

Controls for Lung Dendritic Cell Maturation and Migration during Respiratory Viral Infection¹

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Dendritic cells are ideally suited to orchestrate the innate and adaptive immune responses to infection, but we know little about how these cells respond to infection with common respiratory viruses. Paramyxoviral infections are the most frequent cause of serious respiratory illness in childhood and are associated with an increased risk of asthma. We therefore used a high-fidelity mouse model of paramyxoviral respiratory infection triggered by Sendai virus to examine the response of conventional and plasmacytoid dendritic cells (cDCs and pDCs, respectively) in the lung. We found that pDCs are scarce at baseline but become the predominant population of lung dendritic cells during infection. This recruitment allows for a source of IFN- α locally at the site of infection. In contrast, cDCs rapidly differentiate into myeloid cDCs and begin to migrate from the lung to draining lymph nodes within 2 h after viral inoculation. These events cause the number of lung cDCs to decrease rapidly and remain decreased at the site of viral infection. Maturation and migration of lung cDCs depends on Ccl5 and Ccr5 signals because these events are significantly impaired in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice. cDCs failure to migrate to draining lymph nodes in *Ccl5*^{-/-} or *Ccr5*^{-/-} mice is associated with impaired up-regulation of CCR7 that would normally direct this process. Our results indicate that pDCs and cDCs respond distinctly to respiratory paramyxoviral infection with patterns of movement that should serve to coordinate the innate and adaptive immune responses, respectively. *The Journal of Immunology*, 2007, 179: 1438–1448.

Respiratory viruses are perhaps the most frequent cause of human illness, but immune therapy for these types of viral illnesses is extremely limited. This limitation is due at least in part to our incomplete understanding of the immune response to respiratory viruses. Previous studies of common respiratory viruses such as respiratory syncytial virus (RSV)³ and related paramyxoviruses indicate that the initial immune response is relatively nonspecific and is conducted by airway epithelial cells and the neighboring cells of the innate immune system (1, 2). However, this innate immune response is not likely to fully clear the infection and is unable to provide memory against subsequent infection. Thus, this response leads to an adaptive immune response that is specific for the particular viral pathogen (3). The development of the adaptive immune response depends on conventional lung dendritic cells (DCs) that sample viral Ag, mature into efficient APCs, and migrate to the draining lymph nodes to instruct rare virus-specific T and B cells to proliferate (4, 5). Ultimately, virus-specific CD8 T cells are recruited to the lung to kill

infected host cells (6–8). The cytopathic remnants of the innate and adaptive responses are finally cleared by macrophages that are resistant to virus-induced cell death (2).

Despite the critical position of DC action in the adaptive immune response, we know little about the control of DC function during respiratory viral infection. Because human DCs are difficult to obtain at the various stages of viral infection, a mouse model of respiratory viral infection provides an opportunity to define this process. Mouse conventional DCs (cDCs) can be readily identified by the expression of the β_2 integrin CD11c and can be divided into subtypes based on expression of cell surface markers (9, 10). Myeloid DCs are the reported predominant type of cDCs in the mouse lung and can be identified by expression of β_2 integrin CD11b (11). The other major DC subset is composed of plasmacytoid DCs (pDCs). These cells express B220 and a low level of CD11c and can be identified by expression of mPDCA-1 (12, 13). In several types of viral infection, pDCs are a major source of IFN- α at the site of viral replication; therefore, they could be a critical part of the innate immune response to viral infection (14–16).

Both types of DCs develop in the bone marrow and migrate to the peripheral tissues (including lung tissue) in an immature form (10). At this stage, DCs express low levels of MHC class II (MHC-II) and B7 family costimulatory molecules (such as CD80 and CD86) and are set to efficiently gather Ag using pattern recognition receptors as well as pinocytosis and phagocytosis (11). Once cDCs encounter Ag in the presence of inflammatory stimuli, they mature in a process that includes down-regulation of pattern recognition receptors, a change in homing receptors, and up-regulation of MHC-II, CD80, and CD86. Maturation also includes expression of CCR7 that allows DCs to follow a CCL21 signal to draining lymph nodes (17, 18). Less is known about the chemokine signals that direct immature DCs into the peripheral tissue. A CCL5 antagonist decreased the baseline level of DCs found in rat trachea, but the corresponding chemokine receptor was not defined (19). At least in mice, cDCs express each of the CCL5 receptors (CCR1, CCR3,

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³ Abbreviations used in this paper: RSV, respiratory syncytial virus; DC, dendritic cell; cDC, conventional DC; pDC, plasmacytoid DC; MHC-II, MHC class II; SeV, Sendai virus; i.n., intranasal(ly); PI, postinoculation.

CCR5); therefore, any of these receptors could be responsible for CCL5-dependent traffic of cDCs to the airway (20).

We initiated the present experiments to determine how DCs are called to action in response to respiratory viral infection. We used a mouse model to take advantage of reagents that could better monitor the development of the DC response. Thus, two previous studies used a rat model, but the analysis was limited by the need to identify DCs only by expression of MHC-II (19, 21). RSV would have been a useful choice for study because it is the most common cause of serious respiratory illness in early childhood and is most often associated with the development of childhood asthma. Indeed, several previous studies examined the DC response to RSV in mice (15, 16, 22, 23). Unfortunately, mice are relatively resistant to infection with RSV; therefore, a high threshold inoculum of virus must be given and the resulting all-or-none pattern of illness manifests primarily as alveolitis with viral localization to type I alveolar epithelial cells (Ref. 24 and E. Agapov and M. J. Holtzman, unpublished observations). We therefore use Sendai virus (SeV), which is a mouse parainfluenza virus that is similar to other paramyxoviruses (including RSV) that more commonly infect humans. We have demonstrated that SeV infection in mice causes acute viral inflammation of the small airways that is similar to the comparable condition of viral bronchiolitis in humans (1, 2, 25). In contrast to the previous analysis of RSV infection in mice, we find that SeV infection causes rapid and sustained depletion of cDCs in the mouse lung (23). We further show that the decrease in cDCs is based on migration to draining lymph nodes, and that this process as well as cDC homeostasis at baseline and maturation during infection is mediated at least in part by Ccl5-Ccr5 signaling pathways. The results provide a more complete paradigm for the DC response to respiratory viral infection.

Materials and Methods

Mice

C57BL/6 and CCR5-deficient (*Ccr5*^{-/-}) mice were obtained from The Jackson Laboratory. Mice deficient in CCL5 (*Ccl5*^{-/-}) were developed as described previously (2, 26). Mice 6–12 wk of age were used and were housed for at least 1 wk before use in specific pathogen-free conditions with food and water provided ad libitum. The Washington University Institutional Committee for the humane use of laboratory animals approved all experiments.

Reagents and Abs

Anti-mouse CD16/CD32 Ab (clone 2.4G2; gift from Dr. C. Pham, Washington University, St. Louis, MO) was used for flow cytometry experiments as previously described (27). FITC-, PE-, or allophycocyanin-labeled Abs against murine CD4, CD8, CD11b, CD11c, CD86, B220, MHC-II, Mac-3, CCR7, and isotype control IgGs (rat and Armenian hamster) were obtained from eBioscience and/or BD Pharmingen. PE-anti-murine CD80 was obtained from Serotec. PE- or allophycocyanin-labeled anti-mPDCA-1 was obtained from Miltenyi Biotec. Specific SeV⁺ T cells were detected using tetrameric MHC/peptide reagents for the immunodominant epitope of SeV nucleoprotein (NP_{324–332}) or control OVA peptide (SIINFEKL) complexed with K^b provided by the National Institute of Allergy and Infectious Diseases Tetramer Core Facility (3). One hundred fifty kilodalton FITC-dextran and LPS was obtained from Sigma-Aldrich. CFSE was obtained from Molecular Probes. Blocking goat anti-mouse CCR7 Ab was obtained from Caprologics; control-purified goat IgG was purchased from Jackson ImmunoResearch Laboratories.

Viral inoculation and FITC-dextran or CFSE labeling

Mice were anesthetized with s.c. ketamine/xylazine and 30 μ l of 1 mg/ml FITC-dextran, 5 mM CFSE, PBS, 2×10^5 PFU of SeV (Fushimi strain), or UV-inactivated SeV (SeV-UV) was inoculated intranasally (i.n.). At various time points after inoculation, mice were weighed to verify productive infection. In some experiments, 8 μ g of anti-CCR7 or control IgG was given i.n. with FITC-dextran or CFSE followed 2 h later by SeV, SeV-UV, or 50 μ g of LPS i.n. For repeat SeV-UV administration, SeV-UV was administered i.n. (in 30 μ l of PBS) every 12 h for 3 days. On the indicated

days, mice were sacrificed and lung and lymph nodes were examined for FITC- or CFSE-expressing DCs by flow cytometry.

DC isolation

At various time points after infection, mice were anesthetized and humanely sacrificed by cervical dislocation. An intracardiac injection of sterile PBS was performed to flush out the pulmonary and systemic circulation. Bronchoalveolar lavage was performed to remove any cells in the bronchiolar or alveolar space. Then 1 ml of digest medium was injected intratracheally. The lungs were then carefully dissected from the trachea, main stem bronchi, draining lymph nodes, and surrounding tissue. Once the lungs had been removed from the mouse, they were minced and incubated in digest medium at 37°C for 60 min, during which time the cells were not manipulated. Digest medium consisted of complete DMEM supplemented with 5% FCS, penicillin/streptomycin, 10 mM HEPES, 250 U/ml collagenase I, 50 U/ml DNase I, and 0.01% hyaluronidase (Sigma-Aldrich, Invitrogen Life Technologies, and Worthington Biochemical). During the last 15 min of incubation time, EDTA was added to the medium to a final concentration of 2 mM (Sigma-Aldrich). After digestion, single-cell suspensions were made by passing the cell mixture through a 40- μ m pore cell strainer. Hypotonic lysis was then used to remove erythrocytes. Viable cells were then counted by trypan blue exclusion (Sigma-Aldrich). An analogous procedure was performed for removal of DCs from spleen or lymph node.

Flow cytometry

Samples were analyzed on a FACSCalibur cytometer (BD Biosciences) using linear amplification of the forward/side scatter light signals and logarithmic amplification of the fluorescent light signals with compensation to remove spectral overlap in the fluorescent channels. Data were analyzed using FlowJo software (Tree Star).

Statistical analyses

Student's *t* test was used to assess statistical significance between means. Significance was set at *p* < 0.05. Unless otherwise stated, all data are presented as mean $\pm$ SEM.

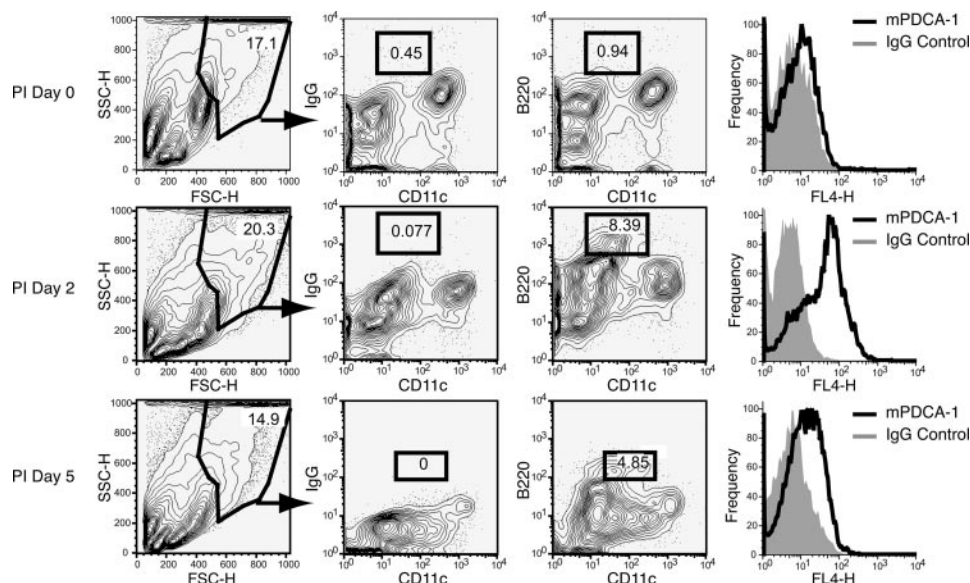
Results

Paramyxoviral respiratory infection alters lung DC levels

To determine the effect of paramyxoviral respiratory infection on lung DC populations, we infected wild-type C57BL/6J mice with SeV and then individually monitored pDC and cDC levels in the lung tissue. We identified pDCs by flow cytometry based on forward and side scatter characteristics, the pattern of high B220 and low CD11c expression, and the presence of mPDCA-1 Ag (Fig. 1). Identification of cDCs depends on high-level expression of CD11c, but this approach to lung cDCs is complicated by alveolar macrophage expression of CD11c. Therefore, we performed bronchoalveolar lavage to remove most of the alveolar macrophages before lung cell isolation. When we used this approach and checked for lung cell expression of Mac-3, a marker of alveolar and lung tissue macrophages (2, 28), we found very few Mac-3⁺CD11c⁺ cells (Fig. 2a). These Mac-3⁺CD11c⁺ cells (which represent alveolar macrophages) exhibited distinct scatter characteristics from Mac-3⁺CD11c⁻ cells (which represent tissue macrophages). In addition, lung cDCs exhibited greater forward scatter than alveolar macrophages, and this scatter profile was set as the cDC gate. Under these conditions, <5% of the cells within the cDC gate were Mac-3⁺CD11c⁺ alveolar macrophages (Fig. 2, a and b). Using this cDC scatter gate, the majority of CD11c⁺ cells did not express either Mac-3 or CD11b (Fig. 2b). Therefore, the CD11c⁺ cells located with this cDC gate were lung cDCs and this approach could be used to assess cDC maturation after SeV infection (Fig. 2c) as developed in the next section.

In the case of pDCs, we found that cell levels were barely detectable at baseline but rapidly accumulated after infection. Indeed, pDCs became the major DC population in the lung by postinoculation (PI) day 2 (Fig. 3a). pDC influx required viral replication, because mice inoculated with inactivated SeV (SeV-UV) showed

FIGURE 1. Gating strategy to identify lung pDCs. Lung pDCs can be identified by scatter characteristics, high expression of B220, and low expression of CD11c. Lung single-cell digests from SeV PI day 0, 2, or 5 were evaluated for expression of B220, CD11c, and the pDC marker mPDCA-1. As can be seen in these representative flow cytometry data, pDCs can be identified from the appropriate scatter gate by either B220⁺CD11c^{low} (quadrant plots) expression or singly mPDCA-1 expression (histograms). Numbers refer to the percentage of cells within a given gate.



no change in pDC levels. In contrast to the behavior of lung pDCs, there were large numbers of cDCs in the mouse lung at baseline ($\sim 2 \times 10^5$ cells per set of mouse lungs). Furthermore, after inoculation with SeV, there was a marked decrease in the number of cDCs in the lung (Fig. 3*b*). The initial decrease in lung cDCs does not require viral replication because SeV-UV inoculation causes a similar decrease in the numbers of lung cDCs. However, SeV infection was associated with continued suppression of the numbers of lung cDCs for at least 7 days, whereas SeV-UV inoculation was followed by a relatively rapid return of lung cDC numbers to baseline that was complete within 3 days.

The virus-induced decrease in lung cDC levels was associated with rapid maturation and differentiation of lung cDCs. At baseline, lung cDCs expressed MHC-II on only a proportion of cells and there was no detectable CD11b expression (Fig. 3*c*). This finding, although similar to what has been reported by at least one other group, contrasts with reports suggesting that lung cDCs were of the myeloid (CD11b⁺) phenotype at baseline (22, 29, 30). With viral inoculation, we found expression of CD11b as well as the costimulatory molecule CD86 and increased expression of MHC-II (Fig. 3*c*). Other markers of cDC differentiation, such as CD4 and CD8, and the costimulatory marker CD80 were not expressed at baseline or during the viral infection (Fig. 3*c*). As expected, B220, a marker for pDCs on CD11c^{low} cells, was not expressed at any time point on CD11c⁺ cDCs. CD86 and MHC-II expression are hallmarks of mature cDCs, normally found in the draining lymph node. Maturation of lung cDCs depended on active infection, because inoculation with SeV-UV failed to cause any changes on lung cDC levels of CD11b, MHC-II, or CD86 (Fig. 3*d*).

We next checked whether the virus-induced changes in the numbers and maturation of lung cDCs was not simply a result of repeated Ag exposure. Thus, we inoculated mice with SeV-UV every 12 h for 3 days, and then checked the level of lung cDCs as well as expression of CD11b, MHC-II, and CD86 on the cell surface. We found no significant difference in either the numbers of lung cDCs or surface expression of these markers in mice given repeated administration of SeV-UV (Fig. 3, *e* and *f*). Taken together, it appears that a productive viral infection causes distinct effects on lung pDC vs cDC populations. In particular, lung cDCs decreased markedly in numbers while undergoing nearly complete maturation into a myeloid phenotype during the period of viral

replication. The period of lung cDC disappearance and maturation corresponded to the period of active viral infection (PI days 1–7), based on the kinetics of viral replication and clearance in this mouse model (25, 31).

Migration of cDCs to draining lymph nodes

To better understand the basis for the decrease in lung cDC levels after viral infection, we next used FITC-dextran to track the location of lung cDCs. FITC-dextran is a high molecular mass carbohydrate that is efficiently ingested by phagocytic cells, including cDCs (29). Thus, $\sim 80\%$ of lung cDCs were FITC⁺ by 2 h after FITC-dextran administration to the lung. The subsequent decrease in lung cDC numbers after SeV inoculation was apparent in both the FITC⁺ and FITC[−] cDC populations (Fig. 4*a*). However, we found a continued decrease in the FITC⁺ population, whereas the FITC[−] cDC population started to increase by PI day 3 and became the predominant lung cDC population by PI day 5. By contrast, mice inoculated with PBS vehicle exhibited no significant change in the FITC⁺ or FITC[−] cDC populations over this entire time period (Fig. 4*b*). We used PBS rather than SeV-UV as a baseline control for steady-state cDC movement because the addition of SeV-UV caused cDC migration out of the lung (Fig. 3*b*). Based on these data, the half-life of a FITC⁺ cDC in a control lung was 8.4 ± 2.4 days, whereas the half-life after SeV infection was decreased to 2.5 ± 0.1 days. These values are similar to what has been reported for other rodent species (19).

In general, cDCs rapidly leave peripheral tissues to migrate to the draining lymph nodes in the setting of infection. In the case of influenza, this migration occurs only within the first 12–24 h after viral inoculation (32). Using FITC-dextran, we were able to identify those cDCs that had migrated out of the lung to the draining lymph node during viral infection (Fig. 4*c*). We found a rapid increase in the number of FITC⁺ cDCs in the draining lymph nodes at 6–12 h after inoculation (Fig. 4*d*). The number of FITC⁺ cDCs remained at this higher level at least until 5 days after inoculation, suggesting that there may be equilibrium between any cell death that may occur and migration of new FITC⁺ cells into the lymph node. We also observed an increase in FITC[−] cDCs in the draining lymph node by PI day 2. This increase may be due to the arrival of newly recruited cDCs that were not present in the lung at the time of FITC-dextran labeling. With SeV infection, there was a dramatic increase in the percentage of cDCs expressing FITC

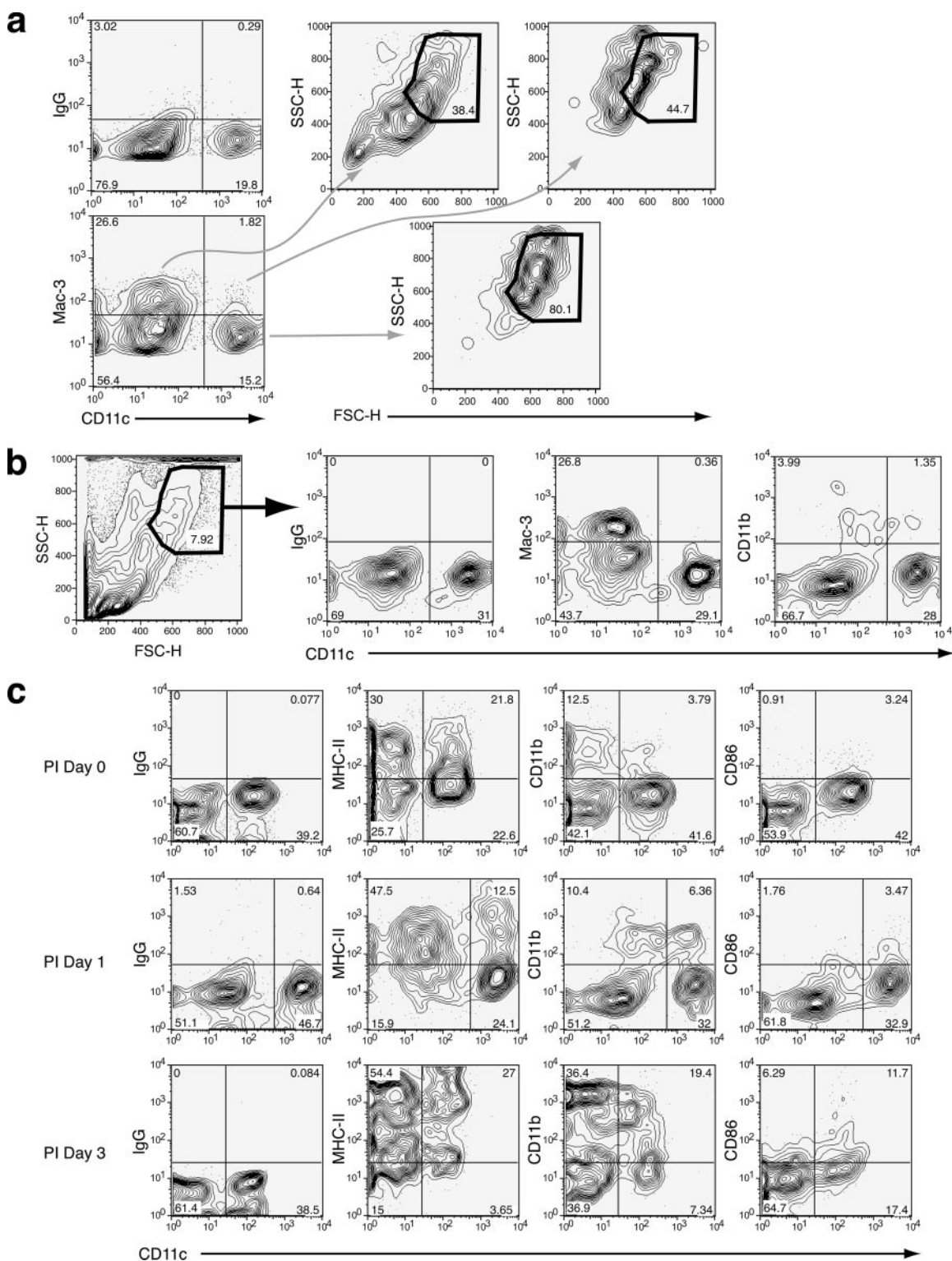
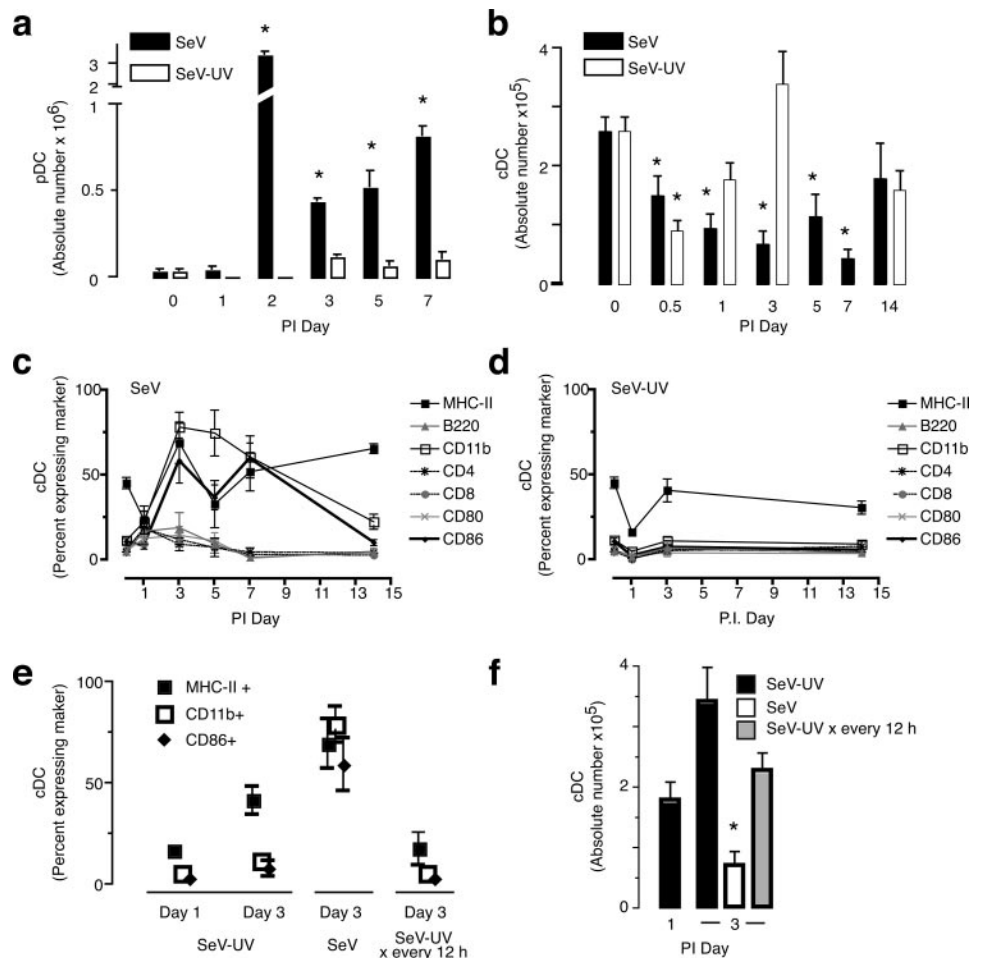


FIGURE 2. Gating strategy to identify lung cDCs. *a*, Representative flow cytometry plots of IgG (*top*) or Mac-3 (*bottom*) vs CD11c expression on uninfected, total lung digest cells reveals two populations of Mac-3⁺ cells: CD11c⁺ (alveolar macrophages) and CD11c⁻ (tissue macrophages). These two populations have slightly different scatter characteristics (*right top panels*) when compared with the Mac-3⁻CD11c⁺ cDC (*right lower panels*). Alveolar macrophage contamination is minimized, as <5% of the CD11c⁺ cells within the cDC gate are Mac-3⁺. Numbers refer to percentage of cells within a given gate or quadrant. *b*, Gating on cells in the cDC scatter gate shows that the CD11c⁺ cells do not express Mac-3 or CD11b. Numbers indicate percentage of cells within the cDC gate (scatter panel, *left*) or from the cDC gate that lies within the given quadrant (*right panels*). *c*, Representative flow cytometry plots showing expression of several cDC surface molecules at SeV PI days 0, 1, and 3. Using the cDC scatter gate, expression of CD11c (x-axis) vs IgG, MHC-II, CD11b, and CD86 (y-axis) is shown. Numbers refer to the percentage of cells in the cDC gate that lie within each quadrant.

in the draining lymph node at 12 h after inoculation (Fig. 4e). Treating mice with vehicle alone caused a substantially slower (and nearly linear) accumulation of FITC⁺ cDCs in the draining lymph node.

FITC-dextran cannot be used for identifying pDCs, since these cells do not ingest significant amounts of this carbohydrate. To track the movement of pDCs, we used the intravital dye CFSE,

FIGURE 3. Effect of paramyxoviral infection on lung DC levels and maturation. *a*, Lung pDCs were quantified by flow cytometry using anti-mPDCA-1 mAb and forward/side scatter characteristics at indicated PI days after SeV or SeV-UV inoculation into wild-type C57BL/6J mice. *b*, Lung cDCs were quantified by flow cytometry using anti-CD11c mAb and forward/side scatter characteristics at indicated PI days after SeV or SeV-UV inoculation. *c*, For conditions in *b*, lung cDCs were also analyzed by flow cytometry for indicated maturation markers at indicated PI days after SeV inoculation. *d*, Similar analysis as in *c*, but at the indicated PI days after SeV-UV. *e*, Same analysis as in *c* and *d* with an additional inoculation with SeV-UV every 12 h for 3 days (SeV-UV $\times$ every 12 h). *f*, For conditions in *e*, lung cDCs were quantified as in *b*. All values represent $n \geq 3$ mice per condition; *, $p < 0.05$ vs PI day 0.



which stains all cells in the lung at the time of dye administration (32). The majority of pDCs in the lung during a viral infection do not contain CFSE (Fig. 4*f*). This finding is consistent with recruitment of unlabeled pDCs to the lung during the course of infection. We also found the rate of decline in CFSE⁺ pDCs and cDCs was similar after viral infection (Fig. 4*g*). The decrease in CFSE⁺ pDCs was caused by the accumulation of CFSE⁻ cells in the lung, whereas the decrease in CFSE⁺ cDCs was likely due to a decrease in CFSE⁺ cells as they left the lung tissue.

pDCs are able to migrate to draining lymph nodes via high endothelial venules. To determine the time course of their arrival in the node, we examined the nodes for the presence of CFSE⁺ pDCs after viral infection. Very few pDCs in the draining lymph node are CFSE⁺ (Fig. 4, *h* and *i*). By examining the percentage of pDC-expressing CFSE in the draining lymph node, we found that there was a transient increase in these cells at PI day 3 (Fig. 4*j*).

Ccl5 and *Ccr5* regulate the baseline level of lung cDCs

Based on the previous observations in rat lung, we evaluated the possible influence of *Ccl5*-*Ccr5* expression on DC traffic in mouse lung as well (19). We initially examined cDC homeostasis by defining the baseline number of cDCs in the lungs of wild-type, *Ccr5*^{-/-}, and *Ccl5*^{-/-} mice. We found significantly increased numbers of cDCs in wild-type lungs compared with both of the transgenic mice, with no difference being noted between *Ccl5*^{-/-} and *Ccr5*^{-/-} mice (Fig. 5*a*). No difference was noted in total lung cell numbers among the three genotypes (data not shown). Although the numbers of cDCs were altered by the presence or ab-

sence of *CCR5* and *CCL5*, there was not a significant difference in DC subsets among wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} mice as assayed by the expression of CD4, CD8, B220, or CD11b on the CD11c-expressing DCs (data not shown). In general, most cDCs in the lung lacked any specific subset marker, having only CD11c highly expressed on their surface (data not shown). We did find that lung cDCs showed more widespread expression of MHC-II in *Ccr5*^{-/-} mice (92.6 $\pm$ 3.1% of DCs, mean $\pm$ SEM; n = 3–8 mice/genotype) than wild-type (50.8 $\pm$ 5.8%) or *Ccl5*^{-/-} mice (46.0 $\pm$ 16.5%). However, no difference was noted in expression of CD80 or CD86 on cDCs from any of the genotypes examined (data not shown).

Ccl5 and *Ccr5* influence maturation but not depletion of lung DCs during viral infection

We next examined the effect of a SeV infection on behavior of lung cDCs in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice. We found that the number of lung cDCs during SeV infection was no different between *Ccl5*^{-/-} and *Ccr5*^{-/-} mice and was similar to the values that we had obtained in wild-type mice (Fig. 5*b* and corresponding experiments in wild-type mice in Fig. 3*a*). Because the baseline numbers of cDCs were different between wild-type and *Ccl5*^{-/-} or *Ccr5*^{-/-} mice, we also calculated the number of lung cDCs as a percentage of the preinoculation values and still found no significant difference between *Ccl5*^{-/-} and *Ccr5*^{-/-} mice and wild-type mice (data not shown).

As with wild-type mice, we also examined the expression of maturation (MHC-II, CD80, CD86) and differentiation (CD4, CD8, B220, CD11b) markers on lung cDCs from *Ccl5*^{-/-} and

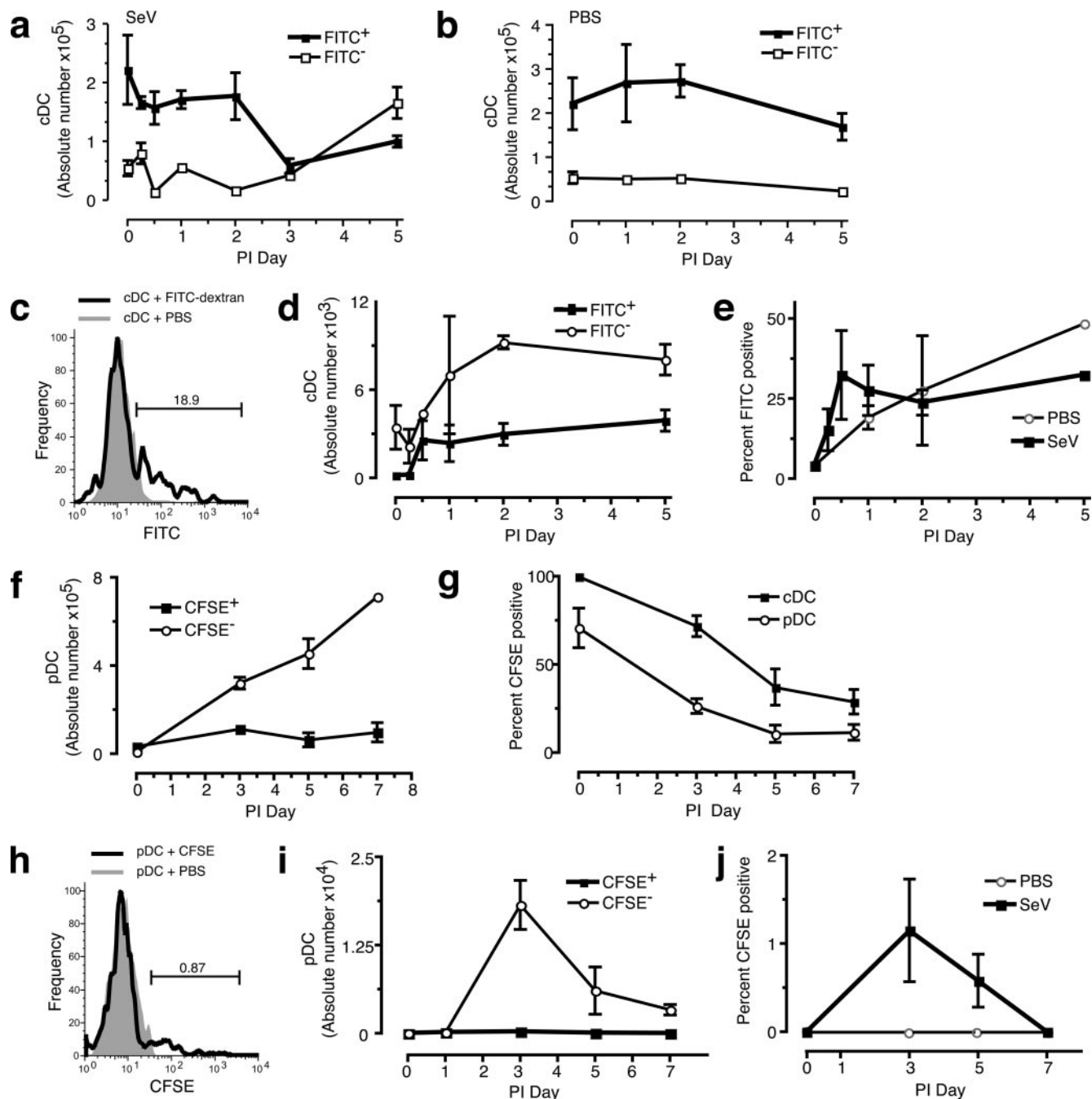
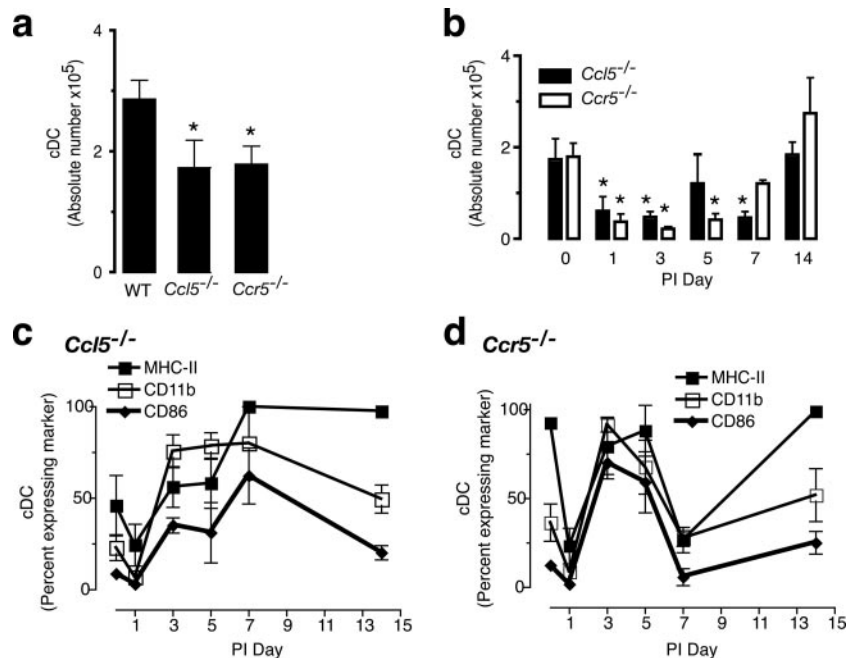


FIGURE 4. Effect of paramyxoviral infection on lung DC traffic to draining lymph nodes. *a*, FITC-dextran was administered 2 h before inoculation with SeV, and FITC⁺ and FITC⁻ cDCs were quantified on the indicated PI days in lung tissue. *b*, For conditions in *a*, corresponding data for control inoculation with PBS. *c*, Representative histogram 1 day PI, showing that FITC⁺ cDCs can be identified in the draining lymph node (FITC expression by CD11c⁺ cDCs shown, number and bar indicate net percent FITC⁺ cDCs). *d*, Using conditions as in *a*, FITC⁺ and FITC⁻ cDCs in the draining lymph node were quantified on the indicated PI days. *e*, Relative level of FITC⁺ cDCs in the draining lymph node after inoculation with SeV or control PBS. *f*, CFSE was administered 2 h before SeV inoculation, and CFSE⁺ and CFSE⁻ lung pDCs were quantified on the indicated PI days. *g*, CFSE was administered as in *e*, and lung cDCs and pDCs were analyzed for the level of CFSE labeling. *h*, CFSE-expressing pDCs can be identified in the draining lymph node 3 days PI. Representative histogram shown with gating on mPDCA-1⁺ cells in the draining lymph node (number and bar indicate the percentage of pDC-expressing CFSE). *i*, Relative level of CFSE⁺ and CFSE⁻ pDCs in the draining lymph node after inoculation with SeV. *j*, Level of CFSE⁺ and CFSE⁻ pDCs in draining lymph nodes. All values represent $n = 3-6$ mice per condition.

Ccr5^{-/-} mice after SeV inoculation. Both *Ccl5*^{-/-} and *Ccr5*^{-/-} mice showed a marked induction of only MHC-II, CD86, and CD11b similar to what was seen with wild-type mice (Fig. 5, *c* and *d*, and data not shown). Interestingly, although cDCs from *Ccl5*^{-/-} mice (like wild-type mice) continued to express these maturation markers at 1 wk after SeV inoculation, cDCs from

Ccr5^{-/-} mice did not. Nonetheless, cDCs from both *Ccl5*^{-/-} and *Ccr5*^{-/-} mice had higher expression of all three maturation markers by 2 wk after inoculation compared with wild-type mice (Figs. 3*c* and 5, *c* and *d*). Similar to wild-type mice, inoculation with SeV-UV failed to induce any maturation markers in either the *Ccl5*^{-/-} or *Ccr5*^{-/-} mice (data not shown).

FIGURE 5. Effect of CCL5 and CCR5 on lung cDC levels and maturation after viral infection. *a*, Baseline levels of lung cDCs were quantified by flow cytometry in wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} mice. *b*, Lung cDC levels were quantified by flow cytometry in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice at the indicated PI days after SeV inoculation. *c*, For conditions in *a*, lung cDCs from *Ccl5*^{-/-} mice were also analyzed by flow cytometry for the indicated maturation markers. *d*, For conditions in *c*, corresponding analysis from *Ccr5*^{-/-} mice. *c* and *d*, No significant changes were found after inoculation with control SeV-UV (data not shown). All values represent $n \geq 3$ mice per genotype; *, $p < 0.05$ vs PI day 0.



FITC-dextran uptake identifies two subsets of lung cDCs

To better define the influence of *Ccl5* and *Ccr5* on cDC function, we next compared the frequency of cDCs capable of ingesting FITC-dextran in wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} lung. We found that cDCs from *Ccr5*^{-/-} mice were significantly less capable of ingesting FITC-dextran than wild-type and *Ccl5*^{-/-} mice (Fig. 6*a*). The FITC⁺ and FITC⁻ cDC populations were then examined using the maturation markers MHC-II and CD86 and the differentiation marker CD11b. For the FITC⁺ cDCs, little difference was noted in the expression of CD11b and CD86 between *Ccl5*^{-/-} and *Ccr5*^{-/-} and wild-type mice (Fig. 6*b*). However, MHC-II expression tended to be decreased in *Ccl5*^{-/-} mice compared to the other two genotypes. Each of the markers was expressed at fairly low levels in all three strains; therefore, the FITC⁺ cDCs appear to be undifferentiated, immature cDCs. By contrast, FITC⁻ cDCs appeared to have matured and differentiated into the myeloid phenotype, as evidenced by increased MHC-II, CD86, and CD11b expression (Fig. 6*c*). This maturation was significantly decreased in *Ccl5*^{-/-} or *Ccr5*^{-/-} mice. These findings suggest that expression of maturation and differentiation markers of cDCs depends in part upon a functional CCL5-CCR5 signaling pathway.

Ccl5^{-/-} and *Ccr5*^{-/-} FITC⁺ and FITC⁻ cDC subsets respond differently to viral infection

We next examined the maturation (expression of MHC-II and CD86) and differentiation (expression of CD11b) status of the FITC⁺ and FITC⁻ lung cDCs 1 day after SeV or SeV-UV inoculation (Fig. 6, *d* and *e*). In wild-type mice, FITC⁻ cDCs were already mature myeloid cDCs and an active viral infection had little additional effect on this cell population (Fig. 6*d*). In *Ccl5*^{-/-} mice, cDCs were less mature and differentiated at the early time point, but had also become mature and differentiated into the myeloid subtype of cDCs by PI day 5. In *Ccr5*^{-/-} mice, cDCs appeared to have an intermediate phenotype at both PI days 1 and 5.

Ccl5 deficiency in the FITC⁺ population led to a much more significant defect in maturation and differentiation (Fig. 6*e*). As noted above, compared with FITC⁻ cells, FITC⁺ cDCs from all three groups were less mature and differentiated at baseline. Indeed, cDCs from the *Ccl5*^{-/-} mice lacked any significant expres-

sion of MHC-II, CD86, or CD11b, even after 1 day of productive viral infection. Interestingly, by 5 days after viral inoculation, the frequency of cDCs expressing these markers in *Ccr5*^{-/-} mice had not changed from PI day 1; however, cDCs from wild-type and *Ccl5*^{-/-} mice strongly increased their expression of all three markers, consistent with the idea that maturation and differentiation of lung cDCs early in a viral infection depends upon CCL5 (acting through a receptor other than CCR5), while later in the infection continued maturation was dependent upon CCR5 expression (and a ligand other than CCL5).

CCL5 and CCR5 mediate lung cDC migration to draining lymph nodes during a paramyxoviral infection

FITC-dextran administration 2 h before instillation of SeV-UV or SeV was also used to track lung cDC migration to the draining lymph nodes. We examined lymph node digests at 1 and 5 days after SeV inoculation for the presence of FITC⁺ cDCs (Fig. 7*a*). As noted above (Fig. 4*e*), ~25% of the cDCs in the draining lymph nodes of wild-type mice were FITC⁺. Surprisingly, very few FITC⁺ cDCs were found in the draining lymph nodes of either the *Ccl5*^{-/-} or *Ccr5*^{-/-} mice during SeV infection, suggesting that the CCL5-CCR5 signaling pathways were involved in the migration of cDCs to draining lymph nodes.

The migration of peripheral tissue cDC to draining lymph nodes depends on expression of CCR7, the receptor for the lymph node homing chemokines CCL19 and CCL21 (17, 20). Therefore, we next examined expression of CCR7 on lung cDCs during SeV infection. We found that *Ccl5*^{-/-} and *Ccr5*^{-/-} mice had significantly decreased frequency of CCR7-expressing cDCs when compared with wild-type mice (Fig. 7*b*). Because of the relative differences in lung cDCs between the three genotypes, we also determined the actual number of CCR7-expressing cDCs and found that the decrease in CCR7-expressing cDCs was significant only in *Ccr5*^{-/-} mice (Fig. 7*c*). These data suggest that CCR5, and to a lesser extent CCL5, may be necessary for induction of CCR7 on cDCs and the subsequent migration of these cells to the draining lymph node.

Although lung cDC migration to the draining lymph node in various allergen challenge models has been shown to depend upon CCR7 expression on cDCs, this requirement has not been shown

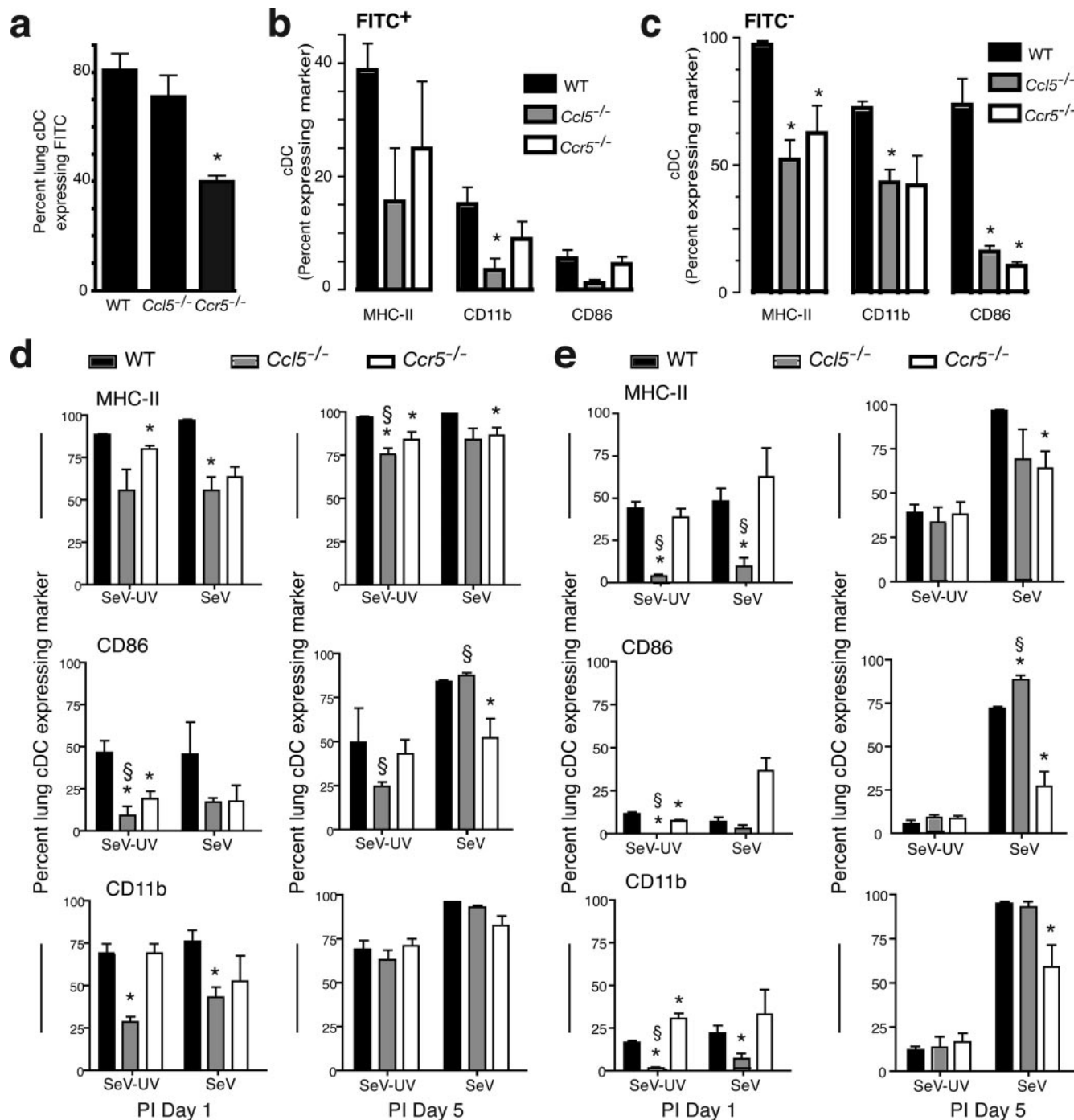


FIGURE 6. Effect of CCL5 and CCR5 on FITC-dextran subpopulations of lung cDCs after viral infection. *a*, Baseline level of FITC-dextran uptake in lung cDCs was quantified by flow cytometry in wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} mice. *b*, Levels of maturation markers on FITC⁺ lung cDCs from wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} mice were quantified by flow cytometry. *c*, For conditions in *b*, corresponding analysis of FITC⁻ lung cDCs. *d*, Lung cDCs were labeled with FITC-dextran as in *a*, and then FITC⁻ cells were analyzed by flow cytometry for the indicated maturation markers at the indicated PI days after SeV or SeV-UV inoculation. *e*, Corresponding values from *d* for FITC⁺ cells. All values represent $n \geq 3$ mice per genotype. *, $p < 0.05$ vs same time point in wild-type mice; §, $p < 0.05$ vs same time point in *Ccr5*^{-/-} mice.

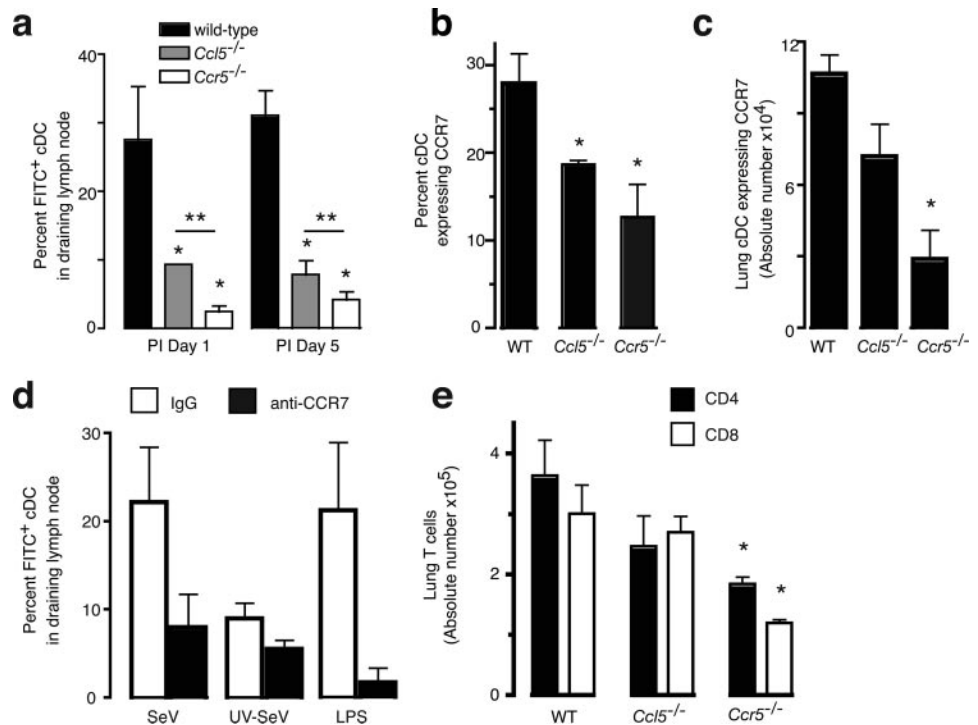
for viral airway disease (33–36). Therefore, we administered FITC-dextran with a CCR7-blocking Ab or isotype control (given intranasally) to wild-type mice followed by inoculation with SeV or SeV-UV or treatment with LPS. On the following day, draining lymph nodes were examined for the presence of FITC⁺ cDC. We found that local blockade of CCR7 decreased the migration of FITC⁺ lung cDCs during SeV infection to the level that was found in the *Ccl5*^{-/-} and *Ccr5*^{-/-} mice (Fig. 7*d*). This finding suggested that cDC migration after viral infection depended on CCR7. The

FITC⁺ cDC migration to the draining lymph node was decreased to a greater extent by CCR7 blockade after LPS treatment than after viral inoculation, suggesting that a component of virus-induced cDC migration may be CCR7 independent.

Decreased cDC migration is associated with decreased lung T cells after viral infection

Given impaired migration of cDCs to draining lymph nodes in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice, we reasoned that an immune defect

FIGURE 7. Effect of CCL5 and CCR5 on migration of lung DCs after viral infection. *a*, Levels of FITC⁺ lymph node cDCs in wild-type, *Ccl5*^{-/-}, and *Ccr5*^{-/-} mice were quantified by flow cytometry on the indicated PI days after SeV inoculation. *b*, Percentage of CCR7⁺ lung cDCs for conditions 2 h PI after SeV inoculation. *c*, For conditions in *b*, corresponding numbers of CCR7⁺ lung cDCs. *d*, Percentage of FITC⁺ cDCs in draining lymph nodes in wild-type mice on PI day 1 after SeV, SeV-UV, or treatment with LPS in the presence or absence of pretreatment with anti-CCR7 mAb or control IgG. *e*, Number of lung CD4⁺ or CD8⁺ T cells in wild-type, *Ccl5*^{-/-}, or *Ccr5*^{-/-} mice on PI day 21 after SeV inoculation. All values represent three to six mice per condition. *, $p < 0.05$ vs wild-type mice; **, $p < 0.05$ *Ccl5*^{-/-} vs *Ccr5*^{-/-}.



might be found after viral infection. We have previously noted that *Ccl5*^{-/-} and *Ccr5*^{-/-} mice showed no decrease in the generation of SeV-specific CD8⁺ T cells (2). Therefore, we examined the possibility that CD4⁺ or CD8⁺ T cell accumulation in the lungs might be adversely affected in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice. Because immune cell-mediated sequelae of airway hyperreactivity and mucous cell metaplasia develop at 21 days after SeV inoculation, we examined T cell accumulation at this time point (37). We found that indeed *Ccr5*^{-/-} mice had significantly fewer CD4⁺ and CD8⁺ T cells in the lungs at PI day 21 compared with wild-type and *Ccl5*^{-/-} mice (Fig. 7e). Therefore, loss of *Ccr5* is associated with a decrease in lung cDC migration to the draining lymph node and subsequent T cell accumulation after SeV infection.

Discussion

Previous work on respiratory viral disease and lung DCs has focused primarily on RSV infection of BALB/c mice (15, 16, 22, 23). In this RSV model, the number of both cDCs and pDCs appear to increase in the airway tissue with viral infection. However, RSV is not a natural pathogen in rodents and therefore replicates poorly in mice. The consequent illness may be different from the one that drives both short- and long-term airway disease in humans. We have therefore chosen to study a natural rodent pathogen, SeV, to evaluate the endogenous cDC and pDC response. SeV causes a severe peribronchial inflammatory response in mice and leads to a 10- to 12-day long respiratory illness that is followed by chronic airway hyperreactivity and mucous cell metaplasia (1, 25, 31, 37). The pattern of viral replication in airway epithelial cells and macrophages and the associated histopathology is similar to the findings in humans with severe RSV infection (38). Previous work has assessed the DC response to SeV in the rat, but these studies were limited because the only marker system for identifying rat DCs was expression of MHC-II on tracheal leukocytes (19, 21). Although these studies showed an increase in MHC-II⁺ airway immune cells during viral infection, the type of cells could not be precisely defined.

In the present report, we use an array of immunocytochemical techniques and show that viral infection leads to a rapid and sustained decrease in lung cDCs. We also demonstrate that this decrease in lung cDCs is associated with a concomitant increase in expression of maturation and differentiation markers on both migrating and resident cDCs. These findings suggest that Ag processing and presentation may be occurring in the lung periphery as well as in the draining lymph node. Indeed, a recent *in vitro* report using bone marrow-derived cDC showed that RSV infection led to activation and increased Ag-presenting function without a change in viability (39). The rapid and sustained decrease in the number of lung cDCs we see after respiratory viral infection may represent a general response to severe infection because it has also been observed in subjects with bacterial sepsis (40). This is supported by the fact that SeV-UV, like SeV, caused an initial decrease in lung cDC numbers; however, with SeV-UV, this decrease was rapidly reversed. Perhaps the controlled suppression with an ongoing inflammatory reaction is an attempt to prevent cDC ingestion and presentation of necrotic tissue, possibly avoiding the generation of an autoimmune response.

In contrast to cDCs, the population of lung pDCs is present only at low levels at baseline. Similarly, while cDC numbers are decreasing in lung tissue, the levels of lung pDCs increase by 100-fold 2 days after viral inoculation. The recruitment of pDCs is followed by a contraction phase, but pDC numbers remain significantly elevated over their baseline values throughout the active period of the viral insult. Given the sudden influx of pDCs with a concomitant drop in cDCs, the pDCs comprise the major DC subtype in the mouse lung during the active immune response to respiratory viral infection. This change in local DC populations is consistent with the critical role of pDCs as the primary cellular source of IFN- α and in turn the central importance of IFN- α in the antiviral response (14–16).

We have also taken advantage of FITC-dextran or CFSE reagents to track cDC and pDC migration, respectively, as these cells move out of the lung and into draining lymph nodes. We identified

two waves of cDC emigration from the lung, an early phase in the first 12–24 h and a later phase at 3 days after viral inoculation. The early phase appears similar to the response to influenza virus (32). This early phase is associated with appearance of cDCs in the lymph node, where they serve to arm the adaptive immune response. However, the later disappearance of cDCs is not associated with an increase in these cells in the draining lymph node. In this case, cDCs could be dying in the periphery or migrating to another lymphoid organ, such as the spleen. Unlike cDCs, the pDCs that were present in the lung at the time of viral inoculation do not migrate to draining lymph nodes. Instead, additional pDCs appear to be recruited to the lung during the antiviral response and then may travel to the lymph node. This pattern of movement would be consistent with the marked increase in CFSE⁺ pDCs found in the draining lymph node at 3 days after viral inoculation. This movement would provide pDCs with an opportunity to skew T cell differentiation, but further studies will be needed to define the origin and the action of pDCs in the lymph nodes.

Our results also help to establish the role of chemokines and chemokine receptors as mediators of DC traffic during homeostasis and inflammatory responses. Previous work had shown that CCL5 blockade with met-RANTES caused a decrease in baseline levels and inflammatory recruitment of cDCs into the rat trachea (19). Because this antagonist can block CCR1, CCR3, and CCR5 signaling, it was unclear which of these receptors influenced DC behavior. Our analysis of *Ccl5*^{-/-} and *Ccr5*^{-/-} mice indicates that baseline levels of lung cDCs depend primarily on intact CCL5 and CCR5 signaling function. In addition, we found that *Ccr5* deficiency significantly decreased the number of lung cDCs that take up FITC-dextran. The lack of FITC-dextran uptake was associated with a more mature surface molecule phenotype in wild-type and *Ccl5*^{-/-} mice. However, the FITC⁺ population exhibited lower expression of these maturation markers in *Ccr5*^{-/-} mice. Therefore, there is a discrepancy between level of maturity and cell phagocytic ability in these mice, but further studies are needed to define the functional relevance of this change.

In addition to an influence over baseline cDC behavior, we also used *Ccl5*^{-/-} and *Ccr5*^{-/-} mice to study the influence of CCL5-CCR5 signaling on the cDC response after SeV infection. As in wild-type mice, *Ccl5*^{-/-} and *Ccr5*^{-/-} mice exhibit a steep reduction in cDC numbers within 1 day of viral infection. We assumed that the virus-induced decrease in cDC numbers was due to migration of cDCs to draining lymph nodes. However, very few cDCs in the lymph node had arrived from the lungs in *Ccl5*^{-/-} or *Ccr5*^{-/-} mice, whereas nearly 25% of cDCs in lymph nodes had trafficked from the lung in wild-type mice. This finding provides the initial evidence of a role for CCL5 and CCR5 in homing of lung cDCs to lymph nodes during viral infection. We are only aware of a role for CCR5 in the movement of pDCs across the high endothelial venule during a *Mycobacterium tuberculosis* infection (41). The mechanism for CCL5-CCR5-dependent homing of cDCs appears to be distinct, because it depends on a decrease in CCR7 expression by cDCs in the lung rather than a defect at the node as seen with pDCs. Macrophages have been shown to suppress DC migration from the lung, and *Ccl5*^{-/-} and *Ccr5*^{-/-} mice have increased numbers of macrophages in their lungs during a paramyxoviral infection (2, 35). However, the CCL5-CCR5 effect on cDCs occurs before macrophage influx into the lung, and macrophage function in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice is compromised by enhanced apoptosis (2). Thus, CCL5-CCR5 signals controlling cDC movement may be distinct from those controlling macrophage survival after viral infection.

Using FITC-dextran we were able to identify lung cDC present at the time of viral inoculation and assess their maturation and

differentiation status. Examining cDC maturation and FITC uptake, we found that cDCs failed to mature or differentiate with viral Ag uptake in *Ccl5*^{-/-} mice; however, this initial defect was overcome by 5 days after viral inoculation. By contrast, cDCs initially responded normally to Ag exposure in *Ccr5*^{-/-} mice, but, unlike wild-type or *Ccl5*^{-/-} mice, their surface expression of these markers was unchanged at 5 days after inoculation. These findings suggest that early maturation of cDCs after initial Ag ingestion depends upon CCL5 acting through a receptor other than CCR5 (e.g., CCR1 or CCR3); however, continued maturation during an ongoing antiviral response requires CCR5 signaling induced by a ligand other than CCL5 (e.g., CCL3 or CCL4). Thus, the signals for initial cDC maturation are distinct from those required for continued expression of MHC-II, CD11b, and CD86.

DCs are the primary APCs, and this function primarily takes place in the draining lymph node. Surprisingly, we found defects in cDC migration to the draining lymph node in *Ccr5*^{-/-} mice infected with SeV, suggesting a possible adaptive immune defect in these mice. However, there are normal levels of virus-specific CD8⁺ T cells in *Ccl5*^{-/-} and *Ccr5*^{-/-} mice after SeV inoculation (2). This finding could suggest that immune cell types other than cDCs are capable of supporting an appropriate virus-specific CD8⁺ T cell response (2). Alternatively, the present findings suggest that mature cDCs remaining in the airway tissue could act locally to present Ag and develop an adaptive immune response. Therefore, while our initial work indicated a predominant role for CCL5-CCR5 signaling to macrophage survival for protection against viral infection, it is still possible that cDC function could contribute to the increased susceptibility of *Ccr5*^{-/-} mice to respiratory viral infection (2). In addition, in the current studies we found that the defect in cDC migration to the lymph node in *Ccr5*^{-/-} mice is associated with decreased numbers of CD4⁺ and CD8⁺ T cell at 21 days after viral inoculation. This T cell response occurs at the time when the sequelae of viral infection begin to develop. Therefore, it is also possible that CCR5 signals participate in driving the airway disease traits of airway hyperreactivity and mucous cell metaplasia that develop long after the initial viral infection.

In conclusion, our data demonstrate that respiratory paramyxoviral infection has profound but precisely orchestrated effects on lung DC populations. Viral infection rapidly stimulates the maturation and emigration of the cDC population and the immigration of pDC population in the lung. The cDCs appear in the draining lymph nodes in the first 6–12 h while pDCs do not arrive until several days after viral inoculation. The sentinel population of immature lung cDCs depend at least in part on CCR5 and CCL5 signals. In addition, the CCL5 and CCR5 signaling pathways have overlapping as well as distinct effects on maturation and migration of lung cDCs in response to respiratory viral infection. For CCR5, there is a striking influence on CCR7-dependent migration of cDCs to draining lymph nodes and consequences for later T cell recruitment to the airway tissues. Together, our results provide for a well-synchronized set of cDC and pDC responses that facilitate the innate and adaptive immune responses to this type of respiratory viral infection.

Disclosures

The authors have no financial conflict of interest.

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