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$CD4^+$ and $CD8^+$ T Cells Exhibit Differential Requirements for CCR7-Mediated Antigen Transport during Influenza Infection[1]

Alex K. Heer,* Nicola L. Harris,† Manfred Kopf,[2,3]* and Benjamin J. Marsland[2,3]*

Upon encounter of viral Ags in an inflammatory environment, dendritic cells up-regulate costimulatory molecules and the chemokine receptor CCR7, with the latter being pivotal for their migration to the lymph node. By utilizing mice deficient in CCR7, we have examined the requirement of dendritic cell-mediated Ag transport from the lung to the draining lymph node for the induction of anti-influenza immune responses in vivo. We found that CCR7-mediated migration of dendritic cells was more crucial for $CD8^+$ T cell than $CD4^+$ T cell responses. While no specific $CD8^+$ T cell response could be detected in the airways or lymphoid tissues during the primary infection, prolonged infection in CCR7-deficient mice did result in a sustained inflammatory chemokine profile, which led to nonspecific $CD8^+$ T cell recruitment to the airways. The recruitment of influenza-specific $CD4^+$ T cells to the airways was also below levels of detection in the absence of CCR7 signaling, although a small influenza-specific $CD4^+$ T cell population was detectable in the draining lymph node, which was sufficient for the generation of class-switched anti-influenza Abs and a normal $CD4^+$ T cell memory population. Overall, our data show that CCR7-mediated active Ag transport is differentially required for $CD4^+$ and $CD8^+$ T cell expansion during influenza infection. ***The Journal of Immunology,*** **2008, 181: 6984–6994.**

Influenza A virus infection is a major cause of morbidity and mortality worldwide. Once inhaled, the virus infects epithelial cells of the respiratory tract and begins to replicate and spread, thereby triggering innate immune mechanisms through pattern recognition receptors such as TLRs or RIG-I (retinoic acid-inducible gene I), and the production of type I IFNs (1, 2). These early innate responses are followed by the initiation of adaptive immunity, a transition that is mediated by dendritic cells (DC)[4] but does not require TLR signals (3, 4). Upon receiving early inflammatory or influenza-derived stimuli, DC up-regulate costimulatory surface molecules, induce cytokine expression, and migrate in a CCR7-dependent manner from the lung and airways to the draining lymph nodes (LN), where they present influenza-specific Ag to naive T cells (5–8).

The clearance of primary influenza infection is not dependent on a single arm of the adaptive immune response but rather a cooperation of $CD4^+$ T cell help, $CD8^+$ CTLs, and Ab production. For instance, mice lacking $CD8^+$ T cells show delayed viral clearance but do not succumb to infection when given a sublethal dose of virus (9). Similarly, CD4-depleted mice demonstrate only a slight delay in viral clearance (10). It is widely accepted that Abs play an important role in protecting mice from lethal type A influenza virus because mice lacking B cells rapidly succumb to infection despite mounting a strong $CD8^+$ T cell response (11, 12). The production of neutralizing Abs occurs in both a $CD4^+$ T cell-dependent and -independent fashion (13, 14), although, in the absence of $CD4^+$ T cells, Ab titers and class switching to isotypes other than IgM are greatly reduced (15, 16). Notably, bystander $CD4^+$ T cell responses can drive class switching to IgA but not to other isotypes (16).

Both initial contact with Ag and subsequent expansion of influenza-specific B and T cells occur in the lung draining LN of wild-type mice; however, whether LN are required for T cell priming, proliferation, and Ab production other than that of the IgM isotype remains controversial (17). Infection with a range of viruses in aly/aly mice, which are devoid of LN and Peyer's patches, resulted in defective activation of naive T cells and defective induction of class switching to IgG isotypes (18). In contrast, other groups reported that mice deficient in lymphotoxin-α, which do not develop any secondary lymphoid organs, can mount a delayed but protective T and B cell response, including class switching to IgG upon influenza infection (19). It was shown later that BALT is induced during influenza infection in the absence of functioning secondary lymphoid organs, possibly compensating for the absence of LN (20).

Although the effector mechanisms providing protection against influenza virus infection have largely been revealed, the requirement of active cell-mediated Ag transport from the lung to the draining LN remains unclear. To study the requirement of active Ag transport for the induction of adaptive immunity

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[4] Abbreviations used in this paper: DC, dendritic cell; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived DC; LN, lymph node; p.i., postinfection; WT, wild type.

against influenza virus, we used mice lacking the chemokine receptor CCR7, a receptor known to be pivotal for cell-mediated Ag transport to the lung draining LN (21). We show that, in the absence of CCR7-mediated migration, the priming and the expansion of influenza-specific $CD8^+$ T cells were below limits of detection, although prolonged inflammation driven by sustained chemokine production in the lung recruited a substantial population of nonspecific memory $CD8^+$ T cells. We further show that $CD4^+$ T cell priming and effector functions are greatly reduced. Surprisingly, however, this minimal expansion of $CD4^+$ T cells was sufficient to induce a virus-specific Ab response (albeit delayed). We conclude that $CD4^+$ and $CD8^+$ T cell expansion has differential requirements for CCR7-mediated Ag transport and that the development of influenza-specific Ab responses requires only a fraction of the normal specific $CD4^+$ T cell effector population.

Materials and Methods

Animals, virus, and infection

CCR7-deficient mice (used with permission from Martin Lipp) were backcrossed at least seven times onto a C57BL/6 background and maintained in specific pathogen-free conditions (BioSupport). C57BL/6 control mice were purchased from Charles River Laboratories. To generate wild-type (WT)/RAG and CCR7/RAG chimeric mice, C57BL/6 mice were irradiated with 950 rad 1 day before being reconstituted with 1.25×10^6 bone marrow cells derived from either C57BL/6 or CCR7-deficient mice and 3.75×10^6 bone marrow cells from RAG-deficient mice. Reconstituted mice were maintained in specific pathogen-free conditions for at least 8 wk before experiments. Influenza virus strain PR8 (A/Puerto Rico/34, H1N1) was originally provided by J. Pavlovic (University of Zurich). Influenza virus strain X31 (A/68, H3N2) was originally provided by Thorsten Wolff (Robert Koch Institute, Berlin). At the age of 6–12 wk, mice were infected intranasally with 50 PFU influenza virus. The mice were briefly anesthetized and inoculated twice with 50 μl virus in endotoxin-free PBS intranasally. For systemic influenza inoculation, mice were given 2×10^5 PFU i.v. All animal experimental procedures were approved by the local animal ethics committee (Zurich).

Bronchoalveolar lavage (BAL)

Mice were sacrificed at time points indicated after infection and were restrained on the back. A small incision on the trachea was performed, through which a catheter was introduced. The lungs were flushed with 1 ml PBS. BAL cells were harvested by centrifugation. Total cell numbers per BAL were determined by Coulter Counter (IG Instrumenten Gesellschaft), and cells were processed for further analysis. BAL fluid was used to measure virus-specific IgA levels.

Determination of virus-specific $CD8^+$ T cells

BAL, LN, or splenic cells (1×10^5) were incubated with 10 μg/ml $NP34^+$ PE-conjugated tetramers for 40 min at 4°C. CD4-FITC and CD8-allophycocyanin (eBioscience) were subsequently added for 20 min at 4°C. Cells were washed and analyzed by flow cytometry (FACSCalibur; BD Biosciences).

Specific restimulation of BAL cells

The day before mice were sacrificed for analysis, 1.5×10^5 bone marrow-derived DC (BMDC) (22) were incubated overnight with 1.6×10^5 PFU UV-inactivated virus (PR8) in 96-well plates. Twelve hours later, these BMDC were pulsed with 1 μg/ml NP34 peptide for 2 h before BAL cells from individual mice were added. After 2 h of incubation at 37°C, monensin (2 μM, Sigma-Aldrich) was added to retain cytokines in the cytoplasm, and cells were again incubated at 37°C for another 3 h. Cells were harvested, stained with FITC-labeled TNF-α (eBioscience), PE-labeled CD4 (BD Biosciences), biotin-labeled CD8 (eBioscience), and allophycocyanin-labeled IFN-γ (eBioscience), as previously described, (4) and analyzed by flow cytometry (FACSCalibur).

Proliferation of precursor $CD4^+$ and $CD8^+$ T cells

Mediastinal LN cells from infected individual mice were incubated with UV-inactivated virus (PR8) in IMDM-10% FCS for 48 h at 37°C with [^{3}H]thymidine added (1 μCi) for the last 12 h. Total [^{3}H]thymidine incorporation was measured with a scintillation reader as an indicator of cell proliferation, as previously described (23).

Histology

Mediastinal LN were carefully removed, put into OCT freezing medium, and snap-frozen. Standard histological techniques were applied. Staining was performed with peanut lectin (agglutinin)-biotin (eBioscience) before streptavidin-AP was added. Sections were developed with a fast blue AP substrate kit (Vector Laboratories) for 10 min before being washed once with TBS and mounted with Immu-Mount (Vector Laboratories). All slides were viewed with a Zeiss Axioplan 2 microscope. Images were taken with a Retiga EXi (QImaging) digitial camera and analyzed with the software Openlab (Improvision).

Detection of virus-specific Abs

At the indicated time points, sera or BAL fluid was measured for virus-specific IgA and IgG Ab isotype levels. Ninety-six-well plates (Maxisorp; Nunc) were coated with UV-inactivated influenza virus (PR8) in PBS overnight at 4°C. Plates were washed and incubated with PBS-1% BSA for 2 h at room temperature for blocking. Sera and BAL fluids from individual mice were serially diluted in PBS-0.1% BSA starting with a 1/2 dilution for BAL fluids and a 1/50 dilution for sera, followed by incubation at room temperature for 2 h. Plates were washed five times and incubated with alkaline phosphatase-conjugated goat anti-mouse Abs to IgG1, IgG2c, or IgA (SouthernBiotech) at a 1/1000 dilution in PBS-0.1% BSA at room temperature for 2 h. Thereafter, plates were washed five times and substrate p-nitrophenyl phosphate (Sigma-Aldrich) was added. Optical densities were measured on an ELISA reader (Bucher Biotec) at 405 nm.

Assessment of mRNA expression

Lungs were taken at the time points indicated after mice were infected with 50 PFU PR8 influenza. RNA was prepared with TRI Reagent (Molecular Research Center) and then treated with DNase (Invitrogen) to avoid genomic DNA contamination before RNA was converted to cDNA by reverse transcription using SuperScript III (Invitrogen). cDNA was quantified by real-time PCR (iCycler; Bio-Rad) using SYBR Green (Stratagene) and samples were normalized with GAPDH expression levels. Primers sequences (forward and reverse, respectively) used were: GAPDH, 5′-GGG TGTGAACCACGAGAAAT-3′ and 5′-CCTTCCACAATGCCAAAGTT-3′; CCL2, 5′-AGGTCCCTGTCATGCTTCTG-3′ and 5′-ATTGGGATC ATCTTGCTGGT-3′; CCL3, 5′-AGATTCCACGCCAATTCATC-3′ and 5′-CCCAGGTCTCTTTGGAGTCA-3′; CCL4, 5′-TTCTGTGCTCCAGG GTTCTC-3′ and 5′-AGCAAAGACTGCTGGTCTCA-3′; CCL5, 5′-CGC ACCTGCCTCACCATA-3′ and 5′-CTGCAAGATTGGAGCACTTG-3′; CXCL9, 5′-TTTTCCTCTTGGGCATCATC-3′ and TGAGGGATTTGT AGTGGATCG-3′; CXCL10, 5′-AAGTGCTGCCGTCATTTTCT-3′ and CCTATGGCCCTCATTCTCAC-3′; CXCL11, 5′-GGATGAAAGCCGT CAAAATG-3′ and 5′-CCAGGCACCTTTGTCGTTTA-3′; CXCL13, influenza PR8 M protein, 5′-GGACTGCAGCGTAGACGCTT-3′ and 5′-CATCCTGTATATGAGGCCCAT-3′ as previously described (24).

Statistical analysis

Student's t test was used to assess the statistical significance between groups. Significance is depicted as $*$, $p < 0.05$ and $**$, $p < 0.01$.

Results

Virus-specific $CD4^+$ and $CD8^+$ T cells cannot be detected in the airways of CCR7-deficient mice during primary influenza infection

It is generally considered that antiviral immune responses develop in the draining LN following active transport of Ag from the site of infection (18, 21). However, it has been reported that in the absence of Ag transport to the LN or when LN structure is disrupted, priming and expansion of T cells can occur in peripheral tissue (20, 25). We sought to determine whether active transport of Ag was required for the generation of a specific T cell response against influenza virus infection. Accordingly, we utilized CCR7-deficient mice where CCR7-mediated DC migration to the LN is absent. Wild-type and CCR7-deficient mice were inoculated intranasally with 50 PFU of influenza virus, and the cellular infiltrate in the airways was analyzed at multiple time points after infection.

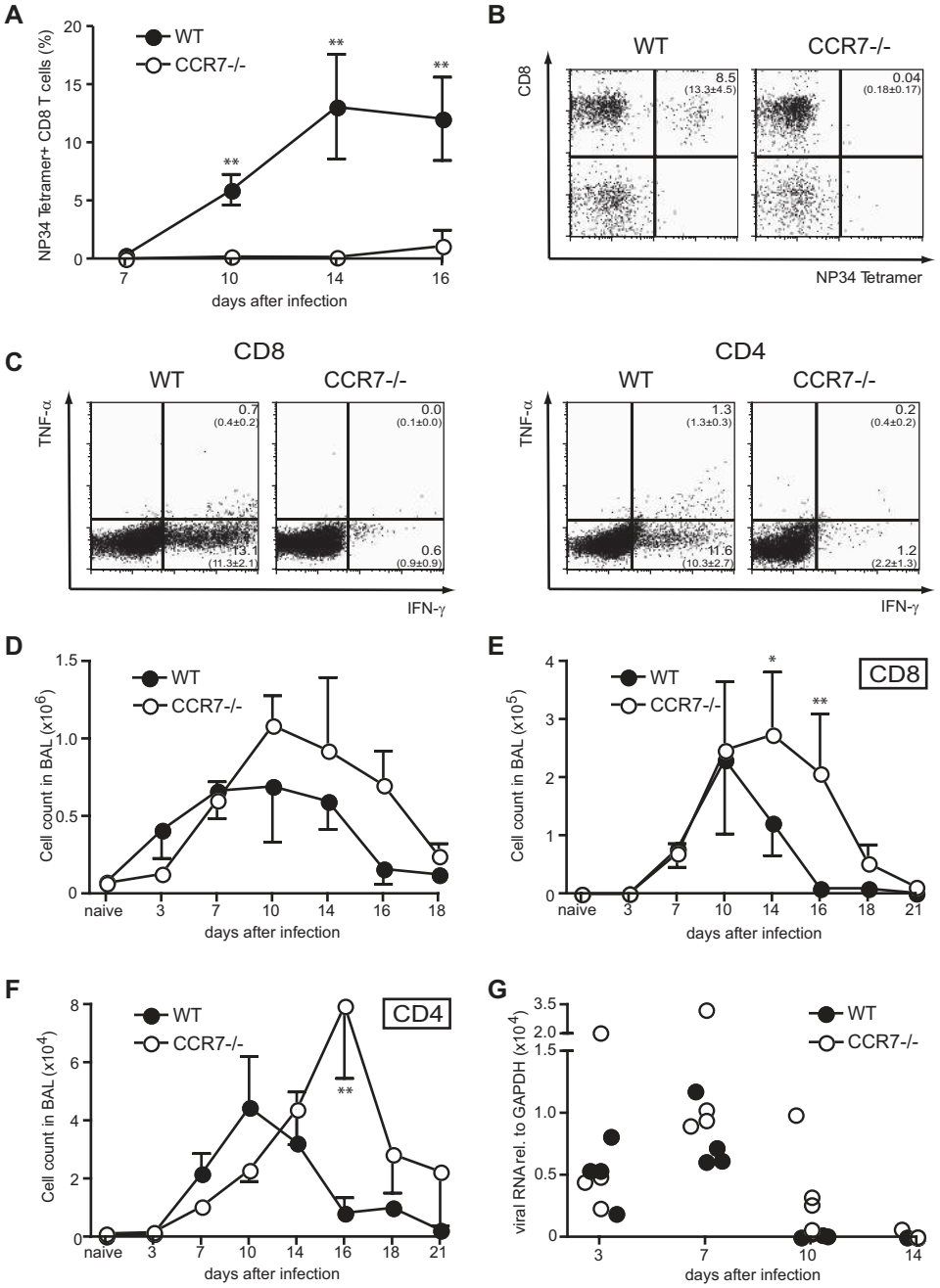

FIGURE 1. Virus-specific T cells are absent in the airways of CCR7-deficient mice but nonspecific cells are nevertheless recruited. CCR7-deficient and C57BL/6 mice were intranasally infected with 50 PFU PR8 influenza virus on day 0, and sacrificed at the time points indicated for analysis. Lung-infiltrating cells were collected by BAL. *A*, Kinetics of the proportion of NP34-tetramer$^+$ cells, gated on CD8$^+$ T cells. *B*, Proportion of NP34$^+$CD8$^+$ T cells at day 14 p.i., gated on lymphocytes. Quadrant proportion, mean, and SD (in brackets) are gated on NP34$^+$CD8$^+$ cells. *C*, BAL cells were restimulated in vitro by APCs for 5 h before fixation, staining, and analysis by flow cytometry. *D*, Kinetics of total cell counts of cellular infiltrate into the airways. *E* and *F*, Kinetics of total CD4$^+$ and CD8$^+$ T cells of cellular infiltrate into the airways assessed by flow cytometry. *G*, Lungs of infected mice were removed at the time points indicated. Viral transcripts were assessed by quantitative real-time PCR. Data are expressed relative to GAPDH expression. Data are representative of at least three independent experiments with three to seven mice per group.

Influenza-specific CD8$^+$ T cells could readily be detected in wild-type mice after day 8 postinfection (p.i.). In contrast, a specific CD8$^+$ T cell response in the airways of mice devoid of CCR7 was below the limits of detection at all points examined (Fig. 1, *A* and *B*, and data not shown). We then assessed the capacity of cells from the airway infiltrate to produce the inflammatory cytokines IFN-γ and TNF-α after in vitro restimulation with influenza virus-presenting DC as an indicator of T cell effector function. In accordance with the lack of virus-specific T cells, we found that CD8$^+$ and CD4$^+$ T cells from CCR7-deficient mice produced baseline levels of IFN-γ and TNF-α upon restimulation, while clear cytokine production by T cells from the wild-type group was observed (Fig. 1*C*). Despite the absence of influenza-specific T cells, the total cell recruitment to the lung was larger in the CCR7-deficient mice as compared with wild-type mice (Fig. 1*D*), and both CD4$^+$ and CD8$^+$ T cell populations were recruited to the lung for a prolonged period (Fig. 1, *E* and *F*). Notably, even in the presence of this robust inflammatory infiltrate there was a delay in the clearance of the virus (Fig. 1*G*). Taken together, these data suggest that the generation of influenza-specific T cells requires CCR7-mediated Ag transport and is not compensated for locally in the lung.

CCR7-dependent cell-mediated Ag transport from the airways to the draining LN is essential for priming and expansion of specific T cells during influenza infection

To ascertain whether the absence of specific T cells in the airways of influenza-infected CCR7-deficient mice reflected a defect in priming and expansion of specific T cells in the draining LN, we infected wild-type and CCR7-deficient mice with 50 PFU of PR8 influenza virus and analyzed the specific T cell response in the draining LN at multiple time points after infection. We first determined the number of DC that had migrated from the airways to the LN 3 days after infection by quantifying the number of

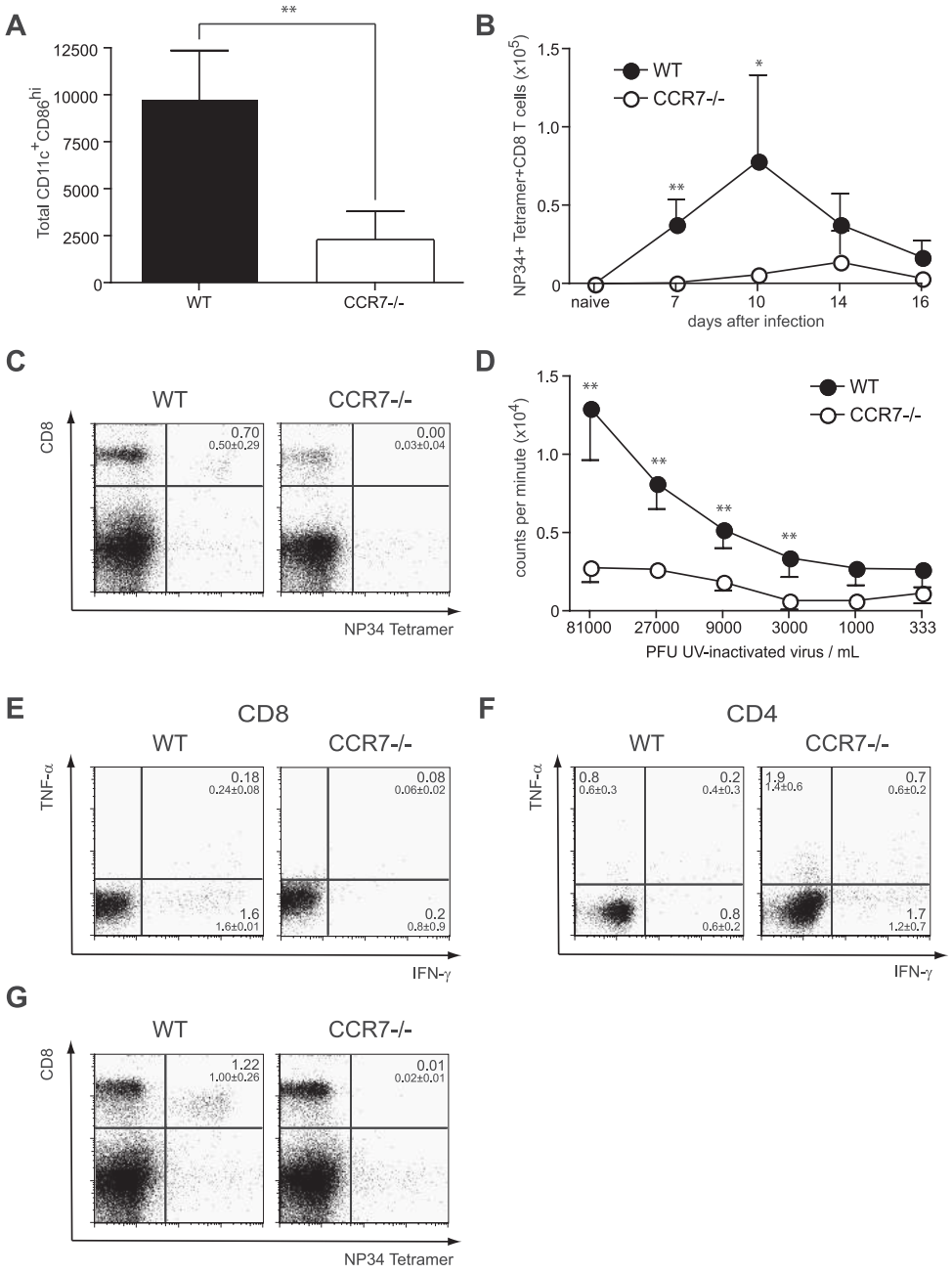

FIGURE 2. Active Ag transport is requisite for triggering anti-influenza T cell responses in draining LN. CCR7-deficient and C57BL/6 mice were intranasally infected with 50 PFU PR8 influenza virus on day 0, and sacrificed at the time points indicated to obtain lung draining LN cells for analysis. *A*, Total number of migratory CD11c$^+$ CD86^{high} DC in the LN at day 3 p.i. *B*, Kinetics of total cell number of NP34-tetramer$^+$-specific T cells. *C*, Proportion of LN NP34$^+$CD8$^+$ T cells at day 10 p.i., gated on live cells. Quadrant proportion, mean, and SD (in brackets) are gated on CD8$^+$ cells. *D*, Total LN cells were cultured in the presence of UV-inactivated virus for 48 h. [^{3}H]thymidine incorporation over the last 12 h of culture was measured as an indicator of cell proliferation. *E* and *F*, LN CD8$^+$ and CD4$^+$ T cells were restimulated in vitro by APCs for 5 h before fixation, staining, and analysis by flow cytometry. *G*, Proportion of splenic NP34$^+$CD8$^+$ T cells at day 10 p.i., gated on live cells. Quadrant proportion, mean, and SD (in brackets) are gated on CD8$^+$ cells. Data are representative of at least two independent experiments with three to seven mice per group.

CD11c$^+$CD86^{high} migratory DC in the draining LN. As expected, the number of migrated DC was significantly ($p < 0.01$) reduced in CCR7-deficient mice (Fig. 2*A*) (21). In line with these data, total cell recruitment to the lung draining LN was greatly reduced in CCR7-deficient mice as compared with C57BL/6 mice at all time points examined (data not shown). We next determined whether NP34-specific CD8$^+$ T cells could be detected in the LN and found that they were below the limits of detection in CCR7-deficient mice until 14 days p.i. when a small population was transiently above background (Fig. 2, *B* and *C*). To determine whether the absence of specific T cells was limited to the CD8$^+$ fraction or also to CD4$^+$ T cells, we next assessed the capacity of LN cells to proliferate ex vivo 7 days p.i. (the peak of CD4$^+$ T cell expansion in wild-type mice) when incubated with UV-inactivated virus. The proliferation of LN cells was greatly reduced in CCR7-deficient mice as compared with the wild-type control group, suggesting that few influenza-specific T cells were present in LN of CCR7-deficient mice at day 7 p.i. (Fig. 2*D*). We also restimulated LN cells with influenza virus-loaded DC at day 10 p.i. and measured cytokine production. In support of the NP34-tetramer analysis (Fig. 2*B*), we could not detect any IFN-γ or TNF-α expression by CD8$^+$ T cells from CCR7-deficient mice at day 10 p.i. (Fig. 2*E*). However, CD4$^+$ T cell cytokine production was above background in LN from CCR7-deficient mice, suggesting that a small pool of virus-specific CD4$^+$ T cells was generated in the draining LN of CCR7-deficient mice during the course of the infection (Fig. 2*F*). To rule out whether the priming and the expansion of specific CD8$^+$ T cells occurs elsewhere, specifically in the spleen of CCR7-deficient mice, we analyzed the frequency of NP34-specific CD8$^+$ T cells in splenocytes by flow cytometry. Again, we could not detect any specific CD8$^+$ T cells in the spleens of CCR7-deficient mice (Fig. 2*G*). In contrast, a virus-specific CD8$^+$ T cell population was prominent in the spleens of wild-type mice as previously reported (Fig. 2*G*) (26). Overall, these data imply that CCR7-mediated Ag transport from the lung is required for the generation of specific CD8$^+$ T cells in draining LN and the spleen,

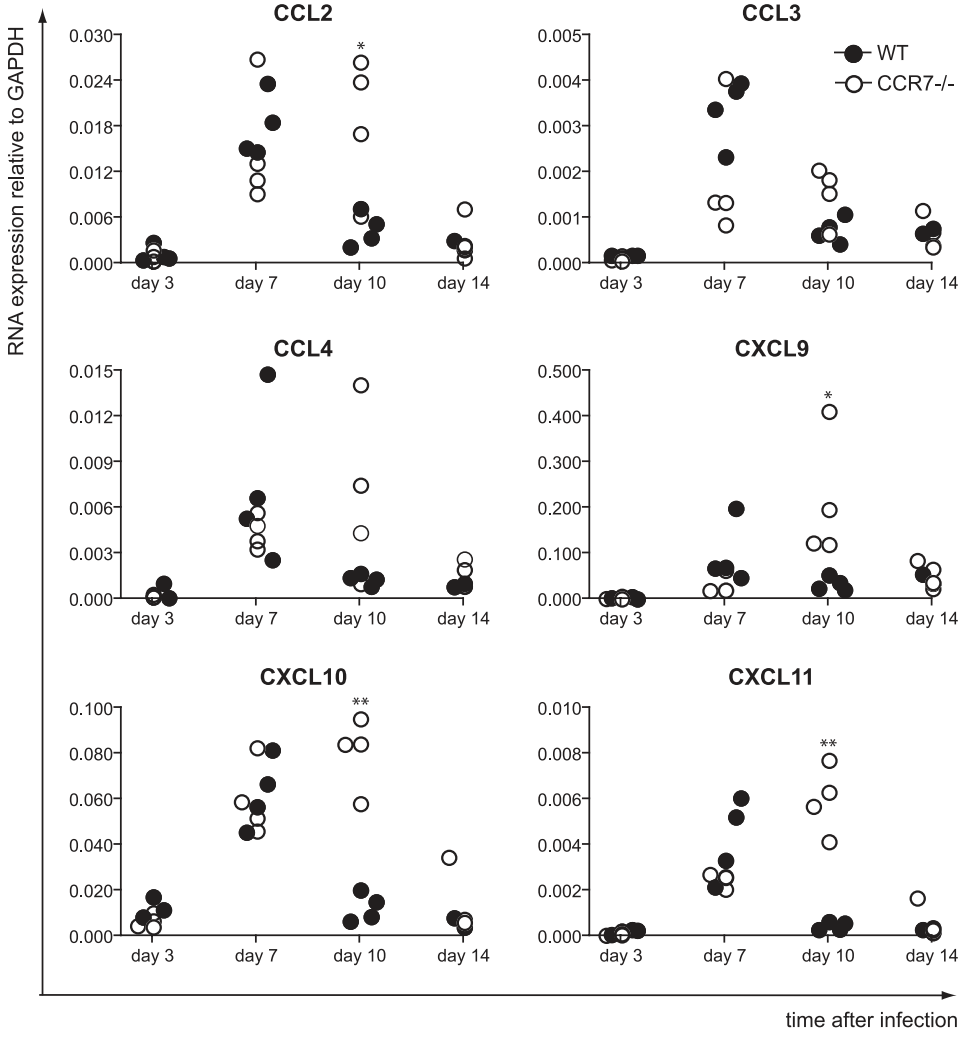

FIGURE 3. Proinflammatory chemokines provide the environment for prolonged recruitment of nonspecific T cells in CCR7-deficient mice. CCR7-deficient and C57BL/6 mice were intranasally infected with 50 PFU PR8 influenza virus on day 0, and sacrificed at the time points indicated for analysis. Lungs of infected mice were removed and mRNA levels were assessed by quantitative real-time PCR. Data are expressed relative to GAPDH expression. Data are representative of two independent experiments with three to four mice per group.

while the generation of influenza-specific $CD4^+$ T cells appears less dependent on CCR7 signaling.

The recruitment of nonspecific T cells to the airways is promoted upon prolonged chemokine expression in the lung

To determine why CCR7-deficient mice display considerable nonspecific cell recruitment to the lungs upon influenza infection, we assessed the levels of several chemokines that are important in T cell attraction under inflammatory conditions by quantitative real-time PCR. CCL2 (MCP-I) was reported to attract macrophages, monocytes, and T cells while CCL3 (MIP-Iα) and CCL4 (MIP-Iβ) are involved in neutrophil chemotaxis, Th1 differentiation, and recruitment of polarized T cells (27–29). CCL2, CCL3, and CCL4 were expressed in the lungs of infected CCR7-deficient mice at comparable levels to wild-type mice on day 7 p.i. Notably, however, the expression of these chemokines was prolonged in CCR7-deficient mice (Fig. 3). We next assessed whether the chemokines CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC), which are associated with memory T cell recruitment, were contributing to the prolonged influx of bystander T cells to the airways of CCR7-deficient mice (30). All three chemokines were significantly ($p < 0.05$) up-regulated in CCR7-deficient mice as compared with wild-type mice on day 10 after infection (Fig. 3). Overall, the prolonged peak of chemokines attracting polarized and memory T cells in CCR7-deficient mice accounts, at least in part, for the increased recruitment of proinflammatory nonspecific T cells during influenza infection.

CCR7-deficient mice develop an influenza-specific IgA and IgG Ab isotype response

A small pool of influenza-specific $CD4^+$ T cells was detectable in the draining LN in the absence of CCR7 (Fig. 2*F*); however, no influenza-specific CD4 T cells could be detected in the airways of CCR7-deficient mice during primary influenza infection. Since specific $CD4^+$ T cell help is critical for the formation of germinal centers and the induction of IgG Ab class switching during influenza infection, we next assessed the capability of CCR7-deficient mice to form germinal centers and to generate influenza-specific class-switched Abs. We infected mice with influenza virus and obtained mediastinal LN for histology, and BAL and serum for analysis of influenza-specific Ab isotypes at multiple time points. Histology sections of mediastinal LN at day 10 p.i. showed clear formation of germinal centers in both CCR7-deficient and wild-type mice (Fig. 4*A*). Analysis of sera and BAL fluid revealed that IgG2c could be detected 7 days p.i. in wild-type mice (Fig. 4*B*). Further analysis of sera from day 10 after infection revealed that class switching to IgG1 and IgG2c was substantially reduced in knockout mice as compared with the wild-type control group (Fig. 4*B*), which is in accordance with the defect in the generation of influenza-specific $CD4^+$ T cells. Surprisingly, however, at day 14 p.i., we found that there was no longer a significant difference in the levels of virus-specific IgG isotypes (Fig. 4*B*), suggesting that a small pool of specific $CD4^+$ T cells is sufficient to induce class switching. Influenza-specific IgA isotype levels in BAL fluid

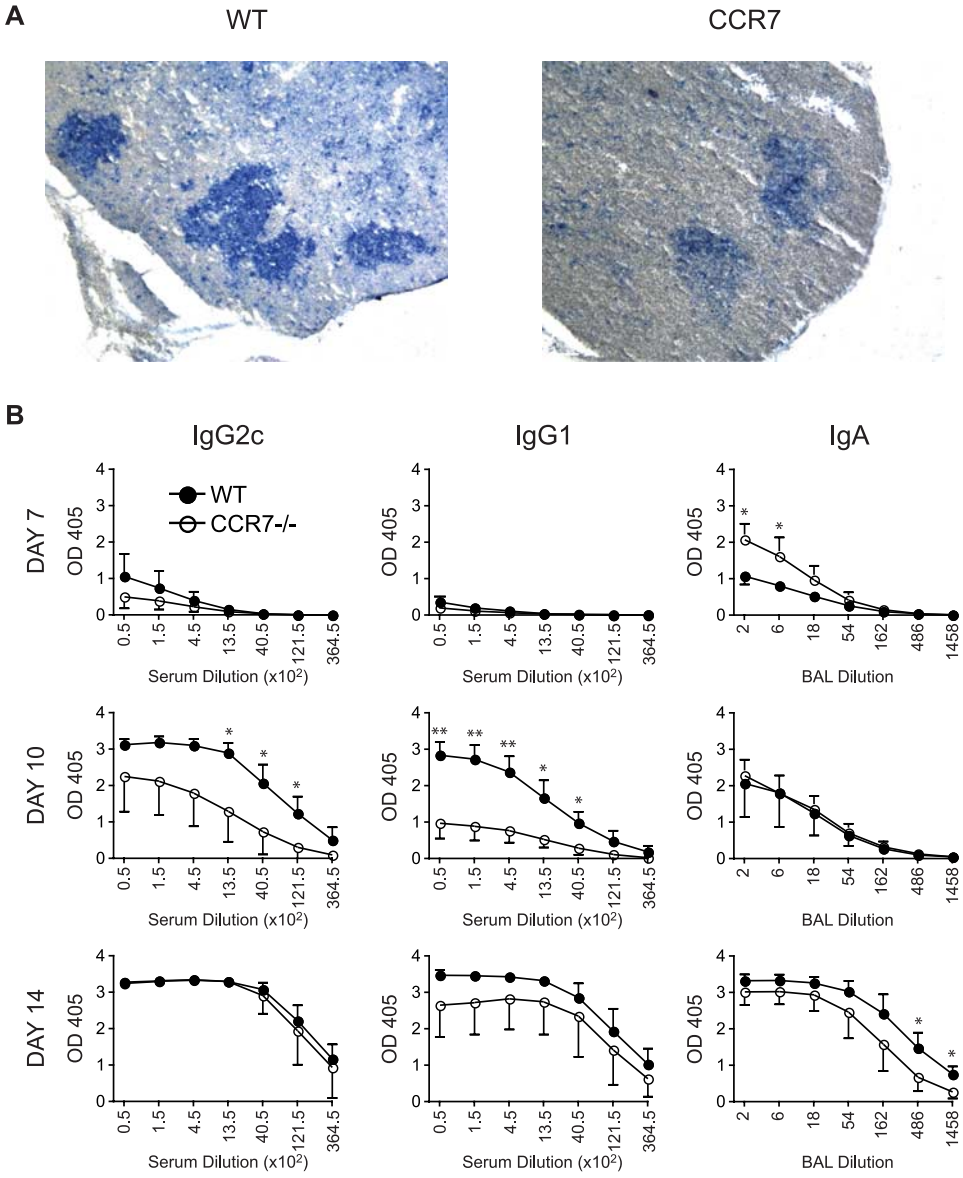

FIGURE 4. Virus-specific B cells from CCR7-deficient mice undergo class switching to IgG and IgA Ab isotypes in germinal centers. CCR7-deficient and C57BL/6 mice were intranasally infected with 50 PFU PR8 influenza virus. Mice were sacrificed or bled at the indicated time points after infection. *A*, Histological sections of mediastinal LN showing PNA^+ areas in dark blue. *B*, IgA from BAL fluid or serum IgG2c and IgG1 Ab isotypes at the indicated time points. Data are representative of two independent experiments with three to seven mice per group.

of CCR7-deficient mice were substantially elevated as compared with wild-type mice at day 7 p.i. (Fig. 4*B*). At day 10 p.i., no difference could be observed in IgA isotype levels between wild-type and CCR7-deficient mice (Fig. 4*B*). This is in line with prior data, showing that class switching to IgA is not dependent on influenza-specific $CD4^+$ T cell help (16). Taken together, these data show that isotype class switching to IgA is not impaired in CCR7-deficient mice and does not depend on cognate T-B interaction, while germinal center formation and class switching to IgG isotypes are delayed but functional in the absence of CCR7.

CCR7 signaling is not required for the generation of an influenza-specific$CD4^+$ T cell memory population

To clarify the differential requirement of CCR7-dependent migration for activation of naive T cells and the generation of an influenza-specificmemory T cell population in CCR7-deficient mice, we assessed the development of anti-influenza T cell recall responses in vivo. T cell recall responses in vivo can be addressed by infecting mice with PR8 influenza A virus followed by infection with the serologically distinct X31 influenza virus (31). Accordingly, mice were inoculated intranasally with 50 PFU of PR8 influenza virus (H1N1). Thirty days later, mice were given X31 influenza virus (H3N2) intranasally and sacrificed 7 days after the secondary infection. Analysis of the in vivo recall response revealed that the total number of $CD4^+$ T cells was similar in CCR7-deficient mice while the total number of $CD8^+$ T cells was significantly reduced when compared with wild-type mice (Fig. 5*A*). To determine whether those $CD4^+$ T cells were specific for influenza virus, we restimulated the cells together with influenza virus-bearing DC. In contrast to the primary T cells response, where specific $CD4^+$ T cells were below the limits of detection in the airways of CCR7-deficient mice (Fig. 1*C*), we found that $CD4^+$ T cells recruited to the lung during secondary infection produced similar levels of the proinflammatory cytokines TNF-α and IFN-γ as compared with wild-type mice. These data suggest that the few specific $CD4^+$ T cells that are generated in the draining LN during the primary response were sufficient to sustain a normal recall response (Fig. 5*B*). We further analyzed the recall response of influenza-specific $CD8^+$ T cells. Contrary to the primary response, where specific $CD8^+$ T cells were below the limits of detection, we found that influenza-specific $CD8^+$ T cells were present in the airways upon secondary infection of CCR7-deficient mice, albeit at significantly reduced total numbers (Fig. 5*C*). These $CD8^+$ T cells were capable of

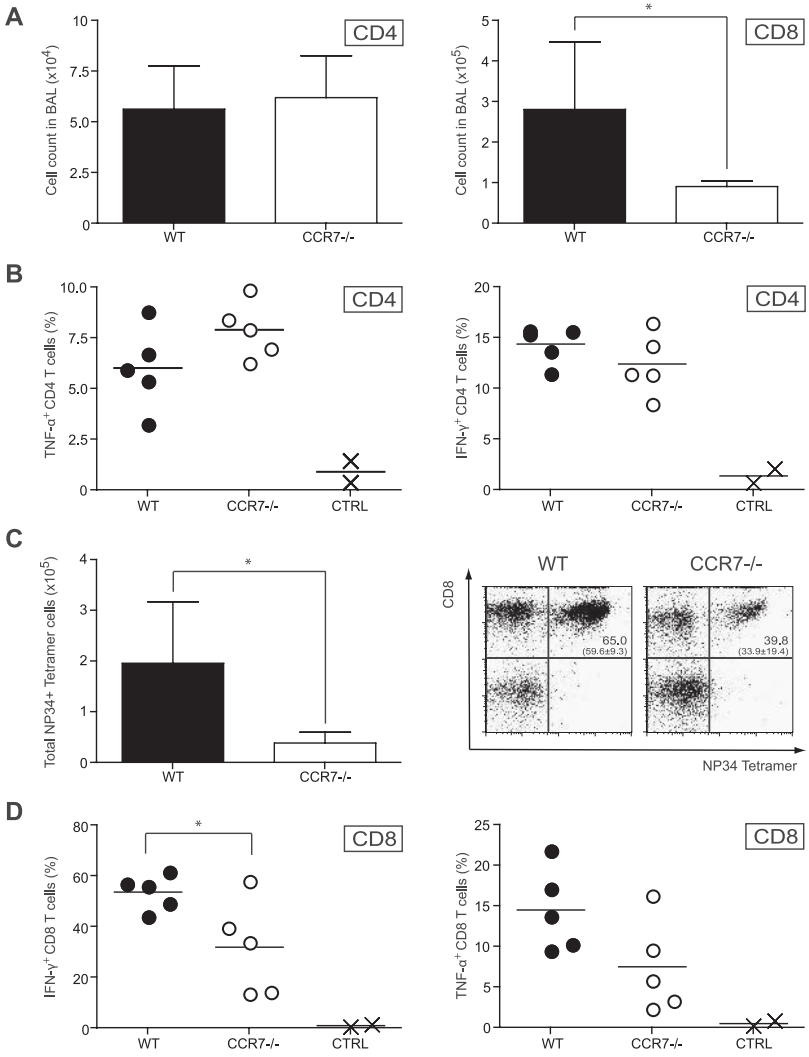

FIGURE 5. CCR7 signaling is not required for the generation of T cell recall responses despite a defective T cell response in primary infections. CCR7-deficient and C57BL/6 mice were intranasally inoculated with 50 PFU PR8 influenza virus. Thirty days later, mice were infected with the serologically distinct X31 influenza virus, and mice were sacrificed 7 days after the second infection. Lung-infiltrating cells were collected by BAL. *A*, Total cell count of infiltrated $CD4^+$ and $CD8^+$ T cells. *B*, BAL $CD4^+$ T cells were restimulated in vitro by APCs for 5 h before fixation, staining, and analysis by flow cytometry. *C*, Total $NP34^+$ T cells, gated on live cells, and proportion of $NP34^+CD8^+$ T cells, gated on lymphocytes, at day 7 after secondary infection. Quadrant proportion, mean, and SD (in brackets) are gated on $CD8^+$ cells. *D*, BAL $CD8^+$ T cells were restimulated in vitro by APCs for 5 h before fixation, staining, and analysis by flow cytometry.

producing TNF-α and IFN-γ upon specific restimulation (Fig. 5*D*). Taken together, these data imply that a small specific $CD4^+$ T cell population was generated in the primary response in the absence of CCR7-mediated active Ag transport, that this specific $CD4^+$ T cell population was sufficient to result in normal recall expansion upon secondary challenge, and that a population of influenza-specific $CD8^+$ (below the limits of detection) was generated during the primary response but was not sufficient to form a normal pool of memory cells.

CCR7-mediated T cell entry and localization in the secondary lymphoid organs is not a critical determinant of T cell priming during influenza infection

CCR7 expression is not only critical for the migration of DC from the periphery into draining LN, but also naive T cells substantially depend on CCR7 signaling to enter secondary lymphoid organs and to localize appropriately within them (7, 32). To address whether CCR7-deficient $CD8^+$ T cells have an intrinsic defect in their activation, we inoculated mice with 2×10^5 PFU PR8 influenza virus via the tail vein to cause systemic distribution of the virus. C57BL/6 and CCR7-deficient mice were sacrificed 10 days p.i. for analysis. We found that the expansion of specific $CD8^+$ T cells in the spleen upon systemic exposure to influenza virus was comparable between the two groups (Fig. 6*A*). Additionally, when splenic $CD8^+$ T cells were restimulated with peptide and influenza virus, the resulting production of IFN-γ was similar in C57BL/6 and CCR7-deficient mice (Fig. 6*A*), indicating that CCR7 expression on $CD8^+$ T cells is not required for their expansion or effector function.

To further test our findings that CCR7-dependent Ag transport by DC was required for the generation of virus-specific $CD8^+$ but not $CD4^+$ T cells, we generated chimeric mice, which were reconstituted with three parts of bone marrow from RAG-deficient mice (no T and B cells) and one part of bone-marrow from either C57BL/6 or CCR7-deficient mice. The resulting mixed bone marrow chimeric mice have competent CCR7-dependent Ag transport by DC together with C57BL/6 lymphocytes or lymphocytes deficient in CCR7. The chimeric mice were infected with 50 PFU PR8 influenza virus and analyzed 12 days p.i. Analysis of the mediastinal LN revealed that the total cell recruitment was significantly ($p < 0.01$) reduced in mice reconstituted with RAG/CCR7-deficient bone marrow as compared with mice reconstituted with RAG/WT bone marrow (Fig. 6*B*). This result is in line with previous reports, which indicate that CCR7 expression on T cells plays an essential role in LN homing. Notably, the proportions of $CD4^+$ and $CD8^+$ T cells were increased in mice bearing only CCR7-deficient T cells (Fig. 6*B*). However, the assessment of the influenza-specific T response showed similar expansion of NP34-specific $CD8^+$ T cells in RAG/WT as compared with RAG/$CCR7^{-/-}$ mice, and restimulation with influenza virus and peptide ex vivo resulted in a comparable production of IFN-γ by $CD8^+$ T cells. IFN-γ by $CD4^+$ T cells was

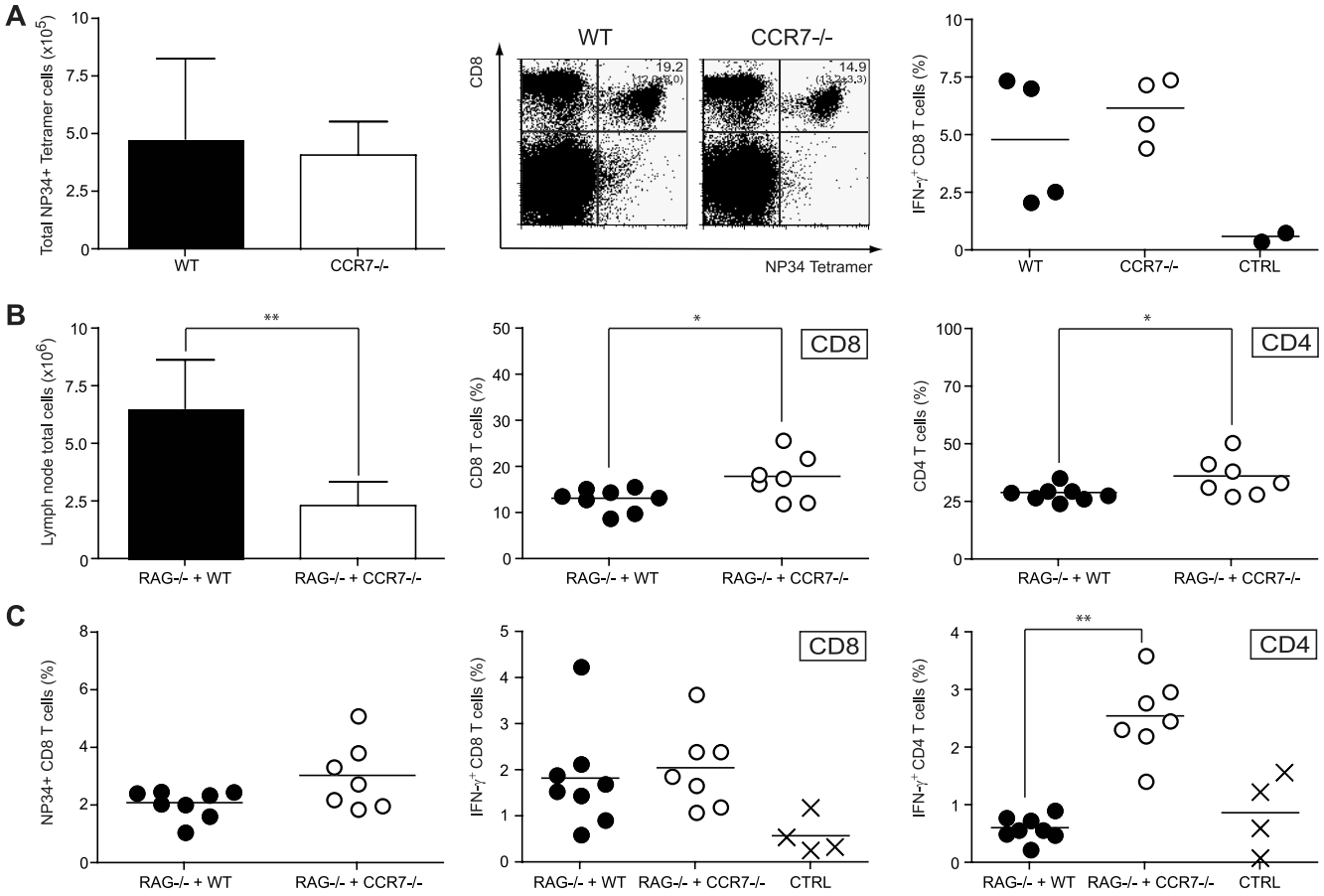

FIGURE 6. CCR7-dependent entry and localization of T cells is not critical for the priming and expansion of specific $CD4^+$ and $CD8^+$ T cells in the LN. *A*, CCR7-deficient and C57BL/6 mice were infected i.v. with 2×10^5 PFU of PR8 influenza virus and sacrificed 10 days p.i. The graphs from left to right show: total number of NP34-tetramer$^+$ T cells in the spleen; proportion of $NP34^+CD8^+$ T cells, gated on lymphocytes; and IFN-γ production by $CD8^+$ T cells after specific restimulation. *B* and *C*, C57BL/6 mice were lethally irradiated and reconstituted with bone marrow from RAG-deficient mice (3/4) and bone marrow from either C57BL/6 or CCR7-deficient mice (1/4). Chimeric mice were infected with 50 PFU PR8 influenza virus intranasally, and lung draining LN were taken 12 days p.i. for analysis. *B*, Total cell count and the proportions of $CD8^+$ and $CD4^+$ T cells in the lung draining LN. *C*, Total number of NP34-tetramer$^+$ T cells from the LN, gated on live cells, and IFN-γ production by $CD8^+$ and $CD4^+$ T cells after specific restimulation. Data are representative of three independent experiments with three to four mice per group. Data in *B* and *C* are pooled from two independent experiments.

higher in RAG/CCR7$^{-/-}$ mice as compared with the control group, further highlighting that CCR7$^{-/-}$ T cells have no inherent defect in their activation (Fig. 6*C*). The significant decrease in total lymphocyte numbers in the draining LN was reflected in reduced total numbers of specific $CD8^+$ T cells in the BAL (data not shown). Overall, these data indicate that $CD4^+$ and $CD8^+$ T cells from CCR7-deficient mice have no intrinsic defect in activation, that CCR7 expression on T cells is critical but not requisite to enter the draining LN, and that the CCR7-dependent localization of T cells within the draining LN does not affect the generation of specific $CD4^+$ and $CD8^+$ T cells. Thus, CCR7-dependent active Ag transport by DC is necessary to induce influenza-specific $CD8^+$ T cells.

Discussion

A number of studies highlight the importance of CCR7-mediated migration of DC from the periphery to the draining LN for presentation of Ag to naive T cells (7, 33). However, the role of CCR7 in directing DC migration from the airways during an infection with influenza virus has not been addressed. Furthermore, the necessity for active transport of influenza-derived Ags to reach the draining LN remains controversial since it has been reported that influenza-specific adaptive immune responses can be initiated locally in the airways (19, 20). We demonstrate in this study that CCR7-mediated Ag transport from the airways to the draining LN is central to the development of an influenza-specific $CD8^+$ T cell response. We further show that a small pool of influenza-specific $CD4^+$ T cells is generated in the draining LN of infected CCR7-deficient mice, leading to a delayed but robust generation of class switched virus-specific Abs. It is notable that effective Ab isotype class switching occurs in this environment where the $CD4^+$ Th response is impaired; clearly, very few $CD4^+$ T cells are required to sustain an anti-influenza Ab response.

Immune responses against influenza infection of the airways develop in the mediastinal LN, where priming and proliferation of $CD8^+$ and $CD4^+$ T cells as well as B cell activation and Ab isotype class switching occur. Analysis of cell cycling has revealed that T cells almost exclusively proliferate in the LN while T cells in the airways do not divide but exhibit effector function upon Ag challenge (34, 35). Thus, it is key to the initiation of a specific T cell response that Ag reaches the LN. Our study confirms these findings by showing that CCR7-mediated migration of airway-derived APCs was largely required for the generation of influenza-specific T cells. Studies with lymphotoxin-α-deficient mice, which lack LN, showed that a

specific T response against influenza virus can develop, albeit delayed, that is sufficient to mediate antiviral protection (19). More recent results by the same group indicated that the absence of lung draining LN leads to the formation of inducible BALT, concluding that the immune responses against influenza infection can take place locally in the lung (20). It was recently shown that CCR7-deficient mice also develop BALT spontaneously (25). Despite the reported presence of BALT in CCR7-deficient mice, we did not find any evidence for a locally induced $CD8^+$ T cell response in CCR7-deficient mice even at late time points p.i., suggesting that CCR7-mediated active Ag transport to the LN was still necessary for a robust influenza-specific T cell response. Considering that lymphotoxin-α-deficient mice develop spontaneous chronic airway inflammation and recruitment of activated lymphocytes (36), it is possible that the BALT observed in these mice is not induced by an influenza infection but rather develops from preexisting lymphoid aggregates. The defect in the generation of influenza-specific $CD8^+$ T cells in CCR7-deficient mice is in line with a recent report showing that plt/plt mice, which lack the CCR7 ligands CCL19 and CCL21, also do not develop an influenza-specific $CD8^+$ T cell response in the airways (37). In our study, specific $CD8^+$ T cells were not only below the limits of detection in the lung and to a large degree in the mediastinal LN in the absence of CCR7, but also in the spleen, indicating that CCR7 signaling is also pivotal for the generation of the large pool of influenza-specific $CD8^+$ T cells in the spleen. However, it still remains to be clarified whether primed $CD8^+$ T cells from the lung draining LN emigrate via efferent lymphatics to the spleen, where they expand, or whether Ag-loaded DC migrate from the airways to the spleen where they prime $CD8^+$ T cells directly. We also demonstrated that the absence of CCR7 signaling leads to prolonged bystander $CD8^+$ T cell recruitment to the airways of infected mice, which we attribute to the inflammatory chemokine profile evident in the course of the influenza infection. Data by Ely et al. indicate that memory $CD8^+$ T cells are rapidly recruited during heterologous respiratory virus infections (38). The chemokine ligands CXCL9, CXCL10, and CXCL11, which share the chemokine receptor CXCR3, were significantly up-regulated at day 10 p.i. in CCR7-deficient mice. CXCR3 is particularly expressed on T cells of the activated/memory phenotype and is associated with the Th1 subset (39, 40). It was recently shown that the total memory $CD8^+$ T cell population has the ability to migrate toward CXCR3 ligands in vitro; however, the key chemokine receptor for Ag-specific memory T cell recruitment in vivo was identified as being CCR5, indicating that prolonged expression of CXCR3 may lead to a general recruitment of memory T cells but not Ag-specific T cells (41). However, it appears that nonspecific $CD8^+$ T cells do not contribute to the clearance of virus (42). Our finding supports this by showing that influenza virus can still be detected at day 10 after infection in CCR7-deficient mice while it was cleared in wild-type mice by this time point. Thus, we suggest that the extended presence of influenza virus in the airways of CCR7-deficient mice leads to prolonged chemokine expression, which results in further nonspecific $CD8^+$ T cell recruitment. Additionally, we show that the number as well as the effector function of $CD4^+$ T cells infiltrating the lung was reduced in CCR7-deficient mice, similar to the deficiency in the generation of specific $CD8^+$ T cells. This indicates that CCR7-dependent migration of DC is also requisite for the priming and expansion of specific $CD4^+$ T cells. Indeed, early work by Levin et al. show that only DC are capable of activating naive $CD4^+$ T cells efficiently (43). In contrast to the airways, we could still detect a small population of specific $CD4^+$ T cells in the draining LN of CCR7-deficient mice, which was sufficient to induce germinal center formation and Ab isotype class switching. Thus, the question remains how Ag reaches the draining LN in the absence of CCR7-dependent cell-mediated transport. It cannot be excluded that cell-free virions reach the LN by drainage via afferent lymph, where they are taken up by LN-resident DC and macrophages and are presented as exogenously derived Ag to $CD4^+$ T cells. In an attempt to address the role of Ag drainage from the periphery to the LN, Shaw and coworkers have applied high-molecular mass molecules subcutaneously into mice and found that these molecules do not localize in the cortical T cell zone of the LN but in the subcapsular sinus before they are drained into efferent lymphatic vessels, even under inflammatory conditions (44). However, a recent report addressing the transport of lymph-borne vesicular stomatitis virus into the LN showed that resident macrophages can take up virus particles from the subcapsular sinus and shuttle them to virus-specific B cells, which then migrate into B cell follicles, but not to the paracortical T cell area (45). Nevertheless, note that the routes of application for most studies investigating Ag drainage are systemic or subcutaneous, which cannot directly be compared with the lung mucosal environment. Whether drainage of cell-free virions or a CCR7-independent cell-mediated Ag transport to the LN accounts for the expansion of influenza-specific $CD4^+$ T cells remains to be clarified.

Interestingly, we found that the small number of specific $CD4^+$ T cells generated in LN of CCR7-deficient mice was sufficient to induce the formation of germinal centers and Ab class switching, which critically depend on specific $CD4^+$ T help via CD40-CD40L interaction (46). The magnitude of the IgG Ab isotype response was similar in CCR7-deficient mice and wild-type mice, although it was delayed, presumably due to defective priming of naive $CD4^+$ T cells. Notably, bystander T cell help is sufficient for class switching to IgA isotypes (16), which is supported by our result demonstrating early production of IgA Abs in the absence of specific T cells.

It was shown by several groups that CCR7 signaling is not solely involved in DC homing to the LN, but also plays an central role in T cell entry into the LN and localization therein (7, 32, 47). Our data with the chimeric mice showed that the differential activation of $CD4^+$ T cells and $CD8^+$ T cells was independent of their expression of CCR7. This suggests that a mechanism other than intranodal $CD4^+$ and $CD8^+$ T cell localization accounts for the defect in the generation of influenza-specific $CD8^+$ T cells. Recent reports suggest that nonmigratory $CD8\alpha^+$ DC are specialized in cross-presentation of exogenous Ags and are therefore the major activator of naive $CD8^+$ T cells (48, 49). However, Belz et al. describe both migratory and LN resident DC populations as being potent inducers of $CD8^+$ T cell priming during influenza infection (50). Moreover, GeurtsvanKessel and colleagues showed that migratory $CD11c^+CD11b^-CD8\alpha^-$ DC presented influenza Ag to both $CD4^+$ and $CD8^+$ T cells while the LN resident $CD11c^+CD11b^-CD8\alpha^+$ presented to $CD8^+$ T cells (51). Our data indicate that while the activation of influenza-specific $CD4^+$ T cells may occur through drainage, and thus LN resident DCs, the migration of airway-derived DC was requisite for activating naive $CD8^+$ T cells, either directly or through the transfer of Ag to LN resident $CD8\alpha^+$ DC.

In conclusion, we demonstrate herein that the absence of CCR7 signaling on DC leads to a severe defect in the generation of influenza-specific $CD8^+$ T cells, which is not compensated for locally in the airways. We further show that delayed clearance of

influenza virus due to the absence of specific T cells results in prolonged recruitment of nonspecific T cells into the airways. Surprisingly, although the generation of specific $CD4^+$ T cells was greatly reduced in CCR7-deficient mice, the few $CD4^+$ T cells specific for influenza in the draining LN were sufficient to induce germinal centers and class switching to IgG isotypes. Taken together, this study reveals that $CD4^+$ and $CD8^+$ T cells exhibit differential requirements for CCR7-mediated active Ag transport during influenza infection and that the key protective mechanism against influenza infection (i.e., specific Abs) is safeguarded by only requiring a fraction of the T cell response to develop normally.

Disclosures

The authors have no financial conflicts of interest.

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