JOURNAL OF VIROLOGY, July 2004, p. 7528–7535 Vol. 78, No. 14
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.14.7528–7535.2004

CC Chemokine Receptor 7 Expression by Effector/Memory CD4$^+$ T Cells Depends on Antigen Specificity and Tissue Localization during Influenza A Virus Infection

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Received 5 January 2004/Accepted 15 March 2004

The lung is an important entry site for respiratory pathogens such as influenza A virus. In order to combat such invading infectious agents, effector/memory T cells home to the lung and other peripheral tissues as well as lymphoid organs. In this process, chemokines and their receptors fulfill important roles in the guidance of T cells into such organs and specialized microenvironments within tissues. In this study, we determined if CD4$^+$ T cells residing in different lung compartments and draining lymph nodes of influenza A virus-infected and naïve mice express receptors allowing their recirculation into secondary lymphoid tissues. We found high levels of L-selectin and CC chemokine receptor 7 (CCR7) expression in lung-derived CD4$^+$ T cells, similar to that detected on T cells in secondary lymphoid organs. Upon influenza A virus infection, the bulk of gamma interferon-positive (IFN-γ^+) and IFN-γ^- CD4$^+$ T cells recovered from lung parenchyma retained functional CCR7, whereas virus-specific IFN-γ-producing T cells were CCR7$^-$. In contrast, a majority of virus-specific IFN-γ^+ T cells in the lung draining lymph node were CCR7$^+$. Independent of infection, CD4$^+$ T cells obtained from the lung airways exhibited the lowest expression level of L-selectin and CCR7, indicating that T cells at this anatomical site represent the most differentiated effector cell type, lacking the ability to recirculate. Our results suggest that effector/memory T cells that enter inflammatory sites retain functional CCR7 expression, which is lost only upon response to viral antigen and after localization to the final effector site.

T cells continuously recirculate throughout the body, ensuring early recognition of and defense against invading viruses. Naïve T cells recirculate between lymphoid tissues and the blood. Upon activation by cognate antigen and antigen-presenting cells in secondary lymphoid tissues, T cells acquire the ability to migrate to peripheral sites of inflammation and infection (reviewed in reference 6). The lung is a peripheral organ that, due to its exposure to the outside air, is continuously threatened by airborne pathogens. Therefore, T cells capable of localizing to anatomical lung compartments are important in the first line of defense against such pathogens.

Influenza A virus infection causes a common and typical respiratory tract infection. Virus replication occurs predominantly in airway epithelial cells, and therefore the infection is usually restricted to the respiratory tract, in both human and murine influenza (16, 33). The immune response against influenza A virus is typically T helper 1 (Th1) dominated (16). It is well established that CD4$^+$ T-effector functions, such as the activation of CD8$^+$ T cells and antigen-presenting cells, gamma interferon (IFN-γ) production, cytolysis of infected cells, and provision of B-cell help for antibody production, are important for viral clearance and long-term protection (4, 5, 16, 20). Murine virus-specific effector/memory T cells persist for several months after viral clearance in the lung airways and function in the first line of defense, demonstrating that T-cell distribution to specific lung compartments is crucial in protection against reinfection (24, 25).

T-cell extravasation from the bloodstream into tissues occurs in specialized postcapillary venules (high endothelial venules in lymph nodes and Peyer's patches) and proceeds through a multistep-adhesion cascade involving chemokines and adhesion molecules. Chemokines function in several steps of this cascade and fulfill further important roles after cells have transmigrated through the endothelium by guiding lymphocytes into and within the underlying tissue parenchyma (reviewed in reference 8).

Entrance of T cells into lymph nodes and Peyer's patches from the blood through high endothelial venules is dependent on their expression of L-selectin and CC chemokine receptor 7 (CCR7), whose ligands, peripheral node addressin and CC chemokines CCL21 and CCL19, are presented on these specialized endothelial cells (43). Accordingly, CCR7 gene-targeted mice and mice carrying the spontaneous mutation *plt* (paucity of lymph node T cells), which lack CCL19 and the lymphoid form of CCL21, display a severely reduced capacity of T cells to enter lymph nodes and Peyer's patches (18, 21). Naïve T cells are uniformly positive for CCR7 and L-selectin, whereas expression of these receptors by antigen-experienced T cells, which can also enter lymph nodes through the afferent lymphatics, is heterogeneous (9, 15, 39, 43). CCR7 and its ligand CCL21 play an important role in guiding mature

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$CCR7^+$ dendritic cells from peripheral tissues into the draining lymph nodes through afferent lymph vessels (18, 21, 22, 38) and may also function in T-cell migration via this route.

In the inflamed lung, the expression of several chemokines that participate in the recruitment of different leukocyte and lymphocyte subsets is induced (14). In contrast, how inflammatory conditions influence CCR7 expression by T cells in the lung and in lymphoid tissues during an active respiratory virus infection is poorly defined. Moreover, it is currently a matter of debate if the capacity of T cells to migrate to peripheral effector sites, like the lung, requires the down-regulation of receptors mediating T-cell entrance into lymphoid tissues, such as CCR7 and L-selectin (9, 15, 28, 37, 39).

Therefore, we systematically analyzed the expression of CCR7 and L-selectin by $CD4^+$ T cells obtained from the lung parenchyma and airways, lung-draining mediastinal lymph nodes (MLN), or spleens of influenza A virus-infected and naïve mice. Our results reveal substantial differences in CCR7 expression among effector/memory T cells from the lung parenchyma and MLN ($CCR7^{high}$) and the airways ($CCR7^{low}$). To analyze the correlation between CCR7 expression and active participation in the antigen response, we compared the CCR7 expression of influenza virus-specific and total effector/memory $CD4^+$ T cells producing IFN-γ upon stimulation. Within the lung, polyclonally stimulated IFN-γ-producing T cells expressed functional CCR7, whereas IFN-γ^+ $CD4^+$ T cells responding to viral antigen were predominantly $CCR7^-$, suggesting a further differentiation within the effector site upon antigen contact.

MATERIALS AND METHODS

Animals and viral infection. Male and female BALB/c mice, 8 to 12 weeks old, were used for all experiments. The animals were purchased from Bundesinstitut für Risikobewertung (Berlin, Germany), and all experiments were performed under specific-pathogen-free conditions in the animal facility of the Deutsches Rheumaforschungszentrum (Berlin, Germany). Animals were ether anesthetized before intranasal (i.n.) infection with a sublethal dose (360 hemagglutinating units) of the influenza A virus strain HKx31 (x31; H3N2) in 30 μl of allantoic fluid. Virus was grown in the allantoic cavities of 11-day-old embryonated chicken eggs for 2 days at 37°C, and virus titers were determined by hemagglutination assays. Virus stocks were stored at −80°C until used for infections. All animal experiments were performed according to institutional and state guidelines.

Cell preparation from airways, lung parenchyma, MLN, and spleens. Lung airway cells were collected through three to five consecutive bronchoalveolar lavages (BAL) with 1 ml of Roswell Park Memorial Institute medium 1640 (RPMI). To clear the lung microvasculature, the lavaged lungs were perfused with 10 ml of phosphate-buffered saline through the right ventricle of the heart before organ preparation. Single-cell suspensions were prepared from spleens, MLN, and lungs in RPMI supplemented with 5% fetal calf serum by passage through a wire mesh. To further purify mononuclear cells from the lung, the cells were resuspended in 40% isotonic Percoll (Biochrome, Berlin, Germany), layered on 70% isotonic Percoll for high-density gradient centrifugation, and subsequently harvested from the interface. Mononuclear cells from the spleen cell suspensions were obtained by high-density gradient centrifugation with Histopaque-1083 (Sigma-Aldrich, St. Louis, Mo.). For all flow cytometric analyses, cells from the respective organs or organ compartments of 10 to 15 animals were pooled.

Chemotaxis assay. Recombinant murine CCL21 was purchased from R&D Systems (Wiesbaden, Germany) and titrated to identify optimal concentrations (15). Mononuclear cells obtained from spleens or lungs of 30 animals were pooled. Lung cells were used directly after preparation (see above) without further purification of $CD4^+$ cells, whereas spleen cells were enriched for cytokine$^+$ $CD4^+$ T cells through depletion of B cells, macrophages, and $CD8^+$ and $CD62L^{high}$-expressing cells by panning as described previously (15). The remaining $CD4^+$ cell population was 50% $CD62L^+$ (intermediate to low expression). The assay was conducted as previously described (15). Briefly, 5×10^5 cells, suspended in 100 μl of assay medium (RPMI plus 0.5% bovine serum albumin), were added per upper well of fibronectin (Invitrogen, Karlsruhe, Germany)-coated 5-μm-pore-size, 24-well tissue culture inserts (Costar, Cambridge, Mass.). CCL21 (100 nM) diluted in assay medium or medium alone was added to the bottom well, and migrated cells were collected after a 90-min incubation at 37°C. The rate of migration was determined by the combined analysis of cell number and subset frequency in the input and migrated population. Triplicates of input and migrated cells were quantified by flow cytometry with fluorescent beads (Fluoresbrite Yellow Green Microspheres; Polysciences, Warrington, Pa.) as an internal standard and antibodies (anti-CD4-cytochrome, RM4-5; BD PharMingen) without washing to set appropriate count gates for $CD4^+$ lymphocytes. The frequencies of cytokine-producing subsets of the input and migrated populations (pools of 8 wells for CCL21 and 24 to 36 wells for the medium control) were analyzed as described below.

Flow cytometry and cell stimulation. Cell samples were stained with the following antibodies: biotinylated or fluorescein isothiocyanate-conjugated anti-CD4 (GK1.5) and allophycocyanin-conjugated anti-CD62L (MEL-14). To prevent unspecific staining, all cells were preincubated with blocking anti-CD16/CD32 antibody 2.4G2/75 and purified rat immunoglobulin G (IgG) (manufactured by Jackson Immunoresearch, West Grove, Pa.; purchased from Dianova, Hamburg, Germany). Peridinin chlorophyll-protein-conjugated streptavidin (BD PharMingen) was used as a second-step reagent. To detect CCR7 surface expression, cells were incubated with the CCR7 ligand CCL19 fused to human IgG1 (42), washed, and further incubated with phycoerythrin-conjugated polyclonal donkey anti-human IgG (Jackson Immunoresearch) that had been preincubated for 30 min with purified mouse gamma-globulin and rat IgG (Jackson Immunoresearch) to reduce nonspecific binding. The specificity of the CCL19-Ig staining was controlled by blocking with 10 μg of recombinant murine CCL19 (R&D Systems)/ml or staining splenocytes from CCR7-deficient mice (18) (data not shown). Gates were set on viable cells based on propidium iodide exclusion.

To detect intracellular cytokines, cells were polyclonally stimulated with 10 ng of phorbol 12-myristate 13-acetate (PMA)/ml and 500 ng of ionomycin (Sigma-Aldrich)/ml for 4 h with the addition of 10 μg of brefeldin A (Sigma-Aldrich)/ml during the last 2 h. Alternatively, cells were stimulated with influenza virus in a procedure similar to that described in reference 40. For the antigen-specific stimulation, 4×10^6 cells of the respective organ or organ compartment/ml resuspended in RPMI–10% fetal calf serum were seeded in 24-well plates for 6 h at 37°C with the addition of UV-inactivated x31 virus in allantoic fluid or UV-treated virus-free allantoic fluid as a control. Optimal virus dilutions were determined by titrations in preliminary experiments (data not shown). For the last 4 h of incubation, 10 μg of brefeldin A (Sigma-Aldrich)/ml was added. After stimulation, the cells were stained for CD4 and surface CCR7 and subsequently fixed with 2% paraformaldehyde. Fixed cells were permeabilized with saponin (Sigma-Aldrich), and intracellular cytokines were detected using the following antibodies conjugated to fluorescein isothiocyanate, phycoerythrin, or allophycocyanin: anti-IFN-γ (AN 18.17.24), anti-interleukin 10 (IL-10) (JES5-16E3), or appropriate isotype controls. In stainings combining CCR7 surface detection with intracellular cytokine analysis, the saponin treatment led to a slightly lower resolution of the CCR7 staining. If not otherwise stated, all staining antibodies were obtained from BD Biosciences, except for biotinylated anti-CD4 and fluorescein isothiocyanate-conjugated anti-IFN-γ, which were kindly provided by H. Hecker and H. Schliemann (Deutsches Rheumaforschungszentrum). Quadrants were set according to the isotype controls. Flow cytometric analyses were performed on a FACSCalibur instrument with CellQuest software (Becton Dickinson), and for each analysis (with the exception of some BAL samples) ≥30,000 $CD4^+$ T cells were acquired.

Statistical analysis. Unless otherwise indicated, all values in the text of the Results section are the mean (± standard deviation [SD]) of all experiments performed, whereas in the figures presented as dot plots show one representative experiment. Data were considered statistically significant when P was ≤0.05 as determined by Student's t test.

RESULTS

CCR7 and L-selectin expression by $CD4^+$ T cells in naïve mice and during influenza A virus infection depends on their tissue localization. To determine the influence of an influenza virus infection on the lymph node-homing phenotype of T cells residing in different lung compartments, we first isolated mononuclear cells from lung parenchyma, lung airways (by BAL),

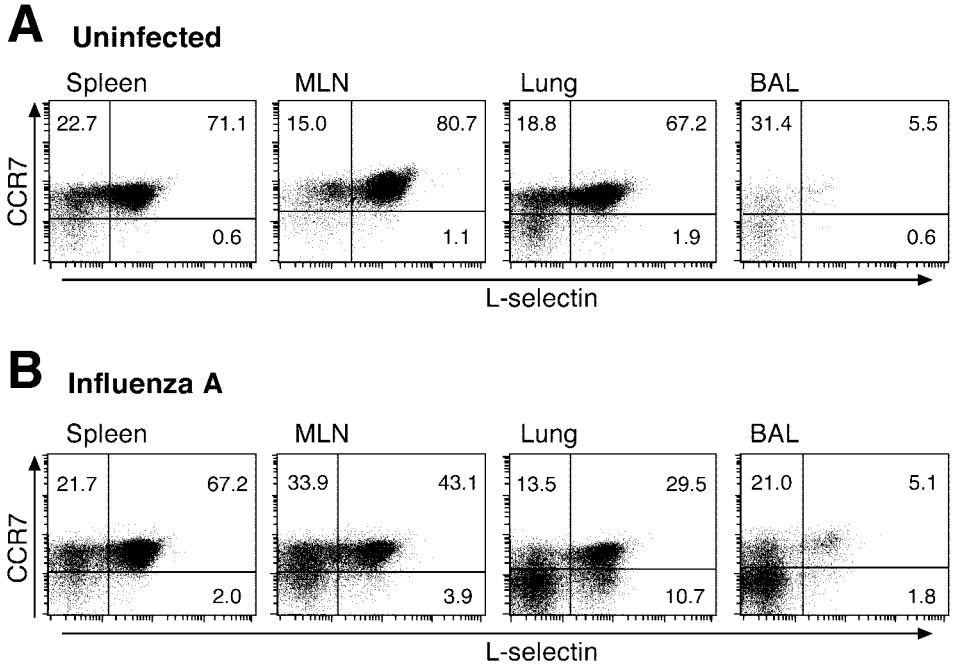

FIG. 1. Flow cytometric analysis of CCR7 and L-selectin surface expression by $CD4^+$ T cells in spleens, MLN, and different lung compartments of naïve and influenza A virus-infected mice. Cells were isolated from the respective organs and organ compartments of naïve BALB/c mice (A) or on day 8 after i.n. infection with the influenza A virus strain x31 (B) and stained for CD4, L-selectin, and CCR7 with the use of monoclonal antibodies and a CCL19-Ig fusion protein, respectively. Dead cells were excluded from the analysis by propidium iodide exclusion. Cells were gated on $CD4^+$ lymphocytes. Representative dot plots of a minimum of three independent experiments with cells pooled from the organs or organ compartments of 10 to 15 mice/experiment are shown. Numbers in dot plots indicate percentages of cells in the respective quadrants.

MLN, and spleens of naïve 6- to 12-week-old specific-pathogen-free BALB/c mice and analyzed surface expression of CCR7 (by CCL19-Ig binding) and L-selectin by $CD4^+$ T cells (Fig. 1A). In the absence of infection, the vast majority of $CD4^+$ T cells from the lung, MLN, and spleen were L-$selectin^{high}$ $CCR7^{high}$. In contrast, only a low frequency of BAL fluid $CD4^+$ T cells expressed L-selectin (7.3% ± 0.3%, mean ± SD of all experiments performed with pooled cells from 10 to 15 mice) or CCR7 (27.2% ± 5%) (representative staining results are shown in Fig. 1A).

Next, we infected BALB/c mice i.n. with a sublethal dose (360 hemagglutinating units) of the pathogenic influenza A virus strain HKx31 (x31; H3N2) and analyzed $CD4^+$ T cells on day 8 of infection. This time point was chosen because most virus is cleared from the lung by this time (30) and the numbers of cytokine-producing $CD4^+$ T cells have peaked (data not shown). The viral airway infection had only marginal effects on the expression of L-selectin and CCR7 by splenic $CD4^+$ T cells (Fig. 1B). The influenza virus infection reduced percentages of L-$selectin^+$ $CD4^+$ T cells in MLN (from 70.3% ± 13.4% of uninfected cells to 47.3% ± 5.1% of cells during infection, $P = 0.04$, Fig. 1) but had no significant effect on CCR7 expression by $CD4^+$ T cells in this compartment (uninfected, 78.9% ± 15%; infected, 80.8% ± 6.4%, Fig. 1B). $CD4^+$ T cells obtained from the lungs of influenza virus-infected mice down-regulated the expression of both L-selectin and CCR7 (L-selectin, from 76% ± 7.8% to 37.4% ± 8.6%, $P = 0.005$; CCR7, from 87.9% ± 7.7% to 46% ± 9.2% in infection, $P = 0.0005$; Fig. 1B). In contrast, the already low frequencies of $CCR7^+$ and L-selectin-expressing $CD4^+$ T cells obtained from the lung airways of naïve mice did not significantly change during infection (Fig. 1B).

Differential expression of CCR7 by IFN-γ-producing $CD4^+$ T cells depends on their tissue localization during influenza A virus infection. It was recently reported that the majority of cytokine-producing $CD4^+$ T cells from mouse spleen and human peripheral blood expresses functional CCR7 (15). In order to address if this was also true for T cells isolated from lung parenchyma, airways, and the lung-draining lymph nodes of naïve and influenza A virus-infected mice, we determined the CCR7 expression of IFN-γ-producing $CD4^+$ T cells from such tissues after 4 h of polyclonal stimulation with PMA and ionomycin. We previously demonstrated that this short-term stimulation only slightly reduces CCR7 surface expression by T cells and can therefore be used to directly assess CCR7 levels by cytokine-producing T cells (15). L-Selectin expression by cytokine producers could not be determined, because stimulation necessary for cytokine detection induces a rapid cleavage of the molecule (11).

In naïve specific-pathogen-free BALB/c mice, the frequency of IFN-γ^+ $CD4^+$ T cells was relatively low in the spleen, MLN, and lung, whereas a higher frequency resided in the airways (BAL) (Fig. 2A). In accordance with our previous results, most IFN-γ-producing $CD4^+$ T cells isolated from MLN and spleens of naïve BALB/c mice stained positive for CCR7 (Fig. 2A and C). IFN-γ^+ $CD4^+$ T cells derived from the lung parenchyma of these mice showed a frequency of $CCR7^+$ cells similar to that of lymphoid tissue-derived $CD4^+$ T cells (Fig. 2A and C). In contrast, IFN-γ^+ $CD4^+$ T cells from BAL fluid were predominantly negative for CCR7 (Fig. 2A and C). These

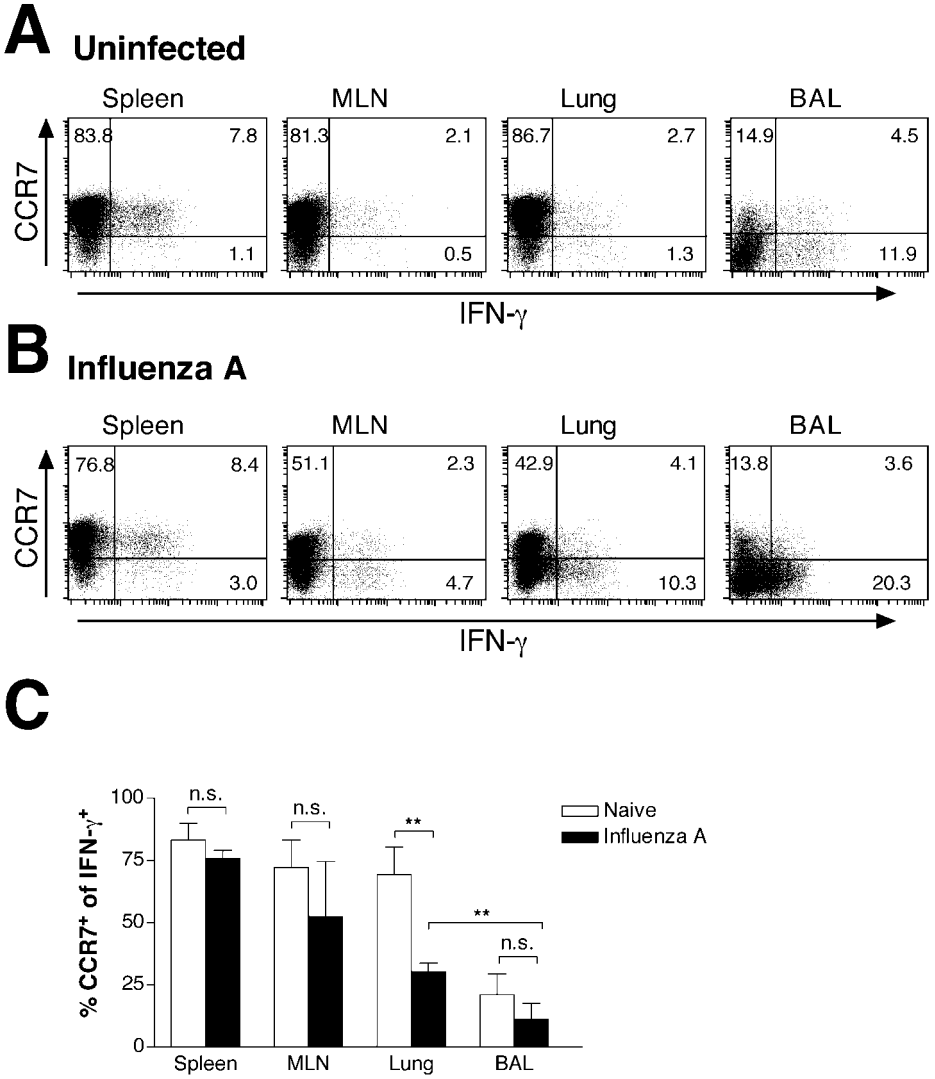

FIG. 2. Flow cytometric analysis of CCR7 surface expression by IFN-γ-producing CD4+ T cells in spleens, MLN, and different lung compartments of naïve and influenza A virus-infected mice. Cells were isolated from the respective organs and organ compartments of naïve BALB/c mice (A) or on day 8 after i.n. infection with the influenza A virus strain x31 (B), polyclonally stimulated for 4 h with PMA-ionomycin, and subsequently stained for surface CD4, CCR7, and intracellular IFN-γ. Cells were gated on CD4+ lymphocytes. Numbers in dot plots indicate percentages of cells in the respective quadrants. One representative experiment (A and B) or a summary of the CCR7 expression by IFN-γ producers in all experiments (C) of a minimum of three independent experiments with cells pooled from the organs or organ compartments of 10 to 15 mice/experiment is shown. Bars represent the means ± SDs. **, $P \leq 0.01$; n.s., not significant.

data reflect differences obtained for total CD4+ T cells in the analyzed organs and/or organ compartments (Fig. 1) and show that effector/memory T cells isolated from a peripheral effector site, namely, the lung parenchyma, express CCR7.

To assess the influence of an ongoing influenza virus infection on CCR7 expression by CD4+ T cells in different lymphoid tissues and lung compartments, we determined the expression of this receptor by IFN-γ+ and IFN-γ− CD4+ T cells in x31-infected mice. The percentages of total IFN-γ+, IFN-γ+ CCR7+, and IFN-γ− CCR7+ CD4+ T cells in the spleen were similar in naïve and infected mice (Fig. 2). As expected, the frequency of IFN-γ-producing CD4+ T cells isolated from the MLN, lung parenchyma, and airways was higher in infected than in uninfected mice (Fig. 2A and B). During influenza A virus infection, IL-4+ CD4+ T cells were not detected in MLN, lung tissue, or BAL fluid (data not shown). In contrast to the other organ compartments analyzed, the percentage of CCR7+ lung-infiltrating IFN-γ+ CD4+ T cells significantly declined during infection ($P = 0.001$) with 30.2% ± 3.62% retaining surface CCR7 (Fig. 2C). In the BAL fluid, only a minority of effector/memory T cells stained positive for CCR7, as was observed in naïve mice (Fig. 2).

The more abundant CCR7 expression by IFN-γ+ CD4+ T cells in lung tissue than in BAL fluid ($P = 0.002$) indicated a greater capacity for recirculation through lymphoid tissues by effector/memory CD4+ T cells originating from the lung parenchyma than by cells from the airways.

IFN-γ-producing CD4+ T cells from the influenza A virus-infected lung migrate toward the CCR7 ligand CCL21. Our findings for IFN-γ+ CCR7+ CD4+ T cells in the lung parenchyma (Fig. 2) differ from recent postulations that effector/memory T cells infiltrating peripheral and inflammatory tissues

were negative for CCR7, which mediates migration into lymphoid tissues (37, 39). Therefore, we performed chemotaxis assays using the CCR7 ligand CCL21 as an attractant to assess the functionality of CCR7 expressed on both the IFN-γ^+ and IFN-γ^- CD4$^+$ T cells isolated from the spleen and lung during an active influenza virus infection. As depicted in Fig. 3A, similar frequencies of IFN-γ^+ cells were detected by intracellular staining after polyclonal stimulation in the CCL21-responsive fraction and in the input cells. In contrast, the frequency of IL-10-expressing CD4$^+$ T cells was reduced in the fraction that migrated (Fig. 3A).

A detailed quantification of the migratory capacity toward CCL21 of the distinct cytokine-positive subsets revealed that, during influenza A virus infection, more than 75% of the splenic IFN-γ single-positive CD4$^+$ T cells (T cells expressing IFN-γ that are negative for IL-10) migrated toward the CCR7 ligand, whereas IL-10 single-positive CD4$^+$ T cells showed a significantly reduced migration to CCL21 ($P = 0.0048$) (Fig. 3B). The data are in accordance with previous results on a high migratory capacity of splenic cytokine producers toward CCL21 (15). A majority of IFN-γ single-positive CD4$^+$ T cells isolated from the lung parenchyma of x31-infected mice also migrated toward CCL21 (58% of input migrating) (Fig. 3B). In contrast, the migration rates of lung-derived IL-10 single-positive and IL-10 IFN-γ double-positive CD4$^+$ T cells toward CCL21 were as low as ~30% of input, which was consistent with decreased CCR7 surface expression as detected by CCL19-Ig (Fig. 3B and data not shown). The migration rate toward CCL21 of total IFN-γ-expressing CD4$^+$ T cells (IFN-γ total, all IFN-γ-expressing CD4$^+$ T cells) was as high as 40% of input (Fig. 3B), reflecting that this population contains high-responsive IFN-γ single-positive and low-responsive IFN-γ IL-10 double-positive CD4$^+$ T cells. The migration rates toward CCL21 correlate well with the frequencies of surface CCR7$^+$ T cells, as detected by staining with CCL19-Ig (Fig. 2B), indicating that this staining method allows identification of functional CCR7 expression of T cells from influenza virus-infected mice.

During an acute influenza A virus infection, virus-specific IFN-γ^+ CD4$^+$ T cells express CCR7 only when they reside in lymphoid tissue. In contrast to our findings of a high frequency of functional CCR7 expression by CD4$^+$ T cells isolated from the lung, it was recently shown that T-cell receptor-transgenic CD4$^+$ T cells recognizing an influenza virus hemagglutinin-derived peptide were negative for CCR7 when isolated from the lung during infection (37). It is possible that effector/memory T cells down-regulate CCR7 upon antigen recognition and differentiation within the inflamed lung. Therefore, influenza virus-specific T cells isolated from the effector site during a viral infection could express lower levels of CCR7 than could the bulk of effector/memory T cells. To test this possibility, we determined the CCR7 expression by virus-specific IFN-γ^+ CD4$^+$ T cells in MLN, lung parenchyma, and BAL fluid.

Cells were isolated from the respective organs or organ compartments on day 8 after i.n. infection with influenza virus, stimulated with inactivated virus or control allantoic fluid for 6 h in the presence of brefeldin A, and subsequently stained for surface CCR7 and intracellular IFN-γ by the use of CCL19-Ig and antibodies, respectively. By this method, variable populations of virus-specific IFN-γ-producing CD4$^+$ T cells could be

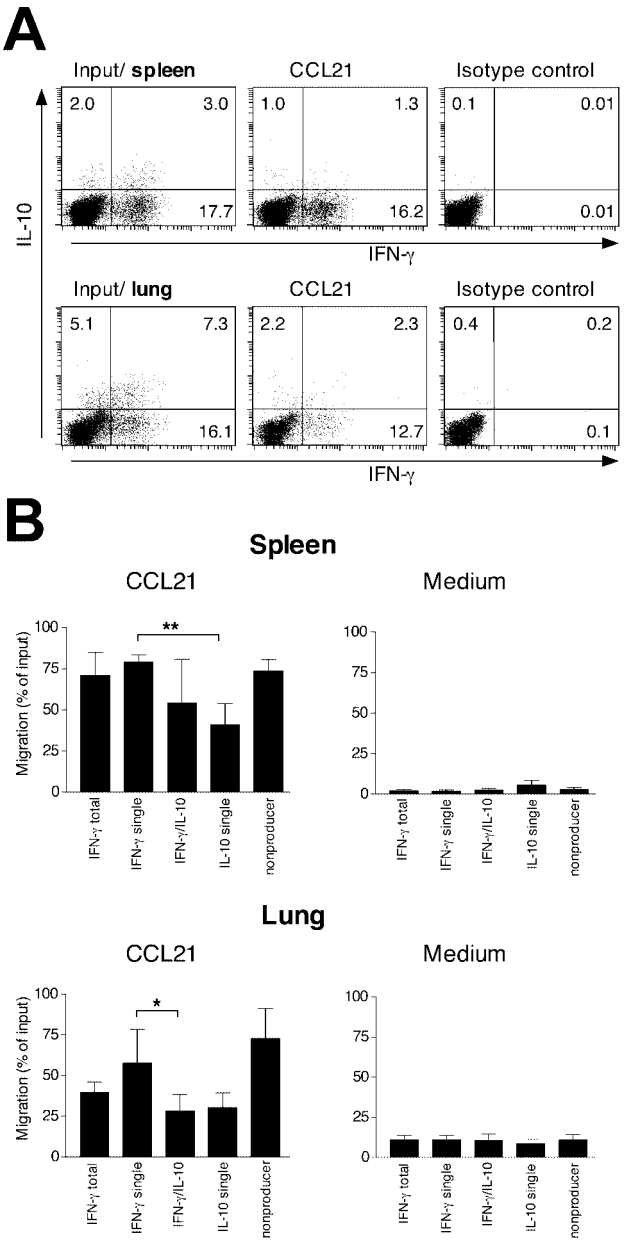

FIG. 3. Chemotactic response profile of IFN-γ- and IL-10-producing CD4$^+$ T cells from spleens and lungs of influenza A virus-infected mice toward the CCR7 ligand CCL21. The migratory response of pooled CD4$^+$ T cells isolated from lungs and spleens of influenza A virus-infected mice toward CCL21 or medium alone was tested in an ex vivo chemotaxis assay, and migration was determined by flow cytometry. For cytokine analysis, input and migratory cells were stimulated with PMA and ionomycin and stained intracellularly for IL-10 and IFN-γ. Results are expressed as the percentages of cells of the respective cytokine subset that migrated to the lower chamber. (A) Flow cytometric analysis of the cytokine profile of the input and migratory cells (gated on CD4$^+$ lymphocytes). Upper row, spleen cells; lower row, lung cells. One experiment representative of four is shown. Numbers in dot plots indicate percentages of cells in the respective quadrants. (B) Migratory response profile of the different cytokine subsets. Subsets marked "single" are positive for the indicated cytokine and negative for the other analyzed cytokine. "IFN-γ total" marks all IFN-γ producers. "Nonproducers" are negative for IL-10 or IFN-γ. Bars represent the means ± SDs of four independent experiments. **, $P \leq 0.01$; *, $P \leq 0.05$.

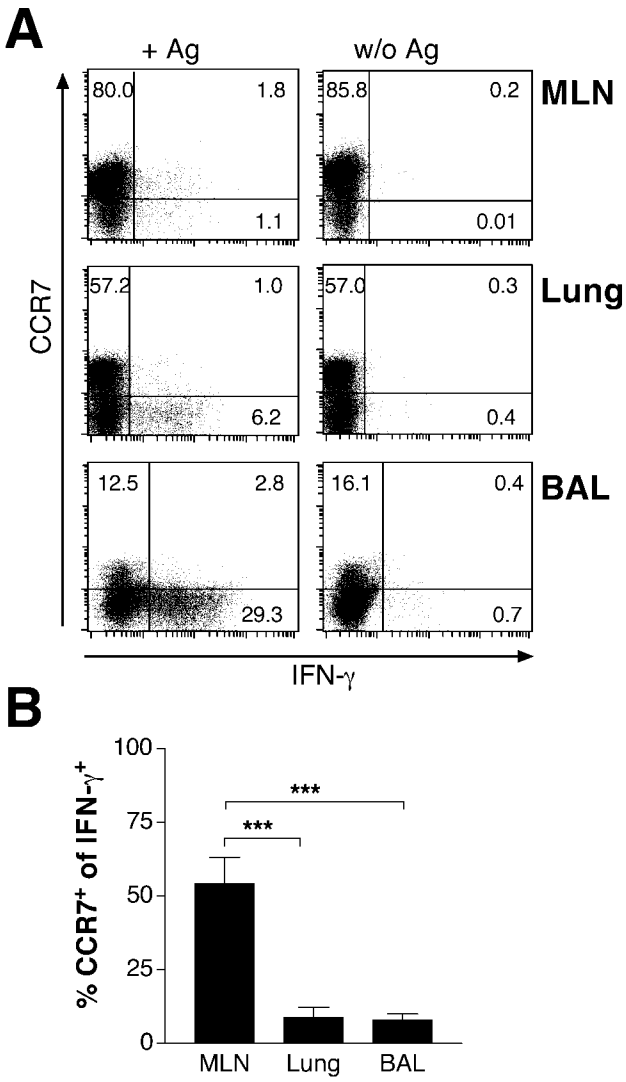

FIG. 4. Flow cytometric analysis of CCR7 surface expression by influenza virus-specific IFN-γ-producing CD4+ T cells. Cells were isolated on day 8 after i.n. infection with the influenza A virus strain x31 from MLN, lung parenchyma, and the lung airways (BAL) and stimulated for 6 h with inactivated virus or control allantoic fluid. Subsequently, the cells were stained for surface CD4, CCR7, and intracellular IFN-γ. Cells were gated on CD4+ lymphocytes. One representative experiment (A) or a summary of the CCR7 expression by IFN-γ producers in all experiments (B) of a minimum of three independent experiments with cells pooled from each organ or organ compartment of 10 to 15 mice/experiment is shown. Bars represent the means ± SDs. ***, $P \leq 0.001$. Ag, antigen. Numbers in dot plots indicate percentages of cells in the respective quadrants.

detected in MLN, lung tissue, and BAL fluid (Fig. 4A), as well as some IL-10-producing CD4+ T cells (data not shown). In contrast, without the addition of virus, very few spontaneous IFN-γ-producing CD4+ T cells were found (Fig. 4A). Virus-specific IFN-γ+ CD4+ T cells were undetectable in the spleen and in tissues from uninfected mice (data not shown). In the BAL fluid, the frequency of IFN-γ+ antigen-specific T cells (Fig. 4A) was even higher than the frequency observed after polyclonal stimulation with PMA-ionomycin (Fig. 2B). This finding may be due to a higher sensitivity to activation-induced cell death (as detected by propidium iodide staining and forward side-scatter changes) by cells of this compartment when such strong stimulatory drugs are used (data not shown). As depicted in Fig. 4, CCR7 expression by antigen-specific IFN-γ+ CD4+ T cells was strongly dependent on the analyzed organ or organ compartment: 54.1% ± 9.1% of the virus-specific IFN-γ+ T cells in the MLN expressed CCR7, whereas only a small fraction of the IFN-γ+ T cells in the lung and BAL fluid were CCR7+ (Fig. 4). We cannot completely rule out that this short-term incubation with inactivated virus used to detect antigen-specific T cells leads to a down-regulation of CCR7; however, the large percentage of T cells from the MLN expressing high levels of CCR7 after in vitro stimulation with viral antigens (Fig. 4A) and the minimal effect of the strong 4-h PMA-ionomycin stimulation on the expression of CCR7 (15) make it very unlikely that our short-term stimulation selectively down-regulates CCR7 expression by virus-specific CD4+ T cells from the lung and BAL fluid but not from the MLN.

The data reveal a different CCR7 expression profile for virus-specific IFN-γ+ CD4+ T cells than for total IFN-γ+ CD4+ cells during influenza A virus infection, suggesting the existence of distinct virus-specific IFN-γ+ effector cell subsets and/or differences in differentiation during an acute infection for T cells at specific anatomical sites.

DISCUSSION

CD4+ T cells play a key role in the primary defense against pulmonary infection and protection against reinfection. Influenza A virus infection can cause severe inflammation of the respiratory tract in a large number of species, in which the Th1-dominated T-cell response is critical in clearing the infection (5, 20).

In this study we asked whether CD4+ T cells that have entered the lung tissue and airways of naïve and influenza virus-infected mice have lost expression of receptors that allow recirculation into lymphoid tissues such as L-selectin and CCR7, an issue still under debate (9, 15, 28, 37, 39). Our results show that the majority of CD4+ T cells isolated from lungs of naïve mice are both L-selectin and CCR7 positive (Fig. 1), which enables them to recirculate back into lymphoid tissues. These results are in contrast to a study with humans where CD4+ T cells from healthy lung tissue were mainly CCR7− (7). It is unclear if this is due to a higher relative age and possible history of lung diseases that may influence the cellular composition of the lung lymphocytes or to species differences.

During influenza virus infection, a high proportion of IFN-γ+ and IFN-γ− lung CD4+ T cells retain the expression of CCR7 and migrate toward its ligand CCL21. This finding clearly argues against a requirement for receptor down-regulation by T cells with immediate effector functions in order to enter inflamed peripheral tissues, as suggested elsewhere (37, 39), and confirms results from studies with humans analyzing T cells from peripheral tissues other than the lung (skin, synovial fluid, and synovium) (9, 28).

In the mouse, the CCR7 ligand CCL21 is constitutively expressed by peribronchial tissue cells (27, 31). Therefore, it is possible that CCR7 is involved in T-cell trafficking to the murine lung, as suggested by others (31). Moreover, CCL21 is expressed by the lymphatic endothelium and participates in the migration of CCR7+ mature dendritic cells from peripheral

tissues into the draining lymph node through the afferent lymphatics (18, 21, 22, 27, 38). It is therefore tempting to speculate that T cells not only require CCR7 to enter most secondary lymphoid tissues from the bloodstream but, like dendritic cells, also use this receptor to leave peripheral tissues and enter afferent lymph vessels to reach the draining lymph node.

$CD4^+$ and $CD8^+$ effector/memory T cells persist in the airways for a long time after infection (23, 25). Moreover, when BAL cells were isolated on day 31 after Sendai virus infection and instilled into the airways of naïve recipients, transferred $CD8^+$ T cells could be found in the BAL fluid but not in lung parenchyma, MLN, or the spleen (23). In these experiments, the same observation has also been made for $CD4^+$ T cells, but not for transferred dendritic cells, which migrated out of the airways and into the MLN (D. L. Woodland, personal communication). In contrast, lymphocytes transferred from MLN or peripheral blood into airways migrated into the lung parenchyma and/or the draining MLN through the afferent lymph vessels, thus reentering the circulation (29, 34). T cells originating from these compartments (lymph nodes and peripheral blood) are known to contain T cells expressing CCR7 and L-selectin (9, 39). These findings indicate that T cells can either leave the airways and reenter circulation or remain sessile in the bronchoalveolar space, depending on their differentiation and receptor profile.

Consistent with this, we found that only a minority of $CD4^+$ T cells from the airways, obtained by BAL, expressed CCR7 or L-selectin. Thus, the lung airways might serve as a highly specialized effector site that is characterized by T cells with low recirculatory capacity (as indicated by $CCR7^{low}$ L-$selectin^{low}$ expression), a more activated phenotype (10, 25), and a stronger ability to produce effector cytokines upon stimulation than that of lung parenchyma-derived T cells (3, 25). These data support the idea that airway T cells are sessile and highly specialized effectors, providing the first line of defense against airborne pathogens by immediate effector function at their entry site.

Recruitment of effector/memory T cells to peripheral tissues is independent of the antigen specificity of the T cells (1), but antigen-specific T cells are trapped at the peripheral site of antigen recognition (36). In the infected lung, both influenza virus-specific and effector/memory $CD4^+$ T cells of other specificities accumulated. Our results suggest that IFN-γ^+ $CD4^+$ T cells of different antigen specificities can enter the lung despite the expression of CCR7. Interestingly, we detected a different CCR7 expression profile for virus-specific IFN-γ^+ $CD4^+$ T cells than for total IFN-γ^+ $CD4^+$ T cells in the inflamed lung; CCR7 expression was barely detectable on influenza virus-specific $CD4^+$ T cells (Fig. 2 and 4). It is tempting to speculate that the loss of CCR7 helps to prevent recirculation and keeps these cells at the site of infection in order to ensure viral clearance. These data are in accordance with a study by Román et al., who detected a lack of CCR7 expression by influenza virus-specific lung $CD4^+$ T cells in a T-cell receptor-transgenic transfer model during infection (37).

Additionally, our analysis revealed that a higher number of virus-specific IFN-γ^+ $CD4^+$ T cells in MLN expressed CCR7 than in the lung parenchyma or airways. These findings suggest the existence of distinct virus-specific effector cell subsets and/or differences in their differentiation or activation stage during the active response to influenza virus at specific anatomical sites. There are several non-mutually exclusive mechanisms that could explain these differences in CCR7 expression by influenza virus-specific IFN-γ^+ T cells from different tissues. (i) The immune response, if initiated in the draining lymph node, could lead to the concurrent development of recirculating $CCR7^+$ IFN-γ^+ and inflammation-seeking $CCR7^-$ IFN-γ^+ effector cell subsets with specialized functions within the MLN (e.g., activation of different classes of antigen-presenting cells, induction of class switch to IFN-γ-induced isotypes, and promotion of Th1/Tc1 development) and the effector site, respectively. (ii) It is conceivable that $CCR7^+$ virus-specific IFN-γ^+ T cells in lymphoid tissue are less differentiated than T cells at the effector site, and they further differentiate into $CCR7^-$ effectors within the lung upon antigen recognition under the influence of infection- and organ-specific factors. (iii) It is possible that an immune response against airborne antigens is initiated not exclusively in the MLN but also in the spleen and respiratory tract-associated lymphoid tissues, such as bronchus- and nasal mucosa-associated lymphoid tissue, and in the lung tissue itself (2, 13, 32, 35, 44), which might lead to the emergence of different phenotypes of effector T cells.

In accordance with our previous data on the chemotaxis of cytokine producers from spleens of naïve mice, the IL-10^+ $CD4^+$ T cells from influenza virus-infected animals exhibited the lowest capacity to migrate toward CCL21 (15). It is known that IL-10 can be immunosuppressive in a number of infections by hampering the clearance of intracellular pathogens (12, 17, 26, 41). On the other hand, the down-modulation of immune responses by IL-10 during infections protects the body from overwhelming destructive inflammatory responses (12, 19, 41). Therefore, the low migratory potential toward CCL21 of IL-10 producers in the lung during influenza may be a mechanism to retain the cells in the lung to prevent severe destruction of the organ during the inflammatory process.

It is still controversial whether CCR7 discriminates different subsets of memory T cells into $CCR7^+$ lymphoid tissue-homing "central memory" T cells that lack the ability to immediately produce effector cytokines upon stimulation and $CCR7^-$ peripheral-homing T cells with immediate effector functions (15, 28, 39, 42). Herein, we provide evidence that CCR7 does not per se distinguish between T cells that reside in lymphoid tissues and those that reside in peripheral tissues. Instead, expression of CCR7 is strongly dependent on the microenvironment from which the cells are isolated, influenced by a local ongoing immune response, and different between bystander and influenza virus-specific T cells.

In conclusion, these data suggest that CCR7 plays a dynamic role in the trafficking of effector/memory T cells through lymphoid and peripheral tissues, where it may function in their entry, retention, or exit. Thus, down-regulation of CCR7 does not simply mark the transition from precursor cells to cytokine-producing effector T cells but rather is the result of antigen-driven differentiation from naïve T cells to circulating effectors and finally late-stage sessile effectors accumulating at distinct peripheral sites.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft, grant SFB421.

We thank Daniel Campbell, Gizette Sperinde, Carrie Arnold, Tohru Sato, and Eugene Butcher for critical comments on the manuscript; Dietmar Zaiss for help with virus production; and Heidi Hecker and Heidi Schliemann for providing antibodies.

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