

Elevated monocyte chemotactic proteins 1, 2, and 3 in pulmonary alveolar proteinosis are associated with chemokine receptor suppression

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Abstract

Pulmonary alveolar proteinosis (PAP) is a rare autoimmune lung disease characterized by abnormal surfactant accumulation within alveolar macrophages, and circulating auto-antibodies against granulocyte-macrophage colony stimulating factor (GM-CSF) resulting in functional GM-CSF deficiency. Monocyte/macrophage chemotactic protein-1 (MCP-1) is elevated in PAP, suggesting association with the pathophysiology. Because PAP has been associated with inflammatory pulmonary changes, we hypothesized that other MCP family chemokines would be present and that Chemokine Chemotaxis Receptor 2 (CCR2) would be elevated on PAP mononuclear cells. Here we show for the first time that MCP-2 and MCP-3, like MCP-1, are highly elevated in PAP. We also confirm that PAP alveolar macrophages and not epithelial cells produce MCP-1, and that MCP-1 from PAP lung has functional chemoattractant activity. Surprisingly, CCR2 expression is diminished in PAP lymphocytes and alveolar macrophages compared to controls. Further, MCP-1 from PAP lung suppresses CCR2 expression *in vitro*, suggesting that in PAP, MCP-1 participates in an autocrine regulatory network *in vivo*.

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Introduction

Pulmonary alveolar proteinosis is a rare lung disease characterized by the accumulation of lipoproteinaceous material in the alveoli indicating inefficient surfactant catabolism by alveolar macrophages [1,2]. Correlation of a GM-CSF deficiency with PAP lung disease came from observations in the GM-CSF knockout mouse. These mice develop a PAP-like lung disease at about 6–8 weeks of age with seemingly no hematopoietic abnormalities [3]. The pulmonary abnormality in these animals can be corrected by the site-directed expression of GM-CSF in the lung [4]. This

was the first evidence to suggest that GM-CSF is essential for normal lung homeostasis. Subsequent to these studies was the observation by Tanaka, Kitamura, and colleagues [5,6] showing that the sera and bronchoalveolar lavage fluid from PAP patients contains neutralizing anti-GM-CSF. The murine [7] and human PAP lung disease both have elevated levels of bronchoalveolar lavage MCP-1 [8,9]. The typical histopathology of idiopathic PAP appears to be a bland filling of the alveolar spaces without significant cellular inflammation, distortion of normal architecture, or fibrosis. Several reports, however, have suggested a bronchoalveolar lavage (BAL) lymphocytosis in PAP [8,10,11] while others have not [9].

Chemokines, including the monocyte chemotactic proteins (MCP), are small chemoattractant proteins of 70–130 amino acids that are produced by a variety of cells including lymphocytes, monocytes/macrophages, smooth muscle cells, epithelial cells, endothelial cells, and fibroblasts

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[12]. These proteins activate G-protein-coupled receptors and induce cells to migrate through a concentration gradient [13]. Some chemokines are homeostatic in nature and are constitutively produced and secreted [14]. Monocyte/macrophage chemotactic factors are a class of -CC- chemokines, which have been associated with cellular recruitment in inflammation, infection, and wound healing [15–17].

MCP proteins are specifically chemotactic for monocytes and lymphocytes [18]. MCP-1, MCP-2, and MCP-3 have considerable structural homology and are redundant in biological properties [19,20]. The major differences among the MCP chemokines are in the concentrations required to mediate their binding properties and biological activities. All three chemokines bind to CCR2. However, MCP-2 and MCP-3 also recognize other receptors whereas MCP-1 appears to interact with only CCR2 [19,20].

Based upon these observations, we propose that other MCP proteins such as MCP-2 and MCP-3, which share the CCR2 receptor, may be elevated in PAP lungs, and that CCR2 itself is elevated. Furthermore, the status of cellular composition of PAP BAL is unclear, despite the tremendously elevated levels of bronchoalveolar lavage MCP-1. We hypothesize that the distribution of mononuclear cells in PAP BAL is regulated by concentration and biological activities of MCPs via effects on CCR2 expression.

Methods

Study population

This protocol was approved by the Institutional Review Board and written informed consent was obtained from all subjects. Healthy controls ($n = 11$) had no history of lung disease and were not on medication. These volunteers underwent bronchoscopy for research purposes only. The diagnosis of idiopathic PAP ($n = 26$) was established by histopathological examination of material from open lung or transbronchial biopsies as previously described [21]. Characteristics of the healthy controls and PAP patients are described in Table 1. None of the patients were on GM-CSF therapy.

Cell collection and culture

Alveolar macrophages and BAL were obtained by fiberoptic bronchoscopy on all PAP and healthy control subjects as previously described [22]. The lavage was obtained from two segments in the right middle lobe and lingula. Saline warmed to 37°C was instilled in 50-ml aliquots (a total of 150 ml per segment) and withdrawn by gentle aspiration. The fluid was separated from cells by centrifugation, aliquoted and stored at -80°C until assayed. The BAL cell pellet was used to determine lavage cell differentials and mRNA gene expression. For culture, BAL cells were plated into 24-well plates (300,000 alveolar

Table 1

Characteristics of patients with alveolar proteinosis and healthy controls

Variable	Alveolar proteinosis ($n = 26$)	Healthy controls ($n = 11$)
Age, years		
Mean \pm SD	45 \pm 10	33 \pm 11
Median (range)	46 (22–65)	36 (20–45)
Race, n (%)		
White	6 (55)	24 (92)
Non-White	5 (45)	2 (8)
Sex, n (%)		
Men	8 (73)	17 (65)
Women	3 (27)	9 (35)
Smoking status		
Nonsmoker	21	11
Active smoker	5	0

macrophages per well) or chamber slides (60,000 cells/well) in RPMI 1640 medium supplemented with 5% human AB serum (Gemini, Calabasas, CA), L-glutamine, and antibiotics. Bronchial epithelial cell preparations were obtained by brushing the upper airway and characterized by Wright stain and immunocytochemistry for cytokeratin content [23]. Peripheral blood mononuclear leukocytes were derived by gradient centrifugation using CPT collection tubes (Becton-Dickinson, Franklin Lakes, NJ).

Chemotaxis assays

Using transwells (Fisher Scientific, 4- μ m filter/24-well transwells) 500 μ l of PAP or healthy control BAL \pm 10 ng/ml recombinant MCP-1 (R&D Minneapolis, MN) were placed in the lower chamber of the transwell as described elsewhere [24]. The healthy control BALs ($n = 4$) used for the chemotaxis assays had no detectable MCP-1. The MCP-1 concentration in the BAL from patients ($n = 4$) was adjusted 10 or 100 ng/ml by dilution with PBS. Identical samples were incubated with 2 μ g/ml anti-MCP-1 neutralizing antibody (R&D Systems). A standard curve of 0–10 ng/ml was run concurrently with and without the antibody. THP-1 cells (1×10^6) were placed in the upper chamber, cultures were incubated for 3 h. Duplicate counts were made from each well and expressed as total cells recruited to the lower chamber.

Flow cytometry analysis

Peripheral blood cells, alveolar macrophages, and THP-1 cells (5×10^5) were stained with FITC-labeled antibodies against HLADR, CD45, CD3, and CD14 (BD-Pharmingen) as well as phycoerythrin-labeled antibodies against CCR2 and isotype controls (BD-Pharmingen) in cold PBS as described previously [25]. Peripheral blood lymphocytes were gated on CD45 and CD3 whereas BAL lymphocytes were gated upon side scatter (SSC) versus forward side scatter (FSC) in addition to and CD3 and CCR2 staining. All cells were analyzed on BD FACscan Analyzer. Results

were expressed as mean percent (%) positive staining cells per 10,000 events. THP-1 assays involved incubations for 1 h with either healthy control or PAP BAL fluid with and without the addition of neutralizing anti-human MCP-1 (2 µg/ml).

Gene array

With the assistance of the Gene Expression Array Core Facility at Case Western Reserve University and University Hospitals Health System, we analyzed three PAP and three healthy control BAL cell samples using the Affymetrix HG-U133A chip. Total RNA was extracted from BAL cells, and an amplified labeled, cRNA was generated from 5–8 µg of total RNA by using the SuperScript kit (Invitrogen) and High Yield RNA transcription labeling kit (Enzo Diagnostics). Fragmented cRNA was hybridized to arrays according to the manufacturer's instructions. Data was analyzed by the MICROARRAY suite (Affymetrix). Each patient was compared to each control giving nine possible comparisons. We selected the most stringent criteria of a twofold or greater difference in the comparisons and data are expressed relative to the healthy controls.

RNA purification and analysis

Total RNA was extracted from BAL cells and PBMC according to manufacturer's directions by RNeasy protocol (Qiagen, Valencia, CA) and PAX tubes (Becton Dickinson), respectively. Expression of mRNA was determined by real time RT-PCR using the ABI Prism 7000 Detection System (TaqMan; Applied Biosystems, Foster City, CA) described previously [25,26]. RNA specimens were analyzed in duplicate using primer sets for a housekeeping gene (GAPDH) and MCP-1 (catalog #HS00L34140-m1), MCP-2 (catalog #HS00428422-g1), MCP-3 (catalog #HS00171147-m1), CCR2 (catalog #HS00704702-s1). Threshold cycle (CT) values for genes of interest were normalized to GAPDH and used to calculate the relative quantity of mRNA expression in PAP samples relative to healthy control values.

Statistics

Data were analyzed by parametric Student's *t* test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $P \leq 0.05$.

Results

MCP-1 levels are not correlated with BAL cell differentials from PAP Patients

The study included 26 patients with PAP (mean BAL differential: alveolar macrophages, $80 \pm 4\%$; lymphocytes, $11 \pm 3\%$, PMNs, $5 \pm 1\%$, eosinophils, $4 \pm 3\%$) and 11

healthy controls (alveolar macrophages; $94 \pm 2\%$; lymphocytes; $5 \pm 2\%$, PMNs, $1 \pm 0.4\%$; Fig. 1). We also evaluated whether the BAL MCP-1 levels (9.5 ± 1.0 ng/ml) correlated with lymphocyte or alveolar macrophage differentials in the BAL. We found no correlation with either lymphocytes ($r^2 = 0.13$), or alveolar macrophages ($r^2 = 0.04$).

MCP chemokine mRNA expression is elevated in PAP BAL cells

In order to gain an overview of chemokine production in PAP lungs, we utilized microarray analysis to survey mRNA expression in BAL cells from PAP patients ($n = 3$) and healthy controls ($n = 3$) using Affymetrix. BAL RNA from PAP patients had significantly increased expression of MCP-1 as compared to healthy control [mean relative increase in mRNA expression was 118 (Table 2)]. The mean fold increase in mRNA expression was 54 for MCP-2 and 14 for MCP-3.

Real-time PCR verification of gene array analysis

In order to verify the presence of increased chemokine mRNA expression from the gene array, we evaluated

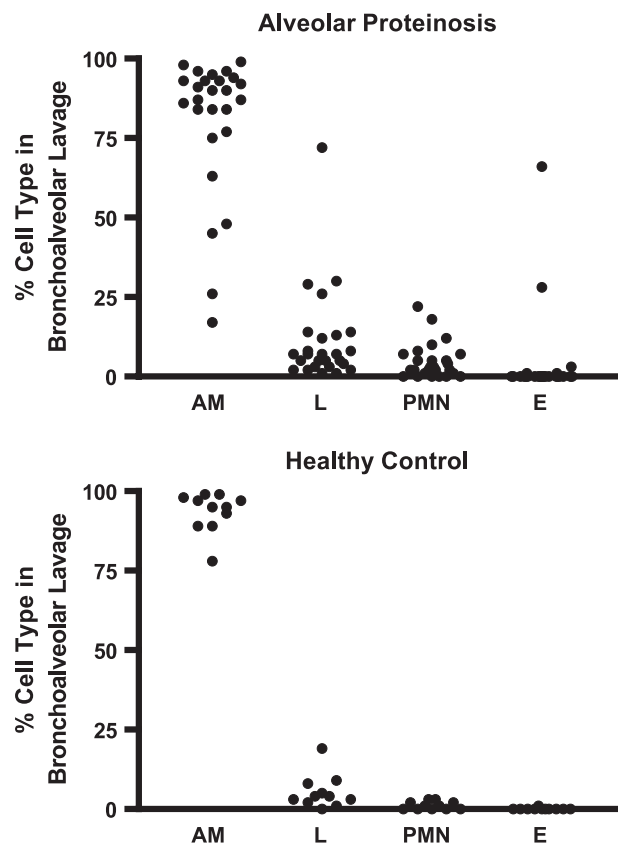


Fig. 1. PAP and healthy control BAL cellular differentials. PAP ($n = 26$) and healthy control ($n = 11$) subjects underwent bronoscopies. BAL cellular differential was obtained from cytopspins using a modified Wright stain. Partial data from 17 of these patients has been published elsewhere [9].

Table 2
Affymetrix GeneChip analysis of MCP chemokine mRNA expression in PAP bronchoalveolar lavage cells^a

	Description	Ratio	
		Range	Mean \pm SEM
MCP-1	Monocyte chemotactic protein 1	9.8–548.7	118 \pm 64
MCP-2	Monocyte chemotactic protein 2	6.5–168.9	54 \pm 20
MCP-3	Monocyte chemotactic protein 3	2.3–19.7	14 \pm 4

^a The ratios were determined by comparing Affymetrix GeneChip values from BAL mRNA derived from three PAP patients with values from three healthy controls for a total of nine comparisons.

chemokines in the BAL cells of PAP patients by real-time PCR using primers specific for MCP-1, MCP-2, and MCP-3. BAL cells from PAP patients had significantly elevated levels of MCP-1 (95 \pm 37 fold change, $P < 0.0001$, $n = 6$), MCP-2 (44 \pm 10, $p = 0.002$, $n = 3$), and MCP-3 (8 \pm 1.5, $p = 0.003$, $n = 3$) relative to healthy controls (Fig. 2).

MCP-1 is synthesized by PAP BAL cells

In addition to correlating the expression of mRNA with protein, we evaluated the ability of PAP BAL cells to secrete MCP-1 in vitro. PAP BAL cells secreted extremely high levels of MCP-1 in vitro during 24-h culture: 101 \pm 30 ng/ml for PAP versus 1.7 \pm 0.3 ng/ml for healthy controls (Fig. 3, $P = 0.003$). Because epithelial cells have been reported to produce MCP-1 (12), we evaluated PAP epithelial cells for their possible contribution to BAL levels. MCP-1 secretion by PAP bronchial epithelial cells (0.1 \pm 0.03 ng/ml, $n = 6$) was considerably lower than that of BAL cells and did not differ from healthy controls (0.4 \pm 0.15 ng/ml, $n = 7$). These results further confirm alveolar macrophages as the source of the elevated MCP-1 protein previously reported in PAP BAL fluid.

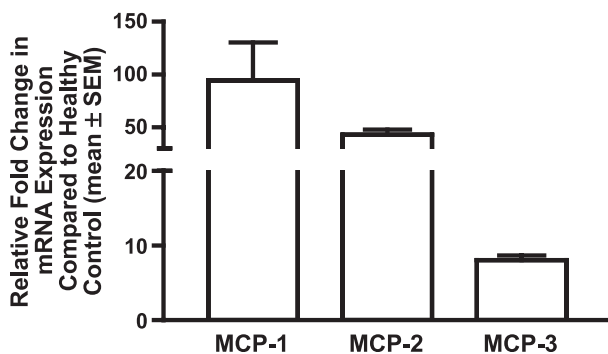


Fig. 2. MCP-1, MCP-2, and MCP-3 mRNA are elevated in BAL cells from patients with PAP. PAP and healthy control mRNA expression was evaluated by real-time RT-PCR. Levels of MCP-1, MCP-2, and MCP-3 mRNA were all significantly elevated relative to healthy controls (all P values < 0.0001).

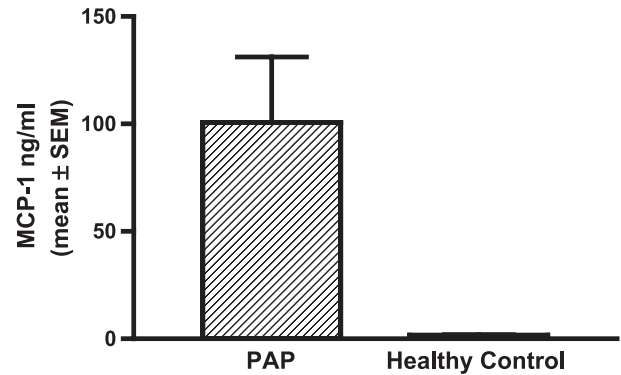


Fig. 3. MCP proteins are elevated in BAL cells from PAP patients. Alveolar macrophages from PAP patients ($n = 6$) and healthy controls ($n = 4$) were cultured in vitro for 24 h. Supernatants were harvested and evaluated for MCP-1 protein. MCP-1 is elevated in PAP supernatants (100 \pm 30 ng/ml) compared to healthy control (1.7 \pm 0.3 ng/ml, $P = 0.003$).

MCP-3 is elevated in PAP BAL fluid

PAP BAL fluid had significantly elevated levels of MCP-3 (2.01 \pm 0.5 ng/ml; range 0.2–10 ng/ml, Fig. 3, $n = 22$, $P < 0.001$) relative to healthy controls (0, $n = 6$). The levels of MCP-3 detected were lower than those for MCP-1 (9.5 \pm 1.0 ng/ml).

MCP-1 is elevated in PAP sera

We also investigated PAP sera to determine whether MCP-1 was elevated systemically. Circulating levels of MCP-1 (2.0 \pm 0.7 ng/ml, $n = 20$) were increased in PAP compared to healthy controls (0.4 \pm 0.6 ng/ml, $n = 5$, $p = 0.001$). Overall, however, MCP-1 levels were higher in BAL fluid than in sera, thus constituting a concentration gradient that might promote chemotaxis to the lung.

MCP chemokines in PAP BAL induce chemotaxis

To examine the functional activity of MCP-1 in PAP BAL fluid, we first evaluated the ability of recombinant MCP-1 to induce chemotaxis of THP 1 monocytic cells across a concentration gradient in the presence and absence of neutralizing anti-MCP 1. Recombinant MCP 1 efficiently induced THP 1 chemotaxis over a concentration range of 2.5–10 ng with 1.5 \pm 0.03 $\times 10^5$ cells recruited by 10 ng/ml of recombinant MCP-1. PAP BAL fluid exhibited significantly greater chemoattractant activity than did healthy control BAL (Fig. 4A, $n = 4$, $P = 0.01$). The enhanced chemotaxis induced by PAP BAL fluid was significantly reduced by addition of a neutralizing anti-MCP-1 antibody in 4/4 chemotaxis experiments ($P < 0.001$). To determine whether the MCP-1 detected in the PAP BAL was efficient at inducing chemotaxis, we diluted PAP BAL fluid from three different patients to 10 and 100 ng/ml. We compared these concentrations of MCP-1 to 10 ng/ml recombinant MCP-1 in their ability to induce chemotaxis of THP-1. The

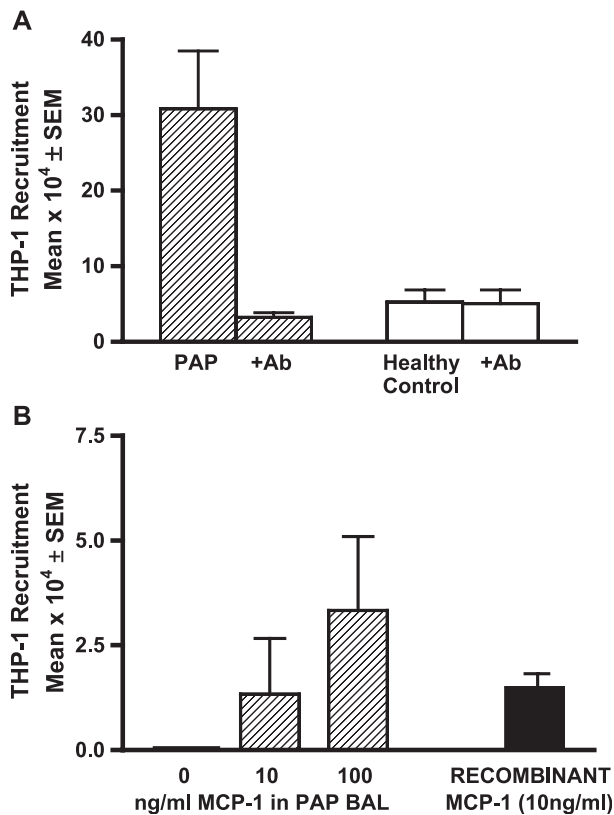


Fig. 4. PAP BAL fluid induces chemotaxis: Chemoattractant activity was determined by measuring THP-1 migration in response to recombinant MCP-1 or BAL fluids in the presence and absence of neutralizing anti-MCP. Results are expressed as mean THP-1 cells recruited. PAP BAL fluid ($n = 4$) contained increased chemoattractant activity compared to healthy control BAL (Fig. 3A, $n = 4$, $P = 0.01$), which was significantly blocked by neutralizing anti-MCP-1 in the chemotactic assays ($P < 0.001$). Fig. 3B, dilutional analysis of PAP BAL fluid, showed that at 10 ng/ml, PAP BAL fluid MCP-1 and recombinant MCP-1 produced similar THP-1 chemotaxis.

diluted endogenous BAL MCP-1 (10 ng/ml) induced chemotaxis activity similar to that 10 ng/ml recombinant MCP-1. These data suggest that the PAP BAL MCP-1 is functional and that MCP-1 was the major mononuclear cell chemokine present in PAP BAL fluid (Fig. 4B, $n = 3$).

CCR2 expression is decreased on PAP cells

To investigate chemokine receptor status, we evaluated BAL and blood mononuclear cells for membrane expression of CCR2 by flow cytometry. CCR2 was highly expressed on PAP blood monocytes ($95 \pm 3\%$, $n = 3$) but did not differ from healthy control ($97 \pm 1\%$, $n = 3$), nor did CD14 or HLADR expression. In contrast, alveolar macrophages from PAP patients displayed significantly less CCR2 (Fig. 5A) ($7 \pm 4\%$, $n = 3$) than did healthy control ($52 \pm 9\%$, $P = 0.013$, $n = 6$) again with no difference in CD14 or HLADR expression. The similarity of CD14 and HLADR expression in PAP and control BAL cells suggests that the ratio of monocytes and macrophages in PAP BAL within control levels. PAP blood lymphocytes also exhibited significantly

less CCR2 expression ($12 \pm 1.5\%$, $n = 5$) than healthy controls ($20 \pm 2\%$; $P = 0.05$, $n = 3$) (Fig. 5B). CCR2 expression of PAP BAL lymphocytes, however ($3.1 \pm 0.8\%$), did not differ from healthy control ($4.7 \pm 1.2\%$) and, overall, lymphocytes from BAL displayed much lower CCR2 expression than those from blood.

PAP BAL fluid down-regulates CCR2 expression

Because of the unexpected finding that CCR2 expression was diminished on PAP cells, we investigated whether PAP BAL fluids might contain CCR2-suppressive activity. THP-1 cells were cultured in PBS, healthy control BAL, or PAP BAL fluids then analyzed for membrane CCR2 expression. After PBS treatment, $76.5 \pm 16.5\%$ of THP-1 cells expressed CCR2 ($n = 4$, Fig. 6). PAP BAL significantly decreased THP-1 CCR2 expression to $31.5 \pm 19.5\%$ ($n = 4$, $P = 0.021$) compared to PBS whereas healthy control BAL treatment had no effect ($58.5 \pm 1.5\%$, $n = 4$, ns). Neutralizing anti-MCP-1 partially reversed the suppressive effect of PAP BAL fluid on THP-1 CCR2 expression by $46 \pm 4.2\%$ ($n = 3$, $P < 0.001$) whereas anti-MCP-1 had no

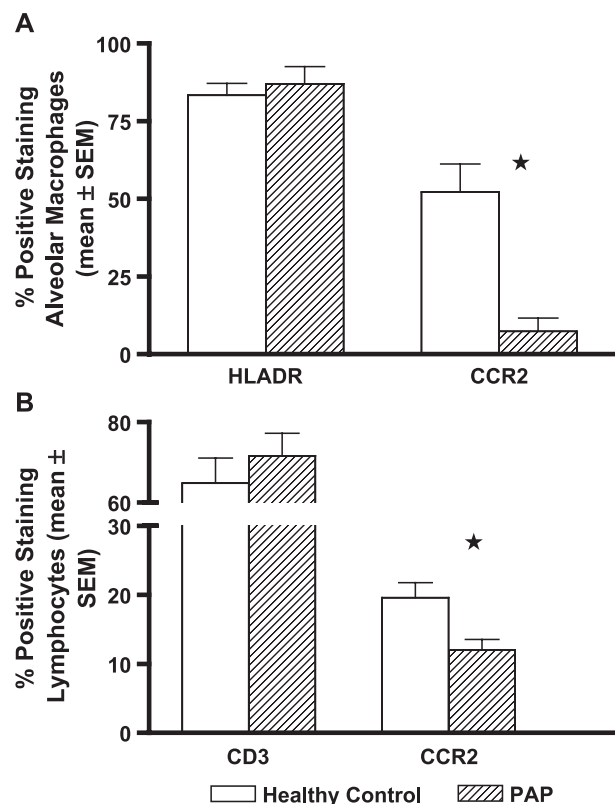


Fig. 5. Decreased expression of CCR2 on PAP alveolar macrophages and circulating lymphocytes. CCR2 surface expression was evaluated on PAP and healthy control alveolar macrophages and circulating lymphocytes by flow cytometry (Fig. 4A). PAP alveolar macrophages exhibited significantly less CCR2 ($7 \pm 4\%$, $n = 3$) than healthy control ($52 \pm 9\%$, $n = 3$, $P = 0.013$) with no difference in HLADR expression (Fig. 4B). CCR2 expression on circulating PAP lymphocytes was decreased ($12 \pm 1.5\%$, $n = 5$) as compared to healthy control ($20 \pm 2\%$, $n = 5$, $P = 0.05$).

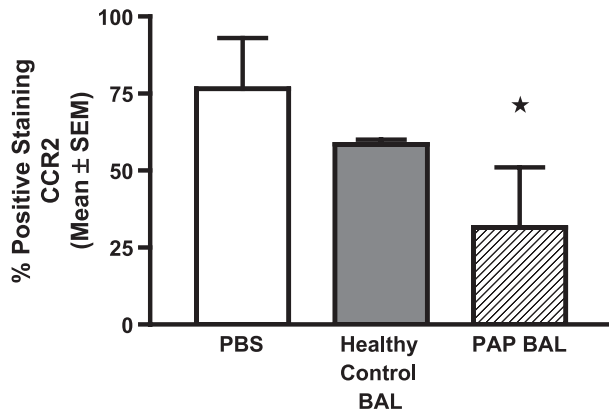


Fig. 6. PAP BAL fluid suppresses CCR2 expression. Surface CCR2 expression was measured on THP-1 cells cultured in PBS, healthy control, or PAP BAL fluid. CCR2 was present on $76.5 \pm 16.5\%$ of PBS-treated THP-1 cells ($n = 7$). After treatment with PAP BAL fluid, only $31.5 \pm 19.5\%$ of THP-1 cells expressed CCR2 ($n = 3$, $P = 0.021$) whereas HC BAL did not alter THP-1 CCR2 expression ($58.5 \pm 1.5\%$, $n = 4$, ns compared to PBS).

effect on THP-1 CCR2 expression after healthy control BAL fluid treatment.

Discussion

These studies demonstrate for the first time that MCP family chemokines, MCP-1, MCP-2, and MCP-3 are all elevated in PAP lung. Importantly, these data are the first to show that PAP BAL fluid exhibits two distinct regulatory properties: (a) chemoattractant activity for mononuclear cells, and (b) suppression of chemokine receptor CCR2. Antibody neutralization experiments indicate that both of these activities associated with PAP BAL fluid in part require the presence of MCP-1. Finally, we also present novel findings that indicate that CCR2 expression is diminished on PAP alveolar macrophages and circulating lymphocytes, potentially due to the autoregulation by MCP-1. These data suggest that in PAP, excess MCP-1 may contribute to CCR2 down-regulation, thus resulting in attenuated chemotactic responses to the lung. Further, the reduced CCR2 expression on alveolar macrophages within the lung where MCP-1 levels are highest, may serve to down-regulate the cellular activation associated with MCP-1 stimulation.

MCP chemokines function predominantly to induce cellular recruitment to sites of inflammation, infection, or injury [20]. However, recent data also suggest that these chemokines may have a more complex role in inflammation due to their ability to induce cellular activation [14]. Literature suggests that there is redundancy between the MCPs especially MCP-1, MCP-2, and MCP-3 in terms of biological function, cellular targets, and cell surface receptors [18]. MCP-1 mediates its actions through CCR2 whereas MCP-2 and MCP-3 interact with CCR2 as well as other chemokine receptors [19,20]. In animal models, there

is considerable evidence that CCR2 regulates MCP-1 expression and that subsequent secretion of MCP-1 down-regulates CCR2 expression [15]. Further, there is considerable evidence that decreased expression of CCR2 and elevated expression of MCP-1 promotes anti-fibrotic cytokine cascades in animal models of pulmonary fibrosis via downstream enhancing effects on TGF- β [27]. Interestingly, in PAP lungs, there is minimal fibrosis suggesting that elevated levels of MCP-1 may protect against development of fibrosis despite long-standing disease [28–30].

BAL fluids from both the GM-CSF knockout mouse model of PAP and patients with PAP contain high levels of MCP-1 [8,9,11,31]. Unlike the GM-CSF knockout mouse, however, where GM-CSF is totally absent, human PAP is due to autoantibody-mediated neutralization of GM-CSF, thus effecting a virtual GM-CSF deficiency [6,9]. These observations suggest that decreased GM-CSF biological activity may have a role in regulating alveolar macrophage expression of MCP-1. This is an area for further investigation. The status of CCR2 expression in the GM-CSF knockout mouse model also remains to be determined. MCP-1 expression has been associated with several autoimmune diseases both in humans [32,33] and animal models [14,34,35]. In human PAP, the potential contribution of autoimmunity to MCP-1-mediated regulation of CCR2 has yet to be established.

The observation that MCP-1 and CCR2 are tightly regulated suggests that the extremely high levels of MCP-1 found in PAP lung, down-regulate alveolar macrophage CCR2 expression. Circulating PAP monocytes, however, demonstrate normal levels of CCR2. These data together with results showing the steep MCP-1 concentration gradient between lung and blood suggest that monocyte recruitment to the lung should occur efficiently in PAP. Once within the lung, however, CCR2 receptors may be rapidly down-regulated, thus allowing for some degree of homeostatic auto-regulation. Interestingly, CCR2 receptors on circulating lymphocytes are severely reduced in PAP, suggesting that entry of lymphocytes to the lung may be limited. The relative sensitivity of lymphocyte CCR2 expression to MCP-1-mediated down-regulation in PAP compared to healthy controls remains to be investigated. In summary, these observations demonstrate that MCP family chemokines are elevated in PAP lung without the presence of a robust change in lung cellular differential. Thus, MCP-1 may play a major role in decreasing cell recruitment to the lung and in regulation of cellular activity within the lung via down-regulation of CCR2 receptors.

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