Induction of Tolerance to Innocuous Inhaled Antigen Relies on a CCR7-Dependent Dendritic Cell-Mediated Antigen Transport to the Bronchial Lymph Node[1]

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Allergic airway diseases such as asthma are caused by a failure of the immune system to induce tolerance against environmental Ags. The underlying molecular and cellular mechanisms that initiate tolerance are only partly understood. In this study, we demonstrated that a CCR7-dependent migration of both CD103^{+} and CD103^{-} lung dendritic cells (DC) to the bronchial lymph node (brLN) is indispensable for this process. Although inhaled Ag is amply present in the brLN of CCR7-deficient mice, T cells cannot be tolerized because of the impaired migration of Ag-carrying DC and subsequent transport of Ag from the lung to the draining lymph node. Consequently, the repeated inhalation of Ag protects wild-type but not CCR7-deficient mice from developing allergic airway diseases. Thus, the continuous DC-mediated transport of inhaled Ag to the brLN is critical for the induction of tolerance to innocuous Ags. *The Journal of Immunology,* **2006, 177: 7346–7354.**

The immune system of the lung has to balance between the induction of a protective immune response against pathogens and the induction of tolerance to harmless aerogenic Ags. The incessant increase in the number of people suffering from allergic airway diseases demands a better understanding of how inflammatory and tolerogenic immune reactions are controlled in the lung. It is assumed that allergic airway diseases are caused by the failure to induce tolerance. The decision for tolerance or protection might be controlled at the level of Ag-presenting dendritic cells (DC)[3] (1, 2). DC have been known for a long time to initiate protective immune responses against invading pathogens under inflammatory conditions. In addition, recent data suggest that DC may also induce tolerance once Ags are presented to T cells under noninflammatory, so-called steady-state conditions (3–8).

Chemokines and their receptors control the migration of immune cells at basically any stage of an immune response, including the recruitment of effector cells to the lung (9). Thus, several chemokine receptors such as CCR3, CCR6, and CXCR4 participate in the inflammatory immune reactions of the lung. They also contribute to the massive influx of effector cells such as eosinophils, thereby provoking the development of allergic airway diseases (10–12). Furthermore, it has been shown that the initiation of inflammatory and allergic airway reactions requires the transport of Ag from the lung to the draining lymph node (DLN) by APCs (reviewed in Ref. 13), a mechanism that depends on the chemokine CCL21 (14). CCR7 and its ligands CCL19 and CCL21 were already observed to be crucially involved in guiding skin DC from the periphery into skin DLN under inflammatory conditions (15, 16). More recently, we demonstrated that CCR7 is also essential for the steady-state trafficking of skin DC to DLN under noninflammatory conditions, suggesting a role for this receptor in the induction of tolerance to self-Ags (5).

In the present study, we provide strong genetic evidence that the presentation of innocuous inhaled Ag within the bronchial lymph node (brLN) and the induction of tolerance toward airborne Ags essentially relies on the continuous, CCR7-dependent, steady-state migration of APCs from the lung to the DLN.

Materials and Methods

Mice

CCR7$^{-/-}$ mice (15) (backcrossed to the C57BL/6 background for seven generations), as well as OT-II Ly5.1, were bred and maintained under specific pathogen-free conditions at the central animal facility of the Hannover Medical School and were used at the age of 8–12 wk. C57BL/6 Ly5.2 and congenic C57BL/6 Ly5.1 were purchased from Charles River Laboratories. All animal experiments were conducted in accordance with local and institutional guidelines.

Generation of bone marrow-derived DC

Bone marrow (BM)-derived DC of wild-type and CCR7-deficient mice were generated as described earlier (5). In brief, BM from femurs and tibiae were cultured in RPMI 1640, supplemented with 30 ng/ml GM-CSF, 10% FCS, and 50 μM 2-ME. At days 1 and 3 of the culture, the medium was changed and the cells were selected for adherence, whereas additional culture medium was added at day 5. At day 7, the cells were harvested, washed, and reseeded in culture medium supplemented with 30 ng/ml TNF (R&D Systems) and 1 μg PGE_2 (Sigma-Aldrich) to induce maturation. In some cases, cells were incubated from day 7 with 0.1 μg/ml OVA peptide specific for the TCR expressed by OT-II cells ($OVA_{323-339}$ ISQAVHAA HAEINEAGR) or 1 mg/ml OVA grade VI (Sigma-Aldrich). All batches of OVA were tested for the presence of LPS (LAL QCL 1000; Cambrex) and only batches containing <5 endotoxin units/mg were used. Fully matured DC were used at day 9.

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[3] Abbreviations used in this paper: DC, dendritic cell; DLN, draining lymph node; brLN, bronchial LN; BM, bone marrow; i.t., intratracheal(ly); LN, lymph node; MHCII, MHC class II; DAPI, 4′,6′-diamidino-2-phenylindole; BAL, bronchoalveolar lavage; CD62L, pDC, plasmacytoid.

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Intratracheal instillation of OVA, Ag-pulsed DC, and latex beads

Mice were anesthetized i.p. with 0.2 mg of ketamine and 0.02 mg of xylazine/g body weight. Subsequently, a blunt cannula (0.7 × 19 mm; Introcan, B. Braun, Melsungen) was instilled through the larynx into the trachea under visual control (Head-worn Loupe KS; Zeiss). Cy5-labeled OVA (1 mg/ml PBS) cells or 0.2- or 1-μm diameter latex beads (Polysciences) were injected intratracheally (i.t.) in a total volume of 60 μl. For in vivo labeling of migrating lung DC, 25 mM CFSE in DMSO was further diluted to 2.5 mM in PBS. Fifty microliters were applied i.t. 2 h before the application of the Cy5-labeled OVA. In some experiments, the far-red fluorescent cell tracker DDAO-SE (60 μl, 2 mM in DMSO diluted to a final concentration of 400 μM in PBS; Molecular Probes) was used for in vivo labeling of migrating lung DC instead of CFSE.

Flow cytometry

To obtain single-cell suspensions of LN, spleen, and lung, organs were cut into small pieces and treated with 25 μg/ml DNase I and 500 μg/ml collagenase D (Roche) for 30 min at 37°C (twice for the lung). The tissue was mechanically minced and the resulting cell suspension was filtered through a nylon mesh and then washed with PBS supplemented with 3% FCS. Cells obtained from lungs were additionally enriched for leukocytes by density gradient centrifugation with Lympholyte M (Cedarlane Laboratories). Cells were stained with the following mAbs against CD3 (17A2; American Type Culture Collection), CD4 (RMCD4-2) and CD8β (RMCD8-2) (17), CD8α (53-6.7), CD11c (HL3), CD16/CD32 (2.4G2), CD45.1 (A20), B220 (RAB-6B2), hamster IgG1, MHC class II (MHCII; AF6-120.1), Ly6c (AL-21), Vα2 (B20.1), Vβ5 (MR9-4), CD103 (M290) (all from BD Biosciences), CD11b (M1/70.15), CD62L (MEL-14), and CD44 (IM7.8.1) (Caltag Laboratories). In vitro-differentiated DC were stained before transfer with mAbs against CD11c, MHC-II, CD86 (RMMP-1; Beckman Coulter), and a CCL19-hIgG fusion protein followed by anti-human IgG-Cy5 Ab ($F(ab')_2$; Jackson ImmunoResearch Laboratories) as described earlier (18). CCR7 expression on LN DC was detected by staining with rat anti-mouse CCR7 (clone 4B12) as described elsewhere (19). To analyze DC subpopulations, single-cell suspensions were blocked with 5% inactivated mouse serum, 5% inactivated rat serum, and 5 μg/ml anti-CD16/CD32 for 15 min at 4°C. Cells were then stained with anti-CD11c-PE, anti-CD4-PerCP or anti-B220-PerCP, anti-CD8α-biotin or anti-Ly6C-biotin, and hamster IgG-allophycocyanin for 20 min at 4°C. Samples were washed twice and subsequently stained with streptavidin-allophycocyanin-Cy7 (BD Biosciences). Directly before analysis, dead cells were stained with 0.2 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI) for 1 min, washed, and resuspended in PBS. For analysis, gates were set on forward scatter (FSC) area/side scatter area histograms followed by FSC height/FSC width histograms to exclude debris and cell duplets. DC were addressed as $CD11c^+$hamster $IgG1^-DAPI^-$ cells. Flow cytometry was done on a FACSCalibur or a LSRII flow cytometer (BD Biosciences). Flow cytometry histograms are depicted on either a five-log (see Fig. 4, *B* and *D*) or a four-log scale (all other figures) as indicated.

Airway histology

For histology, mice were anesthetized and bled to death from the retro-orbital plexus. The thorax was opened and the lung was perfused with cold PBS via the right heart ventricle. The lung was removed, filled via the trachea with OCT Tissue-Tex (Sakura Finetek) diluted 1/4 in PBS, embedded in OCT, and frozen on dry ice. Cryosections were either stained with Alcian blue and nuclear fast red or blocked with rat or mouse serum and stained with anti-CD205 (DEC-205; NLCD145), anti-CD3, or DAPI. Immunohistological analysis of the lung and LN was conducted as described earlier (20) using a motorized Axiovert M200 microscope (Zeiss).

Adoptive transfer of OVA-specific T cells from TCR-transgenic mice

Spleen and mesenteric LN of OT-II mice were minced through a nylon mesh and lymphocytes were separated by density gradient centrifugation on Lympholyte M (Cedarlane Laboratories). Cells were washed twice, resuspended in PBS, and adjusted according to the number of $V\alpha2^+V\beta5^+$ cells. Where indicated, 10^7cells/ml were incubated in RPMI 1640 supplemented with 10% FCS, 25 mM HEPES (pH 7.4), and 5 μM CFSE (Sigma-Aldrich) for 8 min. The reaction was stopped by adding 4 volumes of cold PBS/3% FCS. A 100-μl cell suspension (1×10^6 $V\alpha2^+V\beta5^+$ cells) was injected i.v. into the tail vein of the recipients.

Immunization and aerosol treatment

For immunization, mice were i.p. injected with 100 μg of OVA grade VI (Sigma-Aldrich) in PBS plus 30 μg of CpG 1668 (TIB MOLBIOL). For the induction of allergic lung inflammation, mice were i.p. injected with 150 μg of OVA grade VI (Sigma-Aldrich) in 200 μl of aluminum hydroxide gel adjuvant (2.0% Alhydrogel; Brenntag Biosector). Mice were treated in an aerosol chamber with OVA aerosol (1% in water) or water alone 24 h after adoptive transfer of lymphocytes. OVA solution or water was vaporized using a PariBoy inhalator. Aerosols were dried by mixing with hot air at a final temperature of 30°C. Treatments were performed for 20 min daily and for up to 7 days. On days 1, 3, 5, and 7, numbers of $V\alpha2^+V\beta5^+CFSE^+$ cells in the peripheral blood were determined by flow cytometry. On days 2, 4, 6, and 9, some mice were sacrificed and draining brLN and non-DLN were analyzed by flow cytometry for the presence of proliferating cells using CFSE dilution analysis, and for the expression of the surface markers CD62L and CD44.

In vitro T cell proliferation

Mice received 50 μg of OVA-Cy5 in 50 μl of PBS i.t. After 1 or 24 h, the brLN was removed and $CD11c^+OVA\text{-}Cy5^+$ or $CD11c^+OVA\text{-}Cy5^-$ DC were isolated by flow sorting. Two thousand DC were seeded in a U-bottom 96-well plate and cocultured with 6×10^4 magnetic bead-purified OT-II $CD4^+$ T cells. After 3 days the cells were pulsed with 1 μCi/well [^{3}H]thymidine and further incubated for 16 h. Thymidine uptake was quantified in a beta counter.

Allergic lung inflammation

To induce tolerance, some wild-type and CCR7-deficient mice received OVA aerosol for 7 days, whereas others were treated with a control aerosol. All animals were then immunized i.p. with OVA/alum as described above followed by an OVA-aerosol challenge for 7 consecutive days, starting 2 weeks later. Twenty-four hours after the last aerosol challenge, mice were sacrificed and a bronchoalveolar lavage (BAL) was performed using 3 × 1 ml of RPMI 1640 supplemented with 0.05 mM EDTA. BAL fluid was analyzed by flow cytometry for the presence of immune cells as described elsewhere (21).

Results

Steady-state migration of airway DC to the draining brLN depends on CCR7

Since we had shown earlier that the steady-state migration of skin DC is impaired in CCR7-deficient mice ($CCR7^{-/-}$) (5), we investigated whether the lack of CCR7 affects the continuous migration of DC from the lung to its draining brLN. We first addressed the transport of particulate Ag by lung-resident DC in vivo by i.t. transfer of fluorescent latex particles (200 nm diameter). Histological analysis of the lung revealed that DC of wild-type C57BL/6 (B6) and $CCR7^{-/-}$ mice were equally effective in engulfing latex particles (Fig. 1*A*). Interestingly, although latex particle-carrying DC could be detected in the T cell area of brLN of B6 mice (Fig. 1*B*), we failed to identify latex-bearing DC in the brLN of CCR7-deficient mice (Fig. 1*B*). Consistently, latex-carrying DC were found by flow cytometry in the brLN of wild-type but not $CCR7^{-/-}$ mice (data not shown). These data are in agreement with a recent study suggesting a role for CCR7 in the migration of latex-carrying lung DC to the brLN by use of the *plt/plt* mutant that lacks CCR7 ligands in lymphoid organs (22).

It is a well-established fact that soluble Ag is rapidly carried via afferent lymph to DLN following intradermal application (23, 24). Therefore, we analyzed whether this mechanism also allows Ag transport from the lung toward its DLN. To this end, Cy5-labeled OVA (OVA-Cy5) was applied i.t., and DC of the brLN were investigated for the presence of Ag within 40 min. We assumed that within this short period of time the majority of OVA-carrying DC present in the brLN would have taken up soluble Ag drained into this LN rather then representing DC that entered from the lung. Indeed, in both wild-type and CCR7-deficient animals, we found that 10–20% of all $CD11c^+$ DC were decorated with OVA-Cy5 within this short period of time (data not shown). Sixteen hours

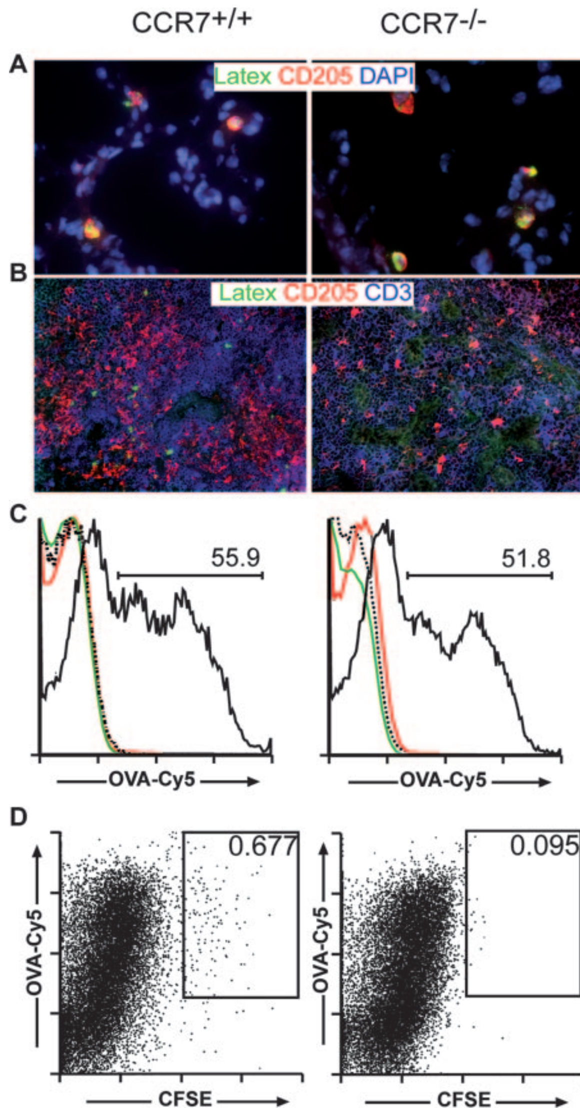

FIGURE 1. Steady-state migration of airway DC to the draining brLN depends on CCR7. *A* and *B,* Twenty hours after i.t. transfer of green fluorescent latex particles (200 nm) wild-type and CCR7$^{-/-}$ mice were sacrificed and cryosections of lungs (*A*) and brLN (*B*) were stained as indicated. Note the absence of latex-carrying DC in the brLN of CCR7-deficient mice. *C,* Sixteen hours after i.t. transfer of Cy5-labeled OVA, brLN of wild-type and CCR7$^{-/-}$ mice were analyzed by flow cytometry. DC were identified by the expression of MHCII and CD11c. Histograms show the amount of OVA-Cy5 taken up by DC (solid black line). DC from brLN of mice that did not receive OVA-Cy5 are shown as dotted black line. MHCII-negative cells of brLN of mice that were i.t. transferred with OVA-Cy5 (red line) or that did not receive any OVA-Cy5 (green line) served as additional controls. Plots are representative of four experiments, analyzing three mice from each group. *D,* Sixteen hours after in vivo staining of lung cells with CFSE and i.t. application of OVA-Cy5, cells of the brLN of wild-type and CCR7$^{-/-}$ mice were isolated and analyzed by flow cytometry. Dot plots show CD11c^{+} cells and numbers indicate percentages of OVA-Cy5^{+}CFSE^{+} cells. Dot plots are representative of three experiments, analyzing three mice from each per group.

after transfer, >50% of all MHCII^{+}CD11c^{+} DC in the brLN carried high amounts of the Ag, whereas MHCII^{-} cells never carried detectable amounts of Ag (Fig. 1*C*). To identify those lung DC that migrate into the brLN, mice were treated i.t. with CFSE before they received OVA-Cy5 via the same route. CFSE treatment stains most of the lung cells including DC (25). Thus, CFSE^{+}OVA-Cy5^{+} DC present in the brLN represent those DC that have migrated from the lung to the DLN. In wild types, ~0.7% of all brLN DC entered this organ via afferent lymphatics from the lung during a 16-h period, whereas CFSE^{+}OVA-Cy5^{+} DC were virtually absent in the brLN of CCR7$^{-/-}$ (Fig. 1*D*). Taken together, these data demonstrate that in CCR7$^{-/-}$ animals, Ag-carrying DC fail to migrate from the lung to the brLN, whereas soluble Ag is drained rapidly to this organ and is taken up by resident brLN DC in both wild-type and CCR7-deficient mice very efficiently.

Intratracheally applied CCR7-deficient DC fail to enter the brLN

To specifically address the role of CCR7 for the migration of APC from the lung to the brLN, we i.t. applied mature, OVA-loaded or fluorescent-labeled BM-derived DC of wild-type or CCR7-deficient mice. After 24 hours, up to 1% of all brLN DC were identified as wild-type DC, whereas it was not possible to find any CCR7$^{-/-}$ DC in this adoptive transfer model (data not shown). We subsequently analyzed the proliferative response of i.v.-transferred CFSE-labeled OT-II CD4^{+} T cells that carry an OVA-specific, MHCII-restricted TCR. We only observed a strong proliferation of the OT-II cells in the brLN (but not in other lymphoid organs) of those recipients that received wild-type OVA-loaded DC (Fig. 2*A*, *upper panel*). In contrast, the application of CCR7$^{-/-}$ DC completely failed to provoke any T cell proliferation in this experimental setup (Fig. 2*A*, *upper panel*). Unloaded DC from wild-type and CCR7$^{-/-}$ mice, which were used as controls, also failed to induce any specific T cell proliferation (Fig. 2*A*, *lower panel*).

To exclude the possibility that CCR7-deficient DC carry an intrinsic defect in Ag presentation, we performed in vitro proliferation assays by applying mature OVA-loaded BM-DC from CCR7-deficient or wild-type donors along with CFSE-labeled OT-II cells. We did not observe any difference between wild type and CCR7$^{-/-}$ DC to induce T cell proliferation in vitro (data not shown). To formally prove that lack of T cell proliferation in the brLN in CCR7-deficient mice is due to impaired migration of DC from the lung into this organ and not due to structural defects of the brLN, we adoptively transferred OVA-loaded mature wild-type DC i.t. into CCR7-deficient recipients. We observed a pronounced proliferation of OT-II cells in the brLN (Fig. 2*B*), demonstrating that the migration of wild-type DC from the lung to the brLN can rescue defective T cell activation in this lymphoid organ in CCR7-deficient mice.

Inhalation of Ag does not induce proliferation of specific T cells in lung DLN in CCR7-deficient mice

The migration of DC from the lung to the brLN seemed to be impaired in CCR7-deficient mice, whereas transport and uptake of soluble Ag by resident brLN DC remained unaffected. Therefore, we used an adoptive transfer model to address the role of passively (soluble) vs actively (DC-carried) transported Ag in T cell activation under noninflammatory, tolerance-inducing conditions. Recipients received dried OVA or control aerosol at daily intervals. Analysis of draining and non-DLN of wild-type mice that had received the OVA aerosol revealed a weak proliferation of OVA-specific OT-II cells in the draining brLN at day 2 and a strong proliferation at days 4 and 6 that was still present at day 9 of the treatment (Fig. 3*A* and data not shown). In contrast, we failed to observe a substantial proliferation of OT-II cells adoptively transferred to CCR7$^{-/-}$ mice following treatment with OVA aerosol at any time point and in any lymphoid organ investigated (Fig. 3*A* and data not shown). In addition, we studied the effect of aerosol treatment on the phenotype of the Ag-specific T cells. Following

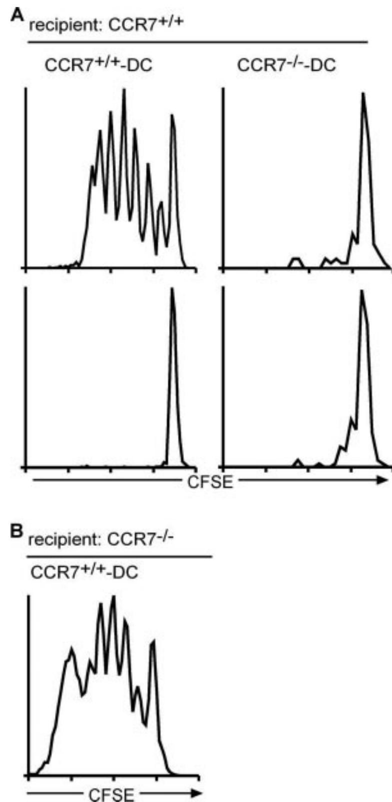

FIGURE 2. Wild-type but not CCR7-deficient, Ag-loaded DC induce T cell proliferation in the brLN following i.t. installation. *A,* BM-derived DC from wild-type or CCR7-deficient mice, either pulsed with OVA-peptide ($OVA_{323-339}$; *upper panel*) or untreated (*lower panel*) were injected i.t. into wild-type mice that had adoptively received CFSE-labeled OT-II cells 24 h earlier. Three days after DC injection, the proliferation of the adoptively transferred T cells in the brLN was analyzed (graphs are representative of three experiments with three mice each for all groups). *B,* TNF-α/PGE-maturated, OVA-loaded BM-derived DC from wild-type mice were injected i.t. into CCR7-deficient mice that adoptively received CFSE-labeled OT-II Ly5.1 cells 24 h earlier. After 3 days the proliferation of the adoptively transferred transgenic $CD4^+$ T cells in the brLN was analyzed (representative data from one of three experiments with three mice each).

four aerosol treatments, OT-II cells isolated from wild-type recipients revealed up-regulation of CD44 and down-regulation of CD62L, indicating that these cells underwent substantial changes once activated under steady-state conditions (Fig. 3*B*). In contrast, OT-II cells kept their naive phenotype when transferred to $CCR7^{-/-}$ recipients (Fig. 3*B*). Because OT-II cells are not impaired in entering LN of CCR7-deficient mice (5), these data suggest that, despite its ubiquitous presence on resident DC, soluble Ag is not adequately presented to allow for T cell activation in $CCR7^{-/-}$ mice.

Our observations also implied that lung-derived DC have to present Ag to allow T cell activation in the brLN. To test this hypothesis, we transferred i.t. nonactivated immature BM-DC from B6 animals into CCR7-deficient mice and subjected the recipients to an OVA-aerosol inhalation 2 h after DC transfer. It is interesting that, under these steady-state conditions, we found a prominent proliferation of OT-II cells in the brLN of CCR7-deficient recipients (Fig. 3*C*), demonstrating that the DC-mediated transport of Ag from the lung to the brLN suffices to induce T cell activation under tolerance-inducing conditions in CCR7-deficient mice.

$CD103^+$ DC are missing in the brLN of CCR7-deficient mice

To characterize the missing DC populations, brLN of wild-type and CCR7-deficient mice were subjected to flow cytometry. We found that in $CCR7^{-/-}$ mice <4% of $CD11c^+$ cells expressed α_E integrin (CD103), whereas this integrin chain was expressed on >30% of all wild-type brLN DC (Fig. 4, *A* and *B*). Furthermore, $CD8^+$ DC accounted for ~7% in wild types, whereas only 4% of all brLN DC showed this phenotype in $CCR7^{-/-}$ animals. Of interest, none of the other populations, such as $CD4^+$, $CD4^-CD8^-$ or $CD4^+CD8^+$ DC, or $Ly6C^+B220^+$ plasmacytoid DC (pDC) were noticeably affected by CCR7 deficiency (Fig. 4*A*). Further analysis of brLN revealed that in both wild-type and CCR7-deficient animals ~30–50% of the pDC expressed CD8, suggesting that the majority of $CD8^+$ cells remaining in the brLN of $CCR7^{-/-}$ mice represent pDC (data not shown).

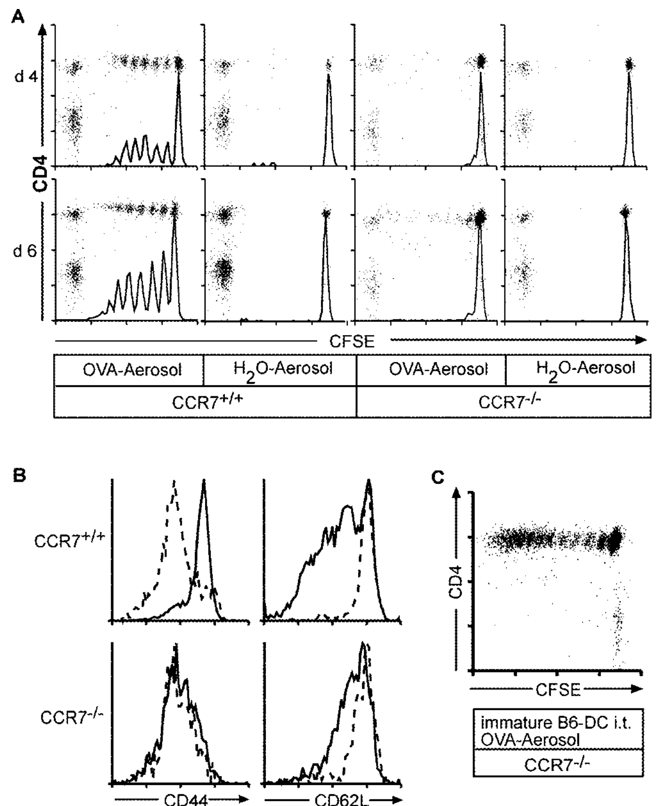

FIGURE 3. CCR7-dependent proliferation of $CD4^+$ T cells and change of phenotype following OVA-aerosol treatment or i.t. DC transfer in the draining brLN. *A,* Wild-type and $CCR7^{-/-}$ mice received CFSE-labeled OT-II cells i.v. 24 h before daily OVA- or control-aerosol treatments. After 4 days (*upper row*) and 6 days (*lower row*) mice were sacrificed and the draining brLN were analyzed for the proliferation of adoptively transferred $CFSE^+$ OVA-specific T cells. $V\alpha2^+$ and $V\beta5^+$ T cells were gated. Superimposed black lines show the CFSE profile of the proliferating cells. Data are representative of six mice from each group. *B,* OT-II Ly5.1 cells adoptively transferred in wild-type and in $CCR7^{-/-}$ recipients were analyzed for the expression of CD44 and CD62L 4 days after starting daily aerosol exposure (solid line, OVA-aerosol treatment; dashed line, water control aerosol) OT-II cells were addressed as $Ly5.1^+V\alpha2^+CD4^+$. Histograms are representative of one of two experiments with three mice from each per group. *C,* Rescue of T cell proliferation. Immature BM-derived DC from wild-type mice were injected i.t. into CCR7-deficient mice that adoptively received CFSE-labeled OT-II Ly5.1 cells 24 h earlier. Mice received OVA aerosols for 4 days before the proliferation of the adoptively transferred transgenic $CD4^+$ T cells was analyzed in the brLN (representative data from one of three experiments with three mice each).

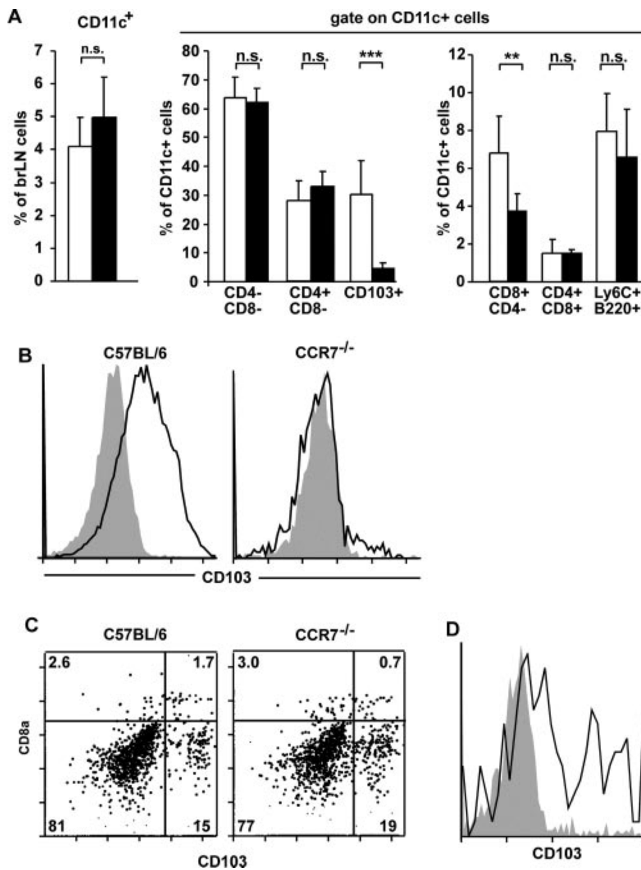

FIGURE 4. Lack of CD103$^+$ DC in the brLN of CCR7$^{-/-}$ (*A* and *B*) and phenotype of lung DC (*C*) and recent lung emigrant DC (*D*). *A,* Percentage of DC in brLN of wild-type (□) or CCR7$^{-/-}$ mice (■) were analyzed. The first panel shows the percentage of CD11c$^+$ cells, all other panels depict the percentage of subpopulations within the CD11c$^+$ cells as indicated. Bars show mean + SD of two experiments analyzing five mice in each group. *B,* Expression of CD103 on CD11c$^+$ brLN from wild-type and CCR7-deficient animals as indicated. Representative of five animals analyzed per genotype (shaded area, isotype control). *C,* Expression of CD8α and CD103 on CD11c$^+$ DC isolated from the lung of wild-type and CCR7-deficent mice. Numbers indicate percentage of cells within each quadrant (mean of three animals each). *D,* Sixteen hours after in vivo staining of lungs with DDAO-SE cells of the brLN of wild-type mice were isolated and analyzed by flow cytometry. Cells were gated on CD11c$^+$DDAO$^+$ and stained for the expression of CD103 (solid line; shaded area, isotype control).

In contrast to the brLN, both CD103$^+$ DC and CD8$^+$ DC are present in the lung of CCR7-deficient mice (Fig. 4*C*). We subsequently characterized the expression of CCR7 on DC in the brLN of wild-type mice and observed that nearly all DC (CD11c$^+$MHCII$^+$) express this chemokine receptor, albeit at varying levels (data not shown). We compared the expression levels of MHCII on CCR7^{neg}, CCR7^{int}, and CCR7^{high} DC and found that the surface level of CCR7 positively correlated with that of MHCII, implying that maturation status correlates with CCR7 expression (data not shown). Because CD103$^+$ DC are missing in the brLN in CCR7$^{-/-}$ mice, we phenotypically characterized DC that entered the brLN from the lung. To this end, the lungs of B6 mice were stained in vivo with the fluorescent dye DDAO. After 16 h the expression of CD103 was assessed on CD11c$^+$DDAO$^+$ cells. It is interesting that 30–50% of the recent lung-derived DC expressed high levels of CD103, suggesting that CD103$^+$ DC residing in the brLN of wild-type mice are lung derived (Fig. 4*D*).

LN DC fail to induce T cell proliferation under tolerance-inducing conditions

Because we knew that only few DC migrate within 24 h from the lung to the brLN under steady-state conditions (Fig. 1*D*), we considered the percentage of DC that arrive in the lung within 30–60 min as negligible when compared with the high number of resident DC taking up soluble OVA-Cy5 locally in this LN (Fig. 1*C*). To directly address whether local uptake of Ag by resident brLN DC allows T cell activation, we sorted OVA-Cy5$^+$ DC as well as OVA-Cy5$^-$ DC from the brLN at 1 and 24 h after i.t. application of the Ag. OVA-Cy5$^+$ DC isolated 1 h after i.t. transfer of Ag exclusively consist of cells that have taken up Ag locally in the LN, whereas cells isolated 24 h after Ag transfer will additionally include DC that have taken up Ag in the lung and subsequently migrated into the brLN. The Ag-presenting capacity of these sorted DC was assessed in in vitro cocultures with Ag-specific OT-II cells. After 3 days, T cell proliferation was tested by [^{3}H]thymidine pulsing. Data shown in Fig. 5 demonstrate that brLN-resident DC completely failed to activate T cells despite carrying high amounts of Ag. In contrast, OVA$^+$ DC isolated 24 h after Ag administration, induced a strong proliferative T cell response. These data strongly support our hypothesis that Ag has to be taken up by lung-resident DC and subsequently carried to the DLN to be presented under tolerance-inducing conditions.

T cells expanded by inhaled Ag do not acquire effector function

Although progressing defensive immune responses are distinguished by a dramatic rise in the frequency of armed Ag-specific effector cells that are able to produce cytokines or to directly lyse target cells, exposure to the same Ag under steady-state conditions results in an expanded pool of Ag-specific T cells lacking effector function (4).

To address the role of CCR7-expressing DC in the induction of tolerance toward inhaled Ag in more detail, we adoptively transferred OT-II Ly5.1 cells to wild type mice. Recipients were then

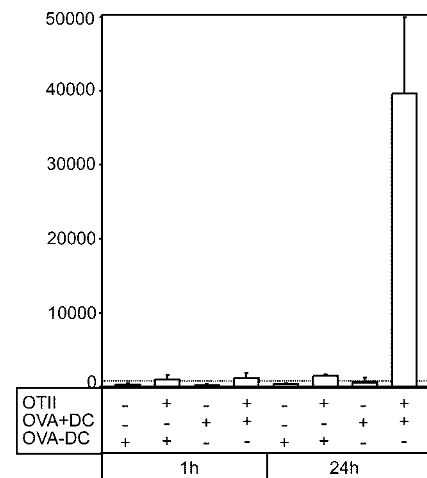

FIGURE 5. Lack of T cell activation by brLN-resident DC under tolerance-inducing conditions. Cy5-labeled OVA was administered i.t. to C57BL/6 mice. Cell suspensions were prepared from collagenase D-digested brLN 1 or 24 h after OVA-Cy5 application. CD3$^-$CD19$^-$CD11c$^+$OVA-Cy5$^+$ and CD3$^-$CD19$^-$CD11c$^+$OVA-Cy5$^-$ subsets were purified by flow sorting. Two thousand DC were cocultured with 6 × 10^4 OT-II cells. After 3 days the cells were pulsed with 1 μCi/well [^{3}H]thymidine and further incubated for 16 h. Thymidine uptake was quantitated in a beta counter. The columns depict the mean + SD from two independent experiments; dotted line, proliferation of OT-II cells in the absence of DC.

treated daily for seven consecutive days with an OVA or a control aerosol. Following this treatment, OT-II cells expanded in the brLN but not in the spleen, whereas i.p. application of OVA plus CpG expanded these T cells in both compartments as expected (Fig. 6*A*). To demonstrate that CD4 cells that are expanded by the inhalation of harmless Ag fail to differentiate into effector T cells, we compared their IFN-γ-expression levels to those that were generated following i.p. immunization with OVA plus CpG. Although both protocols generated approximately equal numbers of OT-II cells in the brLN (Fig. 6*A*), expansion of IFN-γ-producing T cells was only induced once OVA plus adjuvant was applied (Fig. 6*B*).

Lack of tolerance induction in a model of allergic lung inflammation in CCR7$^{-/-}$ mice

Because our data indicated that CCR7-deficient mice might be defective in inducing tolerance to inhaled Ags, wild-type and mutant mice were subjected to a model of allergic lung inflammation. First, wild-type and CCR7$^{-/-}$ mice were sensitized i.p. with OVA/alum. Two weeks later, mice received a daily OVA-aerosol challenge for 7 additional days. Twenty-four hours after the last aerosol challenge, the numbers of eosinophils, neutrophils, and lymphocytes present in the BAL were determined (Fig. 7*A*). Because of this treatment, CCR7$^{-/-}$ mice developed an eosinophilic pneumonia similar to their wild-type counterparts, showing characteristic histological alterations (Fig. 7, *B–E*). We then assessed whether this CD4$^+$ T cell-dominated reaction could be prevented by subjecting the animals to seven daily OVA-aerosol treatments before the i.p. sensitization with OVA/alum and the subsequent challenge. In contrast to the reduction of leukocyte infiltration in wild-type mice, the infiltration in CCR7$^{-/-}$ mice was increased rather than decreased following the preceding tolerance-inducing OVA-aerosol treatment (Fig. 7*A*). Consistently, histological analysis of the lungs showed that the primary OVA-aerosol treatment prevents inflammation in wild-type mice but not in CCR7$^{-/-}$ mice (Fig. 7, *F* and *G*). These data further support our hypothesis that CCR7$^+$ DC are essential for the induction of tolerance toward harmless inhaled Ags. Because the migration of T cells is also affected by CCR7 deficiency, we cannot completely rule out the possibility that this defect also contributes to a lack of tolerance in CCR7-deficient animals observed in this asthma model.

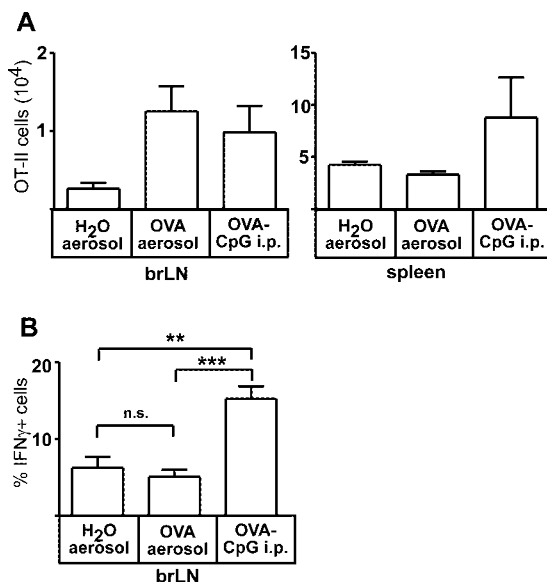

FIGURE 6. CD4$^+$ T cells expanded under tolerance-inducing conditions do not gain effector functions. *A*, OT-II Ly5.1 cells were adoptively transferred in wild-type mice. After 4 days of OVA- or water-aerosol treatment or i.p. immunization with OVA/CpG, OT-II T cells were analyzed in the brLN and spleen. OT-II cells were addressed as Vα2$^+$Vβ5$^+$Ly5.1$^+$CD4$^+$. *B*, IFN-γ was determined by intracellular cytokine staining of OVA-specific CD4$^+$ T cells in the brLN from the OVA/CpG-immunized mice (shown in *A*) compared with unchallenged or OVA aerosol-treated mice. Depicted is the percentage of IFN-γ-producing cells of the Vα2$^+$Ly5.1$^+$CD4$^+$ cells. Experiments shown in *A* and *B* are representative of three independent experiments, each containing three mice per group.

Discussion

Numerous studies have shown that the disposition to develop asthma is continuously increasing among western populations. This chronic airway disease is difficult to treat and knowledge that would help to understand the development of this syndrome is limited. In the present study, we identified several basic mechanisms involved in the induction of tolerance. We provided strong evidence that 1) the steady-state migration of DC from the lung to the draining brLN is CCR7 dependent; 2) the tolerance-inducing activation of T cells by inhaled Ag essentially relies on this steady-state migration; 3) inhaled Ag is very efficiently drained to the brLN by afferent lymphatics and is amply taken up by resident DC; 4) however, in the absence of CCR7, this Ag cannot be efficiently presented by brLN-resident DC to tolerize T cells; and 5) CD103$^+$ DC migrate from the lung to the brLN under steady-state conditions and CD103$^+$ DC are virtually absent in the brLN of CCR7$^{-/-}$ mice. Together, these data strongly suggest that, because of impaired DC migration, CCR7-deficient animals, but not wild-type mice, fail to induce tolerance to inhaled innocuous environmental Ag and, thus, develop allergic lung inflammation. Yet, we cannot completely rule out the possibility that other defects of CCR7-deficient animals, such as impaired T cell recirculation, might contribute to the deficiency in tolerance induction in these animals.

DC are very efficient in presenting Ag, as even a single peptide-MHC complex on their surface suffices to activate naive $\alpha\beta$ T cells (26, 27). Furthermore, secondary lymphoid organs provide the structure to allow an efficient spatial and temporal interaction of Ag-carrying DC and naive T cells specific for a given Ag that are present at the rather low frequency of ~1 in 100,000 (28, 29). This results in the simultaneous presence of ~10 Ag-specific T cells in the brLN in mice at any given time. Furthermore, from our adoptive i.t. transfer of OVA-loaded DC, we experienced that <100 DC had to reach the brLN to efficiently activate T cells (G. Hintzen, L. Ohl, and R. Förster, unpublished observations). Thus, the finding that T cell activation is impaired under noninflammatory conditions in the brLN of CCR7-deficient mice was rather unexpected. Under the experimental conditions described in Fig. 1*C*, ~30,000 DC carried along high amounts of OVA, yet OT-II cells present at numbers between 1,000 and 2,000 in the brLN could be efficiently activated. Based on our observation that resident DC are present in the T cell area in the LN of CCR7$^{-/-}$ mice (G. Hintzen, L. Ohl, and R. Förster, unpublished observations) in combination with the fact that OT-II cells could be activated in CCR7$^{-/-}$ brLN once wild-type DC brought in Ag from the lung, we conclude that only a very small subpopulation of APC, most likely those that transport Ag from the lung under steady-state conditions, are able to induce tolerance. Thus, the observed opulent uptake of inhaled Ag by LN-resident DC seems to be of little relevance for tolerance induction. This hypothesis is supported by our findings and others demonstrating that the DC-mediated transport food Ag or gut commensals to the mesenteric LN is essential for inducing immunological tolerance (30, 31). In a pioneering work, Jenkins et al. (32) found that, following s.c. injection, soluble Ag is rapidly transported to skin-draining LN and taken up by LN-resident DC. It is

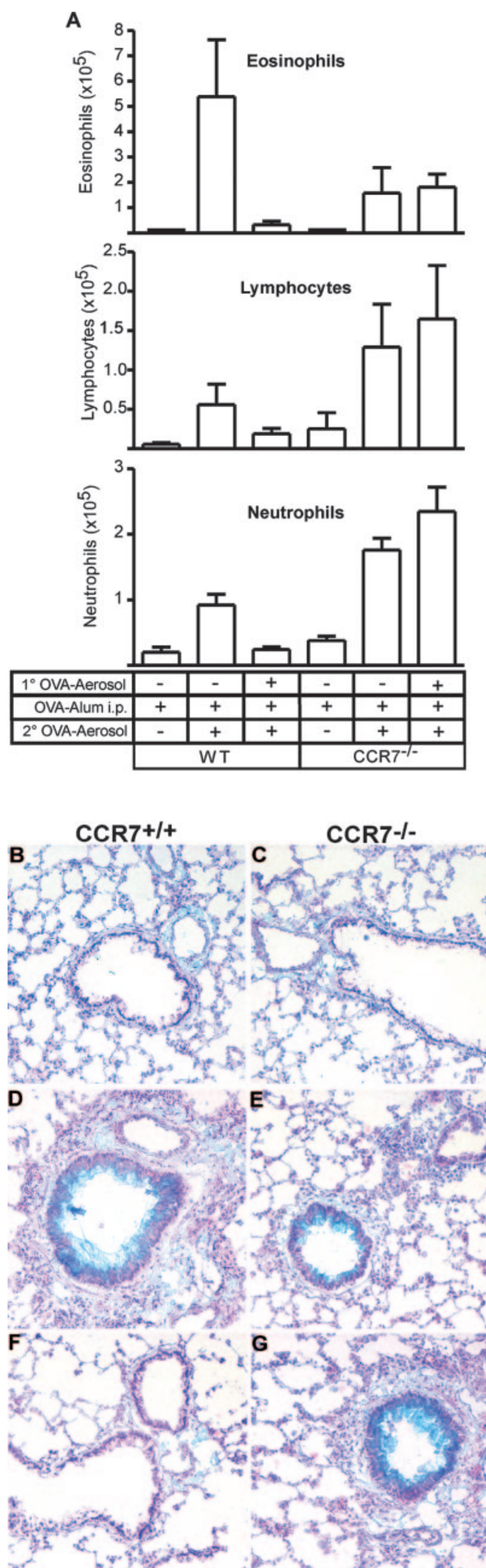

FIGURE 7. Lack of tolerance induction in a model of allergic lung inflammation in CCR7$^{-/-}$ mice. Wild-type and CCR7$^{-/-}$ mice received daily OVA-aerosol treatments for 7 days or remained untreated (1° aerosol). All animals were then immunized i.p. with OVA/alum. Fourteen days later, some animals received another OVA-aerosol treatment for an additional 7 consecutive days (2° OVA aerosol). *A,* The number of eosinophils, lymphocytes, and neutrophils present in the BAL that was taken 24 h after the last aerosol challenge was determined ($n = 8$ for each group from two independent experiments). *B–G,* Lung sections from the mice were stained with Alcian blue and nuclear fast red to show lung inflammation and mucus production. *B*, *D*, and *F,* CCR7$^{+/+}$ mice; *C*, *E*, and, CCR7$^{-/-}$ mice. *B* and *C,* The groups that received control aerosol before and after immunization. *D* and *E,* The mice that received the OVA-aerosol challenge only after immunization. *F* and *G,* Mice that were treated with OVA aerosol before and after the immunization with OVA/alum.

interesting that the ability to take up soluble Ag seems to occur with similar efficacy in skin- and lung DLN (32). Those authors, however, reported that this first wave of Ag suffices to induce T cell proliferation in skin DLN, whereas the induction of delayed-type hypersensitivity requires DC that transport Ag from the skin to the DLN. Along that line, it has been shown that soluble Ag can be targeted to LN-resident DC using hen egg lysozyme coupled to anti-DEC-205 mAb (3, 4). Data presented in this study reveal a different function for brLN DC. Although these DC are very efficient in taking up soluble Ag, they completely fail to efficiently present it to T cells (Fig. 5). However, it should be mentioned that this holds true only for steady-state conditions. Once OVA plus LPS were applied i.t., we observed a profound proliferation of OT-II cells, not only in wild-type, but also in CCR7-deficient recipients (data not shown).

Approximately 85% of the DC population in the lung shows a rapid turnover, being renewed every 36–48 h. This includes the permanent migration of a small population of DC to the DLN presenting inhaled Ags to T cells in the absence of inflammatory stimuli (9). DC migration under such steady-state conditions has been described to induce T cell tolerance (reviewed in Ref. 33). Several subpopulations of DC including immature or semimature DC (reviewed in Ref. 6) or IL-10-producing DC (34) have been suggested to induce tolerance to self or innocuous Ags. Our data confirm that DC migrating under steady-state conditions do not show a unique phenotype regarding the expression of surface molecules. Although steady-state skin-derived DC are CD11c^{high}MHCII^{high} DC (5), a corresponding population showing this phenotype is absent in the brLN of wild-type mice (G. Hintzen and R. Förster, unpublished observation). Furthermore, this study shows that 30–50% of recent lung-derived DC residing in the brLN express CD103. Although CD103$^+$ DC were present to a similar degree in the lungs of wild-type and mutant mice, they were lacking to a large extent in the brLN of CCR7$^{-/-}$ mice. Since CD103$^+$ DC migrate under steady-state conditions from the lung to the brLN in wild-type but not CCR7 mutants, we suggest that impaired steady-state migration of these cells accounts for the observed lack of the corresponding cells in the pool of resident DC in the brLN. It is interesting that intestinal CD103$^+$ DC were recently identified to play an essential role for T cell-mediated regulation of colitis and in generating gut-tropic CD8$^+$ effector cells (35, 36). It is currently unclear whether CD103$^+$ lung-derived brLN DC play a similar role for generating lung-tropic T cells. Data from this work however would suggest that they profoundly contribute to the generation of tolerance toward inhaled environmental Ags.

It is currently unclear where brLN-resident DC in CCR7$^{-/-}$ are derived from and how they entered this organ. In none of the experiments performed did we observe CCR7-independent migration of DC from the lung to the brLN. This is shown by 1) lack of latex-positive DC in the brLN of CCR7$^{-/-}$ mice (Fig. 1*B*); 2) lack of T cell proliferation following adoptive transfer of Ag-pulsed BM-DC from CCR7$^{-/-}$ mice (Fig. 2); 3) lack of CFSE-positive DC in the brLN of CCR7-deficient mice following i.t. application of CFSE (Fig. 1*D*); and 4) lack of proliferation of adoptively transferred T cells in CCR7-deficient recipients following aerosol exposure

(Fig. 3*A*). Based on these observations, we favor the hypothesis that DC present in the brLN of CCR7-deficient animals are blood borne rather than lung derived. Because it is widely accepted that mature DC are extremely inefficient in entering the LN via high endothelial venules (37), we speculate whether some type of DC progenitor cells enter the LN via the blood and then differentiate further. Likewise, it seems possible that some blood-borne DC are missing in the brLN of CCR7-deficient mice. In addition, there is currently little evidence that LN $CD8^+$ DC, although actually present in the lung at low frequency (Fig. 4*C*), contribute much to the pool of migrating DC (25). Experiments performed in this study confirm that only a limited proportion of recent lung-derived DC actually express CD8 (data not shown).

Although CCR7-deficient mice fail to induce tolerance toward inhaled Ags, these animals develop a T cell-dependent eosinophilic pneumonia (Fig. 7). We reported earlier that CCR7 deficiency interferes with the rapid induction of T-dependent immune responses, such as delayed-type hypersensitivity reactions, following a 4-day priming period and that T-dependent B cell responses are strongly impaired in CCR7-deficient mice at day 10 after immunization (15). However, in the latter case, CCR7 deficiency has been compensated to a large extent by day 20, suggesting that expression of CCR7 is only required for the rapid and efficient onset of T-dependent responses (15). Data from eosinophilic pneumonia obtained in the present study support this hypothesis, because a priming period of 14 days allows for the development of the disease in CCR7-deficient mice, although slightly less pronounced than in wild types.

In summary, data presented here provide strong evidence that the CCR7-dependent continuous migration of DC from the lung to the DLN is required for the transport of inhaled Ag and thereby for the proper composition of APCs in the LN. These processes are essential to induce peripheral tolerance of T cells that otherwise would have the capacity to react to harmless airborne Ags by triggering a potentially damaging inflammatory immune response. The hypothesis that maturating DC up-regulate CCR7 to facilitate migration to the DLN subsequent to an inflammatory stimulus (15, 38, 39) is widely accepted. Now there is also strong evidence that DC have to express this receptor to carry innocuous Ag from the periphery into DLN, which is critical for the induction of tolerance.

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Disclosures

The authors have no financial conflict of interest.

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