Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 334 (2005) 254–262
BBRC
www.elsevier.com/locate/ybbrc

Elevated expression of TARC (CCL17) and MDC (CCL22) in models of cigarette smoke-induced pulmonary inflammation

Mirko Ritter, Rolf Göggel, Nveed Chaudhary, Alexander Wiedenmann, Birgit Jung, Andreas Weith, Peter Seither *

Department of Pulmonary Research, Boehringer Ingelheim Pharma GmbH & Co. KG, D-88937 Biberach a.d. Riss, Germany

Received 13 June 2005
Available online 27 June 2005

Abstract

TARC (CCL17) and MDC (CCL22) are well-known chemoattractants for Th2 cells. Here, we evaluated the role of both chemokines for cigarette smoke-induced airway inflammation. The expression profiles of MDC, TARC, and their receptor CCR4 were analyzed in models of acute and chronic cigarette smoke-induced airway inflammation that is characterized by a Th1 immune response. The results were compared to the expression of both chemokines in models of idiopathic pulmonary fibrosis and acute asthma, which are associated with a Th2 immune response. The expression of MDC and TARC was found to be elevated in all lung inflammation models. In contrast to the findings in the asthma and lung fibrosis models, the increased expression of MDC and TARC in the cigarette-smoke model was not associated with an increased infiltration of Th2 cells into smoke-treated lungs. Our data indicate that instead of Th2 cells, airway epithelial cells expressing CCR4 might be the principal targets for MDC and TARC released from alveolar macrophages during cigarette smoke-induced airway inflammation.

Keywords: Cigarette smoke; Airway inflammation; Bronchial epithelium; COPD; CCR4; MDC; TARC

Thymus and activation-regulated chemokine (TARC, CCL17) is a CC chemokine that is constitutively expressed in thymus and is inducible in PBMCs, macrophages, bronchial epithelial cells, endothelial cells, dendritic cells, and keratinocytes by activation of these cells [1–6]. TARC binds to the chemokine receptor CCR4 [7], which is highly expressed on Th2 cells. In agreement with these findings, in vitro studies demonstrate that TARC induces the selective chemotaxis of lymphocytes of the Th2 phenotype [8]. Monocyte-derived chemokine (MDC, CCL22) is another CC chemokine that specifically binds to CCR4 and is described as a potent chemoattractant for Th2 lymphocytes and monocytes [9–11].

Since CCR4 and CCR8 are predominantly expressed by Th2 cells, TARC and MDC are considered to be key mediators of Th2 lymphocyte recruitment in allergic diseases like asthma [12–14] or allergic rhinitis [15]. This is supported by the observation that neutralization of TARC by a specific antibody results in decreased airway eosinophilia and decreased number of infiltrating Th2 lymphocytes in a murine allergic asthma model [16]. Likewise, neutralization of MDC with specific antibodies prevented interstitial lung inflammation and reduced airway hyperreactivity in a model of ovalbumin-induced lung allergic inflammation [17].

Although the roles of MDC and TARC for type-2 inflammations are well established, initial findings also implicate a role of MDC and TARC in a Th1 immune response. Recently, elevated levels of MDC and TARC have been detected in atherosclerotic lesions which are considered to be mainly associated with a Th1-type

* Corresponding author. Fax: +49 7351 54 5991.
E-mail address: peter.seither@bc.boehringer-ingelheim.com (P. Seither).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.06.084

inflammation [18]. In addition, an increased mucosal expression of MDC and TARC was observed within chronic lesions of Crohn's disease [19], which are characterized by an excessive Th1 response. Furthermore, CCR4 has been shown to play a key role in models of sepsis [20].

In these studies, we investigated the expression of MDC and TARC and their common chemokine receptor CCR4 in a model of cigarette smoke-induced acute and chronic airway inflammation, that is associated with a type-1 inflammatory response. The expression profile of MDC, TARC, and CCR4 was compared with other animal models of idiopathic pulmonary fibrosis and acute asthma known to be associated with a type-2 immune response. The expression of MDC and TARC was found to be elevated in all lung inflammation models. This implied prominent roles of MDC and TARC, not only for allergic disease like asthma, but also for the type-1 inflammatory response induced by cigarette smoke. In sharp contrast to the results obtained from asthma and fibrosis models, the increased expression of MDC and TARC in the cigarette smoke-induced lung inflammation model was not associated with an increased infiltration of Th2 cells into the lungs. Our data demonstrate that bronchial epithelial cells express CCR4 and therefore might be principal target cells for MDC and TARC released from alveolar macrophages during cigarette smoke-induced inflammation.

Materials and methods

Asthma model. All animal experiments were performed in accordance with the German Guidelines for Animal Welfare and were approved by the concerned authorities.

In a model for acute asthma male Brown Norway rats were sensitized by intraperitoneal injection of alum/ovalbumin (OVA) (1 mg/kg) on three consecutive days. At days 6 and 7 sensitized rats were challenged by the inhalation of 1% OVA aerosol. In control experiments rats inhaled 0.9% NaCl. Rats were sacrificed at day 8. Lungs were removed, snap frozen in liquid nitrogen, and stored at −80 °C.

Fibrosis model. Male Wistar rats were intratracheally injected with bleomycin sulfate (10 U/kg body weight) using a catheter through the nasal passage. Following bleomycin instillation at days 0, 3, 6, 9, 14, and 21, the rats were killed with a lethal intraperitoneal injection of Narcoren (Pentobarbital Sodium, Rhone Merieux). Lungs were removed, snap frozen in liquid nitrogen, and stored at −80 °C.

Cigarette smoke-induced airway inflammation model. Male Sprague–Dawley rats were exposed to 8–16 regular, non-filter cigarettes (1.2 mg nicotine, 12 mg condensate) a day for 4 days (acute inflammation model). Cigarette smoke was delivered into the cabinet by passing air at a flow rate of 0.3 ml/s through a burning cigarette in a chamber. The combustion time of the cigarette was ~3 min. Fresh air was delivered into the cabinet to remove the smoke. At intervals of 20 min, the smoke of a new cigarette was delivered into the cabinet.

In the long-term cigarette smoke model (chronic inflammation), rats were exposed for 4 weeks to cigarette smoke according to the procedure described above.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) of the right lobe of the lung was performed on day 4 of smoke treatment after the animals had been killed. The lobe was lavaged three times with 2 ml of ice-cold PBS. The cell number was counted with a Casy cell counter (Schaerfe System, Reutlingen, Germany). Differential cell count was undertaken on cytocentrifuged preparations stained with DiffQuik (Dade Behring, Schwalbach, Germany).

Isolation of T-cells and alveolar macrophages. T-cells were purified from the BAL by positive selection using magnetic rat Pan-T-cell microbeads according to the instructions provided by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). An aliquot of the T-cells was used for the preparation of cytospins. Purity of the cell population was determined to be greater than 90% by DiffQuick staining. Cells were lysed in RLT buffer (Qiagen, Hilden, Germany) and used for RNA preparation.

Alveolar macrophages were isolated by adherence. Briefly, BAL cells were washed in PBS/0.1% BSA, resuspended in RPMI-1640 medium, seeded into six-well plastic dishes, and cultivated for 30 min at 37 °C to allow adhesion of macrophages. Non-adherent cells were removed by washing with medium. Adherent macrophages were used for RNA preparation.

ELISA. TARC protein levels were determined using a murine TARC ELISA system (R&D Systems, Wiesbaden, Germany), which exhibits significant cross-reactivity with rat TARC protein. ELISAs were performed according to the manufacturer's instructions.

RNA preparation. RNA extractions from cells were carried out according to the manufacturer's instructions using the RNeasy Mini Kit (Qiagen). Purity and integrity of the extracted RNA was assessed on an Agilent 2100 bioanalyzer with the RNA 6000 Nano LabChip reagent set (Agilent Technologies, Palo Alto, CA).

Real-time quantitative RT-PCR. Primers and probes for real-time RT-PCR are shown in Table 1. The probes used for detection in real-time PCR were labeled with 6-carboxyfluorescein (FAM) at their 5′-terminal and quenched with 6-carboxytetramethylrhodamine (TAMRA) on their 3′-terminal. TaqMan assays for rat CCR5, IFN-γ, CD3, CD4, GAPDH, and human CCR4 were obtained from Applied Biosystems.

TaqMan PCR assays were performed as one-step RT-PCR using the EZ-RT-PCR Reagent Kit (Applied Biosystem) and 40 ng of total RNA. To quantify the results, a calibration curve was used. Serial dilutions of rat lung or spleen RNA were used as standards and were run in parallel to the samples. The results were normalized to endogenous controls (GAPDH, β-actin or cytokeratin-19).

Immunohistochemistry. Rat lung tissues were fixed in formalin and embedded in paraffin. Sections (3 μM) were incubated with a primary goat anti-rat CCR4 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and labeling was detected with a biotin-conjugated anti-goat $F(ab)_2$ fragment followed by an avidin–biotin peroxidase complex amplification step. As controls the anti-CCR4 antibody was blocked by pre-incubation with the peptide encompassing the CCR4 protein sequence used for immunization.

Formalin-fixed, paraffin-embedded sections of human lung tissue were obtained from Biochain (Hayward, CA, USA). Human CCR4 protein was detected using a goat polyclonal anti CCR4 antibody (Abcam, Cambridge, UK). Sections were stained with AEC+ (DAKO, Carpinteria, CA). In controls a normal goat IgG was used instead of the CCR4-specific antibody.

Immunofluorescence. Immunofluorescence staining was performed on human NHBE (primary normal human bronchial epithelial cells) seeded onto collagen-I-coated chamber slides (BD Bioscience, Heidelberg, Germany) and cultivated in completely supplemented BEGM according to the instructions of the provider (Clonetics, San Diego, CA). For staining cells were washed with PBS and fixed in 4% paraformaldehyde. Slides were blocked with 10% rabbit serum in PBS. A goat polyclonal anti-CCR4 (human) antibody (Abcam, Cambridge, UK) was used as the primary antibody. To rule out non-specific staining, a matching isotype negative control was used instead of the CCR4-specific antibody. Binding of the primary antibody was detected using an Alexa488-labeled anti-goat antibody (Molecular Probes, Eugene, OR). Nuclei were stained with propidium iodide. Cells were imaged using a fluorescence microscope (Leica, Bensheim, Germany).

Table 1
Primers and probes used for quantitative real-time RT-PCR

	Sequence	Gene
Forward primer	5′-TGGCTGCCCTGCTTCTG-3′	TARC
Reverse primer	5′-AATGGCCCCCTTGAAGTAGTC-3′	TARC
Probe	5′-CGAGCCACCAATGTAGGCCGAGA-3′	TARC
Forward primer	5′-ACTTCAGACCTCCGATGCA-3′	MDC
Reverse primer	5′-TGTAGTCCTGGCAGCAGATACT-3′	MDC
Probe	5′-TCCCTATGGTGCCAATGTGGAAGA-3′	MDC
Forward primer	5′-CACACATACTGCAAAACCCAGTA-3′	CCR4
Reverse primer	5′-TCCAGGGAGCTGAGGACTT-3′	CCR4
Probe	5′-TCGGTCAACTCGACCACGT-3′	CCR4
Forward primer	5′-CCGTCCCACTGATGGTTT-3′	CCR8
Reverse primer	5′-GTAAAGTTGGAAGCACTGTAGCA-3′	CCR8
Probe	5′-TTACCAGGTGTCCTCTGAAGACGGC-3′	CCR8
Forward primer	5′-CACTTTGAACCAGGTCACAGAA-3′	IL-4
Reverse primer	5′-CCGTAAGGACGTCTGGTACAA-3′	IL-4
Probe	5′-AAGGGACTCCATGCACCGAGATG-3′	IL-4

Data analysis. All results are shown as means ± standard deviation. Statistical significance was determined using a Student's t test, and p values <0.05 were considered statistically significant.

Results

Expression of TARC and MDC in rat models of pulmonary diseases

We analyzed the mRNA expression of TARC and MDC in the lung tissue of different disease models, i.e., in an OVA-induced asthma model (Fig. 1A), a bleomycin-induced fibrosis model (Fig. 1C) and in a model of acute and chronic airway inflammation caused by cigarette smoke (Fig. 1A) using real-time PCR.

In accordance with the well-established Th2 response in acute asthma, we found a strong increase in both MDC and TARC mRNA expression in the lung tissue of the acute OVA-induced asthma model (Fig. 1A). Similarly, TARC and MDC mRNA were strongly upregulated in a rat bleomycin-induced fibrosis model (Fig. 1C) through days 3–9 following the bleomycin challenge with a maximum expression of TARC and MDC at day 6. This is in accordance with previously published data showing a selective induction of MDC and TARC in alveolar macrophages in a rat model of radiation pneumonitis and in human idiopathic pulmonary fibrosis [21].

Cigarette smoke-induced inflammation of the airways and lung parenchyma is widely accepted as the major cause of COPD. To characterize the role of MDC and TARC in the acute inflammatory response caused by cigarette smoke, we analyzed the expression of both chemokines in rats exposed to cigarette smoke for 4 days. The inflammatory component of this model is thought to be Th1 driven and is characterized by an increase in the recruitment of macrophages and neutrophils into the lung, goblet cell metaplasia, and mucus hypersecretion (data not shown). Real-time PCR revealed that the pulmonary inflammation caused by cigarette smoke was associated with a significantly elevated gene expression of TARC and MDC in the lungs of rats exposed to smoke for 4 days (Fig. 1A). In accordance with the elevated TARC mRNA expression, TARC protein levels in lung homogenates and bronchoalveolar fluid (BALF) were found to be strongly elevated in the smoke-treated group (Figs. 2A and C). No elevated TARC serum levels were detected (Fig. 2D) indicating a local, rather than a systemic, effect of cigarette smoke exposure on TARC expression. Since there is no rat MDC ELISA available, MDC protein levels were not measured.

To determine whether the increased mRNA expression of TARC and MDC is the result of an acute inflammatory response or is also detectable in chronically inflamed airways, rats were exposed to cigarette smoke for 4 weeks. This resulted in an increased lung expression of TARC and MDC mRNA indicating an involvement of MDC and TARC in the chronic inflammation induced by sustained cigarette smoke exposure (Fig. 1A).

Alveolar macrophages as major source of TARC and MDC in the lung of cigarette smoke-treated rats

To identify the cell type that is responsible for the cigarette smoke-induced increase of TARC and MDC, the expression of both chemokines was investigated in bronchoalveolar lavage (BAL)-derived cells and in the bronchus from smoke-exposed rats and untreated controls (Figs. 3A and B). We found a strong cigarette smoke-induced increase in TARC and MDC mRNA expression in the BAL cells, whereas none of the chemokines showed an elevated expression in the bronchus of smoke-treated rats. In accordance with the elevated mRNA expression, we found highly increased TARC

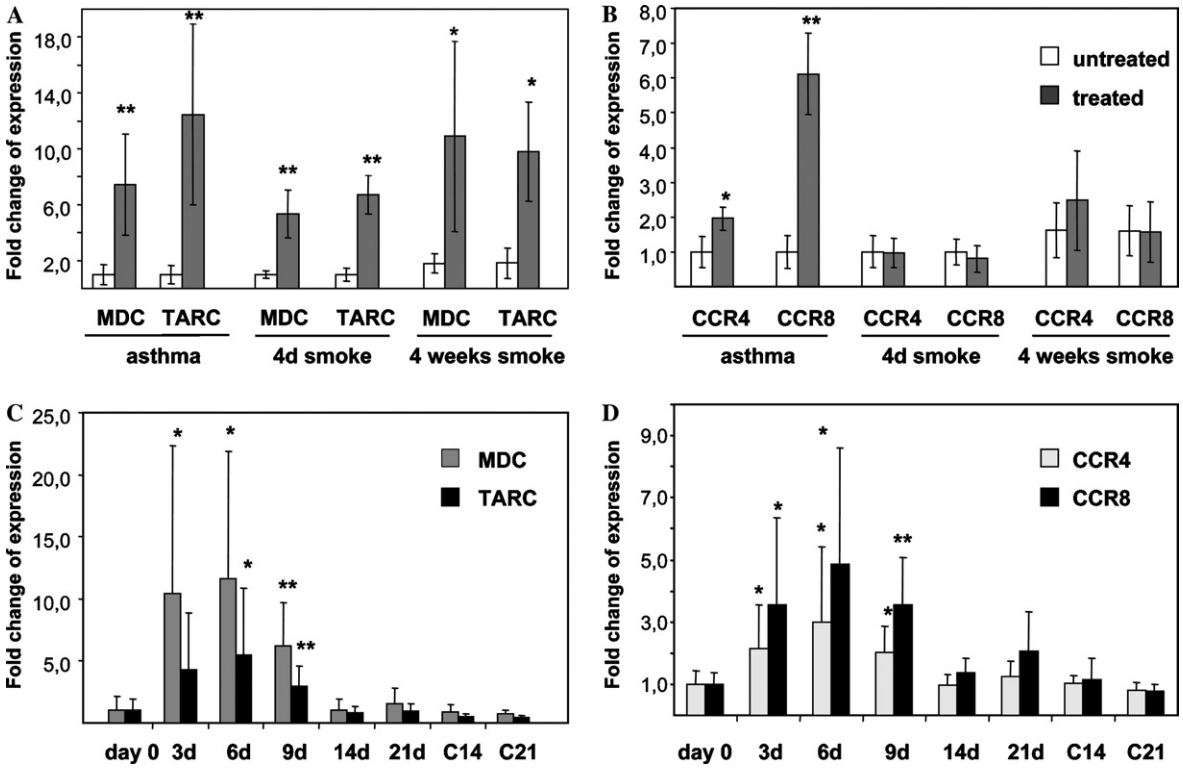

Fig. 1. mRNA expression of MDC, TARC, CCR4, and CCR8 in rat models of pulmonary inflammation. (A) MDC and TARC and (B) CCR4 and CCR8 mRNA expression in rat models of acute asthma and cigarette smoke-induced airway inflammation. In the asthma model ovalbumin (OVA)-sensitized rats were challenged with OVA (gray bars) or NaCl (white bars) for 2d ($n = 6$ per group). In the acute cigarette smoke-model, rats ($n = 5$ per group) were exposed to cigarette smoke for 4 days (gray bars). In the model of cigarette smoke-induced chronic airway inflammation, rats ($n = 5$ per group) were exposed to smoke for 4 weeks (gray bars). Total RNA was extracted from lungs and mRNA expression of (A) MDC and TARC and (B) CCR4 and CCR8 was analyzed by quantitative real-time RT-PCR. Expression data were normalized using cytokeratin-19 as the housekeeping gene. Data are shown as fold changes of mRNA expression relative to the untreated controls (white bars). (C) MDC and TARC and (D) CCR4 and CCR8 mRNA expression in a rat model of pulmonary fibrosis. Lung fibrosis was induced by intratracheal application of bleomycin. Animals were sacrificed at the indicated time points after bleomycin challenge ($n = 10$ per group). mRNA expression of (C) MDC and TARC and (D) CCR4 and CCR8 was analyzed by quantitative real-time RT-PCR. Expression data were normalized using cytokeratin-19 as the housekeeping gene. Data are shown as fold changes in mRNA expression relative to the untreated control group at day 0; C14, C21: NaCl-treated control day 14, day 21 ($n = 5$ per group). *$p < 0.05$; **$p < 0.005$.

protein levels in the lysates of BAL cells of smoke-treated rats compared to the untreated controls (Fig. 2B). Since the BAL fluid contains several cell populations, we isolated macrophages from the BAL to examine which cell type is responsible for the enhanced expression of TARC and MDC. As shown in Fig. 3C, TARC and MDC mRNA expression was strongly upregulated in alveolar macrophages of cigarette smoke-treated animals.

Expression of the chemokine receptor CCR4 in animal models of pulmonary inflammation

To investigate whether an increased expression of TARC and MDC correlates with an enhanced expression of its receptor, we analyzed the expression of CCR4 in the lungs of the asthma, fibrosis, and cigarette smoke models by real-time PCR.

In the lung of the asthma model, the increased expression of TARC and MDC was associated with an elevated mRNA expression of CCR4 and the Th2 lymphocyte markers CCR8 and IL-4 (data not shown), indicating an influx of CCR4 and CCR8-positive IL-4-producing Th2 cells into the lungs (Fig. 1B). Likewise, the elevated expression of TARC and MDC observed in the fibrosis model was accompanied by the increased mRNA expression of the Th2 cell markers CCR4 and CCR8 in lung tissue of bleomycin-treated rats (Fig. 1D). However, in sharp contrast to these results, the increased expression of TARC and MDC in the models of cigarette smoke-induced acute and chronic pulmonary inflammation was not associated with an elevated expression of CCR4 or CCR8 in the lung tissue (Fig. 1B).

No major recruitment of CCR4-positive Th2 cells into lung tissue of smoke-treated rats

Since increased CCR4 and CCR8 mRNA expression in the lungs of smoke-treated rats was not observed,

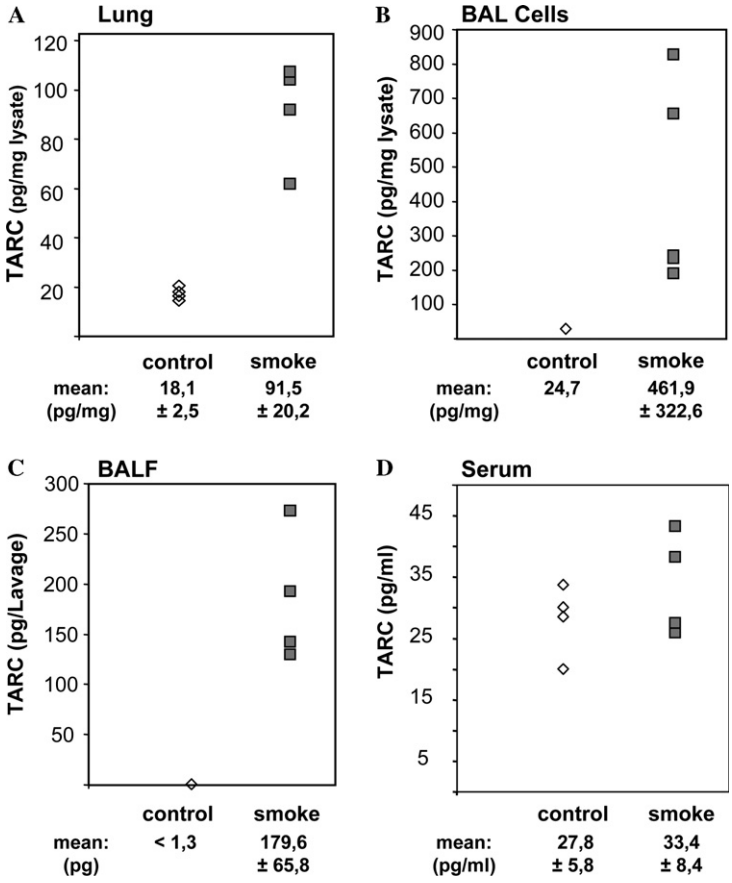

Fig. 2. Measurement of TARC protein levels in rat lung by ELISA. Rats ($n = 4$ per group) were exposed to cigarette smoke for 4 days. Rats were killed and TARC protein levels of (A) total lung, (B) cells obtained by bronchoalveolar lavage (BAL), (C) cell-free bronchoalveolar lavage fluid (BALF), and (D) serum were determined by ELISA. Data were normalized by the protein concentration of cell lysates (lung and BAL cells) or total volume (BALF).

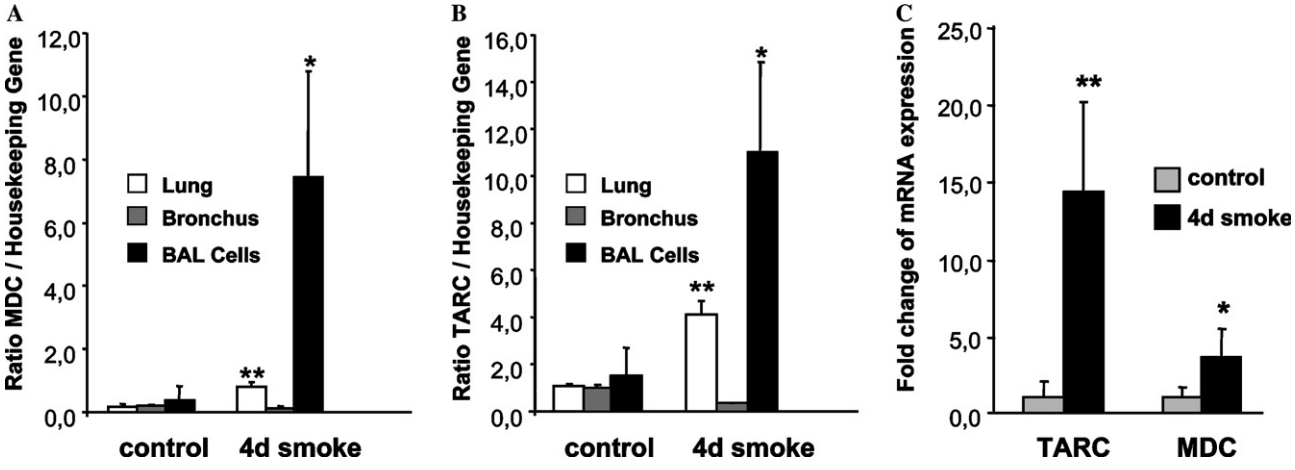

Fig. 3. mRNA expression of MDC and TARC in rat lung, bronchus, BAL cells, and alveolar macrophages. Rats were exposed to cigarette smoke for 4 days and recruited inflammatory cells were isolated by bronchoalveolar lavage (BAL). Total RNA was prepared from lung and bronchus after lavage and from the cells obtained by BAL. mRNA expression of (A) MDC and (B) TARC was analyzed by quantitative real-time RT-PCR ($n = 5$ per group). Expression data were normalized using cytokeratin-19 (lung and bronchus) or GAPDH (BAL cells) as housekeeping genes. (C) mRNA expression of MDC and TARC in rat alveolar macrophages. Rats were exposed to cigarette smoke for 4 days and cells were isolated by bronchoalveolar lavage. Alveolar macrophages were isolated by adhesion to plastic dishes and used for RNA preparation. mRNA expression of TARC and MDC was analyzed by quantitative real-time RT-PCR. Expression data were normalized using GAPDH as an endogenous control. Data are shown as fold changes in mRNA expression relative to the untreated control group. $n = 4$ (control), $n = 6$ (4d smoke) in each group. $^{*}p < 0.05$; $^{**}p < 0.005$.

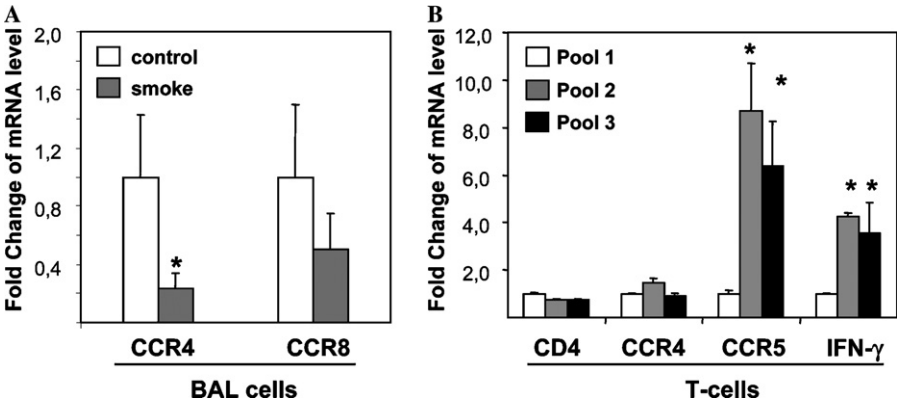

Fig. 4. mRNA expression analysis of total BAL cells and T-cells purified from BAL. Rats were exposed to cigarette smoke for 4 days. (A) Recruited cells were isolated from lung by bronchoalveolar lavage (BAL) and total cells were used for RNA preparation. mRNA expression of TARC and MDC was analyzed by quantitative real-time RT-PCR. Expression data were normalized using GAPDH as the housekeeping gene. Data are shown as fold changes in mRNA expression relative to the untreated control group ($n = 6$ per group). (B) T-lymphocytes were isolated from pooled BAL cells by magnetic beads. Total RNA was extracted from T-cells and mRNA expression of CD4, CCR4, CCR5, and IFN-γ was analyzed by quantitative real-time RT-PCR. Data are shown as fold changes in mRNA expression relative to the untreated control group; pool 1: untreated control ($n = 6$); pool 2 and pool 3: 4d smoke treated ($n = 3$ in each pool). *$p < 0.05$.

we investigated the expression of CCR4 and CCR8 in the BAL cells of smoke-exposed rats (Fig. 4A). Surprisingly, a reduced level of CCR4 and CCR8 mRNA expression in BAL cells of smoke-exposed rats compared to the untreated controls was detected. This is likely to be due to the strong increase in the total number of macrophages and neutrophils following smoke exposure (data not shown), compared to the relative low number of lymphocytes found in the BAL. This finding implies that CCR4 or CCR8-positive cells are not recruited to lungs of smoke-treated rats.

To further characterize the T-cell population found in the BAL of smoke-treated and untreated rats, T-lymphocytes were isolated from BAL fluid and used for mRNA expression profiling. The expression analysis of the isolated T-cells and the T-cell-depleted BAL fraction (i.e., macrophages and neutrophils) demonstrated that among the cells found in the lavage, T-lymphocytes displayed the highest expression of CCR4, whereas CCR4 expression was hardly detectable in the T-cell-depleted fraction (data not shown). However, there was no significant difference in the CCR4 expression of the T-cell population of smoke-treated and untreated rats (Fig. 4B). This indicates that CCR4-positive T-cells (e.g., Th2 cells) are not recruited to the rat lung following smoke exposure. In contrast, we found an increased expression of CCR5 and IFN-γ by the T-cell population of smoke-treated rats (Fig. 4B) indicating the recruitment of Th1 cells rather than Th2 cells to lungs of smoke-treated rats.

CCR4 expression in the bronchial epithelium

To further address the question of which pulmonary cells are possible targets for TARC and MDC in the lung, we examined the expression pattern of CCR4 in rat lung by immunohistochemistry. There was prominent specific staining of bronchial epithelial cells by an anti-CCR4 antibody that could be blocked by the corresponding CCR4 immunizing peptide demonstrating the specificity of the binding (Figs. 5A and D). No difference in CCR4 protein expression was observed between untreated and smoke-treated rats by immunohistochemistry, indicating a constitutive expression of CCR4 in cells of the rat bronchial epithelium (data not shown).

To analyze whether CCR4 is also expressed by human lung epithelium, sections of the human bronchus (Figs. 5B and E) and trachea (Figs. 5C and F) were stained using an anti-human CCR4 or IgG control antibody. These results demonstrated a specific expression of CCR4 in tracheal and bronchial epithelial cells of the human lung. CCR4 protein and mRNA expression was also detected in isolated primary human bronchial epithelial cells (NHBE) by immunofluorescence (Fig. 6A) and RT-PCR (Fig. 6B).

Discussion

We evaluated the role of MDC and TARC, which are known chemoattractants for Th2 cells, in a cigarette smoke-induced airway inflammation model and compared the findings to animal models of asthma and lung fibrosis. Our results demonstrate that MDC and TARC are not only involved in Th2-mediated diseases like asthma but also play an important role for the Th1-mediated immune response like cigarette smoke-induced airway inflammation. In marked contrast to the findings in the asthma and lung fibrosis models, the increased expression of MDC and TARC in the cigarette smoke-induced airway inflammation model is not associated with an increased infiltration of Th2 cells into the lungs.

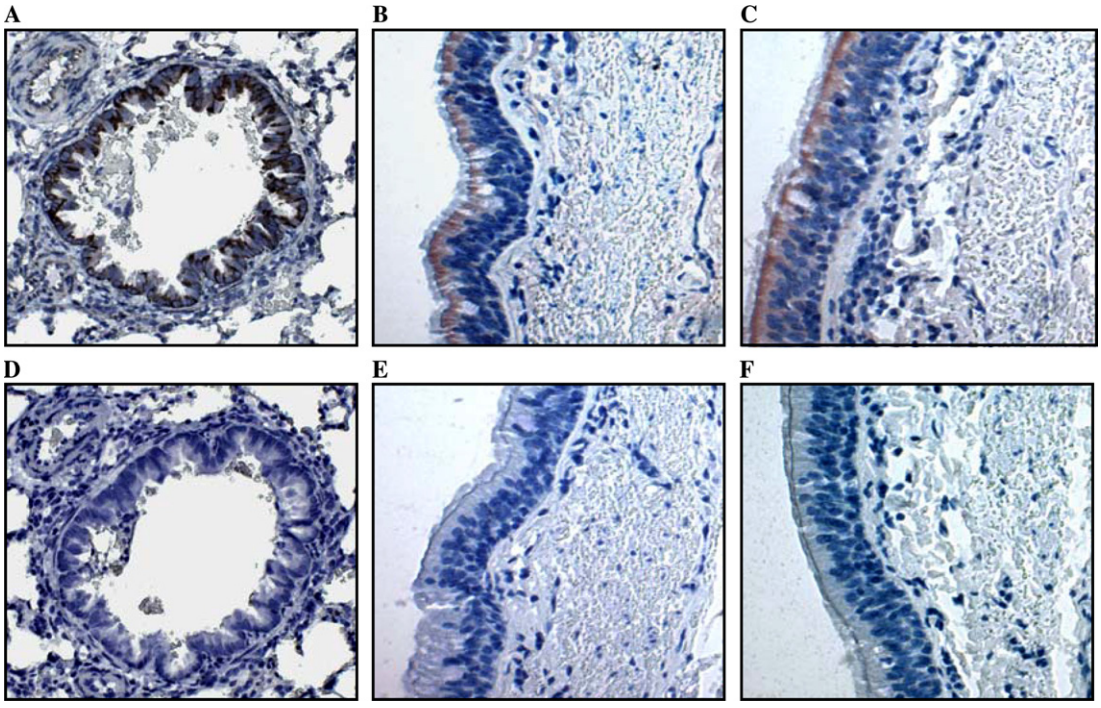

Fig. 5. CCR4 expression in rat and human lung epithelium. (A) CCR4 expression in rat bronchial epithelium was detected in paraffin sections of rat lungs using a polyclonal anti-CCR4 antibody and DAB staining (brown). (D) No staining of rat bronchial epithelia was observed in the presence of a CCR4-blocking peptide. In sections of human lung CCR4 was detected in the epithelium of the (B) bronchium and (C) trachea using a polyclonal anti-human CCR4 antibody and AEC staining (red-brown). No staining of human lung epithelia was observed in the IgG controls (E,F). All sections were counterstained with hematoxylin (blue).

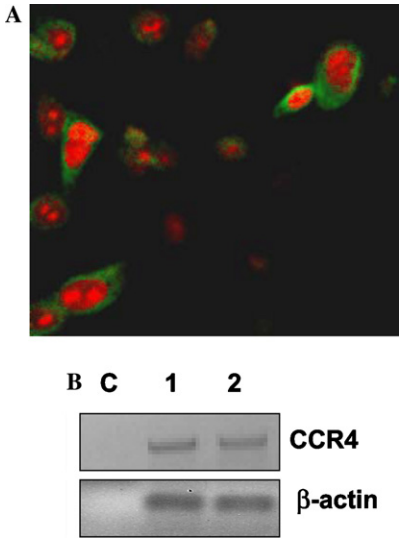

Fig. 6. CCR4 expression in primary bronchial epithelial cells. (A) CCR4 expression (green) of cultivated normal human bronchial epithelial cells (NHBE) was analyzed by immunofluorescence of paraformaldehyde-fixed cells. Nuclei were stained with propidium iodide (red). No staining was observed with an isotype control antibody (data not shown). (B) Detection of CCR4 and β-actin mRNA expression in NHBE derived from two different donors (1 and 2) by RT-PCR (lane C, negative control of RT-PCR). mRNA expression of CCR4 and β-actin was analyzed by quantitative real-time RT-PCR (not shown) and PCR products were separated on an agarose gel.

Our data indicate that instead of Th2 cells, airway epithelial cells expressing CCR4 might be the principal targets for MDC and TARC released from alveolar macrophages during cigarette smoke-induced inflammation. Therefore, our data suggest a novel role for MDC and TARC in type-1 inflammations different from Th2 cell recruitment.

Chronic obstructive pulmonary disease (COPD) is a pulmonary disease closely associated with cigarette smoking. The disease encompasses chronic bronchitis and emphysema and is characterized as a chronic type-1 inflammation involving an increased recruitment of macrophages and neutrophils into the lungs [22]. We analyzed the expression of MDC and TARC in an airway inflammation model based on the cigarette smoke exposure of rats for at least 4 days. Exposure to smoke results in an increased number of neutrophils and macrophages recruited into the lung and leads to goblet cell metaplasia and mucus hypersecretion. Therefore, the model displays several features characteristic of COPD. Our results demonstrate an increased expression of TARC and MDC in the lungs of smoke-treated animals, indicating an important role for both chemokines in cigarette smoke-induced acute and chronic pulmonary inflammation. An increased expression of both chemokines was detected in alveolar macrophages obtained from the smoke-treated animals demonstrating that

these cells are a major source for the elevated levels of MDC and TARC. Alternative sources for MDC and TARC expression in the lung are smooth muscle cells [17] and bronchial epithelial cells [1,6]. Although we cannot exclude that these type of cells contribute to the elevated expression of TARC and MDC in the cigarette smoke model, the observation that the expression of both chemokines in the bronchus of rats is not elevated by smoke treatment suggests that these cells have no major impact on the elevated MDC and TARC levels found in smoke-treated lungs.

In accordance with previous published data, we also detected elevated levels of TARC and MDC expression in models of asthma [16,17] and pulmonary fibrosis [21] emphasizing the type-2 character of these inflammations. However, in contrast to the results obtained from the models of asthma and lung fibrosis, the increased expression of MDC and TARC observed in the cigarette smoke model was not accompanied by an increased expression of Th2 cell markers CCR4, CCR8 or IL-4 in smoke-treated lungs. This was confirmed by the gene expression analysis of T-cells isolated from smoke-treated lungs. These results demonstrated an increased expression of the Th1 markers CCR5 and IFN-γ by T-lymphocytes isolated from smoke-exposed lungs, whereas the T-lymphocyte expression of the Th2 markers CCR4 and IL-4 was not changed by smoke exposure. This clearly indicates an increased recruitment of Th1, rather than Th2, cells into the smoke-exposed lung. Likewise, it is assumed that a Th1 $CD4^+$ and a $CD8^+$ T-cell population is involved in the inflammatory process of COPD in humans [23]. In addition, increased numbers of IFN-γ positive T-cells in the peripheral blood and lungs were reported in patients with COPD [24,25].

There are only few reports pointing to a role of MDC or TARC in type-1 inflammatory diseases. Recently, elevated levels of MDC and TARC have been detected in atherosclerotic lesions [18] and in inflamed mucosa of Crohn's disease patients [19], two diseases known to be associated with a type-1 inflammation. Although the precise role of MDC and TARC in the type-1 inflammatory response is not completely understood, the increased expression of both chemokines might reflect competing programs of Th1 and Th2 immune response within the inflamed tissue.

It has been demonstrated that MDC and TARC can act as chemoattractants for monocytes [10,17]. In our model, there is an increase in the number of alveolar macrophages after smoke treatment, but we could not detect CCR4 protein expression on alveolar macrophages or monocytes by immunohistochemical methods. However, we cannot exclude that there is an increased MDC or TARC-induced recruitment of monocytes into the lung following smoke exposure that is accompanied with the subsequent downregulation of CCR4 expression by inflammatory stimuli or during macrophage differentiation.

Our studies revealed that cells of the bronchial epithelium display a constitutive CCR4 expression indicating that these cells are major targets for MDC and TARC in smoke-induced inflammation. The bronchial epithelium plays a key role in the pulmonary inflammation as a source for many cytokines and chemokines. In addition, bronchial epithelial cells have been shown to express the chemokine receptors CCR3 [26], CXCR3 [27], and CXCR4 [28]. Although the effects of chemokines on the bronchial epithelium are still not understood, it is speculated that they are related to modulation of epithelial function including cell migration, activation, proliferation or apoptosis. Similar effects might be mediated by CCR4. However, further experiments are necessary to reveal the biological and pathophysiological effects of MDC and TARC on airway epithelial cells and the consequences for cigarette smoke-induced inflammatory airway response.

References

[1] M.C. Berin, L. Eckmann, D.H. Broide, M.F. Kagnoff, Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts, Am. J. Respir. Cell Mol. Biol. 24 (2001) 382–389.

[2] J.J. Campbell, G. Haraldsen, J. Pan, J. Rottman, S. Qin, P. Ponath, D.P. Andrew, R. Warnke, N. Ruffing, N. Kassam, L. Wu, E.C. Butcher, The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells, Nature 400 (1999) 776–780.

[3] T. Horikawa, T. Nakayama, I. Hikita, H. Yamada, R. Fujisawa, T. Bito, S. Harada, A. Fukunaga, D. Chantry, P.W. Gray, A. Morita, R. Suzuki, T. Tezuka, M. Ichihashi, O. Yoshie, IFN-gamma-inducible expression of thymus and activation-regulated chemokine/CCL17 and macrophage-derived chemokine/CCL22 in epidermal keratinocytes and their roles in atopic dermatitis, Int. Immunol. 14 (2002) 767–773.

[4] T. Imai, T. Yoshida, M. Baba, M. Nishimura, M. Kakizaki, O. Yoshie, Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein–Barr virus vector, J. Biol. Chem. 271 (1996) 21514–21521.

[5] F. Sallusto, B. Palermo, D. Lenig, M. Miettinen, S. Matikainen, I. Julkunen, R. Forster, R. Burgstahler, M. Lipp, A. Lanzavecchia, Distinct patterns and kinetics of chemokine production regulate dendritic cell function, Eur. J. Immunol. 29 (1999) 1617–1625.

[6] T. Sekiya, M. Miyamasu, M. Imanishi, H. Yamada, T. Nakajima, M. Yamaguchi, T. Fujisawa, R. Pawankar, Y. Sano, K. Ohta, A. Ishii, Y. Morita, K. Yamamoto, K. Matsushima, O. Yoshie, K. Hirai, Inducible expression of a Th2-type CC chemokine thymus- and activation-regulated chemokine by human bronchial epithelial cells, J. Immunol. 165 (2000) 2205–2213.

[7] T. Imai, M. Baba, M. Nishimura, M. Kakizaki, S. Takagi, O. Yoshie, The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4, J. Biol. Chem. 272 (1997) 15036–15042.

[8] T. Imai, M. Nagira, S. Takagi, M. Kakizaki, M. Nishimura, J. Wang, P.W. Gray, K. Matsushima, O. Yoshie, Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells

by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine, Int. Immunol. 11 (1999) 81–88.
[9] D.P. Andrew, M.S. Chang, J. McNinch, S.T. Wathen, M. Rihanek, J. Tseng, J.P. Spellberg, C.G. Elias, STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13, J. Immunol. 161 (1998) 5027–5038.
[10] R. Godiska, D. Chantry, C.J. Raport, S. Sozzani, P. Allavena, D. Leviten, A. Mantovani, P.W. Gray, Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells, J. Exp. Med. 185 (1997) 1595–1604.
[11] T. Imai, D. Chantry, C.J. Raport, C.L. Wood, M. Nishimura, R. Godiska, O. Yoshie, P.W. Gray, Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4, J. Biol. Chem. 273 (1998) 1764–1768.
[12] M.C. Berin, The role of TARC in the pathogenesis of allergic asthma, Drug News Perspect. 15 (2002) 10–16.
[13] T.F. Leung, G.W. Wong, F.W. Ko, C.W. Lam, T.F. Fok, Increased macrophage-derived chemokine in exhaled breath condensate and plasma from children with asthma, Clin. Exp. Allergy 34 (2004) 786–791.
[14] P. Panina-Bordignon, A. Papi, M. Mariani, P. Di Lucia, G. Casoni, C. Bellettato, C. Buonsanti, D. Miotto, C. Mapp, A. Villa, G. Arrigoni, L.M. Fabbri, F. Sinigaglia, The C–C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics, J. Clin. Invest. 107 (2001) 1357–1364.
[15] N. Terada, T. Nomura, W.J. Kim, Y. Otsuka, R. Takahashi, H. Kishi, T. Yamashita, N. Sugawara, S. Fukuda, T. Ikeda-Ito, A. Konno, Expression of C–C chemokine TARC in human nasal mucosa and its regulation by cytokines, Clin. Exp. Allergy 31 (2001) 1923–1931.
[16] S. Kawasaki, H. Takizawa, H. Yoneyama, T. Nakayama, R. Fujisawa, M. Izumizaki, T. Imai, O. Yoshie, I. Homma, K. Yamamoto, K. Matsushima, Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice, J. Immunol. 166 (2001) 2055–2062.
[17] J.A. Gonzalo, Y. Pan, C.M. Lloyd, G.Q. Jia, G. Yu, B. Dussault, C.A. Powers, A.E. Proudfoot, A.J. Coyle, D. Gearing, J.C. Gutierrez-Ramos, Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation, J. Immunol. 163 (1999) 403–411.
[18] D.R. Greaves, T. Hakkinen, A.D. Lucas, K. Liddiard, E. Jones, C.M. Quinn, J. Senaratne, F.R. Green, K. Tyson, J. Boyle, C. Shanahan, P.L. Weissberg, S. Gordon, S. Yla-Hertualla, Linked chromosome 16q13 chemokines, macrophage-derived chemokine, fractalkine, and thymus- and activation-regulated chemokine, are expressed in human atherosclerotic lesions, Arterioscler. Thromb. Vasc. Biol. 21 (2001) 923–929.
[19] F. Jugde, M. Alizadeh, C. Boissier, D. Chantry, L. Siproudhis, S. Corbinais, E. Quelvennec, F. Dyard, J.P. Campion, M. Gosselin, J.F. Bretagne, G. Semana, D. Heresbach, Quantitation of chemokines (MDC, TARC) expression in mucosa from Crohn's disease and ulcerative colitis, Eur. Cytokine Netw. 12 (2001) 468–477.
[20] Y. Chvatchko, A.J. Hoogewerf, A. Meyer, S. Alouani, P. Juillard, R. Buser, F. Conquet, A.E. Proudfoot, T.N. Wells, C.A. Power, A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock, J. Exp. Med. 191 (2000) 1755–1764.
[21] T. Inoue, S. Fujishima, E. Ikeda, O. Yoshie, N. Tsukamoto, S. Aiso, N. Aikawa, A. Kubo, K. Matsushima, K. Yamaguchi, CCL22 and CCL17 in rat radiation pneumonitis and in human idiopathic pulmonary fibrosis, Eur. Respir. J. 24 (2004) 49–56.
[22] P.J. Barnes, Mechanisms in COPD: differences from asthma, Chest 117 (2000) S10–S14.
[23] M.G. Cosio, J. Majo, M.G. Cosio, Inflammation of the airways and lung parenchyma in COPD: role of T cells, Chest 121 (2002) 160S–165S.
[24] S. Grumelli, D.B. Corry, L.Z. Song, L. Song, L. Green, J. Huh, J. Hacken, R. Espada, R. Bag, D.E. Lewis, F. Kheradmand, An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema, PLoS Med. 1 (2004) e8.
[25] M. Majori, M. Corradi, A. Caminati, G. Cacciani, S. Bertacco, A. Pesci, Predominant TH1 cytokine pattern in peripheral blood from subjects with chronic obstructive pulmonary disease, J. Allergy Clin. Immunol. 103 (1999) 458–462.
[26] C. Stellato, M.E. Brummet, J.R. Plitt, S. Shahabuddin, F.M. Baroody, M.C. Liu, P.D. Ponath, L.A. Beck, Expression of the C–C chemokine receptor CCR3 in human airway epithelial cells, J. Immunol. 166 (2001) 1457–1461.
[27] S.G. Kelsen, M.O. Aksoy, Y. Yang, S. Shahabuddin, J. Litvin, F. Safadi, T.J. Rogers, The chemokine receptor, CXCR3, and its splice variants are expressed in human airway epithelial cells, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L584–L591.
[28] J. Eddleston, S.C. Christiansen, B.L. Zuraw, Functional expression of the C-X-C chemokine receptor CXCR4 by human bronchial epithelial cells: regulation by proinflammatory mediators, J. Immunol. 169 (2002) 6445–6451.