

Regulation of CXCR/IL-8 in Human Airway Epithelial Cells

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Key Words

Bronchial epithelium • Interleukin 8 • Inflammation • CXCR • Proteomics

Abstract

Background: Severe asthma is characterized by neutrophilic inflammation and high levels of interleukin (IL)-8. Airway epithelial cells play a pivotal role in the pathogenesis and chronicity of asthma. The objective of this work was to determine whether CXC receptors were involved in human small airway epithelial cell (SAEC) activity by incubating them with IL-8; the investigation also included a proteomic approach. **Methods:** IL-6 and intercellular adhesion molecule-1 (ICAM-1) were assessed by ELISA and flow cytometry, respectively. CXCR-1 and CXCR-2 receptor mRNA and protein expressions were analyzed by RT-PCR, immunocytochemistry and flow cytometry. Cells were incubated with different concentrations (0–100 ng/ml) of IL-8. The involvement of both receptors was assessed using specific antibodies. **Results:** Only the CXCR-1 receptor was expressed in SAECs. IL-8 (50 ng/ml, 12 h) induced the release of IL-6 and had no effect on ICAM-1. Supernatants analyzed by surface enhanced laser desorption ionization time of flight mass spectrometry (SELDI-TOF MS) showed very weak differences in peptide profiles. Interestingly, 4,820-m/z peptide release was detect-

ed in the presence of IL-8 and abolished by CXCR-1 antibody. **Discussion:** The present study illustrated the fact that IL-8 mediated by CXCR-1 increased IL-6. We also highlight the usefulness of SELDI ProteinChip technology to confirm the potential variation of peptide profile. Moreover, we were able to detect the 4,820-m/z peptide secreted in vitro by human airway epithelial cells induced by IL-8 via CXCR-1 receptor. Determination of the protein secretion profile in response to inflammatory stimuli could be an important therapeutic strategy in severe asthma.

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Introduction

Asthma is a chronic inflammatory disorder of the airways characterized by the recruitment of various inflammatory cells and the release of different mediators [1]. It is associated with structural changes in bronchi. Severe asthma was recently described by different subtypes of airway inflammatory infiltration, including the 'classical eosinophilic process', and a neutrophilic phenotype was

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shown on some occasions [2]. High levels of inflammatory cytokines have been detected in the blood of patients with severe asthma [3]. Different cytokines/chemokines such as interleukin (IL)-8, tumor necrosis factor- α , IL-6 and IL-13 may worsen the severity of asthma [4].

IL-8, a CXC chemokine, is a major chemoattractant for neutrophils and other inflammatory cells such as granulocytes [5] and was detected in bronchoalveolar lavage fluid from asthmatic patients [6]. Shute et al. [7] reported that IL-8 is a marker of asthma since this chemokine was undetectable in the serum of control subjects. More recently, we have shown that subjects with a severe neutrophilic asthma phenotype have higher IL-8 levels in supernatants of induced sputum as compared to subjects with mild asthma and controls [8] as suggested by others [9]. In this respect, further insight into the role of IL-8 in severe asthma is required in order to design new therapeutic strategies.

IL-8 mediates its biologic activity through two specific seven-transmembrane G protein-coupled receptors: CXCR-1 and CXCR-2 [10]. CXCR-1 and CXCR-2 bind IL-8 with high affinity. In contrast to CXCR-1, CXCR-2 also binds other CXC chemokines such as growth-regulated oncogene- α , - β and - γ (GRO- α , - β , - γ), neutrophil-activating peptide-2, and epithelial cell-derived neutrophil activating peptide-78 [11]. CXCR-1 and CXCR-2 are known to be expressed on various lung cells, including inflammatory cells and epithelial cells [12]. In neutrophilic asthma, the expression of CXCR-1 and CXCR-2 on peripheral and sputum neutrophils is similar to that in chronic obstructive pulmonary disease and is negatively correlated with the number of neutrophils in the airways [13].

The airway mucosa is lined with a continuous epithelium that forms a vital protective barrier. Epithelial cells are more than a mechanical interface, they also play pivotal regulatory roles in inflammation and host defense [14]. Moreover, bronchial epithelial cells produce and release biologically active compounds, including IL-8, while activating and recruiting inflammatory cells within the airways and actively contributing to the pathogenesis and chronicity of some respiratory diseases such as asthma [15]. However, it is not known whether IL-8 exerts autocrine actions on the airway epithelium and whether IL-8 stimulates the production of other inflammatory mediators by epithelial cells.

In this study, we used an in vitro model of airway epithelium (small airway epithelial cells, SAECs) to investigate the involvement of CXC receptors and study the specificity of the functional activity of IL-8 in airway ep-

ithelial cells. For this purpose, after analysis of the presence of both CXCR-1 and CXCR-2 in our model compared to the in vitro situation using endobronchial biopsies, we analyzed the effect of IL-8 incubation on cytokine production assessed by IL-6 production and on intercellular adhesion molecule-1 (ICAM-1) expression. Moreover, we also analyzed the effect of IL-8 on the regulation of its own receptors, CXCR-1 and CXCR-2, by flow cytometry. Thereafter, we investigated the variation of peptide profiles secreted by epithelial cells incubated with and without IL-8 using a proteomic approach, i.e., surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [16]. Recent reports have identified proteins and peptides that are potent inflammatory markers in respiratory diseases [17–19].

Methods

Fiberoptic Bronchoscopy

Bronchial biopsies were obtained using alligator forceps (Olympus, Tokyo, Japan) on a subsegmental bronchus of the left lower lobe as previously described [20] in 10 normal healthy human volunteers. Our local ethics committee approved the study and all patients agreed to participate by signing a written informed consent.

Human Bronchial Epithelial Cell Culture

Primary SAECs were obtained from Lonza (Basel, Switzerland) and cultured as previously described [21].

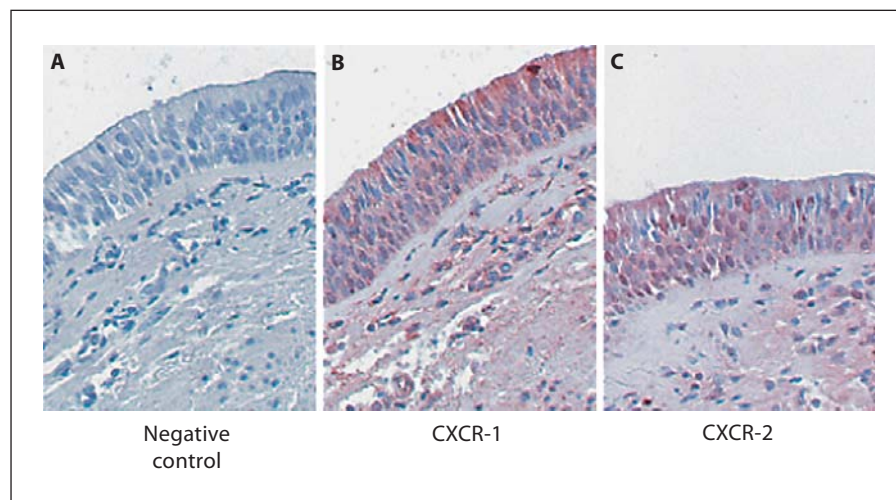
SAEC Stimulation

Cells were cultured in 12-well plates until reaching 80% confluence. Firstly, dose response (0–1,000 ng/ml) and time-course (0–48 h) were assessed to determine the IL-8 concentration (Calbiochem, San Diego, Calif., USA) to be used in subsequent experiments. Supernatants were removed and subsequently analyzed. IL-6 release was measured by ELISA. SAECs were stimulated with IL-8 (50 ng/ml) for 12 h in a medium without bovine serum albumin (for proteomic analysis). In some experiments, specific antibodies directed against CXCR-1 (0.1–1 μ g/ml) and CXCR-2 (1–10 μ g/ml) (R&D Systems, Minneapolis, Minn., USA) were added to the stimulated medium. All results were obtained from 6 different experiments.

RT-PCR Analysis

Cells treated in the presence or absence of IL-8 were harvested and total mRNA was extracted with the RNeasy mini kit according to the manufacturer's protocol (Qiagen, Courtaboeuf, France). cDNA was synthesized with 1 μ g of RNA according to the Ready-To-Go RT-PCR Beads protocol (Amersham Biosciences, Little Chalfont, UK). Human CXCR-1 (sense 5'-CAGATCCACAGATGTGGGAT-3' and antisense 5'-TCCAGCCATTC (AG) CCTTGAGAG-3') and CXCR-2 (sense 5'-AGCTGCTCTTCTGGAGGTGT-3' and antisense 5'-TTAGAGAGTGGAAAGTGTGC-3') (MWG Biotech, High Point, N.C., USA) oligonucleotide primers were

Fig. 1. Immunolocalization of CXCR-1 and CXCR-2 receptors in vivo in human bronchial mucosa from endobronchial biopsies obtained from 10 different healthy volunteers. Slides were stained with a matched IgG antibody (**A**), an anti-CXCR-1 (**B**) or an anti-CXCR-2 (**C**) receptor antibody. Red deposits show the positive expression of both receptors. Magnification $\times 200$.



used to amplify 257- and 1,154-bp products, respectively. The annealing temperatures and number of cycles used for CXCR-1 and CXCR-2 mRNA amplification were 58°C, 35 cycles and 60°C, 36 cycles, respectively. Then PCR products were detected using 1% agarose gel electrophoresis and ethidium bromide. β -Actin was used as the housekeeping control gene. All results were obtained from 6 different experiments.

Immunocytochemistry and Immunohistochemistry

CXCR-1 and CXCR-2 expression was studied both on human endobronchial biopsy sections and SAECs. Biopsies were fixed in 10% formaldehyde and embedded in paraffin blocks. Tissue sections (4 μ m) were fixed on microscope slides. SAECs were seeded on 16-well LabTek glass slides, cultured to confluence and briefly fixed in acetone/methanol (1/1). An immunostaining procedure was performed with mouse monoclonal anti-human CXCR-1 and CXCR-2 antibodies (10 μ g/ml) (R&D Systems), and the LSAB 2 (Dako Cytomation, Glostrup, Denmark) visualization system (HRP labeled) using 3-amino-9-ethylcarbazole (AEC) as a chromogen. The cells containing red deposits of AEC were considered as positive. Mouse immunoglobulin (IgG) (Dako) was used as negative control. The counterstaining was done with Mayer's hematoxylin (Dako), after rinsing with distilled water, slides were mounted in Faramount mounting medium (Dako).

Flow Cytometry

Cells were incubated with anti-human CXCR-1, CXCR-2 or ICAM-1 (1 μ g/ 10^6 cells, 20 min) (R&D Systems) on ice, washed twice in 1% BSA-PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody (Zymed, San Francisco, Calif., USA) on ice (1 μ g/ 10^6 cells, 20 min). After two washes in 1% BSA-PBS, cells were fixed in 1% paraformaldehyde. The background immunofluorescence level was determined by incubating cells with FITC-conjugated mouse IgG. Cells were analyzed with an FACSCalibur flow cytometer (Becton Dickinson, Le Pont-de-Claix, France). Each analysis involved at least 10,000 events. All results were obtained from 6 different experiments.

SELDI-TOF MS Analysis of Cell Supernatants

CM10 ProteinChip arrays, weak cation exchange (Ciphergen Biosystems, Fremont, Calif., USA) were preequilibrated with 200 μ l of binding buffer (100 mM ammonium acetate, pH 5, 0.1% Triton) using a 96-well bioprocessor and incubated for 5 min with gentle agitation. After removing the binding buffers from the wells, 5 μ l of each sample diluted in 20 μ l of binding buffer were incubated for 15 min on an ELISA plate shaker at room temperature. This step was repeated seven times. Arrays were washed twice with 100 mM ammonium acetate, pH 5, with 0.1% Triton and once with 100 mM ammonium acetate, pH 5, for 5 min, followed by a final brief HEPES rinse. The bioprocessor was removed and the ProteinChip array air-dried. Finally, 0.8 μ l of saturated sinapinic acid solution was applied twice to each spot and chips were allowed to air-dry.

SELDI-TOF-MS and Data Analysis

Mass-spectrometric analysis was performed by SELDI-TOF-MS in a PBS-II ProteinChip reader (Ciphergen Biosystems) according to automated settings for the measured region (low mass = 2–20 kDa and high mass = 20–200 kDa). Within a single comparative experiment, all conditions were kept the same for data collection (calibration, focusing mass, laser intensity and detector sensitivity). Each spectrum was an average of at least 100 laser shots and externally calibrated with the All-in-1 Protein Standard II. Spectrum analysis was carried out using the ProteinChip software, version 3.2. The background was subtracted using the default software settings. All mass spectra were normalized using a total ion current that averages the intensity and adjusts the intensity scales for all spots.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed using the nonparametric Kruskal-Wallis and Wilcoxon tests since data were not normally distributed. Differences were considered significant at the $p < 0.05$ level.

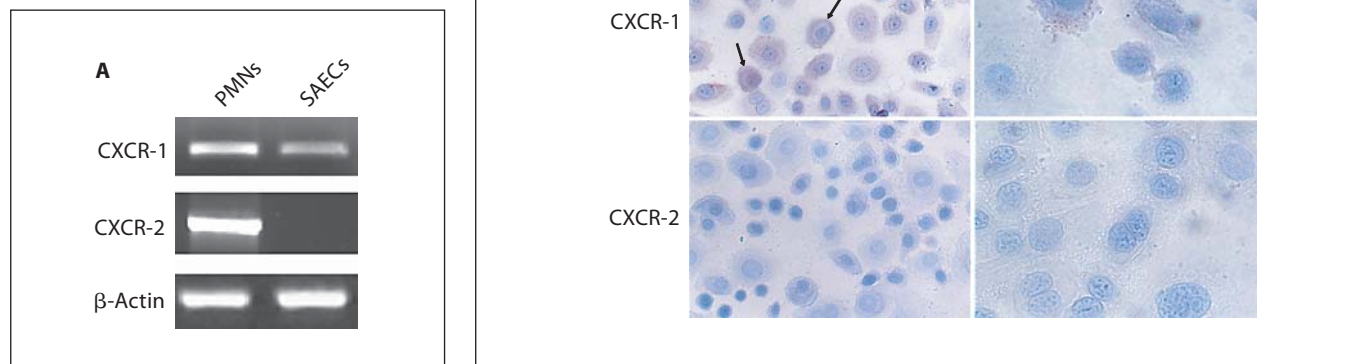


Fig. 2. Expression of CXCR-1 and CXCR-2 receptors by SAECS. **A** Representative RT-PCR analysis of CXCR-1, CXCR-2 and β -actin (housekeeping gene) mRNA expression. Human PMNs were used as positive control. **B** Representative pictures of CXCR-1 and

CXCR-2 immunoreactivities in SAECS. Red deposits show positive expression (arrows indicate stained cells). Immunostaining with a mouse IgG was used as negative control. Magnification $\times 200$ (left column) and $\times 400$ (right column).

Results

Expression of CXCR-1 and CXCR-2 by Bronchial Epithelium

In order to determine CXCR-1 and CXCR-2 expressions in human SAECS, we performed immunohistochemical staining in human bronchial biopsies obtained from 10 different donors (fig. 1). We observed that both receptors are expressed in bronchial epithelium (fig. 1B, C, respectively) as compared to the negative control (fig. 1A). CXCR-1 and CXCR-2 immunostaining was mainly localized in the epithelium, with positive staining in basal and columnar cells, but CXCR-1 was strongly expressed on the apical surface of the epithelium (fig. 1B). Infiltrated inflammatory cells within the submucosa showed positive staining for CXCR-1 and CXCR-2.

CXCR-1 and CXCR-2 Expression in SAECS

We demonstrated CXCR-1 mRNA expression but no CXCR-2 mRNA expression in SAECS (fig. 2A). Immunocytochemical analysis also demonstrated that only CXCR-1 protein was expressed in SAECS (fig. 2B). Moreover,

FACS analysis showed that SAECS weakly expressed CXCR-1 with 12% of positive staining (fig. 2Ce) while CXCR-2 was not expressed (fig. 2Cf). An epithelial antigen was used to confirm the SAEC phenotype (97% of cells were positive) (fig. 2Cd). Expression on neutrophils isolated from blood was used as positive control with 96% of positive staining (fig. 2Cb).

IL-8-Induced IL-6 Release by SAECS

We showed that IL-8 induced IL-6 release by SAECS in a concentration-dependent manner (fig. 3A). Moreover, IL-8-induced IL-6 secretion was statistically significant from 50 to 1,000 ng/ml compared to the control condition using the Wilcoxon test. Closer to physiologic conditions, we decided to use 50 ng/ml of IL-8 in further experiments. The increase in IL-6 production was inhibited using an anti-CXCR-1 antibody (fig. 3B).

IL-8 Had No Effect on ICAM-1 and CXCR-1 and CXCR-2 Expressions

As shown in figure 4A, there was a weak shift in the expression of anti-CXCR1, which demonstrated that

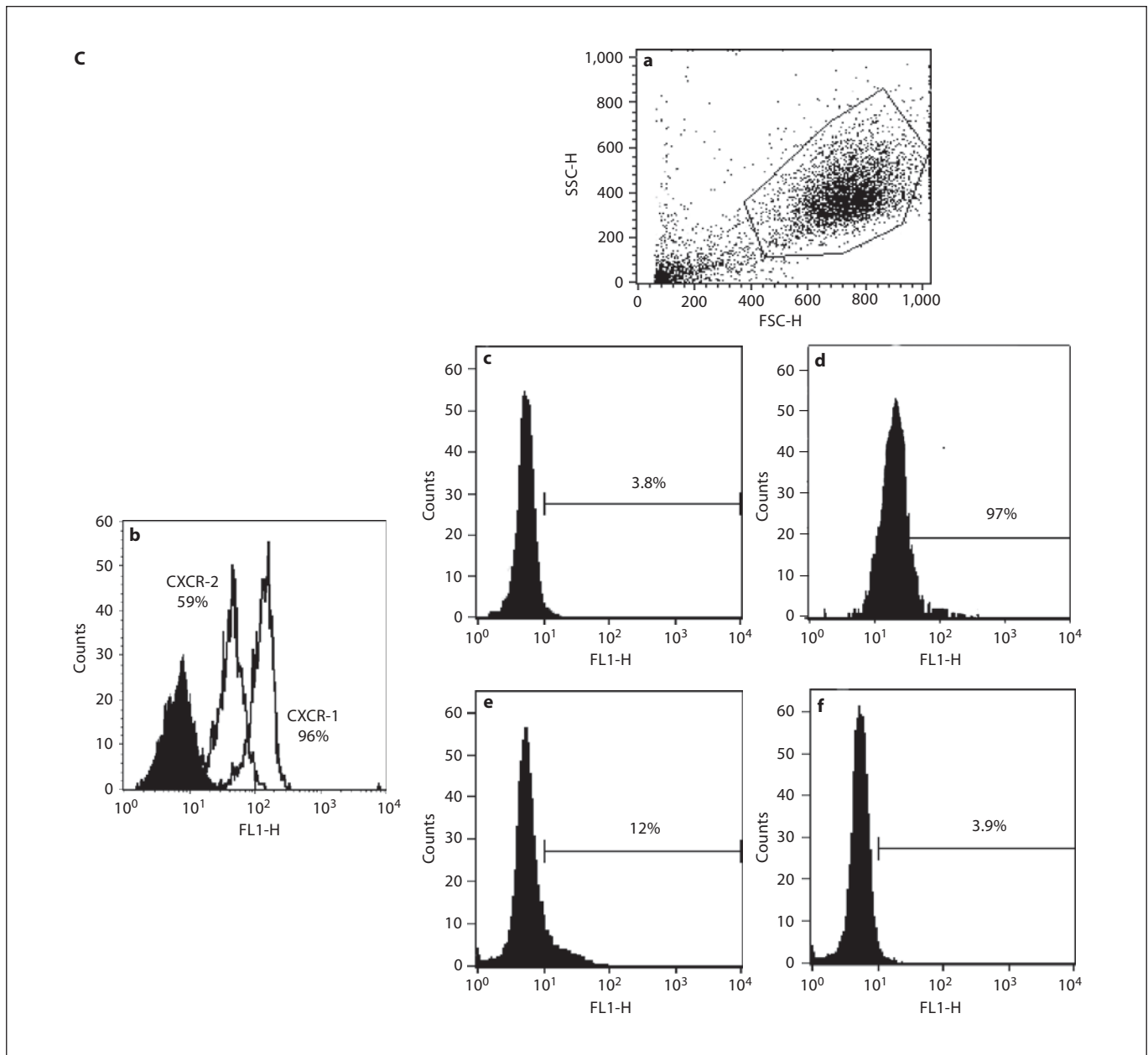


Fig. 2. Expression of CXCR-1 and CXCR-2 receptors by SAEs. **c** Flow cytometry analysis of CXCR-1 and CXCR-2 receptor surface expression in SAEs. **a** Scattergram. **b–f** Representative histograms. **b** PMNs as positive control with a 96% positive stain for

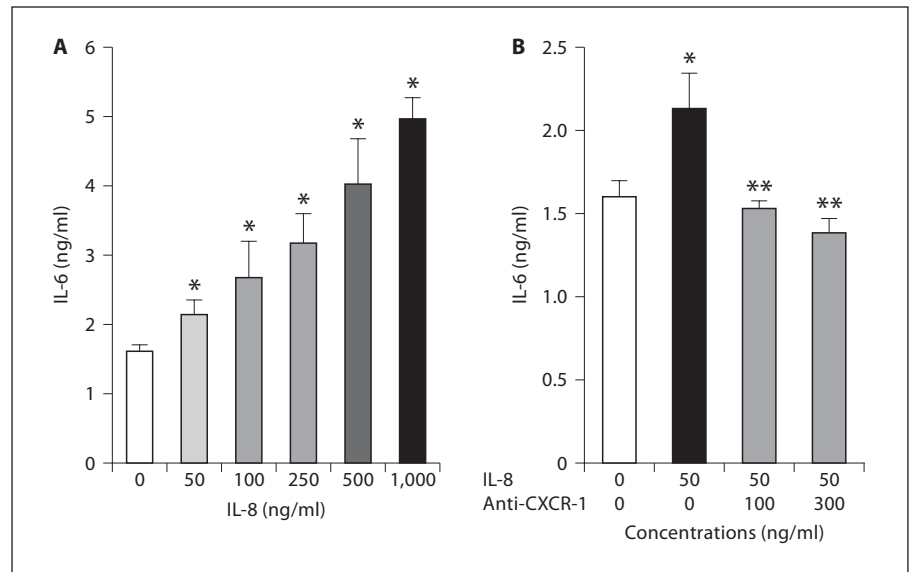
CXCR-1 and 59% for CXCR-2. **c** Mouse IgG as negative control. **d** Epithelial antigen antibody with 97% staining. **e** CXCR-1 with 12% staining. **f** CXCR-2 (no positive staining).

SAECs weakly express CXCR-1 and do not express CXCR-2 (fig. 4B). Membrane ICAM-1 was expressed by SAEs (fig. 4C). When cells were incubated with IL-8 for 24 h, there was no effect of IL-8 either on the expression of both CXCR-1 and CXCR-2 or on the expression of ICAM-1.

SELDI-TOF Analysis of SAEC Secretion Products Induced by IL-8

SELDI-TOF profiles were very similar in stimulated and nonstimulated cells as illustrated in figure 5. Indeed, calculation of Pearson factors (fig. 5A) revealed that the variations of spectra between the stimulated and non-

Fig. 3. Effect of IL-8 on IL-6 release by SAECs. **A** Dose response of IL-8 on IL-6 release. SAECs were incubated for 24 h with IL-8 (0–1,000 ng/ml). **B** Inhibition of IL-8-induced IL-6 release using anti-CXCR-1. SAECs were pretreated with anti-CXCR-1 (100 or 300 ng/ml) for 15 min prior to stimulation with IL-8 (50 ng/ml). IL-6 level was measured by ELISA. Results are expressed as mean $\pm$ SEM concentration (ng/ml for 4 experiments. * $p < 0.05$ unstimulated versus IL-8 stimulation; ** $p < 0.05$ IL-8 versus IL-8 + anti-CXCR-1.



stimulated conditions were similar to the variation between stimulated and nonstimulated spectra analyzed together (nonstimulated vs. nonstimulated and stimulated vs. stimulated). A statistical analysis of the SELDI-TOF profile by nonhierarchical clustering (fig. 5B) clearly showed that stimulated and nonstimulated conditions did not segregate together.

IL-8-Induced Specific Release of a Peptide Entity via CXCR-1 Receptor

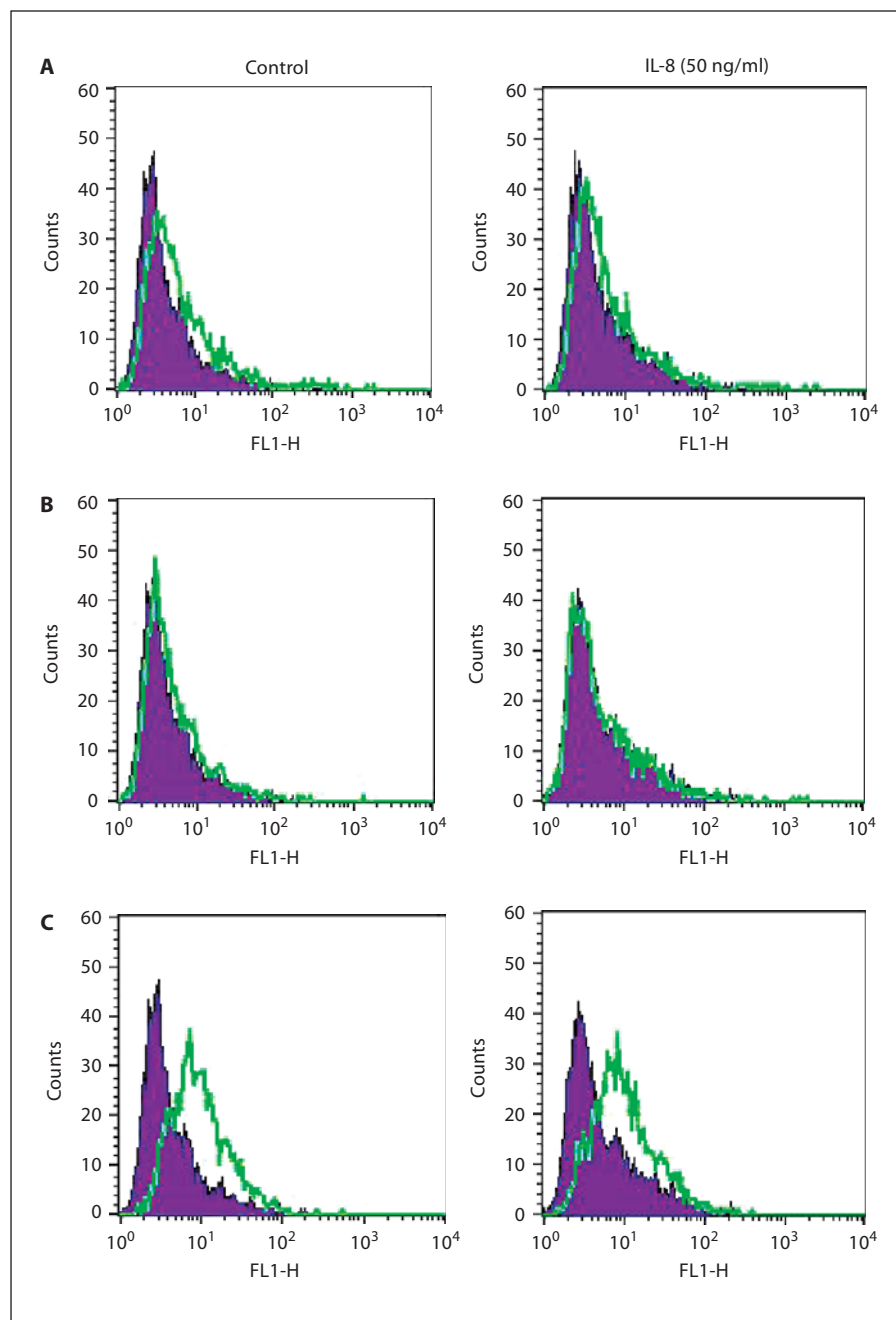
Peptides/proteins detected by SELDI-TOF in the media of cultured cells in the presence or not of 50 ng/ml of IL-8 are shown in figure 6. To test the specificity of this result, we analyzed the medium alone or with exogenous IL-8 (fig. 6A). We focused on a 4,820-m/z peptide whose detection was dependent on specific IL-8 concentrations. We showed that an increase in the 4,820-m/z peptide occurred in cell supernatants incubated with IL-8 and was absent in the culture medium or in medium containing exogenous IL-8. To test the reproducibility of our experiments and to avoid intraspecific effects, we confirmed our results in two different batches of cells sampled from two different donors (fig. 6B). Moreover, IL-8 induced an increase in a 4,820-m/z peptide which was statistically significant (1.76 ± 0.13 vs. 0.56 ± 0.74 ; $n = 4$; $p = 0.03$, Student's *t* test) (fig. 6C). Incubation of SAECs with a CXCR-1 antibody completely abolished IL-8-induced 4,820-m/z peptide release (fig. 6D) whereas a CXCR-2 antibody had no effect (fig. 6D).

Discussion

Incubation of SAECs with IL-8 as performed in the present study demonstrated for the first time that CXCR receptors were implicated in SAEC activities. The study also included a proteomic approach using SELDI-TOF-MS. We found that in contrast to endobronchial biopsies, only CXCR-1 receptor was expressed in SAECs. Moreover, IL-8 induced the release of IL-6, which was blocked by addition of anti-CXCR1 antibody and had no effect on ICAM-1. IL-8 did not modulate the expression of either CXCR. Supernatants analyzed by SELDI-TOF-MS showed a very weak difference in peptide profile. However, significant 4,820-m/z peptide release was detected in the presence of IL-8 and abolished by CXCR-1 antibody.

IL-8 is a major cytokine involved in various types of inflammatory reactions, including asthma [22]. IL-8 expression was also increased in bronchial epithelium of asthmatic patients [23]. In addition, we have shown that subjects with severe neutrophilic asthma have higher sputum IL-8 levels as compared with those of patients with milder asthma and control subjects [8]. Several studies have reported that IL-8 could modulate inflammatory responses, epithelial cell proliferation, migration, cell growth, metastasis and cell functions of different malignant epithelial cells of various origins [24, 25]. However, this effect of IL-8 was not univocal as some studies reported opposite findings [26]. To the best of our knowledge, normal human bronchial epithelium responses to IL-8 have never been characterized before. In our exper-

Fig. 4. Effect of IL-8 on CXCR-1, CXCR-2 and ICAM-1 expression assessed by flow cytometry. Flow cytometry analysis of surface expression of CXCR-1 receptor (**A**), CXCR-2 receptor (**B**) or adhesive molecule ICAM-1 (**C**) on SAECs before (control) and after IL-8 (50 ng/ml) stimulation (left and right columns, respectively). Results are shown by representative histograms. Mouse IgG was used as negative control and is represented in purple. Specific staining is shown in green.



iments, we showed that an IL-8 concentration (50 ng/ml) similar to those detected in biologic fluids of patients with severe asthma could change the secretion profile of human epithelial cells in vitro.

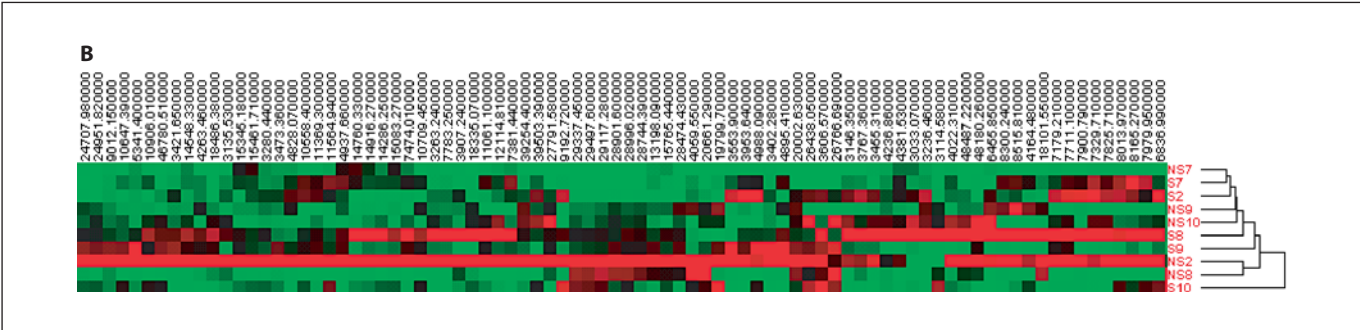
IL-8 mediates its activity by two specific receptors, i.e. CXCR-1 and CXCR-2, which both bind IL-8 with high affinity, but CXCR-2 also binds other CXC cytokines. We investigated IL-8 receptor expression in vivo in human

bronchial biopsies from normal subjects and in primary cultures of SAECs using three different methods. In contrast to a previous study showing that only CXCR-2 was expressed in human bronchial tissue from COPD patients [12], we demonstrated that both receptors were expressed in vivo in normal human bronchial epithelium. CXCR-1 seemed to be highly expressed on the apical surface of epithelial cells while CXCR-2 was expressed in

Fig. 5. Global statistics of proteomic analysis by SELDI-TOF-MS. **A** Pearson's correlation factor between spectra (nonstimulated vs. nonstimulated, NS vs. NS stimulated vs. stimulated, S vs. S, and nonstimulated vs. stimulated, NS vs. S) (n = 8). **B** Nonhierarchical clustering showed no segregation of peptidic spectra under stimulated and nonstimulated conditions in 4 different experiments.

A

	Mean	Max	Min
NS vs. NS	0.660	0.990	0.303
S vs. S	0.742	0.989	0.474
Ns vs. S	0.719	0.993	0.348



both basal and columnar cells. In another study, the authors found that both receptors were weakly expressed in human bronchial epithelial cell lines in vitro [27]. However, in our primary cell culture system, only CXCR-1 receptors were expressed as already reported in an intestinal epithelial cell line [28]. Neither CXCR-2 mRNA nor protein was detected in SAECs.

In order to identify proteins that could be regulated by the IL-8 pathway in SAECs, we investigated IL-6 and ICAM-1 expression. IL-6 (as well as other cytokines) is involved in the acute phase of the inflammatory response [29]. In this case, IL-6 acts as a proinflammatory cytokine. However, other in vitro and in vivo studies have shown that IL-6 could also have an anti-inflammatory effect and protective properties [30]. Indeed, inhibition of proinflammatory mediator production (tumor necrosis factor- α , IL-1 and MCP-2) by IL-6 was described, as well as increased release of anti-inflammatory mediators [31]. IL-6 also decreases neutrophil sequestration, superoxide anion production and activates matrix metalloprotease inhibitor production [32]. There are only a few studies on the effect of IL-6 on bronchial epithelial cells and they show an antiproliferative effect on tumoral cells [33], activation of SP-B production [34] and an antioxidative role [35]. In our study, we demonstrated that IL-8 dose-dependently increases the secretion of IL-6. Moreover, IL-6 release was dependent on CXCR-1 since the addition of the antibody against CXCR-1 was able to inhibit this effect. However, the anti- or proinflammatory effect of IL-8-induced IL-6 release should be clarified. On the other

hand, ICAM-1 is an adhesive molecule with proinflammatory properties and many studies have shown increased expression of ICAM-1 in bronchial epithelial cells in airways of asthmatic patients [36]. There is a soluble form of ICAM-1 (sICAM-1) as well, but its function is not very well known. sICAM-1 is increased in serum and bronchoalveolar lavage fluid of subjects with atopic asthma [37]. In our study, ICAM-1 expression was shown on SAECs at the basal level, but the addition of IL-8 did not modify this expression on the membrane of epithelial cells. These results are in agreement with another in vitro study on human normal bronchial epithelial cells and the human bronchial epithelial cell line 16-HBE140 [38]. We have also measured sICAM-1 release in SAEC supernatants after IL-8 stimulation, but this form was undetectable (data not shown). These results suggest that IL-8 is not involved in the increased expression of ICAM-1 observed in asthma.

In order to analyze a wide spectrum of peptides that could be involved, we decided to use an original approach analyzing protein profiles in biological fluids using MS. Protein expression patterns based on SELDI-TOF-MS have been used to discover early diagnostic markers, predict therapeutic responses and diagnose malignancies [39, 40] while also identifying early diagnostic markers of infection [41, 42]. To our knowledge, no information has been published on the use of SELDI-TOF-MS to assess changes in airway epithelial cell secretion in vitro. In the current study, we used a weak cation exchange chip surface and a pH 5 binding buffer, so unique cationic pro-

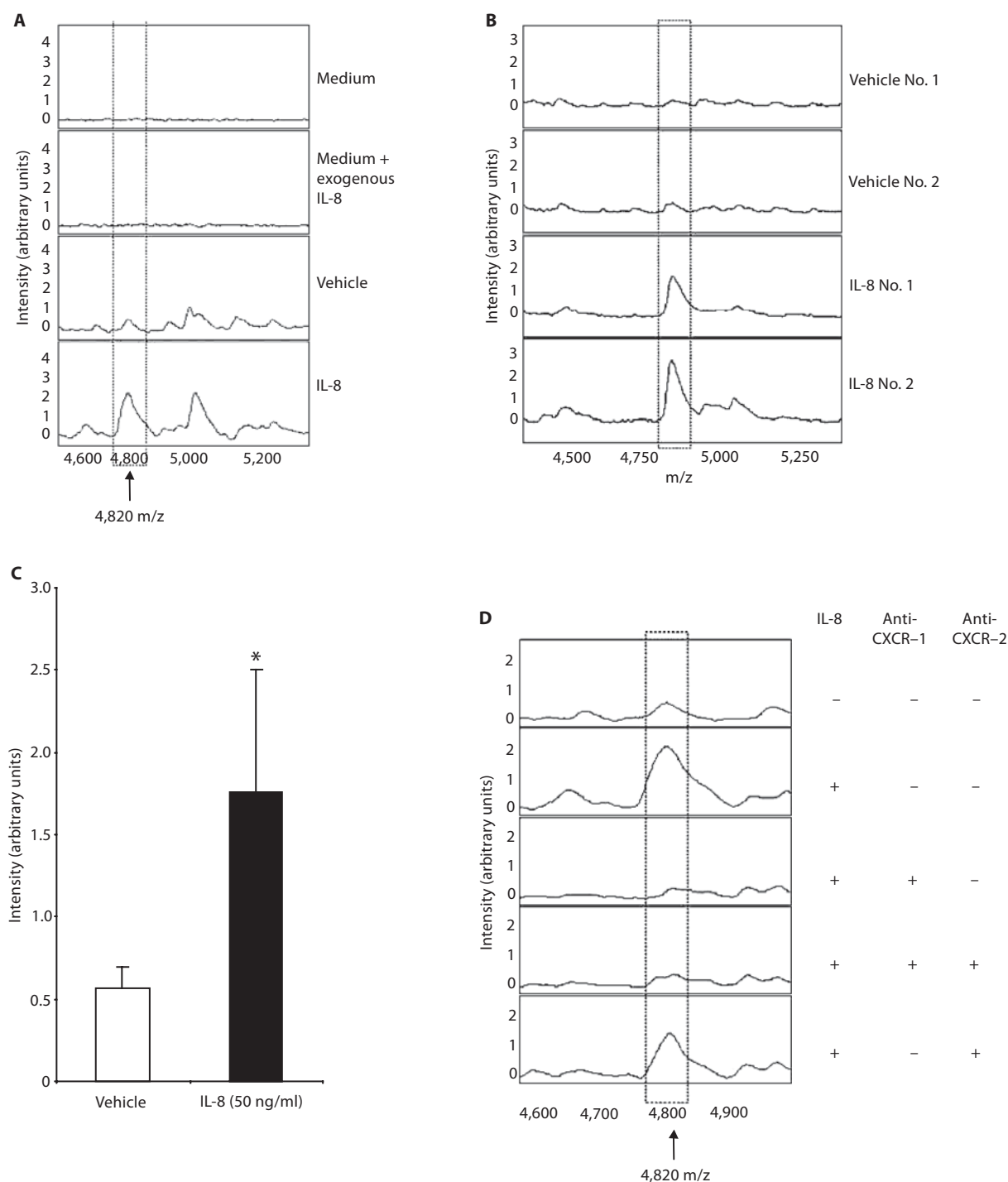


Fig. 6. Expression of proteins released from SAECs incubated with IL-8. **A** Differential display of the 4,820-m/z protein comparing the results obtained with culture media alone in the presence of exogenous IL-8 or using cell supernatants treated with vehicle and IL-8 (50 ng/ml). **B** Differential display of the 4,820-m/z protein comparing cell culture supernatants from two batches. **C** IL-

8-induced 4,820-m/z peptide release. Data are expressed as mean $\pm$ SD intensity ($n = 4$, $* p = 0.03$). **D** Representative SELDI protein spectra of SAEC culture media incubated with IL-8 (50 ng/ml) and/or CXCR-1 and CXCR-2 receptor antibodies showing that the increase in the levels of 4,820-m/z peptide was inhibited by anti-CXCR-1 but not anti-CXCR-2 antibodies.

teins were detected within 12 h of IL-8 challenge at this pH. When analyzing the peptide profile under the two different conditions (with and without IL-8 incubation), we found a very similar pattern using Pearson factors. According to statistical analysis, nonhierarchical clustering did not segregate the two conditions, meaning that a very weak difference exists in the secretion products under these two conditions. Meanwhile, using this method, we demonstrated a specific increase in a 4,820-m/z peptide secreted by SAECs. Hence, using a specific antibody directed against CXCR-1, we were able to inhibit the release of the 4,820-m/z peptide while anti-CXCR-2 had no effect. This suggests that secretion of this peptide was dependent on the CXCR-1 pathway and thus specific to IL-8 stimulation in our model. Unfortunately, due to technical difficulties, we have not yet been able to identify our

peptide of interest despite the very high reproducibility of our experimental results.

In conclusion, this study highlights the poor physiologic effect of IL-8 on human epithelial cells despite the presence of its receptor CXCR-1. This lack of dramatic changes may suggest a regulatory component used by the epithelium in order not to amplify a devastating effect that could be enhanced by the very high levels of IL-8 secreted in the airways of patients with severe asthma. On the other hand, based on the data presented here, we would like to emphasize that potent effects of IL-8 are more dependent on CXCR-2 than CXCR-1, and that the lack of expression of CXCR-2 may explain the weak effects on epithelial cells. One other hypothesis would be that the very low expression of CXCR-1 is a regulatory process to avoid amplification of the inflammatory secretion.

References

- Chung KF, Barnes PJ: Cytokines in asthma. *Thorax* 1999;54:825–857.
- Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, Chu HW: Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* 1999;160:1001–1008.
- Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ: Neutrophilic inflammation in severe persistent asthma. *Am J Respir Crit Care Med* 1999;160:1532–1539.
- Fahy JV, Kim KW, Liu J, Boushey HA: Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. *J Allergy Clin Immunol* 1995;95:843–852.
- Kunkel SL, Standiford T, Kasahara K, Strieter RM: Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 1991;17:17–23.
- Chanez P, Enander I, Jones I, Godard P, Bousquet J: Interleukin 8 in bronchoalveolar lavage of asthmatic and chronic bronchitis patients. *Int Arch Allergy Immunol* 1996;111:83–88.
- Shute JK, Vrugt B, Lindley IJ, Holgate ST, Bron A, Aalbers R, Djukanovic R: Free and complexed interleukin-8 in blood and bronchial mucosa in asthma. *Am J Respir Crit Care Med* 1997;155:1877–1883.
- Vachier I, Bonnans C, Chavis C, Farce M, Godard P, Bousquet J, Chanez P: Severe asthma is associated with a loss of LX4, an endogenous anti-inflammatory compound. *J Allergy Clin Immunol* 2005;115:55–60.
- Gibson PG, Simpson JL, Salto N: Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;119:1329–1336.
- Holmes WE, Lee J, Kuang WJ, Rice GC, Wood WI: Structure and functional expression of a human interleukin-8 receptor. *Science* 1991;253:1278–1280.
- Ahuja SK, Murphy PM: The CXC chemokines growth-regulated oncogene (GRO) α , GRO β , GRO γ , neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. *J Biol Chem* 1996;271:20545–20550.
- de Boer WI: Perspectives for cytokine antagonist therapy in COPD. *Drug Discov Today* 2005;10:93–106.
- Pignatti P, Moscato G, Casarini S, Delmastro M, Poppa M, Brunetti G, Pisati P, Balbi B: Downmodulation of CXCL8/IL-8 receptors on neutrophils after recruitment in the airways. *J Allergy Clin Immunol* 2005;115:88–94.
- Nathan C: Points of control in inflammation. *Nature* 2002;420:846–852.
- Chanez P: Severe asthma is an epithelial disease. *Eur Respir J* 2005;25:945–946.
- Issaq HJ, Conrads TP, Prieto DA, Tirumalai R, Veenstra TD: SELDI-TOF MS for diagnostic proteomics. *Anal Chem* 2003;75:148A–155A.
- Merkel D, Rist W, Seither P, Weith A, Lenter MC: Proteomic study of human bronchoalveolar lavage fluids from smokers with chronic obstructive pulmonary disease by combining surface-enhanced laser desorption/ionization-mass spectrometry profiling with mass spectrometric protein identification. *Proteomics* 2005;5:2972–2980.
- de Torre C, Ying SX, Munson PJ, Meduri GU, Suffredini AF: Proteomic analysis of inflammatory biomarkers in bronchoalveolar lavage. *Proteomics* 2006;6:3949–3957.
- Candiano G, Bruschi M, Pedemonte N, et al: Proteomic analysis of the airway surface liquid: modulation by proinflammatory cytokines. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L185–L198.
- Vachier I, Chiappara G, Vignola AM, et al: Glucocorticoid receptors in bronchial epithelial cells in asthma. *Am J Respir Crit Care Med* 1998;158:963–970.
- Carayol N, Campbell A, Vachier I, Mainprice B, Bousquet J, Godard P, Chanez P: Modulation of cadherin and catenins expression by tumor necrosis factor- α and dexamethasone in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 2002;26:341–347.
- Remick DG: Interleukin-8. *Crit Care Med* 2005;33(suppl):S466–S467.
- Marini M, Vittori E, Hollemborg J, Mattoli S: Expression of the potent inflammatory cytokines, granulocyte-macrophage colony-stimulating factor and interleukin-6 and interleukin-8, in bronchial epithelial cells of patients with asthma. *J Allergy Clin Immunol* 1992;89:1001–1009.

- 24 Inoue K, Slaton JW, Eve BY, et al: Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res* 2000;6:2104–2119.
- 25 Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS: Interleukin-8 stimulates cell proliferation in non-small cell lung cancer through epidermal growth factor receptor transactivation. *Lung Cancer* 2007;56:25–33.
- 26 Wang J, Huang M, Lee P, Komanduri K, Sharma S, Chen G, Dubinett SM: Interleukin-8 inhibits non-small cell lung cancer proliferation: a possible role for regulation of tumor growth by autocrine and paracrine pathways. *J Interferon Cytokine Res* 1996;16:53–60.
- 27 Farkas L, Hahn MC, Schmoczer M, Jentsch N, Kratzel K, Pfeifer M, Schulz C: Expression of CXC chemokine receptors 1 and 2 in human bronchial epithelial cells. *Chest* 2005;128:3724–3734.
- 28 Sturm A, Baumgart DC, d’Heureuse JH, Hotz A, Wiedenmann B, Dignass AU: CXCL8 modulates human intestinal epithelial cells through a CXCR1 dependent pathway. *Cytokine* 2005;29:42–48.
- 29 Baumann H, Gauldie J: The acute phase response. *Immunol Today* 1994;15:74–80.
- 30 Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK: IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. *J Clin Invest* 1998;101:311–320.
- 31 Tilg H, Trehu E, Atkins MB, Dinarello CA, Mier JW: Interleukin-6 (IL-6) as an anti-inflammatory cytokine: induction of circulating IL-1 receptor antagonist and soluble tumor necrosis factor receptor p55. *Blood* 1994;83:113–118.
- 32 Ward NS, Waxman AB, Homer RJ, Mantell LL, Einarsson O, Du Y, Elias JA: Interleukin-6-induced protection in hyperoxic acute lung injury. *Am J Respir Cell Mol Biol* 2000;22:535–542.
- 33 Takizawa H, Ohtoshi T, Ohta K, Yamashita N, Hirohata S, Hirai K, Hiramatsu K, Ito K: Growth inhibition of human lung cancer cell lines by interleukin 6 in vitro: a possible role in tumor growth via an autocrine mechanism. *Cancer Res* 1993;53:4175–4181.
- 34 Yan C, Naltner A, Martin M, Naltner M, Fangman JM, Gurel O: Transcriptional stimulation of the surfactant protein B gene by STAT3 in respiratory epithelial cells. *J Biol Chem* 2002;277:10967–10972.
- 35 Kida H, Yoshida M, Hoshino S: Protective effect of IL-6 on alveolar epithelial cell death induced by hydrogen peroxide. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L342–L349.
- 36 Vignola AM, Campbell AM, Chanez P, Bousquet J, Paul-Lacoste P, Michel FB, Godard P: HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. *Am Rev Respir Dis* 1993;148:689–694.
- 37 Stanciu LA, Djukanovic R: The role of ICAM-1 on T-cells in the pathogenesis of asthma. *Eur Respir J* 1998;11:949–957.
- 38 Relova AJ, Shahana S, Makeeva N, Roomans GM: Effect of cytokines on ICAM-1 and ZO-1 expression on human airway epithelial cells. *Cell Biol Int* 2005;29:768–777.
- 39 Ornstein DK, Rayford W, Fusaro VA, et al: Serum proteomic profiling can discriminate prostate cancer from benign prostates in men with total prostate specific antigen levels between 2.5 and 15.0 ng/ml. *J Urol* 2004;172:1302–1305.
- 40 Carrette O, Demalte I, Scherl A, Yalkinoglu O, Corthals G, Burkhard P, Hochstrasser DF, Sanchez JC: A panel of cerebrospinal fluid potential biomarkers for the diagnosis of Alzheimer’s disease. *Proteomics* 2003;3:1486–1494.
- 41 Papadopoulos MC, Abel PM, Agranoff D, et al: A novel and accurate diagnostic test for human African trypanosomiasis. *Lancet* 2004;363:1358–1363.
- 42 Gravett MG, Novy MJ, Rosenfeld RG, et al: Diagnosis of intra-amniotic infection by proteomic profiling and identification of novel biomarkers. *JAMA* 2004;292:462–469.