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Cigarette smoke-induced pulmonary inflammation, but not airway remodelling, is attenuated in chemokine receptor 5-deficient mice

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Experimental
Allergy

Summary

Background Chronic obstructive pulmonary disease (COPD) is characterized by a chronic inflammatory response of the airways and lungs to noxious particles and gases, mostly cigarette smoke (CS). Pathological changes characteristic of COPD include airway wall thickening, peribronchial fibrosis, peribronchial lymphoid follicles and destruction of lung parenchyma (emphysema). The recruitment of inflammatory cells into the lung in response to CS is thought to play an important role in the development of COPD.

Objective Our aim was to study the contribution of chemokine receptor 5 (CCR5) to the pathogenesis of COPD and specifically whether the development of airway remodelling is a direct result of airway inflammation or rather occurs through an independent mechanism.

Methods In this study, C57BL/6 wild-type mice and CCR5-deficient mice were subjected to sub-acute (4 weeks) and chronic (24 weeks) CS exposure.

Results Both sub-acute and chronic CS exposure significantly increased CCR5 mRNA expression and protein levels of CCR5 ligands [macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β and regulated upon activation, normal T expressed and secreted (RANTES)], and induced the recruitment of neutrophils, macrophages, dendritic cells, and lymphocytes to the bronchoalveolar lavage (BAL) of wild-type mice. Chronic CS exposure also increased the number and extent of peribronchial lymphoid follicles. In CCR5 knockout (KO) mice, these CS-induced increases in CCR5 ligands, inflammatory cells in BAL and peribronchial lymphoid follicles were all significantly attenuated compared with wild-type animals. Importantly, chronic CS exposure induced airspace enlargement in wild-type mice, while CCR5 KO mice were partially protected against the development of emphysema. However, CCR5 deficiency did not affect CS-induced airway wall remodelling, because chronic CS exposure induced a similar increase in airway wall thickness, smooth muscle mass and peribronchial deposition of collagen and fibronectin in both wild-type and CCR5 KO mice.

Conclusion Our data suggest that CCR5 contributes to pulmonary inflammation and to the development of emphysema in response to CS. CCR5 is, however, not implicated in CS-induced airway wall remodelling, suggesting that the mechanisms that lead to airway inflammation are distinct to those responsible for airway remodelling.

Keywords COPD, CCR5, cigarette smoke, emphysema, remodelling

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Introduction

Chronic obstructive pulmonary disease (COPD) is the fifth leading cause of death world-wide and is an important cause of chronic disability [1, 2]. Further increases in its

prevalence and mortality are expected in the coming decades. The disease is characterized by a chronic inflammation as well as obstruction of small airways and destruction of lung parenchyma, causing enlargement of alveolar spaces (emphysema) [3]. Patients with COPD also show pathologically distinct structural alterations of the small airways, referred to as airway wall remodelling [4].

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Cigarette smoking is by far the most important risk factor for COPD. However, the molecular and cellular mechanisms involved in cigarette smoke (CS)-induced inflammation, pulmonary emphysema and airway remodelling in COPD are still unclear.

The chronic inflammation in COPD is characterized by increased numbers of macrophages, dendritic cells (DCs), neutrophils, and CD8⁺ T lymphocytes [3, 5, 6]. Several cytokines and chemokines (e.g. TNF- α and IL-8) may play a key role in recruiting and activating inflammatory cells in the lungs of COPD patients [7, 8]. Furthermore, smokers who develop COPD show increased amounts of interstitial collagen in the small airways, referred to as airway wall fibrosis [4]. The excess deposition of connective tissue in the smaller conducting airways in COPD occurs preferentially in the adventitial compartment [9]. This peribroncholar fibrosis might contribute to fixed airway obstruction by restricting the enlargement of airway calibre that occurs with lung inflation. It is still open to debate whether this airway remodelling occurs as a consequence of the chronic inflammation in COPD, or rather through direct induction of growth factors by CS and thus independent of inflammation.

CC chemokines are thought to be involved in COPD. Macrophage inflammatory protein-1 α (MIP-1 α)/CC chemokine ligand 3 (CCL3) and MIP-1 β /CCL4 are markedly elevated in bronchoalveolar lavage (BAL) and sputum of COPD patients [10]. MIP-1 α mRNA levels are higher in the bronchial epithelium of smokers with airflow limitation than in smokers without airflow limitation or in never-smokers [11]. Strong gene expression of regulated upon activation, normal T expressed and secreted (RANTES)/CCL5, also belonging to the CC chemokine family, was observed in bronchial biopsies of COPD patients [12].

Chemokine receptor 5 (CCR5), a seven-transmembrane, G-protein-coupled receptor, belonging to the β -chemokine receptor family, is a receptor for MIP-1 α , MIP-1 β and RANTES. CCR5 is expressed on granulocytes, macrophages, immature DCs, CD8⁺ T lymphocytes and memory CD4⁺ T lymphocytes [13, 14], and is an important co-receptor for HIV [15]. It is well known that CCR5 influences the recruitment of monocytes/macrophages, DCs and T cells [14]. Prior studies have shown that CCR5 plays an important role in various inflammatory processes in the lungs [16–18]. Schuh et al. [19] demonstrated that CCR5 deficiency reduced the hallmarks of allergic asthma, including airway remodelling. Importantly, CCR5 was reported to be increased in bronchial biopsies of COPD patients with mild/moderate disease compared with control smokers [20] and high levels of T lymphocytes, expressing CCR5, were associated with loss of lung function and emphysema in COPD patients [21]. J. Elias and co-workers elegantly demonstrated that IFN- γ is a potent stimulator of CC chemokines, and that CCR5 plays an important role in the pathogenesis of both IFN- γ - and CS-

induced inflammation and emphysema [22]. Recently, we demonstrated that CCR6 and its ligand MIP-3 α /CCL20 may be involved in the pathogenesis of COPD [23]. To further elucidate the functional role of the CCR5 pathway in COPD, we used the murine model of CS-induced pulmonary inflammation and emphysema [6]. In the present paper, we report the pulmonary effects of sub-acute (4 weeks) or chronic (24 weeks) exposure to CS in wild-type and CCR5 knockout (KO) mice. We analysed the inflammatory responses in both the BAL fluid and lungs, and performed morphometric analysis to quantify the extent of pulmonary emphysema and airway remodelling after chronic exposure to CS.

Materials and methods

Animals

Mice with a targeted deletion of the entire CCR5 gene and backcrossed for 10 generations to the C57BL/6 genetic background were obtained from the Section of Molecular Genetics and Microbiology, University of Texas, Austin, Texas [24], and bred in the animal facility at the Ghent University Hospital. Males (8 weeks old) were used for the described experiments. C57BL/6 wild-type males (also 8 weeks old) were used as control mice (Harlan, Zeist, the Netherlands). The local Ethics Committee for animal experimentation of the faculty of Medicine and Health Sciences (Ghent, Belgium) approved all *in vivo* manipulations.

Smoke exposure

Mice ($n = 8$ /group) were exposed, whole body, to CS as described previously [6]. Briefly, groups of eight mice were exposed to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without a filter, University of Kentucky, Lexington, KY, USA) four times a day with 30-min smoke-free intervals, 5 days a week for four (sub-acute exposure) or 24 weeks (chronic exposure). An optimal smoke:air ratio of 1:6 was obtained. The control groups were exposed to air. Carboxyhaemoglobin in the serum of smoke-exposed mice reached a non-toxic level of $8.3 \pm 1.4\%$ [compared with $1.0 \pm 0.2\%$ in air-exposed mice ($n = 7$ for both groups)], which is similar to carboxyhaemoglobin blood concentrations of human smokers [25].

Bronchoalveolar lavage

Twenty-four hours after the last smoke exposure, mice were weighed and killed with an overdose of pentobarbital (Sanofi, Libourne, France) and a tracheal cannula was inserted. Three times 300 μ L followed by three times 1 mL of Hank's balanced salt solution (HBSS), free of ionized

calcium and magnesium but supplemented with 0.05 mM sodium ethylene diamine tetraacetic acid (EDTA), were instilled via the tracheal cannula and recovered by gentle manual aspiration. The six lavage fractions were pooled, centrifuged and the cell pellet was washed twice and finally resuspended in 1 mL of HBSS. A total cell count was performed in a Bürcker chamber and the differential cell counts (on at least 400 cells) were performed on cytocentrifuged preparations using standard morphologic criteria after May–Grünwald–Giemsa staining. Flow cytometric analysis of BAL cells was performed to enumerate DCs and CD4⁺ and CD8⁺ T cells.

Preparation of lung single-cell suspensions

Following BAL, the pulmonary and systemic circulation was rinsed. The left lung was used for histology, and the right lung for the preparation of a cell suspension as detailed previously [26]. Briefly, the lung was thoroughly minced, digested, subjected to red blood cell lysis, passed through a 50 µm cell strainer and kept on ice until labelling. Cell counting was performed with a Z2 Beckman-Coulter particle counter (Beckman-Coulter, Ghent, Belgium).

Labelling of bronchoalveolar lavage cells and lung single-cell suspensions for flow cytometry

Cells were pre-incubated with an F_c-receptor blocking antibody (anti-CD16/CD32, clone 2.4G2) to reduce non-specific binding. The monoclonal antibodies used to identify mouse DC populations were: biotinylated anti-CD11c (N418) and phycoerythrin (PE)-conjugated anti-IA^b (AF6-120.1). We discriminated between macrophages and DCs using the methodology described by Vermaelen and Pauwels [27]. After gating on the CD11c-bright population, two peaks of autofluorescence could be distinguished. Macrophages are identified as the CD11c-bright, high autofluorescent population, and do not express MHCII. DCs are identified as CD11c-bright, low autofluorescent cells, which strongly express MHCII. DCs enumerated by these criteria correspond to myeloid DCs. The following antibodies were used to stain mouse T cell subpopulations: fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (L3T4), FITC-conjugated anti-CD8 (Ly-2) and biotinylated anti-CD3 (145-2C11) monoclonal antibodies. The additional marker used for activation was PE-conjugated anti-CD69 (H1.2F3). Biotinylated anti-CD11c and anti-CD3 were revealed by incubation with streptavidine-allophycocyanine (SAv-APC). All monoclonal antibodies were obtained from BD Pharmingen (San Diego, CA, USA), except anti-CD11c (N418 hybridoma, a gift from Prof. M. Moser, Brussels Free University, Belgium).

As a last step before analysis, cells were incubated with 7-amino-actinomycin (7-AAD or viaprobe, BD Pharmingen)

for dead-cell exclusion. All labelling reactions were performed on ice in FACS-EDTA buffer.

Flow cytometry data acquisition was performed on a dual-laser FACS VantageTM flow cytometer running CELL-QuestTM software (Becton Dickinson, Mountain View, CA, USA). FlowJo software (www.Treestar.com) was used for data analysis.

Histology

The left lung was fixed by gentle infusion of 4% paraformaldehyde through the tracheal cannula [6]. After excision, the lung was immersed in a fresh fixative for 2 h. The lung lobe was embedded in paraffin and cut in to 3 µm transverse sections. Lung tissue samples were stained with hematoxylin and eosin, and examined by light microscopy for histological sections. For each animal, 10 fields at a magnification of ×200 were captured in a blinded fashion using a Zeiss KS400 image analyzer platform (KS400, Zeiss, Oberkochen, Germany).

Quantification of emphysema

Emphysema is a structural disorder characterized by destruction of the alveolar walls and enlargement of the alveolar spaces. We determined destruction of the alveolar walls by measuring the destructive index (DI) [28] and enlargement of alveolar spaces by quantifying the mean linear intercept (L_m) after 24 weeks of smoke exposure, as described previously [6, 29], using image analysis software (Image J 1.33). Only sections without cutting artefacts, compression or hilar structures (airway or blood vessel with a diameter larger than 50 µm) were used in the analysis. The L_m was measured by placing a 100 × 100 µm grid over each field. The total length of each line of the grid divided by the number of alveolar intercepts gives the average distance between alveolated surfaces, or the L_m . The L_m was measured by two independent observers, with a strong positive correlation ($R^2 = 0.82$, $P < 0.01$). DI was measured by superimposing a grid with 42 points on the lung field. Structures lying under these points were classified as normal (N) or destroyed (D) alveolar and/or duct spaces. Points falling over other structures, such as duct walls, alveolar walls, etc., did not enter into the calculations. The DI was calculated from the formula: $DI = D/(D+N) \times 100$.

Morphometric quantification of lymphoid follicles

To evaluate the presence of lymphoid follicles in lung tissue after 24 weeks of smoke exposure, lung sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to the following immunohistological CD3/B220 double staining: at first, sections were incubated with Boehringer blocking reagent with triton and

primary antibody anti-CD3, followed by goat-anti-rabbit biotin (both obtained from DakoCytomation, Heverlee, Belgium). Then, slides were incubated with streptavidin horseradish peroxidase and coloured with DAB. In a second step, sections were stained with anti-B220-biotin after Boehringer blocking (with triton). Finally, slides were incubated with streptavidin alkaline phosphatase (DakoCytomation) and coloured with Vector blue (Vector Laboratories Inc., Burlingame, CA, USA). Lymphoid follicles were defined as accumulations of at least 50 cells and counted in the tissue area surrounding the airways (airway perimeter 0–2000 µm). Results were expressed as counts relative to the numbers of airways per lung section.

Airway remodelling

Collagen in the airway wall was stained using Sirius Red, and the amount of fibronectin with a goat anti-rat fibronectin antibody (Calbiochem, Badsoden, Germany) using the streptavidin–biotin peroxidase method [30]. Airway smooth muscle was revealed by staining with a rabbit anti-mouse α -smooth muscle actin antibody (Abcam, Cambridge, UK). For each experimental group, three lung sections per animal were examined. Morphometrical parameters [31] were marked manually on the digital representation of the airway: the length of the basement membrane (Pbm), the area defined by the basement membrane (Abm) and the area defined by the total adventitial perimeter (Ao). The total bronchial wall area (WAt) was calculated ($WAt = Ao - Abm$) and normalized to the squared length of the basement membrane. For the quantification of collagen and fibronectin deposition and smooth muscle hypertrophy, the area in the airway wall covered by the stain was determined by the software (KS400; Zeiss) and its value was calculated as described previously [30]. The area of collagen (WCt), fibronectin (WFt), or α -smooth muscle actin (WAc) was normalized to Pbm. All airways with a Pbm smaller than 2000 µm and cut into reasonable cross sections (defined by a ratio of minimal to maximal internal diameter >0.5) were included.

Measurement of cytokines and chemokines

Using commercially available ELISA kits (R&D Systems, Abingdon, UK), MIP-1 α , MIP-1 β , RANTES, macrophage chemotactic protein-1 (MCP-1), KC (mouse IL-8) and activated TGF- β 1 protein levels were determined in BAL fluid after 4 and/or 24 weeks of CS exposure.

After 4 weeks of CS exposure, the protein levels of cytokines in BAL fluid were determined by FACS using the Cytometric Bead Array (CBA – Mouse Th1/Th2 cytokine kit; BD Biosciences, San Diego, CA, USA), as described previously [32]. A mixture of five capture bead populations, each with distinct fluorescence intensities and

coated with antibodies specific for IL-2, IL-4, IL-5, IFN- γ and TNF- α were prepared. The CBA capture beads were incubated together with PE-conjugated detection antibodies, test samples or standards, to form sandwich complexes. Following acquisition of sample data using the flow cytometer (FACSCalibur™ flow cytometer; BD Biosciences), the sample results were generated in graphical and tabular format using the CBA Analysis Software (BD Biosciences).

Reverse transcriptase-polymerase chain reaction analysis

Total lung RNA was extracted with the RNeasy Midi Kit (Qiagen, Hilden, Germany). Expression of CCR5 mRNA, relative to hypoxanthine guanine phosphoribosyltransferase (hprt) mRNA, was analysed with the Assays-on-Demand™ Gene Expression Products (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed on a Light-Cycler 480 system (Roche, Basel, Switzerland) with MuLV RTase (Applied Biosystems). Reverse transcription was performed at 48 °C for 30 min, followed by a 10-min incubation at 95 °C for denaturation of RNA–DNA heteroduplexes, and 50 cycles of 95 °C for 10 s and 60 °C for 15 s. Monitoring of the RT-PCR occurred in real time using an FAM/TAMRA probe. All reactions were performed starting from 10 ng of total RNA.

Statistical analysis

Reported values are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed with Sigma Stat software (SPSS 11.0 Inc, Chicago, IL, USA) using non-parametric tests (Kruskal–Wallis, Mann–Whitney *U*). *P*-values <0.05 were considered to be significant.

Results

Increased chemokine receptor 5 mRNA expression in lung tissue upon cigarette smoke exposure

RT-PCR analysis on mRNA extracted from total lung tissue of wild-type mice revealed a significant increase in CCR5 expression upon both sub-acute (4 weeks) and chronic (24 weeks) exposure to CS, compared with air-exposed littermates (Fig. 1).

Chemokine expression in the bronchoalveolar lavage fluid following cigarette smoke exposure is chemokine receptor 5 dependent

Because the levels of the CCR5 ligands (MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5), MCP-1/CCL2 and IL-8 are augmented in the sputum and BAL of COPD-patients, we determined the protein levels of these chemokines in the BAL fluid of wild-type and CCR5 KO mice.

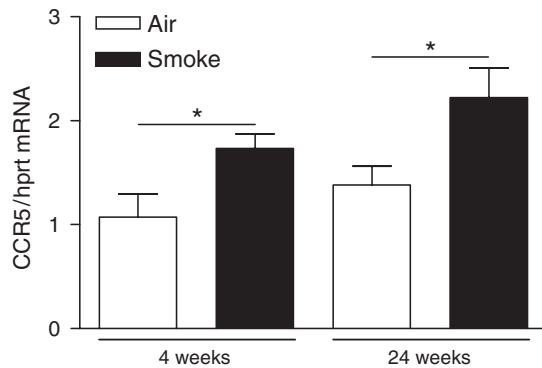


Fig. 1. Effect of cigarette smoke exposure on chemokine receptor 5 (CCR5) mRNA expression in total lung tissue. mRNA levels of CCR5 in total lung tissue of wild-type mice upon sub-acute (4 weeks) and chronic (24 weeks) exposure to air or cigarette smoke, as measured by RT-PCR. Results are expressed as a ratio with hprt mRNA (mean $\pm$ SEM). $N=5$ animals/group (* $P < 0.05$). hprt, hypoxanthine guanine phosphoribosyl-transferase.

Sub-acute (4 weeks) and chronic (24 weeks) exposure to CS clearly induced levels of MIP-1 α , MIP-1 β , MCP-1, and KC in BAL fluid of both wild-type and CCR5 KO mice, compared with air-exposed littermates (Fig. 2). However, significantly smaller increases of MIP-1 α , MIP-1 β , MCP-1 and KC were observed in CS-exposed CCR5 KO mice vs. CS-exposed wild-type animals (Fig. 2). The protein levels of RANTES were significantly augmented in the BAL fluid of wild-type animals upon chronic (24 weeks) CS exposure. In contrast, no differences in RANTES levels were observed in chronic CS-exposed CCR5 KO mice vs. air-exposed CCR5 KO littermates (Fig. 2c).

Cigarette smoke-induced increase of inflammatory cells in bronchoalveolar lavage fluid and lungs is chemokine receptor 5 dependent

In both wild-type and CCR5 KO mice, sub-acute (4 weeks) or chronic (24 weeks) exposure to CS caused a significant increase in absolute numbers of total BAL fluid cells, including neutrophils, monocytes/macrophages, DCs and lymphocytes (Figs 3a–e). However, at both timepoints, these increases in inflammatory cells were significantly smaller in CS-exposed CCR5 KO mice, compared with wild-type animals (Figs 3a–e). In more detail, exposure to CS significantly increased the number of both CD4⁺ and CD8⁺ T lymphocytes in wild-type and CCR5 KO mice, compared with air-exposed control mice (Figs 3f and g). Again, these increases were significantly impaired in CCR5 KO mice vs. wild-type littermates (Figs 3f and g).

In lung homogenates, chronic CS exposure induced – similar to the BAL compartment – a significant increase in the numbers of DCs, macrophages and activated CD4⁺ and CD8⁺ T cells in both wild-type and CCR5 KO mice (data not shown). However, the CS-induced increase in the

numbers of activated CD4⁺ and CD8⁺ T lymphocytes in the lung was smaller in CCR5 KO mice compared with wild-type mice ($P < 0.05$).

Cigarette smoke-induced increase of cytokines in bronchoalveolar lavage fluid of wild-type and chemokine receptor 5 knockout mice

To decipher whether CS exposure modifies the T helper type 1 (Th1)/Th2 balance, several cytokines were measured in the BAL fluid by Cytometric Bead Array. Sub-acute (4 weeks) CS exposure significantly increased the BAL levels of TNF- α and IL-2 in wild-type mice compared with air exposure (Figs 4a and b). In contrast, no differences in the protein levels of TNF- α and IL-2 were found between BAL fluid of air- and CS-exposed CCR5 KO animals. IFN- γ levels tended to increase in the BAL fluid of wild-type and CCR5 KO animals upon CS exposure (Fig. 4c). The levels of IL-4 and IL-5 were below the detection limit in almost all BAL fluid samples, irrespective of mouse genotype or exposure regimen (data not shown).

Partial protection against emphysema in chemokine receptor 5 knockout mice upon chronic cigarette smoke exposure

Pulmonary emphysema is characterized by the destruction of alveolar walls due to damage to the lung parenchyma, leading to enlargement of alveolar spaces. Therefore, we quantified emphysematous lesions by measuring the L_m and the DI. Chronic (24 weeks) exposure to CS clearly induced pulmonary emphysema in wild-type animals, as evidenced by a significant increase in the L_m (air 37.6 ± 0.3 vs. CS 40.0 ± 0.2 μ m, $P < 0.05$) and DI (air 25.6 ± 0.7 vs. CS 33.7 ± 0.4 , $P < 0.05$). In CCR5 KO mice, the difference in L_m and DI upon chronic CS exposure did not reach statistical significance (L_m : air 37.2 ± 0.3 vs. CS 39.0 ± 0.2 μ m, NS; DI: air 26.1 ± 0.7 vs. CS 30.9 ± 0.5 , NS). The significant airspace enlargement due to chronic CS exposure in wild-type mice and the partial protection against emphysema in CCR5 KO mice is demonstrated on haematoxylin and eosin-stained lung tissue sections (Figs 5a–d).

Cigarette smoke-induced increase of peribronchial lymphoid follicles is chemokine receptor 5 dependent

Immunohistochemistry using anti-CD3 and anti-B220 monoclonal antibodies to stain T lymphocytes and B-lymphocytes, respectively, revealed the presence of only a few small lymphoid follicles in the lung areas surrounding the airways of air-exposed wild-type and CCR5 KO mice (Fig. 6a). Chronic (24 weeks) CS exposure significantly augmented the number of peribronchial lymphoid follicles in the lungs of both wild-type and CCR5 KO mice.

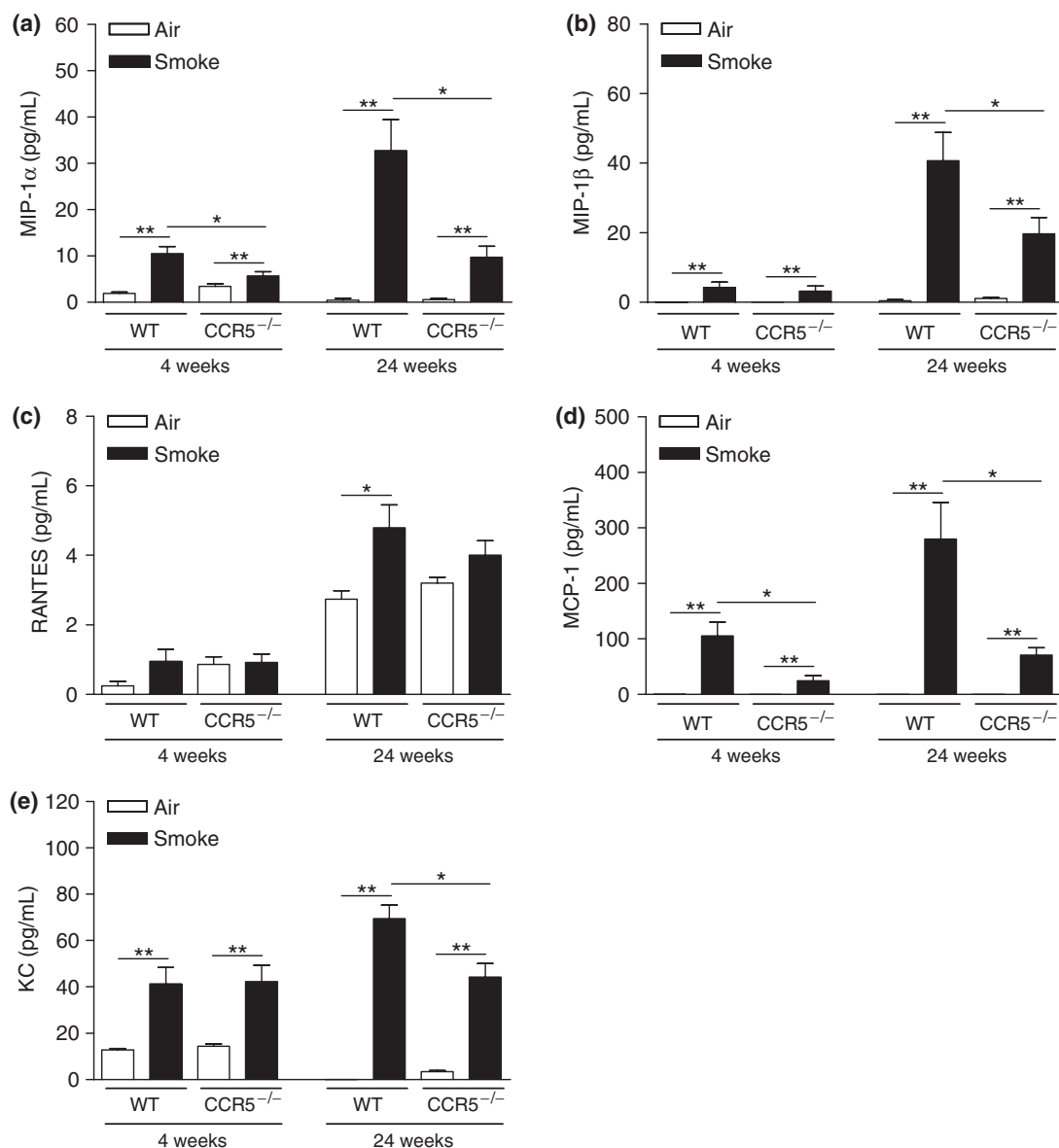


Fig. 2. Effect of cigarette smoke exposure on the protein levels of chemokines in bronchoalveolar lavage (BAL) fluid. Protein levels of chemokines in the BAL of wild-type and chemokine receptor 5 knockout (CCR5 KO) mice upon sub-acute (4 weeks) and chronic (24 weeks) exposure to air or cigarette smoke, as measured by ELISA: (a) MIP-1 α , (b) MIP-1 β , (c) RANTES, (d) MCP-1 and (e) KC. Results are expressed as pg/mL (mean $\pm$ SEM). $N = 8$ animals/group (* $P < 0.05$ and ** $P < 0.01$). MIP-1 α , macrophage inflammatory protein-1 α ; MIP-1 β , macrophage inflammatory protein-1 β ; RANTES, regulated upon activation, normal T expressed; MCP-1, monocyte chemoattractant protein-1; KC, mouse IL-8; WT, wild-type.

However, this increase in lymphoid follicles was significantly attenuated in the CCR5 KO mice (Fig. 6a). Moreover, these peribronchial lymphoid follicles appeared to be larger and denser in wild-type mice, compared with CCR5 KO animals (Figs 6b–e).

Cigarette smoke-induced airway remodelling is similar in wild-type and chemokine receptor 5 knockout mice

To investigate the effects of chronic (24 weeks) CS exposure on the deposition of extracellular matrix in the

airway wall, lung sections were stained with anti-fibronectin and Sirius Red to reveal fibronectin and collagen, respectively. To study smooth muscle hypertrophy, lung sections were stained with anti- α -smooth muscle actin. In wild-type animals, chronic (24 weeks) CS exposure significantly increased the amount of fibronectin present in the peribronchial area (Figs 7a–e). CCR5 deficiency did not affect the CS-induced increase in peribronchial fibronectin deposition (Figs 7a–e). Similarly, chronic CS exposure induced a significant increase in peribronchial collagen deposition in both wild-type and CCR5 KO mice

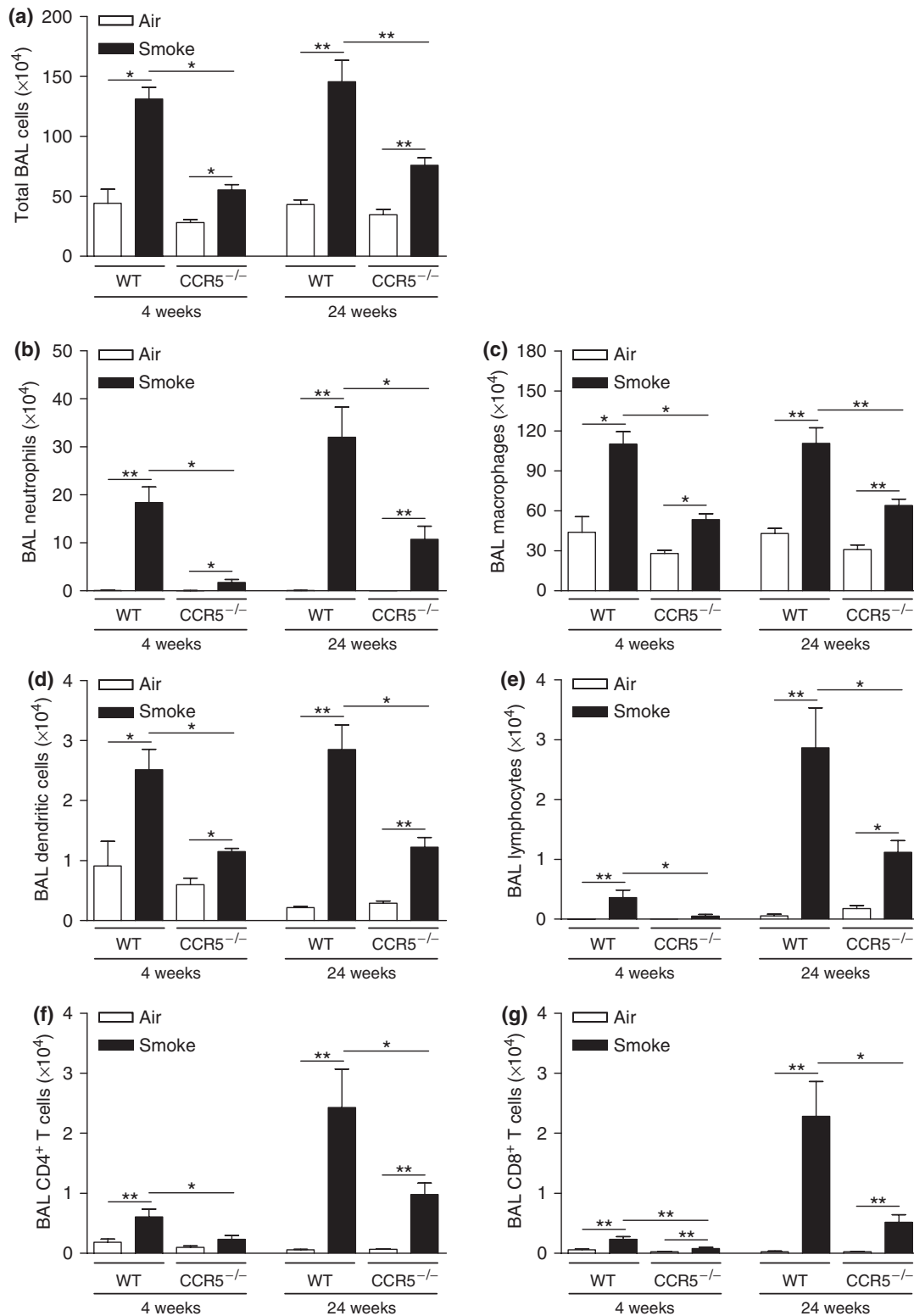


Fig. 3. Effect of cigarette smoke exposure on cell differentiation in bronchoalveolar lavage (BAL) fluid. Total BAL cells and cell differentiation in BAL fluid of wild-type and chemokine receptor 5 knockout (CCR5 KO) mice upon sub-acute (4 weeks) and chronic (24 weeks) exposure to air or cigarette smoke: (a) Total BAL cells, (b) neutrophils, (c) macrophages, (d) dendritic cells, (e) lymphocytes, (f) CD4⁺ T lymphocytes and (g) CD8⁺ T lymphocytes. Results are expressed as means $\pm$ SEM. $N = 8$ animals/group (* $P < 0.05$, ** $P < 0.01$). WT, wild-type.

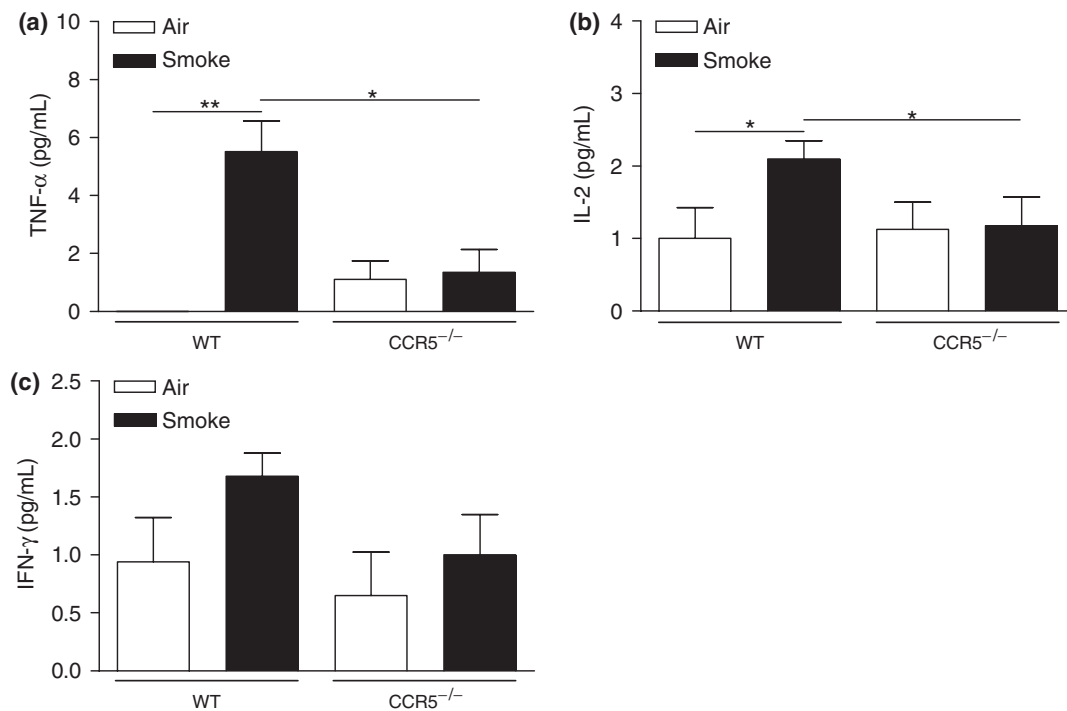


Fig. 4. Effect of cigarette smoke exposure on the levels of cytokines in bronchoalveolar lavage fluid (BALF). Protein levels of cytokines in the BALF of wild-type and chemokine receptor 5 knockout (CCR5 KO) mice upon sub-acute (4 weeks) exposure to air or cigarette smoke, as measured by cytometric bead array: (a) TNF- α , (b) IL-2 and (c) IFN- γ . Results are expressed as pg/mL (mean $\pm$ SEM). $N = 8$ animals/group (* $P < 0.05$ and ** $P < 0.01$). WT, wild-type.

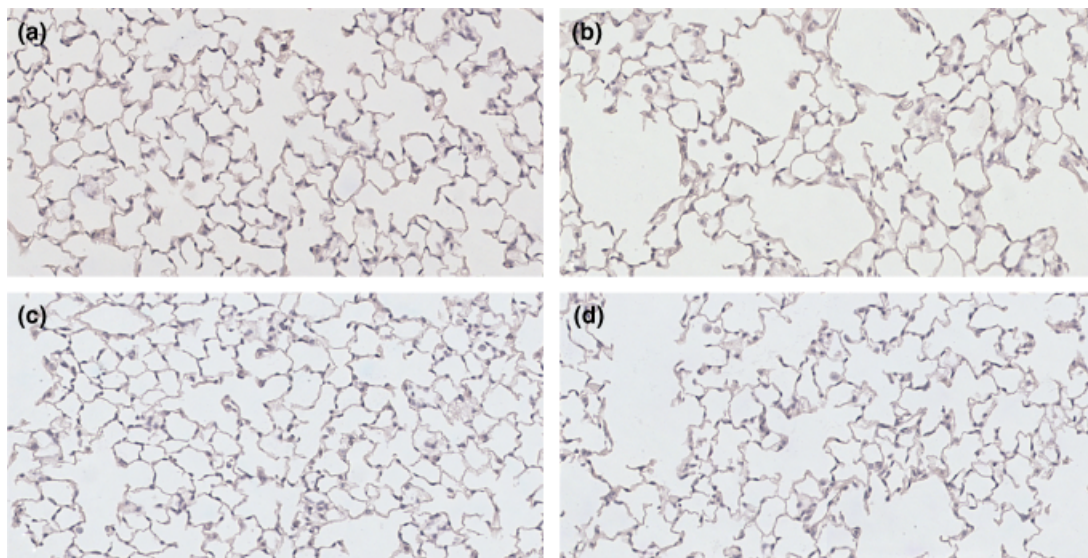


Fig. 5. Pulmonary emphysema upon chronic cigarette smoke exposure. Photomicrographs of haematoxylin and eosin-stained lung tissue of air and cigarette smoke (CS)-exposed wild-type and chemokine receptor 5 knockout (CCR5 KO) mice at 24 weeks (chronic exposure; magnification $\times 200$): (a) air-exposed wild-type mice, (b) CS-exposed wild-type mice, (c) air-exposed CCR5 KO mice and (d) CS-exposed CCR5 KO mice. WT, wild-type.

(Figs 7f–j). Smooth muscle mass was significantly increased in the airways of chronic CS-exposed wild-type and CCR5 KO mice (Figs 7k–o), but again there was no influence of the CCR5 deletion. Importantly, the increased

peribronchial deposition of collagen and fibronectin and the smooth muscle hypertrophy contributed to a significant increase in the airway wall thickness upon chronic CS exposure in both genotypes (Fig. 7p).

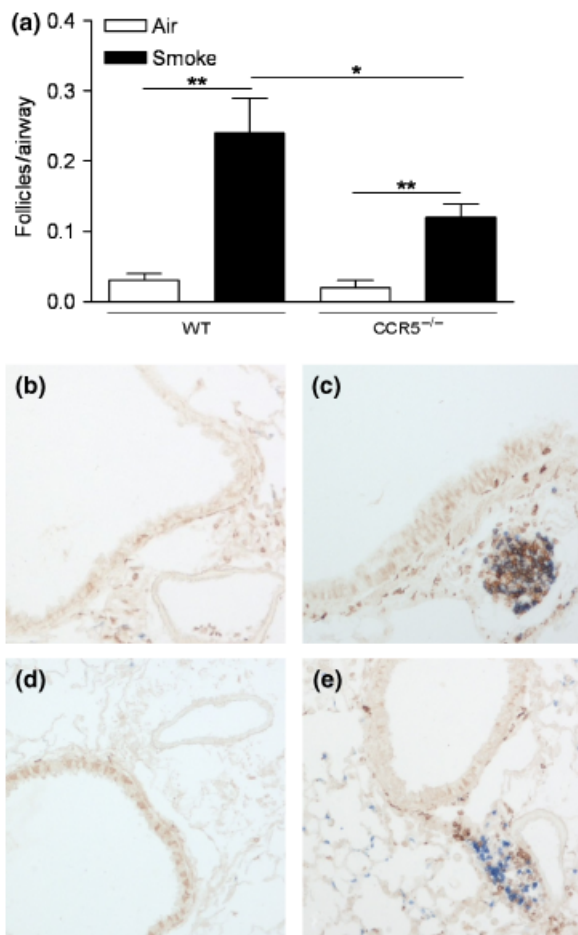


Fig. 6. Quantification of pulmonary lymphoid follicles upon chronic cigarette smoke exposure. Peribronchial lymphoid follicles in lung tissue of wild-type and chemokine receptor 5 knockout (CCR5 KO) mice upon chronic (24 weeks) exposure to air or cigarette smoke (CS) (a). Results are expressed as means $\pm$ SEM. $N=8$ animals/group ($*P < 0.05$, $**P < 0.01$). Photomicrographs of peribronchial lymphoid follicles in lung tissue of air and CS-exposed wild-type and CCR5 KO mice at 24 weeks (chronic exposure; magnification $\times 200$): (b) air-exposed wild-type mice, (c) CS-exposed wild-type mice, (d) air-exposed CCR5 KO mice and (e) CS-exposed CCR5 KO mice. WT, wild-type.

Cigarette smoke-induced increase of transforming growth factor- β 1 in bronchoalveolar lavage fluid of wild-type and chemokine receptor 5 knockout mice

To obtain a mechanistic explanation for the observed airway wall remodelling in wild-type and CCR5 KO mice, we measured the protein levels of activated TGF- β 1 in BAL fluid by ELISA. Sub-acute exposure to CS significantly increased the protein levels of TGF- β 1, in both wild-type and CCR5 KO mice (Fig. 8).

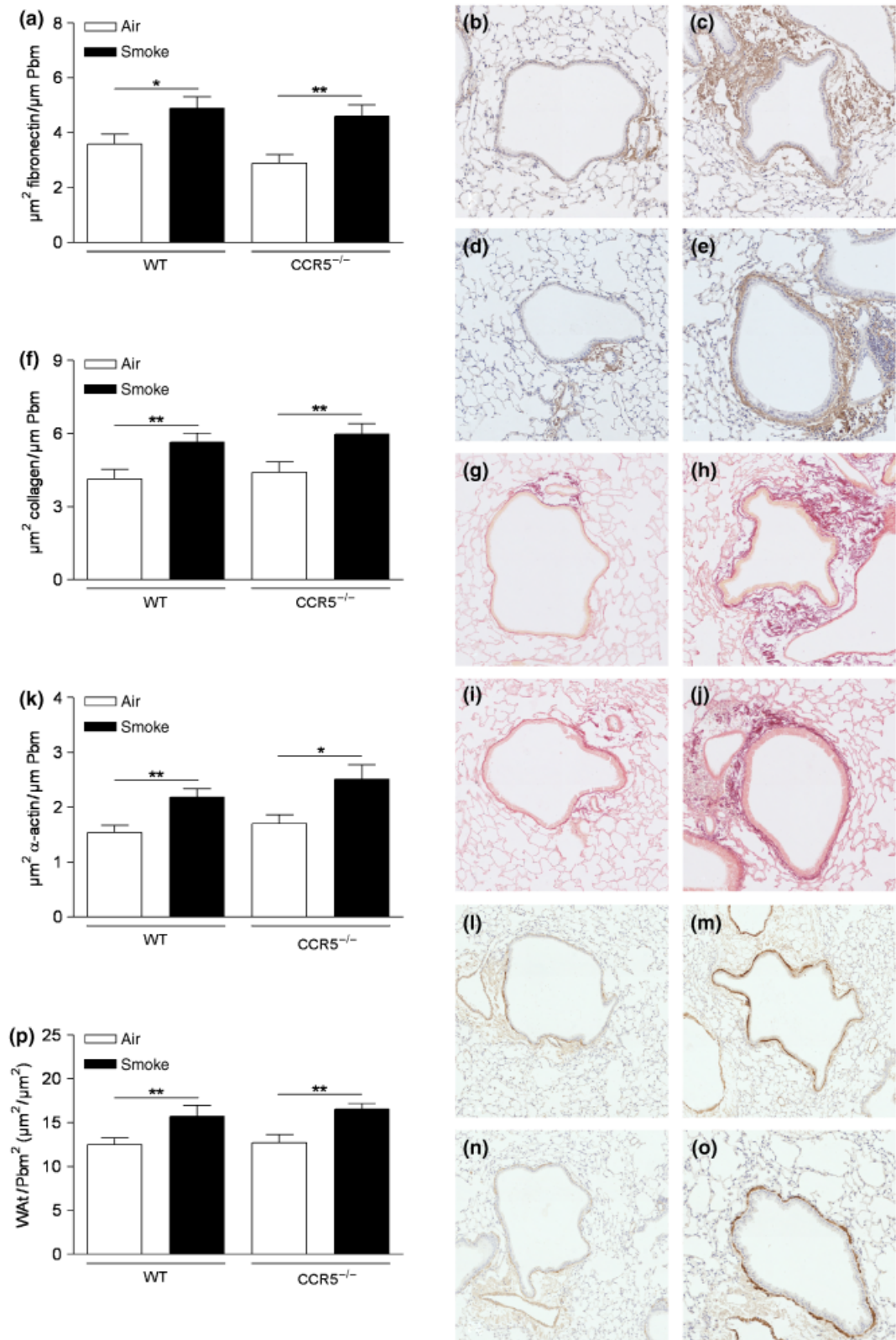
Discussion

Exposure to CS is the main risk factor for the development of COPD, a disease that is characterized by pulmonary

inflammation, destruction of lung parenchyma and remodelling of the small airways. In this study, we show that CCR5 deficiency attenuates the CS-induced increase in inflammatory cells of both the innate and the adaptive immune system, and offers a partial protection against the development of pulmonary emphysema. In contrast, CCR5 deficiency does not affect airway wall remodelling, induced by chronic CS exposure.

CS can activate lung resident cells, including epithelial cells, macrophages and DCs, which then release several inflammatory mediators that are capable of recruiting inflammatory cells [3, 33–36]. After both sub-acute and chronic CS exposure, increased protein levels of MIP-1 α /CCL3, MIP-1 β /CCL4 and RANTES/CCL5 in the BAL fluid of wild-type mice were associated with a high influx into the BAL and lungs of monocytes/macrophages, DCs and T lymphocytes, which all express the corresponding chemokine receptor CCR5 [13, 14, 20]. The expression of the CCR5 receptor in lung homogenates of wild-type mice was significantly increased upon both sub-acute and chronic CS exposure. In contrast, smaller numbers of macrophages, DCs and T lymphocytes were observed in the BAL and lungs of CS-exposed CCR5 KO mice. Moreover, in these CCR5-deficient mice, we observed a significantly impaired increase of all three CCR5 ligands upon CS exposure. This was also documented by Ma et al. [22] and can be explained as a downstream effect of the CCR5 deficiency, where the impaired pulmonary inflammation leads to a reduced release of inflammatory mediators. Indeed, the CS-induced increase in the production of chemokines MCP-1 and KC and cytokines TNF- α and IL-2 that we observed in wild-type mice was significantly attenuated in CCR5 KO mice. As a consequence of this attenuated inflammation, CCR5 KO mice were partially protected against the development of pulmonary emphysema, which also confirms the observations of Ma et al. A logical explanation would be that the reduced numbers of MMP-12-releasing macrophages or DCs [37, 38], and neutrophil elastase-producing neutrophils [3] in CCR5 KO mice, influence the proteinase/antiproteinase balance in favour of antiproteolytic activity.

We demonstrated that chronic CS exposure leads to an increase in peribronchial lymphoid follicles. This correlates with human COPD patients, where Hogg et al. [39] demonstrated that progression of COPD is associated with increasing percentages of airways that contained lymphoid follicles. CCR5 KO mice had significantly less of these lymphoid accumulations around the airways upon CS exposure. Interestingly, Castellino et al. [40] showed in a recent study that inflammation increases the expression of CCR5 on CD8⁺ T cells, permitting them to be attracted to sites of CD4⁺ T cell–DC interaction. Together with the reduced numbers of lymphocytes and more specific activated CD4⁺ and CD8⁺ T cells in the airways and lungs,



this underscores the contribution of CCR5 in T cell migration and activation.

Airway wall remodelling is another hallmark of COPD, and contributes to the airflow obstruction. Our group has shown for the first time that exposure to CS leads to airway wall remodelling in a mouse model of COPD. Although, compared with asthma, the structural alterations of the airways in COPD are less pronounced [41], we were able to measure several features of the remodelling process. Firstly, we observed an increase in peribronchial deposition of collagen and fibronectin upon chronic CS exposure. Secondly, we showed an increase in airway smooth muscle mass upon CS exposure. And thirdly, this smoke-induced deposition of fibronectin and collagen and smooth muscle hypertrophy resulted in a significant thickening of the airway walls. Interestingly, we demonstrated that although the CCR5 deletion had a significant effect on CS-induced pulmonary inflammation, it had no effect on airway wall remodelling. This suggests that smooth muscle hypertrophy and the peribronchial deposition of collagen and fibronectin is not a downstream effect

of the ongoing pulmonary inflammation, but rather occurs through independent processes. This has also been proposed in asthma, where activation of the epithelial-mesenchymal trophic unit leads to release of growth factors, resulting in airway remodelling [42, 43].

We provided a similar mechanistic explanation for the observed airway wall remodelling in COPD by demonstrating an increase in activated TGF- β 1 protein levels in BAL fluid of both CS-exposed wild-type and CCR5 KO mice. It has been described that TGF- β 1 drives airway remodelling in CS-exposed tracheal explants [44], and recently Churg et al. [45] presented data on increased TGF- β 1 gene expression in airway walls isolated from lungs of mice upon a single CS exposure. This last observation confirms the ability of CS to directly induce the release of growth factors, independent of the pulmonary inflammation. Interestingly, we have also observed this development of airway wall remodelling regardless of the degree of inflammation in CCR6-deficient mice [23].

Therapy for COPD and emphysema is currently limited to pharmacologic bronchodilation or treatment with inhaled glucocorticosteroids, but these drugs do not affect the natural history of the disease (e.g. accelerated decline in lung function). Therapeutic interventions that target chemokines and chemokine receptors are now in clinical development for a variety of pathological inflammatory processes and some of these might be effective in blocking the influx of inflammatory cells into the airways and lung parenchyma in COPD. Our experimental findings suggest that CCR5 antagonists, currently in development as HIV entry inhibitors, might be useful for the treatment of COPD [46]. However, one caveat when applying this approach is the absence of an effect of CCR5 deficiency on airway wall remodelling. Therefore, it would be interesting to determine whether the incidence, onset and severity of COPD are altered in individuals who are naturally deficient in CCR5.

In conclusion, we have demonstrated that CCR5 deficiency leads to an impaired recruitment of inflammatory cells into the lungs and a partial protection against emphysema following chronic CS exposure. Our *in vivo*

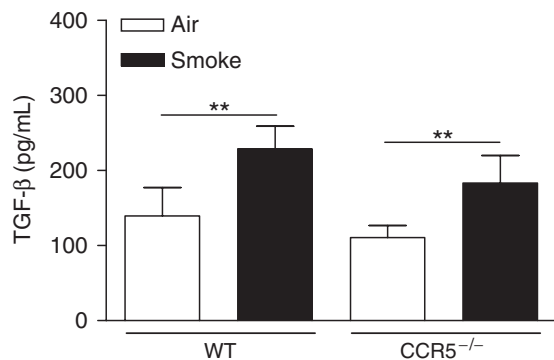


Fig. 8. Effect of cigarette smoke exposure on the protein levels of activated TGF- β 1 in bronchoalveolar lavage fluid. Protein levels of activated TGF- β 1 in the bronchoalveolar lavage fluid of wild type and chemokine receptor 5 knockout (CCR5 KO) mice upon sub-acute (4 weeks) exposure to air or cigarette smoke, as measured by ELISA. Results are expressed as pg/mL (mean $\pm$ SEM). $N = 8$ animals/group (** $P < 0.01$). WT, wild-type.

Fig. 7. Quantification of airway wall remodelling upon chronic cigarette smoke exposure. Peribronchial fibronectin deposition in lung tissue of wild-type and chemokine receptor 5 knockout (CCR5 KO) mice upon chronic (24 weeks) exposure to air or cigarette smoke (CS) (a). Results are expressed as means $\pm$ SEM. $N = 8$ animals/group (* $P < 0.05$, ** $P < 0.01$). Photomicrographs of peribronchial fibronectin deposition in lung tissue of air- and CS-exposed wild-type and CCR5 KO mice at 24 weeks (magnification $\times 100$): (b) air-exposed wild-type mice, (c) CS-exposed wild-type mice, (d) air-exposed CCR5 KO mice and (e) CS-exposed CCR5 KO mice. (Pbm, length of basement membrane). Peribronchial collagen deposition in lung tissue of wild-type and CCR5 KO mice upon chronic (24 weeks) exposure to air or cigarette smoke (CS) (f). Results are expressed as means $\pm$ SEM. $N = 8$ animals/group (** $P < 0.01$). Photomicrographs of peribronchial collagen deposition in lung tissue of air- and CS-exposed wild-type and CCR5 KO mice at 24 weeks (magnification $\times 100$): (g) air-exposed wild-type mice, (h) CS-exposed wild-type mice, (i) air-exposed CCR5 KO mice and (j) CS-exposed CCR5 KO mice. Airway smooth muscle mass in lung tissue of wild-type and CCR5 KO mice upon chronic (24 weeks) exposure to air or cigarette smoke (CS) (k). Results are expressed as means $\pm$ SEM. $N = 8$ animals/group (* $P < 0.05$, ** $P < 0.01$). Photomicrographs of smooth muscle in the airways of air- and CS-exposed wild-type and CCR5 KO mice at 24 weeks (magnification $\times 100$): (l) air-exposed wild-type mice, (m) CS-exposed wild-type mice, (n) air-exposed CCR5 KO mice and (o) CS-exposed CCR5 KO mice. Airway wall thickness (WAt) of wild-type and CCR5 KO mice upon chronic (24 weeks) exposure to air or CS (p). Results are expressed as means $\pm$ SEM. $N = 8$ animals/group (** $P < 0.01$). WAt, total bronchial wall area; WT, wild-type.

studies in this murine model of COPD indicate that CCR5 contributes to the pathogenesis of CS-induced pulmonary inflammation, peribronchial lymphoid follicle formation and emphysema, but is not implicated in airway wall remodelling.

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