Lack of CCR7 induces pulmonary hypertension involving perivascular leukocyte infiltration and inflammation

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Lack of CCR7 induces pulmonary hypertension involving perivascular leukocyte infiltration and inflammation. Am J Physiol Lung Cell Mol Physiol 301: L50–L59, 2011. First published April 15, 2011; doi:10.1152/ajplung.00048.2010.—The chemokine receptor CCR7 regulates lymphocyte trafficking, and CCR7 deficiency induces infiltration of T and B cells adjacent to vessels in mouse lungs. Perivascular infiltration of T and B cells has also been found in human pulmonary arterial hypertension, and downregulation of the CCR7 receptor in circulating leukocytes of such patients has been observed. To investigate whether changes in the CCR7 system contribute to the pathogenesis of pulmonary hypertension, we utilized mice deficient of the CCR7 receptor. The cardiopulmonary and inflammatory responses of CCR7 depletion were evaluated in CCR7-deficient and wild-type mice. Measurements of cytokines upregulated in the animal model were also performed in patients with pulmonary hypertension and controls and in vascular smooth muscle cells. We found that mice lacking CCR7 had increased right ventricular systolic pressure, reduced pulmonary artery acceleration time, increased right ventricular/tibial length ratio, Rho kinase-mediated pulmonary vasoconstriction, and increased muscularization of distal arteries, indicating pulmonary hypertension. These mice also showed increased perivascular infiltration of leukocytes, consisting mainly of T and B cells, and increased mRNA levels of the inflammatory cytokines interleukin-12 and CX3CL1 within pulmonary tissue. Increased serum levels of interleukin-12 and CX3CL1 were also observed in patients with pulmonary hypertension, particularly in those with pulmonary hypertension associated with connective tissue disorder. In smooth muscle cells, interleukin-12 induced secretion of the angiogenic cytokine interleukin-8. We conclude that these results suggest a role for CCR7 in the development of pulmonary arterial hypertension, at least in some subgroups, possibly via pulmonary infiltration of lymphocytes and secretion of interleukin-12 and CX3CL1.

PULMONARY HYPERTENSION is defined as a mean pulmonary artery pressure >25 mmHg at rest or >30 mmHg during exercise.

Pulmonary arterial hypertension (PAH), a syndrome resulting from restricted flow through the pulmonary arterial circulation, ultimately leading to right-sided heart failure and premature death, is one of five groups of pulmonary hypertension (35). Accumulating evidence indicates important roles of inflammatory mechanisms in the pathogenesis of PAH, especially in PAH associated with connective tissue disorder and other forms of immune-mediated diseases (e.g., human immunodeficiency virus infection) (17, 22). Lung histology from patients with associated PAH (APAH) has revealed perivascular infiltrates of inflammatory cells consisting of T and B cells and macrophages, as well as other markers of immune-mediated pathology (e.g., enhanced pulmonary expression of inflammatory cytokines) (3, 6, 11). The role of inflammation seems not to be restricted to APAH, and increased pulmonary expression of various inflammatory mediators has also been found in other forms of PAH, such as idiopathic PAH (IPAH) (13, 46). However, at present, the potential role of inflammation, including inflammatory cells within pulmonary tissue, in the pathogenesis of PAH is not clear (6, 17).

The migration of lymphocytes from the bloodstream into peripheral tissues, such as the lungs, is, at least partly, regulated by chemokines. This activity seems not to be restricted to the classical inflammatory chemokines. The regulatory or homeostatic chemokines CCL19 and CCL21 are through their receptor CCR7 centrally involved in immune surveillance and regulation of leukocyte movement during homeostasis. Their primary function is thought to be the control of homing of naïve T cells and antigen-presenting dendritic cells to lymph nodes. Recently, however, a role for this chemokine system has also been revealed in inflammation and T-cell homing into nonlymphoid tissue, as well as in the regulation of regulatory T-cell (Tregs) trafficking, implying a more complex role of CCR7 in immune responses (36, 39). It has been observed that mice lacking the CCR7 receptor have perivascular accumulation of lymphocytes in the lungs (19, 30), suggesting a role for CCR7 in the regulation of normal pulmonary leukocyte homeostasis. The perivascular accumulation of lymphocytes resembles findings in patients with PAH. In a study of subgroups of PAH patients, analysis of gene expression profiles from circulating mononuclear cells showed reduced CCR7 gene
expression (5). Although this finding was not confirmed by polymerase chain reaction, it may potentially suggest impairment of the CCR7 system in PAH. Furthermore, patients suffering from IPAH have a reduction in naïve CCR7-positive T cells and an increase in CCR7-negative cytotoxic T cells in the circulation (2), the latter being able to release cytokines and induce inflammation. These patient data show that changes in the CCR7 system occur in PAH, and thus we were prompted to study whether the CCR7 receptor may play a role in the pathogenesis of pulmonary hypertension. For this purpose, mice lacking CCR7 (CCR7−/−), the receptor for CCL19 and CCL21, were examined with regard to signs of pulmonary hypertension. We hypothesized that the lack of CCR7 could induce pulmonary hypertension via induction of perivascular infiltration of leukocytes in the lungs and increased release of cytokines and chemokines within the pulmonary tissue.

MATERIALS AND METHODS

Animal model. The animals were housed with a 12:12-h day-night cycle at 21°C, and food and water were available ad libitum. A total of 40 11-wk-old and 12 4-wk-old male CCR7−/− mice with the C57Bl/6 background (19) and 38 11-wk-old and 12 4-wk-old C57Bl/6 wild-type (WT) mice were included. The CCR7−/− mice were constructed at the Max-Delbrück-Center for Molecular Medicine (Berlin, Germany) and bred at the animal facility of the Institute for Experimental Medical Research at Oslo University Hospital (Oslo, Norway), according to institutional and state guidelines. Cardiac pressure measurements and Doppler echocardiography were performed under general anesthesia with inhalation of 1.5% isoflurane (32). Blood samples were drawn quickly from the inferior vena cava under general anesthesia (31). For histological examinations, mice were anesthetized and bled to death by cutting vena cava inferior, and 4% paraformaldehyde was infused into the lung at 20-cmH2O perfusion pressure via a tracheal tube (54). Subsequently, the lungs were removed and fixed for 24 h in 4% paraformaldehyde. Lung tissue for mRNA isolation was obtained from mice euthanized by dislocation of the neck in isoflurane anesthesia. The heart and lungs were rapidly excised, the right ventricular (RV) and left ventricular (LV) free walls were separated from the septum, weighed, and snap-frozen in liquid nitrogen. Ventricular weights were normalized to tibial length (TL) to measure changes in the two ventricles separately, as previously described (32, 49). Lung weights were also related to TL (32, 49). There were no significant changes in TL (16.7 ± 0.1 vs. 16.8 ± 0.1, CCR7−/− and WT group, respectively, \( P = 0.23 \)).

Hemodynamic measurements. Doppler echocardiography was utilized to measure the pulmonary artery acceleration time (PAAT) at the pulmonary artery root and cardiac output in the ascending aorta in CCR7−/− and WT mice (32). The RV systolic pressure (RVSP) was assessed with a micropressure transducer (Samba Preclin 420 LP transducer, Samba Sensors AB, Västra Frölunda, Sweden) inserted in the RV via the right internal jugular vein (59), and the cutoff for recording of heart rate was set at 400 beats/min. Data from 10 consecutive beats were recorded by a custom-made program designed in LabView (National Instruments, Monchengladbach, Germany) and analyzed by using DIAdem 10.2 (National Instruments). These hemodynamic measurements were performed in a blinded manner. To explore a possible pulmonary vascular reactivity, RVSP measurements were performed in CCR7−/− and WT mice, which were tracheotomized and connected to a rodent ventilator (model 874092, B. Braun, Melsungen, Germany) at baseline (room air) and after inhalation of 40 ppm nitric oxide (NO) (37) for 5 min using the INOvent delivery system (Datex-Ohmeda, Madison, WI) (28). The injector module of NO was adapted into the circuit, and gas samples were taken immediately before the ventilator to control that inhaled NO was 40 ± 1 ppm. To test the NO inhalation system, three WT mice inhaled 10% oxygen (hypoxia) for 5 min (37), followed by additional inhalation of 40 ppm NO for 5 min, which resulted in significant reduction in RVSP (35 ± 3 vs. 26 ± 1 mmHg, hypoxia and hypoxia + NO, respectively, \( P < 0.05 \)). RVSP measurements were also performed with the vasodilator fasinil before (baseline) and 5 min after a bolus injection of 30 mg/kg fasinil (LC Laboratories, Woburn, MA) (15) into the left jugular vein by using a CMA/100 microinjection pump (CMA/Microdialysis, Solna, Sweden).

Measurement of pulmonary RhoA activity. Pulmonary levels of GTP-loaded RhoA were assessed with the G-LISA RhoA Activation Assay (luminescence based; Cytoskeleton, le Pery-en-Yvelines, France), as suggested by the manufacturer (15). Total levels of RhoA were determined using Western blotting, as previously described (61), using a monoclonal RhoA antibody (no. 2117; Cell Signaling Technology, Beverly, MA).

Histology. For histological and morphometric examinations, six lungs from CCR7−/− and WT mice were fixed in 4% paraformaldehyde and embedded in paraffin. The lungs were sectioned transversely (5 μm) and stained with hematoxylin and eosin.

Immunohistochemistry and morphometric analysis. Formalin-fixed, paraffin-embedded sections were deparaffinated, rehydrated, and de-masked in a microwave oven for 24 min in 0.01 M sodium citrate buffer (pH 6.0), Tris/ETDA buffer (pH 9.1), or target retrieval solution (pH 6.00–6.20) (Dako, Glostrup, Denmark). The sections were subjected to staining by using DAKO autostainer (Dako) and the following primary antibodies: polyclonal rabbit anti-mouse CD3 (1:50) and rabbit anti-human smooth muscle α-actin (1:500; both from Abcam, Cambridge, MA), monoclonal rat anti-mouse CD45R/B220 (1:4,500), monoclonal rat anti-mouse forkhead-winged transcriptional factor box protein 3 (FoxP3, 1:50), and monoclonal rat anti-mouse F4/80 (1:100; all from eBioscience, San Diego, CA), and polyclonal rabbit anti-human von Willebrand factor (1.500, Dako). Differential cell counts of leukocytes in perivascular infiltrates were performed manually. The total number of peripheral arteries at alveolar duct and wall level was calculated as the number of arteries positive for the endothelial cell marker von Willebrand factor per 100 alveoli, based on assessments of five fields. Muscularization of arteries was identified by a positive immunostaining with antibody against smooth muscle α-actin and categorized as fully (75–100% of medial layer covered by anti-smooth muscle α-actin staining), partially (51–74% of medial layer covered by anti-smooth muscle α-actin staining) muscularized alveolar wall and alveolar duct arteries (magnification ×200 for assessments of muscularization and number of arteries) (18). Muscularization was assessed by comparing fully and partially muscularized alveolar wall and duct arteries to the total number of peripheral arteries (37). All microscopic assessments were performed in a blinded manner.

Quantitative real-time PCR. A real-time quantitative PCR system (ABI 7900HT Fast Real-Time PCR System, PE Biosystems, Foster City, CA) was used to measure the mRNA amounts of interleukin (IL)-1β, IL-6, IL-10, IL-12, IL-17, IL-18, IL-23, IL-27, interferon-γ, tumor necrosis factor-α, keratinocyte-derived chemokine (KC), transforming growth factor-β, activin A, CX3C1L1 (fraktalkine), CX3CR1 (fraktalkine receptor), and endothelin-1 in the left lung from CCR7−/− and WT mice. Total mRNA was isolated by using spin total RNA isolation system (Promega, Madison, WI). All RNA samples were quality assessed by Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) and RNA integrity numbers. The RNA samples were reverse transcribed by using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Specific mRNA transcripts were quantified by Taqman GA assays (Applied Biosystems, Foster City, CA). All samples were tested in triplicate, and average values were used for quantification. The average value of each cytokine and chemokine was calculated as the number of arteries positive for the endothelial cell marker von Willebrand factor per 100 alveoli, based on assessments of five fields. Muscularization of arteries was identified by a positive immunostaining with antibody against smooth muscle α-actin and categorized as fully (75–100% of medial layer covered by anti-smooth muscle α-actin staining), partially (51–74% of medial layer covered by anti-smooth muscle α-actin staining) muscularized alveolar wall and alveolar duct arteries (magnification ×200 for assessments of muscularization and number of arteries) (18). Muscularization was assessed by comparing fully and partially muscularized alveolar wall and duct arteries to the total number of peripheral arteries (37). All microscopic assessments were performed in a blinded manner.

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OptiMem (Invitrogen, Paisley, UK), with and without IL-12 (30 ng/ml, R&D Systems, Minneapolis, MN), IL-1β (1 ng/ml, R&D Systems), or a combination thereof. After 24 h, cell-free supernatants were harvested and stored at −80°C until further analyses. In the proliferation experiments, the cells were stimulated with IL-12 (30 ng/ml) and/or IL-1β (1 ng/ml), and [3H-methyl]thymidine (2.5 μCi/ml; Amersham Biosciences, Buckinghamshire, UK) was added simultaneously. After an additional 24 h, cells were harvested and analyzed as previously described (61). In all experiments, vehicle was used as controls.

Enzyme-linked immunosorbent assay. The serum levels of CX3CL1 and IL-12p40 were assayed in CCR7−/− and WT mice and in patients with pulmonary hypertension and healthy controls by enzyme-linked immunosorbent assays (ELISAs) obtained from R&D Systems. Concentrations of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 in pulmonary arterial SMC supernatants were measured by ELISA (R&D Systems). Concentrations of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 in pulmonary arterial SMC supernatants were measured by ELISA (R&D Systems). All intra- and interassay coefficients of variance were <10%.

Patients and controls. Twenty-two patients with pulmonary hypertension (mean pulmonary arterial pressure > 25 mmHg at rest, with a normal pulmonary capillary wedge pressure < 12 mmHg), in New York Heart Association functional classes III-IV, referred to our tertiary center for the characterization of suspected chronic precapillary pulmonary hypertension, were recruited consecutively in the time period between 2000 and 2006. The study population was divided into three groups according to the type of pulmonary hypertension: 1) patients with IPAH (n = 7; 2 men and 5 women, 38 ± 18 yr); 2) patients with PAH (n = 9; 4 men and 5 women, 46 ± 12 yr; connective tissue disorder (n = 8) and human immunodeficiency virus infection (n = 1)); and 3) patients with chronic thrombotic and/or embolic pulmonary hypertension (CTEPH) disease (n = 6; 2 men and 4 women, 61 ± 12 yr) verified with pulmonary angiograms. Nine sex- and age-matched individuals (4 men and 5 women, 50 ± 14 yr) underwent right-sided heart catheterization during electrophysiological studies of supraventricular arrhythmias, but with otherwise normal hemodynamic function and myocardial structure, were control subjects. All patients and controls were Caucasians, and age values are means ± SD. Serum samples were obtained from the femoral artery (9).

Ethics. The animal experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and were approved by the Norwegian National Animal Research Committee. The regional ethical committee approved the study on PAH patients and healthy controls according to the Declaration of Helsinki, and informed consent was obtained from each person.

Statistical analysis. Data are presented as means ± SE, unless otherwise stated. Comparisons between two groups were made by unpaired Student’s t-test or the nonparametric Mann-Whitney rank sum test, depending on the distribution of data (SigmaStat 3.1.1, Systat Software, Richmond, CA). When more than two groups were compared, correction for multiple testing was performed using Dunn’s method. Differences were considered significant for P < 0.05.

RESULTS

CCR7−/− mice have pulmonary hypertension. Eleven-week-old CCR7−/− mice had significantly increased RVSP compared with WT mice, as determined by RV catheterization (Fig. 1A). Also, Doppler echocardiography showed that the CCR7−/− group had a significant reduction in PAAT compared with the WT mice (Fig. 1B), without any significant changes in cardiac output (25.0 ± 2.1 vs. 26.3 ± 1.7 ml/min, CCR7−/− and WT group, respectively; P = 0.63), indicating pulmonary hypertension. The CCR7−/− group also had increased RV weight-to-TL ratio and increased lung weight-to-TL ratio compared with WT mice (Fig. 1, C and D), without any differences in the LV weight-to-TL ratio (data not shown), further supporting increased RV afterload and pulmonary hypertension in CCR7−/− mice. Consistent with the elevated RVSP in CCR7−/− mice, there was increased muscularization of distal arteries at alveolar duct and alveolar wall level in CCR7−/− mice (45 ± 6 vs. 21 ± 2% muscularization of distal arteries, CCR7−/− and WT group, respectively, P < 0.05, n = 6/6, Fig. 2, A and B), without any changes in the density of peripheral arteries (7.5 ± 0.7 vs. 8.8 ± 0.5 arteries/100 alveoli, CCR7−/− and WT group, respectively, P = 0.17). Interestingly, 4-wk-old CCR7−/− mice had no significant changes in PAAT (17.7 ± 0.9 vs. 19.5 ± 2.3 ms, CCR7−/− and WT group, respectively, P = 0.47, n = 6/6) or RV weight-to-TL

![Fig. 1. Pulmonary hemodynamics, right ventricular (RV), and lung weights. A: RV systolic pressure (RVSP) in CCR7−/− (open bars) and wild-type (solid bars) mice (n = 10 and 5, respectively). B: pulmonary artery acceleration time (PAAT) (n = 6 each). C: RV weight-to-tibial length ratios (n = 15 and 11, respectively). D: lung weight-to-tibial length ratios (n = 15 and 11, respectively). Values are means ± SE, *P < 0.05 vs. wild-type mice.](http://ajplung.physiology.org/)

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CD3+ T cells (61% of the leukocytes) (Fig. 3B). Also B cells (B220+) contributed substantially to the accumulation of lymphocytes (34% of the leukocytes) (Fig. 3D). FoxP3+ T cells, indicating Tregs, were scattered throughout infiltrates (Fig. 3F), and macrophages (F4/80+) were found in the vicinity of the leukocyte infiltrates (Fig. 3H).

Expression of cytokines and chemokines in lung tissue. Our findings so far suggest that CCR7 deficiency induces a mild, but significant, degree of pulmonary hypertension, accompanied by perivascular infiltration of B cells and, in particular, of T cells in the lungs. To investigate whether the observed infiltration of leukocytes in the lungs of CCR7−/− mice may alter the expression of selected mediators, mRNA levels of 16 cytokines and chemokines were measured (see MATERIALS AND METHOD section).

Fig. 3. Cellular composition of perivascular pulmonary lymphoid infiltrates from CCR7−/− (B, D, F, and H) compared with wild-type (A, C, E, and G) assessed by immunohistochemical staining. A and B: T cells (CD3+). C and D: B cells (B220+). E and F: regulatory T cells (FoxP3+). G and H: macrophages (F4/80+). Original magnification, ×400 (A–H).

ratio (0.6 ± 0.02 vs. 0.6 ± 0.03 mg/mm, CCR7−/− and WT group, respectively, P = 0.39, n = 6/6), indicating that CCR7−/− mice had not developed pulmonary hypertension at this time point.

To test if pulmonary vasoconstriction contributed significantly to the pulmonary hypertension in CCR7-deficient mice, pulmonary vascular reactivity was tested by inhalation of NO, and notably NO induced a significant reduction in RVSP in CCR7−/− mice (31 ± 1 vs. 24 ± 1 mmHg, baseline and NO, respectively, P < 0.05, n = 6). No significant changes were found in WT mice (25 ± 1 vs. 24 ± 1 mmHg, baseline and NO, respectively, n = 6). In line with this, the Rho-kinase inhibitor fasudil induced a significant decrease in RVSP in CCR7−/− mice (31 ± 1 vs. 25 ± 1 mmHg, baseline and fasudil), respectively, P < 0.05, n = 6). No significant changes were measured in WT mice (26 ± 1 vs. 25 ± 1 mmHg, baseline and fasudil, respectively, n = 6). This prompted us to investigate pulmonary RhoA activity, and the levels of GTP-loaded RhoA were increased in CCR7−/− mice (241 ± 18 vs. 100 ± 4%, CCR7−/− and WT group, respectively, P < 0.001, n = 8/10). The amount of RhoA was not significantly changed in CCR7-deficient mice (108 ± 7 vs. 100 ± 6%, CCR7−/− and WT group, respectively, n = 8/10). These findings indicate that RhoA/Rho kinase-mediated vasoconstriction contributes to the pulmonary hypertension in CCR7 deficiency.

CCR7−/− mice have perivascular lymphoid infiltrates. Histological analysis showed considerable perivascular infiltration of leukocytes in the CCR7−/− group, which was not present in the WT group (Fig. 2, C and D). Morphologically, the majority of the cells in these infiltrates were mature lymphocytes, which surrounded pulmonary arteries and bronchi, and by immunohistochemistry we showed that the majority of these cells were...
METHODS). As shown in Fig. 4, pulmonary tissue from CCR7-deficient mice showed enhanced mRNA levels of IL-12 p40, CX3CL1 and its corresponding receptor CX3CR1, KC (the mouse homolog of human IL-8 and growth-related oncogene-α), and IL-10, compared with pulmonary tissue from WT mice. None of the other measured cytokines/chemokines showed differences between CCR7−/− and WT mice (data not shown).

Levels of circulating IL-12p40 and CX3CL1. Our laboratory and others have previously studied circulating levels of IL-8 and IL-10 in patients with pulmonary hypertension (9, 33, 56). To further explore the relevance of the upregulated mediators in lung tissue in CCR7−/−, we examined serum levels of IL-12p40 and CX3CL1 in patients with pulmonary hypertension. As shown in Fig. 5, serum levels of IL-12p40 and CX3CL1 were significantly raised in the APAH group (n = 9), and of IL-12 p40 also in the IPAH group (n = 7), compared with controls (n = 9). The concentrations of IL-12p40 and CX3CL1 were not changed in the CTEPH group compared with controls (n = 6, Fig. 5, A and B). In CCR7−/− mice (n = 9), serum levels of IL-12 p40, but not of CX3CL1, were markedly increased compared with levels in WT mice (n = 9, Fig. 5, C and D).

IL-12 induces IL-8 secretion in pulmonary artery SMC. Our findings show markedly enhanced expression of IL-12 p40, both in the circulation and in pulmonary tissue in CCR7−/− mice, as well as in serum from patients with APAH and IPAH. In contrast to CX3CL1 (3, 42), which was upregulated in pulmonary tissue from CCR7−/− mice and in serum from APAH patients, IL-12 has previously not been examined with regard to the pathogenesis of pulmonary hypertension. We, therefore, investigated the effect of IL-12 on proliferation and cytokine secretion (i.e., IL-6, IL-8, and MCP-1, all with relevance to PAH pathology) (9, 23, 25, 27, 48) in vascular SMC from pulmonary arteries. While IL-12 had no effect on cell proliferation (data not shown), IL-12 stimulation of SMC for 24 h resulted in increased secretion of IL-8 and MCP-1, although the increase in MCP-1 did not reach statistical significance (P = 0.06; Fig. 6, A and B). IL-1β is a potent activator of vascular SMC and has been related to the pathogenesis of PAH (21), and, interestingly, IL-12 augmented the IL-1β-induced release of MCP-1 (Fig. 6B). IL-12 had no significant effect on the IL-1β-induced release of IL-8 (Fig. 6B) and had no effect on IL-6 either alone or in combination with IL-1β (Fig. 6C).

Fig. 4. Pulmonary mRNA levels of selected mediators. A: the amount of interleukin (IL)-12p40 mRNA normalized to RPL32 mRNA in CCR7−/− mice (open bar) was compared with wild-type (solid bar), which was set to 100%. B: CX3CL1 (fraktalkine). C: CX3CR1 (fraktalkine receptor). D: keratinocyte-derived chemokine (KC). E: IL-10. Values are means ± SE (n = 6 wild type and 6 CCR7−/− for all). *P < 0.05 vs. wild-type mice.
In the present study, we show that CCR7-deficient mice develop perivascular lymphocyte infiltrates in pulmonary tissue consisting primarily of B and, in particular, of T cells. The leukocyte infiltrates were accompanied by an increase in pulmonary arterial pressure compared with WT mice, as shown by 1) elevated RV pressure, 2) reduced PAAT, 3) increased RV weight, 4) increased muscularization of peripheral pulmonary arteries, and 5) pulmonary arterial vasoconstriction. In addition, pulmonary tissue from CCR7⁻/⁻ animals showed enhanced mRNA levels of IL-12 p40, CX3CL1, CX3CR1, KC, and IL-10, supporting a net inflammatory pulmonary phenotype in CCR7-deficient mice. Increased IL-12 and CX3CL1 levels were also seen in a subgroup of PAH patients (i.e., APAH), also known to have perivascular leukocyte infiltrates in their lungs. Our findings further suggest a role for inflammation in the pathogenesis of PAH, potentially involving a dysregulated CCR7 system, at least in certain diagnostic subgroups.

Inflammatory mechanisms seem to play an important role in the pathogenesis of PAH (10, 43, 55). Thus studies in animal models, as well as some clinical studies, have suggested that...
inflammatory cytokines and chemokines are involved in the development of pulmonary hypertension (3, 12, 16, 34, 53). In human PAH, the identification of perivascular inflammatory cell infiltrates consisting of T cells, B cells, and macrophages additionally support the concept of inflammatory mechanisms in some types of PAH (3), especially in APAH. In the present study, the pulmonary hypertension found in CCR7-deficient mice was accompanied by increased amounts of T and B cells in perivascular leukocyte infiltrates, consistent with findings in subgroups of human PAH (6, 22). Interestingly, depletion of CD4⁺ T cells inhibited pulmonary arterial muscularization in a mouse model of antigen-induced pulmonary arterial remodeling (8), indicating that T cells play a role in the process of muscularization in pulmonary arteries. Our finding further supports a role for CCR7 in the regulation of lymphocyte trafficking in pulmonary tissue, and the lack of this homeostatic chemokine receptor seems to result in an inflammatory phenotype within the lung.

Several studies have suggested a role for chemokines in the pathogenesis of PAH (3, 11, 42, 46), and our data suggest that this should not be restricted to the so-called inflammatory chemokines. Previous studies have demonstrated that an imbalanced regulation of CCR7 may influence, not only lymphocyte trafficking to and within secondary lymphoid organs, but also migration of T and B lymphocytes through nonlymphoid tissues (19, 30). The present study suggests that the lack of this receptor could result in an inflammatory phenotype within pulmonary tissue, potentially contributing to development of pulmonary hypertension. The perivascular leukocyte infiltrates in the lungs may together with enhanced muscularization of peripheral pulmonary arteries, and a tendency to higher density of peripheral arteries, account for a higher lung weight in mice depleted of the CCR7 receptor.

A major finding in the present study was that CCR7⁻/⁻ mice showed enhanced pulmonary expression of IL-12 p40 and CX3CL1, and, importantly, increased expression of these cytokines was also found in clinical PAH, with increased serum levels in patients with APAH, and for IL-12p40, also in IPAH, which is in accordance with recent studies in which increased serum level of IL-12p70 predicted survival in IPAH patients (50, 57). In contrast, CTEPH patients, who do not have perivascular infiltration of T and B lymphocytes in the lungs (38), had no significant changes in the circulating levels of IL-12p40 and CX3CL1, indicating that the upregulated mediators in IPAH and APAH were not merely a response to elevated pulmonary arterial pressure. Even though the serum concentration of CX3CL1 in CCR7-deficient mice was not changed in venous blood, which can be due to dilution in the blood, local effects of CX3CL1 on lung vessels may occur.

While several studies have suggested a pathogenic role for CX3CL1 in PAH (3, 42), the role of IL-12 in pulmonary hypertension is, to our knowledge, not known. IL-12 plays an important role in the inflammatory interaction between T cells and macrophages (20), but the effect of IL-12 is not restricted to leukocytes and leukocyte-derived cells. Thus, in our study, we found that IL-12 induced enhanced release of IL-8 and augmented the IL-1β-mediated increase in MCP-1 in pulmonary artery SMC. We do not know whether similar results would be found in SMC from PAH patients, which is a limitation of our study. Additionally, it has been shown that
adenoviral IL-12 gene transduction in bronchial epithelial cells leads to increased production of IL-8 and IL-6 (40), which have both been claimed to play a role in the pathogenesis of PAH (7, 47, 48, 51). In our study, the pulmonary mRNA levels of IL-6 and IL-1β were not altered in CCR7−/− mice, which is in contrast to increased levels of these cytokines in, for instance, the monocrotaline model of pulmonary hypertension (4, 14). However, in that model, adventitial macrophage accumulation has been suggested to be of importance, and to a lesser extent infiltration of lymphocytes (52, 58), being different from the T- and B-cell infiltration in CCR7−/− mice, which developed pulmonary hypertension spontaneously. Interestingly, recent studies suggest that CX3CR1 is preferentially expressed on Th1 cells that induce the production of IL-12, suggesting a link between CX3CR1 and IL-12 in CCR7-deficient mice. Moreover, we found that the pulmonary expression of KC, the mouse ortholog of IL-8, was increased in CCR7−/− mice. IL-8 is an angiogenic cytokine that acts as a chemoattractant and mitogen for SMC (29, 62). It is possible that the inflammatory interaction between IL-12 and IL-8 may contribute to vascular remodeling and the development of pulmonary hypertension in CCR7−/− mice. In addition to being angiogenic, IL-8 also has proinflammatory properties (45). IL-8 has the potential to activate RhoA (60), and it is possible that the increased RhoA activity in CCR7−/− mice could be related to the high pulmonary levels of the IL-8 analog KC observed in these mice. Our experiment with Rho kinase inhibition indicates that the pulmonary hypertension observed in CCR7 deficiency can be related to RhoA/Rho kinase-mediated vasoconstriction. Thus there might be a link between increased RhoA/Rho kinase signaling and pulmonary inflammation, involving the proinflammatory cytokine IL-8. Nonetheless, our findings clearly underscore the complexity of the cytokine network that could contribute to the pathogenesis of pulmonary hypertension.

In addition to several inflammatory cytokines, CCR7−/− mice also showed higher expression of IL-10 within the lungs. In a rat model of pulmonary hypertension, IL-10 delivery reduced vascular SMC proliferation and prevented the development of pulmonary hypertension (24), and another experimental study indicates that IL-10 could be a mediator in the treatment of pulmonary hypertension by utilizing a type of endothelial progenitor cells (41). However, although CCR7−/− mice showed increased expression of IL-10 within pulmonary tissue, these mice seem to have a net inflammatory phenotype, as shown by increased expression of IL-12 p40, CX3CL1, CX3CR1, and KC in the lung. Moreover, while IL-10 is regarded as a prototypical anti-inflammatory cytokine, persistent IL-10 activation may promote inflammation through B-cell-mediated mechanisms (1, 44), a cell type that was abundant within pulmonary tissue in CCR7−/− mice. Finally, increased IL-10 levels could potentially reflect increased activation of Tregs, but the lack of increase in pulmonary transforming growth factor-β, another Treg-derived cytokine, makes this conclusion more unlikely.

Our findings in the present study may suggest the involvement of CCR7-related mechanisms in the pathogenesis of PAH. This is supported by the finding of downregulation of the CCR7 receptor in circulating mononuclear cells from PAH patients (5) and also by the increase in CCR7-negative cytotoxic T cells in IPAH (2). Interestingly, patients suffering from connective tissue disorders, who may develop APAH, can have a rare mutation in the promoter region of the CCR7 gene (26). Kahllmann et al. (26) tested whether this genetic variant had any effect on CCR7 expression by cloning a fragment of the WT and the variant promoter into a firefly luciferase reporter plasmid, and they found that the activity of the mutant promoter was reduced by 50% compared with a WT promoter, indicating that the mutation can lead to reduced function of the CCR7 receptor, as in CCR7−/− mice. Further genetic studies searching for mutations in the CCR7 gene, especially in patients with APAH, would be of interest to clarify a possible role of the CCR7 receptor in human PAH.

In summary, mice that lack the chemokine receptor CCR7 express infiltrates of inflammatory cells surrounding pulmonary vessels and show a net inflammatory phenotype within pulmonary tissue, accompanied by pulmonary hypertension. These findings further underscore a role for CCR7 in the regulation of pulmonary inflammation, and our results also suggest the involvement of CCR7-related mechanisms in the pathogenesis of PAH, at least in certain diagnostic subgroups. In the authors’ opinion, further studies linking CCR7 deficiency, pulmonary inflammation, and PAH are needed.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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