A and B) and recall (Fig. 9, C and D) NP-specific CD8 T cell responses in μMT mice, it was unable to restore either the primary (Fig. 9, A and B) or memory responses (Fig. 9, C and D) in μMT.FcγR$^{-/-}$ mice. Thus, influenza-specific Abs modulated the primary and recall NP-specific CD8 T cell responses in an FcγR-dependent fashion.

Figure 9. Flu-specific Abs enhance NP-specific CD8 primary and memory recall responses by a FcγR-dependent mechanism. B6, μMT, and μMT.FcγR$^{-/-}$ mice were infected with X31 and treated with Ab+ flu immune serum (IS) or Ab[neg] control serum (CS) on days 7, 14, and 21 after infection. Mice were analyzed on day 20 after infection (A and B) or were challenged on day 60 with PR8 virus and analyzed 6 d after challenge (C and D). The frequencies (A and C) and absolute numbers (B and D) of NP+ CD8+ T cells in the medLN were assessed using flow cytometry. Values for control B6 mice are shown for comparison. Data are representative of at least two independent experiments (mean ± SD of 5 mice per group; *, P < 0.01 vs. control serum).
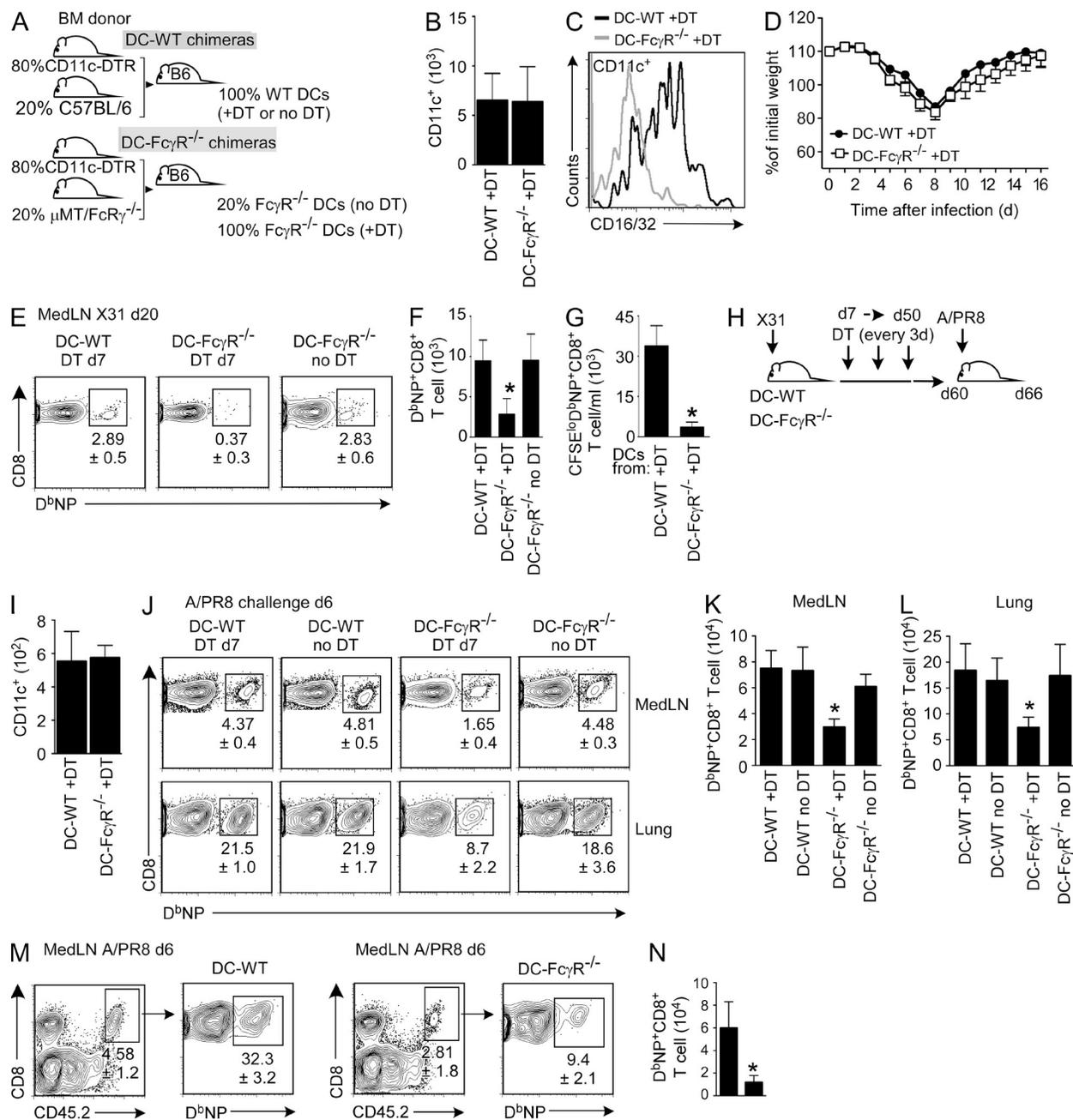
Figure 10. FcγR–expressing DCs extend the duration of NP Ag presentation to CD8 T cells and increase the size of NP–specific primary and secondary CD8 T cell responses. (A–G) B6 mice were irradiated and reconstituted with an 80:20 mixture of BM from CD11c-DTR and B6 donors (DC-WT chimeras) or from CD11c-DTR and μMT.FcγR$^{-/-}$ donors (DC-FcγR$^{-/-}$ chimeras). Reconstituted chimeras (A) were infected with X31 and injected with PBS or DT beginning on day 7 and continuing every 3 d to eliminate DCs derived from the CD11c-DTR progenitors. The frequency and numbers of CD11c$^+$ DCs (Fig. S3; B) and the expression of FcR CD16/32 on CD11c$^+$ DCs from medLNs (C) were assessed on day 20 after infection. Body weight changes (D) were monitored for 2 wk after infection. The frequencies (E) and absolute numbers (F) of NP$^+$ CD8 T cells in the medLN on day 20 after infection were evaluated. (G) CD11c$^+$ DCs from medLN of the day 12 X31-infected DC-WT and DC-FcγR$^{-/-}$ chimeras were co-cultured for 72 h with CFSE-labeled CD8 T cells isolated from day 7 X31-infected B6 mice. The total numbers of CFSE^{lo} NP$^+$ CD8$^+$ T cells were determined using flow cytometry. (H–K) DC-WT and DC-FcγR$^{-/-}$ chimeras were infected with X31 and injected with PBS or DT beginning on day 7 after infection and continuing every 3 d until day 50. 10 d after the last DT injection, the numbers of repopulating medLN CD11c$^+$ DCs were determined (I). The remaining chimeras were challenged with PR8 virus, and the frequencies (J) and absolute numbers (K and L) of NP$^+$ CD8$^+$ T cells in the medLN (J and K) and lung (J and L) were assessed at 6 d after challenge. (M and N) Memory CD8$^+$CD44^{hi} T cells were sorted from the spleens of DT-treated DC-WT and DC-FcγR$^{-/-}$ chimeras 10 wk after primary X31 infection. 5 × 10^3 NP$^+$ CD8$^+$CD44^{hi} T cells were transferred into naive CD45.1$^+$ mice. Recipient mice were infected with PR8 24 h later. The frequencies (M) and numbers (N) of donor NP$^+$ CD8 T cells in the medLN were determined 6 d later. Data are representative of two independent experiments (mean ± SD of 4–5 mice per group; *, P < 0.01 vs. DT control).

FcγR–expressing DCs control the NP-specific primary and memory CD8 T cell responses

Given the reliance of the NP-specific primary and recall CD8 T cell responses on NP-specific Ab, FcγRs, and DCs, we hypothesized that FcγR expression by DCs was required for the extended Ag presentation by the DCs and subsequently for the development of fully functional memory CD8 T cells. To test this hypothesis, we reconstituted lethally irradiated B6 mice with either an 80:20 mix of BM from CD11c-DTR and μMT.FcγR$^{-/-}$ mice (DC–FcγR$^{-/-}$ chimeras) or with an 80:20 mix of BM from CD11c-DTR mice and B6 mice (DC–WT chimeras; Fig. 10 A). 8 wk after reconstitution, we infected the mice with X31 and then treated both groups every 3 d with DT from days 7 to 50 to ablate DCs. Using this approach, DCs derived from the CD11c-DTR precursors were ablated in both groups of mice, leaving only DCs and DC precursors derived from either the WT or FcγR$^{-/-}$ progenitors. Importantly, the numbers of DCs in the medLN of both groups of DT-treated chimeras were comparable (Fig. 10 B and Fig. S3 A). However, the DCs from the DT-treated DC–FcγR$^{-/-}$ chimeras did not express CD16/32 (Fcγ III/II Receptor), whereas the DCs in the DT-treated DC–WT chimeras expressed CD16/32 (Fig. 10 C). Both groups of infected chimeras showed similarly transient weight loss and recovery over the first week after infection (Fig. 10 D). As predicted, the frequencies and numbers of NP-specific CD8 T cells on day 20 after infection were significantly decreased in the medLN of DT-treated DC–FcγR$^{-/-}$ chimeras relative to those from DT-treated DC–WT chimeras or untreated DC–FcγR$^{-/-}$ chimeras (Fig. 10, E and F). Thus, DCs expressing FcγRs delayed the contraction and extended the duration of the primary NP-specific CD8 T cell response.

Next, to test the capacity of FcγR$^{-/-}$ DCs to present NP at later time points during the primary immune response, we infected DC–WT and DC–FcγR$^{-/-}$ chimeras with X31, administered DT on days 7 and 10, purified CD11c$^+$ DCs from the medLN on day 12, and co-cultured them for 3 d with CFSE-labeled CD8 T cells from day 7 X31-infected B6 mice. We found that FcγR$^{-/-}$ DCs failed to expand NP-specific CD8 T cells when compared with WT DCs (Fig. 10 G). Thus, FcγR expression by DCs was required to expand NP-specific CD8 T cells in vitro during the later phase of the primary response, suggesting that DCs expressing a stimulatory FcγR are required for extended Ag presentation to CD8 T cells during the primary infection.

To evaluate whether the loss of FcγR expression on DCs during the primary response to influenza infection compromised the NP-specific CD8 T cell recall response, we infected DC–WT and DC–FcγR$^{-/-}$ chimeras with X31 and treated a cohort of the chimeras with DT every 3 d from day 7 until day 50 (Fig. 10 H). We found that the numbers of NP-specific memory CD8 T cells were equivalent in the LNs and lungs of both groups of mice and that the DC pool was repopulated with normal numbers of WT DCs (Fig. 10 I and Fig. S3 B). Mice were then challenged with PR8 and the accumulation of NP-specific CD8 T cells was analyzed 6 d later. We found that the frequencies and numbers of responding NP-specific memory CD8 T cells were significantly decreased in the medLN (Fig. 10, J and K) and lung (Fig. 10, J and L) in DC–FcγR$^{-/-}$ chimeras relative to those in DC–WT chimeras or untreated DC–FcγR$^{-/-}$ chimeras.

To ensure that the defective memory CD8 T cell response was not due to any FcγR$^{-/-}$ DCs remaining at the time of challenge, we purified CD44^{hi} CD8 T cells from CD45.2$^+$ DT-treated DC–WT and DC–FcγR$^{-/-}$ chimeras 10 wk after primary X31 infection and transferred equivalent numbers of NP-specific CD8 T cells into CD45.1$^+$ recipients. We infected the recipients with PR8 and monitored the CD8 recall response 6 d later. We found that the frequencies and numbers of donor NP-specific CD8 T cells were significantly decreased in the medLNs of mice that received T cells from DT-treated DC–FcγR$^{-/-}$ donors compared with mice that received T cells from DT-treated DC–WT donors (Fig. 10, M and N). Taken altogether, the data support a model in which primary and secondary CD8 T cell responses to a subset of influenza Ags are regulated by ICs that target those Ags to FcγR–expressing DCs. This process promotes extended Ag presentation by DCs, which delays the contraction of primary CD8 T cell responses and facilitates the development of a high-quality memory CD8 T pool that is poised to rapidly respond to challenge infection.

DISCUSSION

Although a brief Ag-dependent stimulation is sufficient to initiate expansion and differentiation of virus-specific naive CD8 T cells (Kaech and Ahmed, 2001; van Stipdonk et al., 2001), DCs continue to present Ag to CD8 T cells in both the medLN and infected peripheral tissues for extended periods of time, in many cases even after resolution of the viral infection (Zammit et al., 2006; Kim et al., 2010). Presentation of viral Ags by DCs to CD8 T cells at these later times enhances the proliferation, survival, differentiation, and migration of primary Ag-specific CD8 effectors (Zammit et al., 2006; McGill et al., 2008; Ballesteros-Tato et al., 2010; Kim et al., 2010). Our data extends these results to show that the magnitude and quality of the CD8 T cell secondary recall response are also highly dependent on sustained Ag presentation by the LN DCs, but not lung DCs, during the primary response. These results are in accordance with previous studies (Kim et al., 2010), showing that DCs isolated from the LNs are unique in their ability to present residual viral Ag to CD8 T cells. More importantly, our data demonstrate that ICs, composed of virus proteins and isotype-switched virus-specific Abs, are captured by FcγR-expressing DCs, allowing these DCs to continue to present Ag to CD8 T cells after virus clearance. This Ab-dependent extended Ag presentation delays the contraction of the primary CD8 T cell response and programs the development of memory CD8 T cells that can produce multiple cytokines, proliferate rapidly, and protect after challenge infection. Thus, we have identified a novel B cell–dependent mechanism for establishing fully functional memory CD8 T cells that becomes important after the pathogen is cleared and Ag and inflammation become limiting.

Our results are somewhat unexpected because early studies using B cell–deficient mice suggest that memory CD8 responses require DCs (Belz et al., 2004) but not B cells (Asano and Ahmed, 1996; Di Rosa and Matzinger, 1996). Likewise, a recent study suggests that CD8 T cell responses are not controlled in an Ab-dependent fashion (Whitmire et al., 2009). However, other publications demonstrate that the development of CD8 primary effectors, memory cells, and secondary effectors is attenuated in B cell–deficient mice (Homann et al., 1998; Bergmann et al., 2001; Christensen et al., 2003; Shen et al., 2003; Brodie et al., 2008). The opposing outcomes of these studies are often attributed to the use of B cell–deficient µMT or JHD mice, which have defects in lymphoid tissue organogenesis and T cell homeostasis (Lund and Randall, 2010). However, primary CD8 T cell responses to tumor and transplantation Ags are also impaired in adult mice that are transiently depleted of B cells (DiLillo et al., 2010, 2011), suggesting that the role for B cells in CD8 T cell responses is unlikely to be due solely to developmental defects in lymphoid organs. Our experiments using transient B cell depletion demonstrate a role for B cells—independently of lymphoid tissue organogenesis—in improving the quality of the recall response of memory CD8 T cells. Our data also establish a mechanistic basis for the regulation of CD8 T cell responses by Ab-producing B cells and show that, although the initial priming and expansion of NP-specific CD8 T cells occurs normally in the absence of B cells, the contraction phase occurs prematurely. Likewise, although memory CD8 T cells develop in normal numbers in the absence of B cells, the quality of those memory CD8 T cells, their capacity to expand and produce effector cytokine after secondary challenge, and their ability to confer protection is highly dependent on the production of Ab during the primary response. Thus, some of the discordant results concerning the role of B cells in CD8 T cell responses may be due to the times chosen for analysis and whether the memory CD8 T cells were simply enumerated or tested for functionality after Ag reexposure.

Our data also clearly show that not every CD8 memory recall response is dependent on the presence of B cells or Ab. Given that the role of Ab in programming memory CD8 T cells is to facilitate the FcR-mediated acquisition and cross-presentation of viral Ags at later times when virus is cleared, then CD8 T cell responses to Ags that do not induce a robust class-switched Ab response within the first week of the primary infection are less likely to be affected by the presence or absence of Ab. For example, the humoral immune response to influenza is dominated by Abs to abundant viral proteins, including hemagglutinin (HA), NA, and NP (Gerhard, 2001), suggesting that CD8 T cells responding to epitopes in these proteins would be more likely to sustained in an Ab-dependent fashion. In support of this hypothesis, we found that the primary and secondary CD8 T cell response to epitopes in the viral NA protein were dependent on B cells and virus-specific Ab. In contrast, the primary and secondary responses to the less abundant PA protein were not dependent on B cells or Ab. Interestingly, we found that the primary CD8 T cell response to PA contracts more rapidly than the NP or NA response in normal mice. Furthermore, although the NP, PA, and NA CD8 responses are co-dominant at the peak of the primary response and in the resting memory cell stage, the PA recall response is always much smaller and subdominant to the NP response after challenge infection (Belz et al., 2000; Crowe et al., 2003). The difference in the magnitude of the NP and PA responses was shown to be dependent, at least in part, on the differences in the abundance of NP and PA Ags (La Gruta et al., 2006). It is tempting to speculate that ICs containing virus proteins and the virus-specific class-switched Abs, which extend the duration of Ag accessibility to the DCs, may also contribute to the immunodominance profile of the memory CD8 recall response.

How might Ab and FcR–mediated Ag acquisition by DCs influence the duration or quality of the CD8 T cell primary and secondary responses? One possibility is that Ab–Ag ICs may simply target the Ag to FcγR-expressing DCs (Amigorena and Bonnerot, 1999) late in the primary response as the pathogen burden declines. Indeed, DCs take up ICs more efficiently than soluble Ag (Gil-Torregrosa et al., 2004), allowing for enhanced Ag processing and cross-presentation at lower Ag concentrations (Regnault et al., 1999). Ag presentation when Ag and inflammation are both limiting may also influence the type of memory CD8 T cells that develop. For example, naive T cells activated under conditions of modest inflammation differentiate into memory cells more quickly and without the need to go through the effector stage of differentiation (Badovinac et al., 2004). Furthermore, CD8 T cells that are primed by DCs under conditions of low inflammation have high proliferative potential after rechallenge (Pham et al., 2009). Because late Ag presentation by IC-binding FcγR-expressing DCs coincides with viral clearance and a gradual reduction in inflammation, one could envision that the encounter with Ag-presenting DCs during conditions of limited inflammation may be essential for programming memory CD8 T cells with strong secondary proliferative potential.

Another intriguing possibility is that DCs activated via ICs binding the stimulatory FcγRs (Amigorena and Bonnerot, 1999; Baker et al., 2013) are qualitatively different than DCs that are activated by cytokine receptor and/or pathogen recognition receptor signals. For example, the IC-activated DCs may delay the contraction of the primary CD8 T cell response by providing signals like IL-7 (Kaech et al., 2003), IL-15 (Becker et al., 2002; Schluns et al., 2002), or CD70 (Hendriks et al., 2000; Dolfi et al., 2008) that are known to enhance the survival of CD8 T cells. Alternatively, IC-activated DCs could directly deliver distinct programming signals to the CD8 T cells that are required for the development of functional CD8 memory T cells but are dispensable for the initial expansion of the primary CD8 T cell response. These signals may facilitate endogenous IL-2 secretion (Williams et al., 2006; Feau et al., 2011) or the expression of CD27 by the responding CD8 T cells (Hendriks et al., 2000; Dolfi et al., 2008; Ballesteros-Tato et al., 2010; Feau et al., 2012) and, although not directing memory CD8 cell development per se, may prevent the premature apoptosis of the activated NP^{+} CD8 cells and influence the quality or type of memory cells that develop.

Although we do not yet know how the IC-binding FcγR–expressing DCs change the programming of the developing memory CD8 T cells, the data are clear that Ags capable of inducing a rapid class-switched Ab response by B cells can be efficiently targeted to FcγR-expressing DCs in the form of Ab-containing ICs. The data also highlight the functional importance of nonneutralizing Abs directed against internal epitopes of influenza. Influenza vaccines are designed to elicit Abs to surface proteins like HA, NA, and even the external domain of M2 (M2e) because Abs to epitopes on these proteins can neutralize or reduce the efficiency of infection. However, natural influenza infections also elicit strong Ab responses to proteins like NP that are localized inside the virus or inside infected cells (Carragher et al., 2008; LaMere et al., 2011a). Unlike neutralizing Abs, Abs against NP are unable to prevent infection. However, these Abs effectively reduce morbidity and mortality in mice when present before infection (Carragher et al., 2008; LaMere et al., 2011b). Interestingly, anti-NP Abs are most effective in combination with a CD8 T cell response (LaMere et al., 2011a), consistent with the conclusion that one major role of nonneutralizing Abs is to facilitate Ag capture by Ag-presenting DCs. This has significant practical applications for T cell–directed antiviral or antitumor vaccines. In these settings, primary immunizations with ICs rather than Ag alone may be a more effective way to induce functional memory CD8 T cells. In addition, immunization protocols that use Ags and adjuvants that induce a rapid B cell–dependent class-switched Ab response are more likely to induce the development of fully functional CD8 memory cells.

Finally, our data suggest that B cell depletion therapies (BCDTs), which are already approved for treatment of rheumatoid arthritis and systemic lupus erythematosus and are being tested in the context of solid organ transplantation, may influence CD8 T cell responses. Indeed, as T cells cause many of the pathological manifestations in the diseases that are responsive to BCDT, it was proposed that BCDT may work, at least in part, by modulating T cell–dependent autoimmune responses (Lund and Randall, 2010). Although recent studies demonstrate that transient BCDT significantly attenuates primary and memory CD4 T cell responses to pathogens and auto-Ags (Lund and Randall, 2010; Barr et al., 2012), the impact of BCDT on autoreactive CD8 T cells has not been examined in detail. Given the findings presented here, it will be important to assess whether BCDT also, over time, prevents autoreactive T cell epitope spreading and exacerbation of disease. In addition, it will be important to evaluate whether patients chronically treated with BCDT generate functional CD8 memory cells after exposure to new viral pathogens or antiviral CD8 vaccines. In the organ transplantation setting, BCDT is used to prevent the development of neo antigraft Ab responses. Perhaps equally important, our data suggest that BCDT may also attenuate the development and expansion of neo graft-specific CD8 effector and effector memory cells that are major contributors to graft rejection. Thus, BCDT may provide an unexpected dual benefit in autoimmune and transplant patients by attenuating the autoreactive and graft-specific B cell– and CD4 T cell–dependent humoral immune responses as well as the DC- and CD8 T cell–dependent cellular immune responses.

MATERIALS AND METHODS

Mice and infections. The mouse strains used in these experiments include: C57BL/6J (B6), B6.SJL-*Ptprc^a Pep^cb*/BoyJ (CD45.1+ B6 congenics), B6.129S2-*Ighm^{tm1Cgn}*/J (µMT), B6.FVB-Tg (Itgax-DTR/EGFP)57Lan/J (CD11c-DTR), C57BL/6-Aicda^{tm3.1Mnz}/J (*Aicda^{−/−}*), B6.129S2-*Ighm^{tm1Cgn}*/B6.129P2-*Fcer1g*^th1Rav/J (µMT.FcγR^{−/−}), and C57BL/6-Tg(TcraTcrb)^{1100Mjb/J} (OT-I). *Aicda^{−/−}* mice (Muramatsu et al., 2000) were originally obtained from the laboratory of T. Honjo (Kyoto University, Kyoto, Japan) and µMT.FcγR^{−/−} mice were generated by intercrossing the µMT strain with the *Fcer1g*^th1Rav/J strain (both from The Jackson Laboratory). All other mice were originally obtained from The Jackson Laboratory and were bred in the University of Rochester animal facility. Adult mice were infected i.n. with 0.1 LD$_{50}$ of influenza H3N2 A/X31 or A/WSN/33 (WSN)-OVA and challenged i.n. with 1 LD$_{50}$ of influenza H1N1 A/PR8/34. In some experiments, µMT mice were infected with a dose of PR8 virus that is lethal to this susceptible strain of mice (∼0.25 LD$_{50}$ for B6 mice). µMT mice receiving a lethal dose of virus were monitored 3×/day and were euthanized when the weight loss of the mice exceeded 30% or when the animals were unresponsive to external stimuli or unable to obtain food or water. The University of Rochester and UAB Institutional Animal Care and Use Committees approved all procedures involving animals.

BM chimeras. Recipient mice were irradiated with 950 rad from a ^{137}Cs source delivered in a split dose and reconstituted with 10^7 total BM cells. CD11c-DTR BM chimeras were generated by reconstituting B6 recipients with 100% CD11c-DTR BM. To generate mice that lacked FcγR expression specifically in the DC compartment, B6 recipients were reconstituted with 80% CD11c-DTR BM + 20% FcγR^{−/−} BM. For controls, B6 recipients were reconstituted with 80% CD11c-DTR BM + 20% B6 BM. Mice were allowed to reconstitute for at least 8–12 wk before influenza infection and DC depletion.

In vivo depletion of B cells and DCs and Ab adoptive transfers. To deplete CD20+ B cells, mice were injected i.p. 4 d before flu infection with 250 µg mouse anti–mouse CD20 (Hamel et al., 2008; clone 18B12, IgG2a isotype; provided by J. Browning and R. Dunn, Biogen Idec, Boston, MA) or isotype control Ab (clone 2B8). For depletion of CD11c+ cells, CD11c-DTR BM chimeras (described above) were infected and treated i.p. with 60 ng DT (Sigma-Aldrich) beginning at specific time points between 7 and 21 d after primary infection. Mice received additional injections of DT every 3 d up to the experimental time point or to 50 d after primary infection. To produce flu Ab+ immune sera and flu Ab^neg control sera, B6 and µMT mice were infected with X31 influenza virus. Control (µMT) or immune (B6) serum was collected on days 10, 15, and 20 and pooled. In some experiments, flu Ab+ immune sera and flu Ab^neg control sera were collected from WSN-OVA–infected mice on days 15 and 20 after infection. NP-specific IgG-secreting hybridomas (clones IC5-2A10-G1, IgG2b; IC6-1H5-G2a, IgG2a; and H19-L2-1-G2b, IgG2b) were obtained from W. Gerhard and K. Mozanowska (Wistar Institute, Philadelphia, PA). mAbs were purified from hybridoma supernatants by Bio X Cell. Isotype-matched Abs (MOPC-21, MPC-11, and CI.18) were purchased from Bio X Cell. Mice were injected i.p. with immune or control serum (500 µl) or with a mixture of anti–NP IgG mAbs (300 µg of each mAb) or isotype control mAbs on days 10, 14, and 21 after X31 infection or on days 15 and 21 after WSN-OVA infection.

Cell preparation and flow cytometry. Lungs from infected mice were isolated, 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 lungs, medLN, or spleens were mechanically disrupted by passage through a wire mesh. Red blood cells were lysed with 150 mM NH$_4$Cl, 10 mM KHCO$_3$, and 0.1 mM EDTA. FcRs were blocked with 5 µg/ml anti–mouse CD16/32 (BD), followed by staining with MHC class I tetramers or fluorochrome-conjugated Ab. The

H-2K^b SIINFEKL and H-2D^b NP$_{366-374}$ or PA$_{224-233}$ MCHI tetramers were generated by the National Institutes of Health Tetramer Core Facility (Atlanta, GA). Fluorochrome-labeled anti-CD8α (53–6.7), anti-CD44 (IM7), anti-CD62L (IM7), anti-CD27 (PD61), CD127 (SB/199), anti-CD62L (MEL-14), anti-CD45.2 (104), anti–MHC class II (AF6-120.1), anti-CD16/32 (2.4G2), anti-CD11c (HL3), anti–IL-2, and anti–IFN-γ were obtained from BD and anti-KLRG1 was obtained from eBioscience. For intracellular cytokine staining, single-cell suspensions of medLN cells were stimulated with 2 μg/ml NP$_{366-374}$ peptide and 40 U rIL-2 in the presence of 10 μg/ml Brefeldin A for 3 h. FcR blocking reagent was not used before staining with CD16/32 Ab. Flow cytometry was performed on FACSCanto II (BD) and C6 (Accuri) instruments in the Flow Cytometry Core Facility at the University of Rochester or in our laboratory at UAB.

Cell purifications, T cell transfers, and in vitro cultures. DCs from pooled medLN or lung of day 12 X31-infected mice were purified on LS columns using anti-CD11c MACs beads (Miltenyi Biotec). CD8 T cells were isolated by MACs from the medLNs of day 7 X31-infected B6 mice or spleen from naive OT-I mice. All preparations were >95% pure as determined by flow cytometry. In some experiments, CD8 T cells were labeled for 10 min at 37°C with 5 μM CFSE (Molecular Probes). 2×10^4 or 10^5 DCs and 10^5 CFSE-labeled CD8 T cells were cultured in 200 μl of complete medium in round-bottomed 96-well plates for 72 h at 37°C. In some experiments OVA protein, OVA peptide (OVA$_{257-264}$), or NP peptide (NP$_{366-374}$) were added to the cultures. Complete medium included RPMI 1640 supplemented with sodium pyruvate, Hepes, nonessential amino acids, penicillin, streptomycin, 2-mercaptoethanol, and 10% heat-inactivated FBS (Gibco). Memory CD8$^+$CD44^{hi} T cells were sorted from donor mice using a FACSAria (BD) from preparations of splenocytes after positive selection with anti-CD8 MACS beads. An aliquot of sorted CD8$^+$CD44^{hi} memory T cells was stained with flu NP MHC class I tetramers to calculate the number of NP$^+$ T cells present in the sorted population. Equivalent numbers of CD8$^+$CD44^{hi} NP$^+$ T cells were transferred i.v. into 5×10^3 naive CD45.1 recipient mice or 5×10^4 μMT recipient mice.

Statistical analyses. Prism software (version 5.0a; GraphPad Software) was used for data analysis. Data were analyzed using the unpaired Student's t test. Values of $P \leq 0.05$ were considered significant.

Online supplemental material. Fig. S1 shows flow cytometry gating strategy for identification of NP-specific memory CD8 T cell subpopulations. Fig. S2 shows gating strategy for DC subsets in the medLN of flu-infected B cell depleted animals. Fig. S3 shows gating strategy to identify DCs in the medLN of DT-treated DC-WT and DC-FcγR$^{-/-}$ chimeras after flu infection. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131692/DC1.

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