

Differential Proteomic Analysis of Bronchoalveolar Lavage Fluid in Asthmatics following Segmental Antigen Challenge*[§]

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Allergic asthma is characterized by persistent airway inflammation and remodeling. Bronchoalveolar lavage conducted with fiberoptic bronchoscopy has been widely used for investigating the pathogenesis of asthma and other lung disorders. Identification of proteins in the bronchoalveolar lavage fluid (BALF) and their expression changes at different stages of asthma could provide further insights into the complex molecular mechanisms involved in this disease. In this report, we describe the first comprehensive differential proteomic analysis of BALF from both asthmatic patients and healthy subjects before and 24 h after segmental allergen challenge. Our proteomic analysis involves affinity depletion of six abundant BALF proteins, SDS-PAGE fractionation, protein in-gel digestion, and subsequent nano-LC-MS/MS analysis in conjunction with database searching for protein identification and semiquantitation. More than 1,500 distinct proteins were identified of which about 10% displayed significant up-regulation specific to the asthmatic patients after segmental allergen challenge. The differentially expressed proteins represent a wide spectrum of functional classes such as chemokines, cytokines, proteases, complement factors, acute phase proteins, monocyte-specific granule proteins, and local matrix proteins, etc. The majority of these protein expression changes are closely associated with many aspects of the pathophysiology of asthma, including inflammation, eosinophilia, airway remodeling, tissue damage and repair, mucus production, and plasma infiltration. Importantly a large portion of these proteins and their expression changes were identified for the first time from BALF, thus providing new insights for finding novel pathological mediators and biomarkers of asthma. *Molecular & Cellular Proteomics* 4: 1251–1264, 2005.

Asthma is a chronic lung disease characterized by reversible airway obstruction, inflammation, and bronchial hyperre-

sponsiveness. The most common form of the disease, allergic asthma, results from inappropriate immune responses to common allergens in genetically susceptible individuals. After exposure to an allergen, such as ragweed or timothy grass, two phases of asthmatic responses can be distinguished (1–5). The early phase of bronchoconstriction reaction occurs within minutes of the allergen challenge and is initiated by smooth muscle contraction as a consequence of the activation and mediator release from local mast cells. A few hours after allergen exposure, a second and more severe response occurs that is accompanied by a long and increasing nonspecific bronchial airway hyperresponsiveness. This late stage response is characterized by the infiltration and accumulation of lymphocytes such as macrophages, neutrophils, and particularly eosinophils.

Pathologies of allergic asthma are complex, involving many cells for different functions (1, 3, 5). The study of pathogenic mechanisms requires a thorough understanding of functional roles of different proteins involved in normal pulmonary homeostasis as well as the specific disease process. Bronchoalveolar lavage (BAL)¹ conducted with fiberoptic bronchoscopy has been widely used to collect cells and other soluble components from the thin layer of epithelial lining fluid that covers the airway and the alveoli (6). Bronchoalveolar lavage fluid (BALF) thus collected contains different cell types as well as a wide variety of proteins that either originate from the blood stream or are released locally by epithelial and inflammatory cells (7–9). Due to the diverse origin of BALF proteins, analysis of BALF may reveal important pathological mediators and enable more accurate characterization of many lung diseases

¹ The abbreviations used are: BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; SAC, segmental allergen challenge; 2D, two-dimensional; FEV₁, forced expiratory volume in 1 s; GO, Gene Ontology; MMP, matrix metalloproteinase; HMG, high mobility group protein; CRISP-3, cysteine-rich secretory protein-3; TIMP-1, tissue inhibitors of metalloproteinase 1; MBL, mannose-binding lectin; SP, surfactant protein; IGF-1, insulin-like growth factor-1; HGFL, hepatocyte growth factor-like protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; antigen PD₂₀, provocative dose that caused a 20% reduction in FEV₁; PNU, protein nitrogen units; HSA, human serum albumin; IL, interleukin; NCBI, non-redundant NCBI protein database.

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at the molecular level. In conjunction with bronchoscopy, segmental allergen challenge (SAC) is a research technique to study the temporal relationship between allergen challenge and inflammatory responses in a controlled setting *in vivo* (6, 9–11). It allows repeated sampling within the same subject at different time points. Moreover the dose of allergen delivered into the airway can be precisely controlled.

Mass spectrometry-based proteomics has emerged as a powerful technique for global protein profiling. Historically protein expression and identification were studied by using two-dimensional (2D) gel electrophoresis. However, recently the method of using automated multidimensional HPLC in combination with MS has gained increasing popularity mainly because of its capability of detecting low abundance proteins and ease of automation (12–14). It has been utilized with great success for large scale analysis of complex protein mixtures such as cell lysates (12). Several recent studies have also demonstrated its utility and potential for global quantitative protein profiling of human body fluids in the search for novel biomarkers (14–17). Generally for the purpose of accurate relative quantitation, proteins or their corresponding peptides from samples to be compared are differentially labeled with stable heavy isotopes either metabolically or externally with various isotope-coded tagging reagents. Subsequently the relative abundances of proteins are determined by the ratio of intensities of their corresponding isotope-labeled peptide pairs measured by MS (18). However, metabolic labeling is not suitable for the analysis of human tissue samples. On the other hand, protein profiling using external labeling often results in limited coverage of complex proteomes due to the reliance on the small percentage of cysteine-containing peptides detectable by MS or the increased complexity due to non-stoichiometric labeling of residues other than cysteines. Moreover such labeling technologies can only compare a few samples without increasing the overall data complexity and dynamic range dramatically. Recently several groups have demonstrated the feasibility of protein relative quantitation across multiple samples without using an isotope-tagging internal standard (16, 19–21). Chelius *et al.* (19) have shown that chromatographic peak areas of peptide precursor ions calculated from the alternating MS full scans in LC-MS/MS experiments were closely correlated with protein concentrations even in complex proteomes such as human serum. More recently, Liao *et al.* (16) also reported that the ion intensity information from MS scan in data-dependent LC-MS/MS experiments can be compared at least semiquantitatively to identify differentially expressed protein biomarkers in synovial fluids from rheumatoid arthritis patients. Interestingly their findings have been confirmed by an independent proteomic study based on 2D gel electrophoresis (22).

There have been numerous reports on the characterization of BALF proteins by proteomic approaches in attempts to uncover biomarkers and pathological mediators for various pulmonary disorders such as idiopathic pulmonary fibrosis,

cystic fibrosis, acute lung injury, nonallergic asthma, and coronary obstructive pulmonary disease (7, 9–11, 23–33). To this end, 2D gel electrophoresis separation followed by mass spectrometry identification has been most widely used. However, due to the huge dynamic range of concentrations of BALF proteins and the inherent limitation of the technique, the 2D gel electrophoresis-based approach was only able to identify a limited number of relatively high abundance proteins without prior sample fractionation. Identification and semi-quantitation of interesting proteins that are present at nanogram per milliliter levels in body fluids such as BALF remain challenging, and there have been no reports on the comparative proteomic analyses of BALFs from both allergic asthmatic patients and healthy volunteers (7, 9–11, 23–33). To provide a more comprehensive BALF protein database and understand their involvement in allergic asthma, here we report the first differential proteomic analysis of BALFs both from asthmatic patients and healthy subjects before and after segmental allergen challenge using affinity depletion of six abundant proteins, SDS-PAGE separation of the remaining proteins, and nano-HPLC-MS/MS analysis for protein identification and semiquantitation.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—A multiple affinity protein removal column (4.6×50 mm) and corresponding buffer solutions were purchased from Agilent (Wilmington, DE). The affinity column was used to remove six abundant proteins (serum albumin, IgG, IgA, haptoglobin, transferrin, and antitrypsin) using affinity-purified polyclonal antibodies packed into an LC column. Molecular weight filters (Microcon; molecular weight cutoff, 3,000) were purchased from Millipore (Bedford, MA). A 10% Tricine SDS-PAGE gel was obtained from Invitrogen. Sequencing-grade modified trypsin was from Promega (Madison, WI). ELISA assay kits for MMP-9 were purchased from R&D Systems (Minneapolis, MN). All other chemicals were purchased from Sigma and used without further purification.

Study Design—Four patients with mild asthma and three healthy control subjects were recruited for the study at The Cleveland Clinic (Table I summarizes the characteristics of the study population). Asthma was defined by the National Institutes of Health guidelines including episodic respiratory symptoms, reversible airflow obstruction (documentation of variability of FEV₁ by 12% and 200 ml, either spontaneously or after inhaled albuterol), or a positive methacholine challenge test (34). All subjects underwent allergy prick skin testing with a standard panel of 10 aeroallergens (Bayer, Spokane, WA), normal saline solution (negative control), and histamine (positive control). A positive reaction was defined as a wheal ≥ 3 mm in diameter. The prick needle was a two-pronged lancet from Allergy Laboratories of Ohio, Inc. Skin tests were read after 15 min. Allergic asthmatic patients had at least two positive skin tests with one of them as ragweed or timothy grass. Healthy controls were non-smokers and skin test-negative, and they had a negative methacholine provocation test (according to the method of Chai *et al.* (35), decrease in FEV₁, $<20\%$ with maximum concentration of methacholine of 25 mg/ml over five stages). All subjects had a base-line FEV₁ $>50\%$ predicted, no asthma exacerbation, or respiratory tract infection within 1 month.

At the initial screening visit, subjects were classified as allergic asthmatics or non-allergic healthy controls. Each subject underwent a medical history, physical examination, and pulmonary function testing. Allergic asthmatic patients underwent whole-lung aerosol aller-

TABLE I
Asthmatic and healthy control subjects included in the study

Inclusion criteria for asthmatic and healthy controls are described under "Experimental Procedures." For protein depletion and subsequent proteomics analysis, all the sample volumes were adjusted so that an equivalent apparent epithelial lining fluid was processed for all samples. Ag, antigen; A-PD₂₀, antigen PD₂₀; WLAC, whole lung antigen challenge; Max Ag, maximum antigen; F, female; M, male; NA, not applicable.

Patient	Age	Sex	Ag for SAC	Days after aerosol challenge	Response to SAC	FEV ₁ base line	FEV ₁ predicted	A-PD ₂₀ by WLAC	Fall in FEV ₁ at Max Ag	SAC dose
	yr						%	μg/ml	%	PNU
Asthmatic (MM)	32	F	Ragweed	85	Good	3.89	102	139	54	31.6
Asthmatic (HB)	23	F	Timothy grass	33	Good	3.34	101	55.27	42	10
Asthmatic (KG)	28	M	Timothy grass	29	Fair	3.20	81	183	59	31.6
Asthmatic (JH)	50	F	Ragweed	57	Poor	1.96	78	167	67	31.6
Healthy (SW)	30	F	Ragweed	28		3.50	101	NA	103	100
Healthy (JC)	27	M	Ragweed	29		4.86	112	NA	103	100
Healthy (KW)	45	F	Ragweed	48		3.30	115	NA	112	100

gen challenge with ragweed or grass to demonstrate antigen responsiveness and to determine the provocative dose that caused a 20% reduction in FEV₁ (antigen PD₂₀). A stock solution of allergen (10,000 protein nitrogen units (PNU)/ml, Greer Laboratories, Lenoir, NC) was diluted with saline solution to produce six concentrations (1, 3.16, 31.6, 100, 1,000, and 3,162 PNU/ml). The allergen preparations at the concentrations used were all below the USP acceptable limit of endotoxin for injection (0.25 endotoxin units/ml) as determined by the limulus amebocyte lysate assay. The patient took 5 breaths at each concentration per the method of Chai *et al.* (35) through a Rosenthal-French dosimeter. The antigen PD₂₀ in PNU was determined. The dose used for segmental allergen challenge was 10% antigen PD₂₀ diluted into 10 ml of saline solution into each segment. The healthy non-allergic controls were challenged with 100 PNU allergen. At least 4 weeks (see Table I, column "Days after aerosol challenge") after the aerosol challenge, a bronchoscopy was performed to obtain a baseline BAL. Three 50-ml aliquots of normal saline solution warmed to 37 °C were instilled in the lingula and withdrawn by gentle hand aspiration. Processing of BAL has been described previously (36). Allergen (in 10 ml of saline solution) was instilled into two subsegments of the right middle lobe. Subjects underwent a second bronchoscopy 24 h later to obtain a BAL of 150 ml from each of the two allergen-challenged subsegments. The BALFs thus obtained were filtered, and cells were separated by centrifugation at 400 × *g* for 10 min at 4 °C. The BALF supernatants were further concentrated with Centricon (molecular weight cutoff, ~3,000) and were stored at -80 °C. Because the amount of BALF proteins is highly variable among individuals and can be dramatically different before and after SAC due to the enhanced airway leakage (8), for quantitative proteomic analysis, we chose to normalize the samples based on the apparent epithelial lining fluid (per milliliter of epithelial lining fluid) with urea as a dilution marker during BAL collection (4, 10). This study was approved by the institutional review board of the Cleveland Clinic Foundation, and informed consent was obtained from all subjects.

Depletion of Abundant Proteins by Immunoaffinity Chromatography—All chromatographic fractionations were performed according to the manufacturer's instructions at ambient temperature on an Agilent 1100 HPLC system with automated sample injector and fraction collector both set at 4 °C. Briefly the BALF samples from asthmatic patients and healthy controls were normalized to the equivalent volume of initial BALF volume and then diluted accordingly to a final volume of 250 μl with sample loading buffer (buffer A) of the Agilent depletion system. The samples were injected automatically onto the affinity column in buffer A at a flow rate of 0.25 ml/min for 9 min. The proteins bound to the column were eluted with buffer B at a flow rate

of 1.0 ml/min for 3.5 min, and the column was regenerated by equilibrating with buffer A for 7.5 min with a total run cycle of 20 min. The depleted BALF (flow-through) and proteins bound to the column (column retentate) were collected at 1.75–5 min and 10.25–12 min, respectively.

SDS-PAGE Separation and In-gel Digestion—The collected fractions were concentrated in Microcon centrifugal concentrators (molecular weight cutoff, 3,000) and were buffer-exchanged into ~30 μl of solution containing 50 mM Tris-HCl and 0.1% SDS at pH 8.5. Proteins were reduced with 20 mM DTT at 60 °C for 1 h and alkylated with 50 mM iodoacetamide at ambient temperature in the dark for 30 min. The samples were mixed with 10× SDS sample loading buffer prior to SDS-PAGE separation in a 10% Tricine minigel. The gel was stained using colloidal Coomassie Blue. The whole gel lane was cut evenly into 20 slices. Each slice was minced into 1 × 1-mm size pieces and subsequently subjected to in-gel digestion with modified trypsin (0.53 μg/gel slice) in a digestion robot (DigestPro, ABIMED, Analysen-Technik GmbH, Langenfeld, Germany). Finally the tryptic digests were concentrated in a SpeedVac to a final volume of ~40 μl before MS analysis.

Nano-LC-MS/MS Analysis—Tryptic digests were analyzed by an automated nano-LC-/MS/MS system using a Famos autosampler (LC Packings, San Francisco, CA) and an Agilent 1100 HPLC binary pump coupled to an LTQ ion trap mass spectrometer equipped with a nanospray ionization source (ThermoFinnigan, San Jose, CA). 10 μl of the digest solution were injected onto a reversed-phase PicoFrit column packed with Magic C₁₈ (5-μm particle, 200-Å pore size, 75 μm × 12 cm; New Objective, Woburn, MA). Peptides were eluted from the column with a 90-min linear gradient from 2 to 55% B at a flow rate of 0.2 μl/min (mobile phase A: 0.1% formic acid aqueous solution; mobile phase B: 90% acetonitrile and 0.1% formic acid). The spray voltage was 1.8 kV, the heated capillary temperature was maintained at 180 °C, and the collision energy for MS/MS was set at 35 units. Automated data-dependent MS analysis was carried out using the dynamic exclusion feature built into the MS acquisition software (Xcalibur 1.3, ThermoFinnigan). Each MS full scan (*m/z* 350–2,000) was followed by MS/MS scans of the first three most intense peaks detected in the prior MS scan to obtain as many CID spectra as possible.

Protein Identification and Semiquantitation—Proteins from each BALF sample were identified using SpectrumMill (version 3.1, Agilent) by searching against the mammalian subset of the National Center for Biotechnology Information (NCBI) non-redundant protein database (updated as of March 31, 2004). Search parameters included a static modification on cysteine residues (carbamidomethylation), a 50% minimum matched peak intensity, ±2.5-Da tolerance on precursor

ions and ± 0.7 -Da tolerance on product ions, one missed tryptic cleavage, and ESI-ion trap scoring parameters as defined by the searching algorithm. To address the database redundancy issue, the proteins that share common peptides were grouped together and displayed as a single protein group in "Protein Centric Columns" mode as described in the SpectrumMill search engine. Within any given protein group, the human protein with the highest score was selected as the most likely correct search result. If the scores were the same, the proteins were listed in an alphabetic order by the search software, and the human proteins listed on top were selected. In a few cases where human proteins are not found in a protein group, then no protein was selected from the group. All of the database search results were further validated by applying the designated protein and peptide scores as well as the following user-defined criteria: 1) protein validation mode: protein score, >18 ; peptide scored percent intensity, $>70\%$ for all charge states; peptide score, >7 for peptide charge $+1$, >8 for peptide charge $+2$, and >9 for peptide charges $\geq +3$; 2) peptide validation mode: peptide scored percent intensity, $>70\%$; peptide score, >13 for all charge states. In short, a protein can be considered positively identified if either multiple spectra of moderate quality or better or at least one spectrum of high quality was obtained. The searching criteria used here would result in a false positive rate of $<4\%$ as justified by Liao *et al.* (16). To estimate the protein relative abundance, the peak areas of the extracted ion chromatograms for each peptide precursor ion in the full scan (total peptide ion current) was calculated in the region ± 1.4 m/z and ± 75 scans using SpectrumMill. As data acquisition was so fast (0.4 s/spectrum average), there are always enough data points to ensure accurate measurement of chromatographic peak areas of the peptides. The variation for protein quantitation using such a method has been shown to be about 20% by separate groups when using protein standards (19–21). The abundance of an individual protein was then calculated as the sum of the total ion current measured for all peptide precursor ions derived from that protein. Thus the relative concentration of each protein was determined by comparing total MS intensities of all identified peptides from that protein in one sample and those from the other samples. ELISA assay of MMP-9 in each sample was also performed according to the protocol recommended by the vendor.

Functional Categorization of BALF Proteins—The annotation of protein cellular localization and biological function was performed using its Gene Ontology (GO) biological process terms (17, 37). Among the 1,592 proteins identified from NCBI non-redundant database, about two-thirds have Swiss-Prot records of which 63% had GO process terms associated with them. However, more than 95% of the differentially expressed proteins had corresponding GO process terms.

RESULTS

Affinity Depletion of Abundant Proteins—BALF contains an abundance of serum proteins permeated through the air-blood barrier. This is especially true in allergen-challenged samples due to SAC-induced plasma protein leakage (8). The existence of highly abundant serum proteins masks the detection of physiologically relevant proteins that are usually present at very low levels. An affinity column with mixed polyclonal antibodies was used to remove serum albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin, which represent the six most abundant proteins in human plasma. Fig. 1 shows the SDS-PAGE images of proteins in an asthmatic BALF (patient MM) before (Lane A) and after protein depletion (Lane B) as well as proteins bound to the affinity column (column retentate) (Lane C). The undepleted BALF (Lane A) is

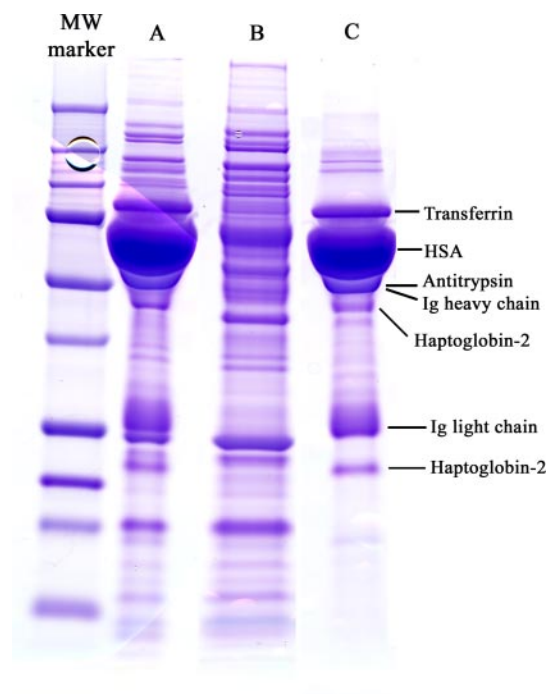


FIG. 1. Typical SDS-PAGE images of BALF proteins without affinity depletion (A), a depleted sample (B), and proteins bound to affinity depletion column (C). Sample loaded to Lane B is equivalent to 2.5-fold volume of the BALF loaded to Lanes A and C.

dominated by HSA and different IgGs. After depletion, about 90% of the total protein mass was removed, and the mixture was greatly simplified (Lane B). Accordingly the dynamic range of the protein mixture was reduced roughly by 2 orders of magnitude. In-gel digestion followed by LC-MS/MS analysis of undepleted BALF proteins (entire Lane A) resulted in the identification of 441 distinct proteins of which 281 were identified from two or more peptides and 56 of them were immunoglobulins. Whereas a total of 889 distinct proteins were identified from the depleted BALF (entire Lane B) with 686 proteins identified by two or more peptides and 10 of them as immunoglobulins. The efficiency of the depletion is also reflected in the significant reduction of the total peptide ion intensity and the abundance rank of the six target proteins in the depleted BALF sample compared with undepleted sample and column retentate (Table II). From the comparison of total peptide ion intensities of column flow-through and retentate, we estimated that $\sim 90\%$ of the IgA and haptoglobin were depleted, and the removal efficiency for the other four proteins was greater than 95%. The specificity of the depletion column was evaluated by the identification of in-gel digested proteins bound to the column. Among a total of 118 nonspecifically bound proteins identified from the column, most of them were also found in flow-through with much higher abundance. There were many protein bands below the major HSA spot at 66 kDa, but they were mostly identified as HSA degradation products.

TABLE II
Total peptide ion intensity (TPI) and protein abundance rank for six target proteins identified from original BALF (without depletion), depleted BALF, and affinity column retentate

Abundance rank of a protein in a mixture is listed based on the total ion counts of all identified peptides.

Entry name	Molecular mass	Accession ^a no.	Without depletion		Depleted BALF		Column retentate		Depletion
			TPI	Rank	TPI	Rank	TPI	Rank	
	<i>kDa</i>								%
Serum albumin precursor	69.4	4502027	6.9E+09	1	2.8E+08	5	4.1E+10	1	>99
IgG κ light chain VLJ region	29.3	21669377	2.3E+09	2	4.6E+07	118	2.7E+09	3	98
Serotransferrin precursor	77.1	4557871	1.2E+09	3	1.4E+08	8	2.4E+09	4	95
IgG κ heavy chain	52.7	27370813	6.7E+08	4	4.1E+07	54	3.4E+09	2	99
Ig λ chain precursor	24.7	87890	6.5E+08	5	2.1E+07	119	4.6E+08	6	95
Antitrypsin	46.7	15990507	2.5E+08	6	3.1E+06	30	1.3E+09	5	>99
Haptoglobin-2 precursor	45.2	4826762	1.4E+08	7	2.1E+07	23	2.7E+08	8	93
IgA	53.2	22760231	8.7E+07	8	4.3E+07	85	3.7E+08	7	90

^a Non-redundant NCBI protein database (NCBIInr).

Protein Profiling in BALFs of Asthmatics and Healthy Control Subjects—Analysis of BALF samples by LC-MS/MS in combination with database searching using SpectrumMill against NCBI non-redundant database resulted in long lists of proteins identified from each sample. In addition, the information about the number of peptides identified as well as the total peptide MS ion intensities for each protein in each sample was also generated by SpectrumMill. The most noticeable similarity between the patterns of protein expression across different samples is that, as expected, plasma proteins are always present as predominant components. The majority of the proteins identified were represented across all samples with little intensity or abundance change with a subset of them changing in either asthmatic samples or challenged asthmatic samples. The basis for the selection of interesting candidates that could be potential pathogenic mediators or biomarkers for asthma (see below for more detailed selection criteria) are the differences in total peptide ion intensity of a particular protein as well as the frequency of its appearance between asthmatics and healthy subjects or between asthmatics before and after challenge. Altogether a total of 1,592 distinct proteins representing a wide spectrum of functional classes was identified with high confidence in 14 discrete BALFs from four asthmatic patients and three healthy subjects. The majority of these proteins on the list have not been previously reported in BALF (Supplemental Table I).

Differential Expression of BALF Proteins—To evaluate the relative changes in the protein expression responding to SAC, the protein levels before and 24 h after SAC were compared. Protein expression levels from the asthma group were also compared with those from the control group to find proteins that are specific to asthmatics. Only proteins identified from three or more unique peptides were qualified for the selection. Proteins were considered to be differentially expressed if either of the following three criteria was met: 1) the protein displays more than 5-fold change in three or more asthmatics and in no more than one healthy subject before and after SAC; 2) the protein has more than 5-fold change in two or more asthmatics and in no healthy subject before and after SAC; 3)

expression in asthmatics is at least 100-fold higher than that in healthy controls. For example, calgranulin B was detected in all four asthmatics and three control samples. Its expression in asthmatics was significantly higher after SAC (27-, 52-, 1-, and 6-fold increase, respectively) but to a much lesser extent in healthy controls (0.6-, 5-, and 4-fold, respectively). In another example, eosinophil granule major basic protein, eosinophil-derived neurotoxin, and eosinophil cationic protein were selected because they were all detected in two challenged asthmatics (MM and HB) that had good response to SAC but in none of the healthy controls. By comparing the protein profiles of four asthmatics and three healthy controls with the above criteria, about 160 proteins were selected that were differentially expressed in challenged asthmatic patients. These proteins are summarized in Table III based on their cellular functions and localizations. Most of the proteins in Table III exhibited elevated expression after SAC except a small group of proteins that were down-regulated after challenge. The differentially expressed proteins listed here represented a wide range of biological categories including serum proteins such as apolipoprotein, metabolic enzymes such as protein prostaglandin-H₂ D-isomerase, calcium-binding proteins such as calgranulin C, cytokine/chemokines such as high mobility group protein 1 (HMG-1) and CCL17, matrix metalloproteinase such as MMP-9 and its inhibitor tissue inhibitors of metalloproteinase 1 (TIMP-1), cellular signaling proteins such as neutrophil gelatinase-associated lipocalin, and proteins involved in lung remodeling such as hepatoma-derived growth factor and its inhibitor as well as a number of proteins whose properties and functions have not been well characterized.

To evaluate the accuracy of relative quantitation of proteins directly based on their total MS signals, the concentration of MMP-9 in each sample was also measured by ELISA and was plotted against the total MS ion intensities of MMP-9 from the same samples. As shown in Fig. 2, a relatively good linear correlation ($R^2 = 0.9827$) was observed between the concentration of MMP-9 measured by ELISA (x axis) and the total MS ion intensity of MMP-9 measure by MS (y axis), reflecting the

TABLE III
List of up- and down-regulated proteins following segmental allergen challenge

All listed proteins were identified with three or more unique peptides and met the following three criteria: 1) displays more than 5-fold change in three or more asthmatics and in no more than one healthy; 2) has more than 5-fold change in two or more asthmatics and in no healthy subject; 3) has more than 100-fold higher expression in asthmatics than that in healthy controls (for detailed expression pattern of each protein in this table in all 14 samples, see Supplemental Table II). MASP, MBL-associated serine protease. HNRPC, human nuclear ribonucleoprotein particle.

Protein name	Molecular mass	NCBI nr accession no.	No. unique peptides
	<i>kDa</i>		
Up-regulated proteins			
Cytokines/chemokines, growth factors, and related proteins			
Hepatocyte growth factor-like protein	80.4	123114	13
Monocyte differentiation antigen CD14	40.1	4557417	12
Hepatocyte growth factor activator (HGFA)	70.7	4504383	10
Insulin-like growth factor-binding protein 2 (IGFBP-2)	35.1	124058	7
Insulin-like growth factor-binding protein 3 (IGFBP-3)	31.7	4504617	6
Mimecan (osteoglycin) (osteoinductive factor)	33.9	7661704	3
Small inducible cytokine A17 (CCL17)	10.5	4506829	4
Insulin-like growth factor I	14.7	8100793	4
Adiponectin	26.4	4757760	3
Insulin-like growth factor-binding protein 6 (IGFBP-6)	25.3	11321593	3
Eosinophil and neutrophil granule proteins			
MMP-9	78.4	4826836	21
Neutrophil gelatinase-associated lipocalin (NGAL)	22.6	1171700	16
Myeloperoxidase (EC 1.11.1.7), splice form H14	92.4	88180	14
Glycosylphosphatidylinositol phospholipase D	92.4	20269065	12
Eosinophil lysophospholipase	16.5	547870	11
Eosinophil granule major basic protein (MBP)	25.2	30410754	9
CRISP-3	27.6	5174675	6
Calgranulin C (neutrophil S100 protein)	10.6	5032059	7
Eosinophil-derived neurotoxin	18.3	11139038	5
Eosinophil cationic protein polymorphism 3	16.3	17064146	4
Complement factors			
Complement factor H (H factor 1)	139.1	4504375	87
Complement factor B preproprotein	85.6	13278732	52
Complement C6 (22)	104.8	105741	38
Complement component C8 α chain	65.2	4557389	20
Complement component C8 γ chain	22.2	4557393	13
Complement factor D (EC 3.4.21.46)	26.2	67580	13
Complement component C1q	25.7	27363488	7
Proteases and inhibitors			
Inter- α -trypsin inhibitor heavy chain H1	101.4	2851501	34
α_1 -Antichymotrypsin (ACT)	47.7	112874	32
Serine (or cysteine) proteinase inhibitor	48.5	21361302	23
Serine (or cysteine) proteinase inhibitor	46.3	12653501	22
Cathepsin D	44.6	4503143	21
Proteasome activator complex subunit 1	28.7	5453990	16
Fetuin-B (IRL685) (16G2)	42.1	7657242	11
Similar to carboxypeptidase N 83-kDa chain	65.2	37550267	13
Inter- α -trypsin inhibitor heavy chain 3	99.1	422961	12
Carboxypeptidase B2 (plasma, carboxypeptidase U)	48.4	13937897	9
CAP18	19.3	4757904	8
Proteasome activator complex subunit 2	27.4	18203506	7
Carboxypeptidase N catalytic chain	52.3	4503011	7
Tissue inhibitor of metalloproteinases	18.8	220125	7
MASP-2	75.7	5459324	4
Other enzymes			
Catalase	59.8	4557014	25
Pyruvate kinase, M1 isozyme	57.9	33286418	22
Transketolase (TK)	67.9	4507521	22
Paraoxonase 1	39.7	19923106	19
L-Lactate dehydrogenase B chain (LDH-B)	36.6	4557032	16
N-Acylsphingosine amidohydrolase (acid ceramidase)	46.5	30089930	16

TABLE III—continued

Protein name	Molecular mass	NCBI nr accession no.	No. unique peptides
	<i>kDa</i>		
Uracil-DNA glycosylase	35.5	35053	11
Nucleoside-diphosphate kinase B (NDK B)	17.3	4505409	12
Alcohol dehydrogenase class III χ chain	39.7	113408	10
Alcohol dehydrogenase	39.9	1223739	11
Unnamed protein product	32.6	21758578	11
Glutathione reductase, mitochondrial (GR) (GRase)	56.3	14916998	10
Thioredoxin (surface-associated sulfhydryl protein)	11.7	135773	9
N-Acetylglucosamine kinase (GlcNAc kinase)	37.4	24638065	8
Pyridoxal kinase (pyridoxine kinase)	35.1	4505701	7
Ribonuclease 4	13.8	5730372	7
Glutathione peroxidase	25.5	2160390	7
Glycogen phosphorylase, liver form	97.1	4506353	7
Glutathione peroxidase 4	22.1	4504107	7
Angiogenin (ribonuclease 5)	16.3	14189876	5
Prostaglandin-H ₂ D-isomerase	21	32171249	5
4a-Hydroxytetrahydrobiopterin dehydratase	12.1	476908	4
Putative 42–9–9 (protein thioredoxin-like 5; thioredoxin)	13.9	14249348	4
Cytidine deaminase (cytidine aminohydrolase)	16.2	11386157	3
Local matrix proteins			
C20orf114 (LPLUNC1 protein)	52.5	37182312	21
Chitinase 3-like 2	43	11993935	18
Zinc- α -2-glycoprotein	33.9	141596	15
Chitinase 3-like 1 (cartilage glycoprotein-39)	42.6	14919433	15
Surfactant, pulmonary associated protein B	42.1	15021771	15
bG174L6.2 (MSF, megakaryocyte-stimulating factor)	151.1	13559026	15
Ficolin 3 isoform 1	32.9	27754776	10
dJ564F22.1 (lipopolysaccharide-binding protein)	53.4	31652249	8
Mannose-binding lectin	26.1	5911809	6
Thrombospondin 1	129.4	135717	5
Peptidoglycan recognition protein (PGRP-S)	21.7	4827036	4
Palate lung and nasal epithelium clone protein	26.7	7706119	3
Intelectin (endothelial lectin HL-1)	35	31542986	3
Ficolin 2	34	4758348	2
Additional acute phase proteins			
Orosomucoid 1	23.5	20070760	19
Calgranulin B (S100 calcium-binding protein A9)	13.2	4506773	15
Calgranulin A (S100 calcium-binding protein A8)	10.8	21614544	16
Serum amyloid A-4 protein (C-SAA)	14.8	10835095	14
Serum amyloid P-component (SAP)	25.4	4502133	13
Calvasculin (S100 calcium-binding protein A4)	11.7	4506765	8
Lipopolysaccharide-binding protein	53.4	31652249	8
C-reactive protein	25	117486	6
Apolipoproteins			
Apolipoprotein B	515.6	178730	221
Apolipoprotein A-I (apoA-I)	30.8	4557321	44
Apolipoprotein A-IV	45.4	4502151	41
Apolipoprotein E (apoE)	36.2	4557325	24
Clusterin (complement lysis inhibitor, SP-40)	52.2	32891795	23
Apolipoprotein H (apo-H)	38.3	543826	20
Apolipoprotein L1	44	17433279	18
Apolipoprotein D (apoD)	21.3	4502163	12
Apolipoprotein A-II (apoA-II)	11.2	4502149	12
Apolipoprotein M (apoM)	21.3	22091452	11
Apolipoprotein C-I (apoC-I)	9.3	4502157	6
Apolipoprotein C-II (apoC-II)	11.3	32130518	5
Apolipoprotein C-III (apoC-III)	10.9	4557323	5
Apolipoprotein C-IV (apoC-IV)	14.6	4502161	3
Other abundant serum proteins			
Ceruloplasmin	115.5	1620909	70

TABLE III—continued

Protein name	Molecular mass	NCBI nr accession no.	No. unique peptides
	<i>kDa</i>		
Plasminogen [contains angiostatin]	90.6	4505881	59
Vitamin D-binding protein/group-specific component	53	455970	56
Fibrinogen α/α -E chain	95	4503689	52
Unnamed protein product (lactoferrin)	78.1	34412	50
AMBP protein (α -1 microglycoprotein)	39	4502067	22
Histidine-rich glycoprotein	59.6	4504489	22
Thyroxine-binding globulin	46.3	1351236	19
α -2-HS-glycoprotein (Ba- α -2-glycoprotein)	39.3	4502005	18
Angiotensinogen	53.1	11118375	18
Unnamed protein product (fibulin 1)	70.6	22761800	14
Leucine-rich α -2-glycoprotein	38.2	21707947	13
Coagulation factor IX	51.8	4503649	10
Vitamin K-dependent protein C	52.1	4506115	7
Coagulation factor X (Stuart factor)	54.7	4503625	6
Myoglobin	17.2	4885477	4
Thrombospondin 4	105.8	549139	4
Cytoskeletal proteins			
Tubulin β -2 chain	49.8	5174735	14
Coactosin-like protein	15.9	21624607	13
Tubulin α -1 chain (α -tubulin 1)	50.2	34740335	13
Similar to common salivary protein 1	18.9	21687060	11
Myosin light chain alkali, smooth muscle isoform	17	2842665	7
Actin-related protein 2/3 complex subunit 4	19.7	5031595	6
Actin-related protein 2/3 complex subunit 1B	41	5031601	5
Desmoplakin I	310	2134996	5
Actin-related protein 3 (actin-2)	47.4	5031573	3
Intracellular proteins and others			
Annexin A3 (annexin III) (lipocortin III)	36.4	4826643	27
ADP-ribosylation factor 3	20.6	4502203	11
Sex hormone-binding protein	41.4	36448	9
CD5L	38.1	37182111	9
Transforming protein RhoA	21.8	16923986	7
GTP-binding nuclear protein RAN (TC4)	24.4	5453555	7
Ras suppressor protein 1 (Rsu-1) (RSP-1)	31.5	6912638	6
HMG-1	24.9	4504425	5
A-kinase anchor protein 9	434.1	34786919	7
40 S ribosomal protein S12	14.5	14277700	5
Annexin A11 (annexin XI)	54.4	4557317	4
Chaperonin 10-related protein	10.3	4028622	4
κ -B motif-binding phosphoprotein	51	1083569	4
Selenoprotein P	41.7	34783648	4
Putative GTP-binding protein PTD004 homolog	44.7	21313144	4
Similar to 40 S ribosomal protein S10	28.4	34935399	4
Guanine nucleotide-binding protein β subunit-like	35.1	5174447	4
Dynein light chain 2A, cytoplasmic	10.9	7661822	3
Activated RNA polymerase II transcription cofactor 4	14.4	19923784	3
MTRANCD5	22.3	2065179	3
HNRPC protein	33.6	14250048	3
Myeloid cell nuclear differentiation antigen	45.8	4505227	3
Down-regulated proteins			
Neural adhesion molecule NB-3	114	7657361	27
Contactin precursor (glycoprotein gP135)	113.3	28373117	24
Angiotensin-converting enzyme (ACE)	149.7	4503273	24
Sushi domain (SCR repeat)-containing	90.2	10092665	19
Hepatocyte growth factor activator inhibitor 1	58.4	32313599	15
Decay-acceleration factor	37.8	1857867	15
Protein tyrosine phosphatase, receptor type	217.1	19743919	15
Plexin D1	212.1	28933451	11
G-protein-coupled receptor GPR116	144.5	24475630	11

TABLE III—continued

Protein name	Molecular mass	NCBI nr accession no.	No. unique peptides
	<i>kDa</i>		
Neurotrimin precursor (hNT)	38	7705413	9
Sodium-dependent phosphate transporter isoform-3b	75.8	5453752	8
Neuropilin 1	71.9	7271465	8
Pirin	32.1	4505823	6
Osteoclast-associated receptor hOSCAR-M1	28.8	19557668	4

capability of the MS-based method for gross relative protein quantitation in the global analysis of complex protein mixtures such as the BALF proteome.

Gene Ontology Classification—Proteins identified in BALF were categorized by GO to define their molecular functions and cellular localization as well as biological processes. For this purpose, all 1,592 proteins from the entire 14 experimental data sets as well as the differentially expressed proteins were summarized separately as an overview (Fig. 3, A and B) to show the distribution of BALF proteins using the GO indexing system. The total protein complement identified in the BALF (Fig. 3A) covered a broad range of protein functional classes associated with diverse biological processes such as proteolysis, immune response, inflammatory responses, cell adhesion, cell mobility, cell proliferation, metabolism, and signal transduction. In addition to extracellular proteins, a number of membrane and intracellular proteins were also identified. However, the differentially expressed proteins (Fig. 3B) are primarily extracellular/secreted proteins that are involved in various aspects of immune responses and airway damage and protection such as inflammation, proteolysis, response to oxidative stress, apoptosis, or defense response to bacteria.

DISCUSSION

Controlled provocation of airway inflammation using bronchoscopy and segmental allergen challenge is a powerful tool for the *in vivo* study of airway inflammation. The unique features and conclusions from our study are as follows. 1) This is the first comprehensive differential proteomic analysis of bronchoalveolar lavage fluid from healthy subjects as well as mild asthmatic patients at base line and 24 h after segmental allergen challenge. 2) Over 1,500 distinct proteins were identified of which about 10% displayed significant up-regulation specific to the asthmatic patients after SAC (Supplemental Table I). 3) A large proportion of these proteins and their expression changes were identified for the first time from BALF; many of these have biological relevance to the pathophysiology of asthma. In this study, we utilized a high throughput proteomic approach to study the differential expression of proteins in BALFs. In contrast to traditional biochemical approaches that can only study one or a few particular protein species, proteomics is able to analyze thousands of proteins and thus provides comprehensive BALF proteome information that is essential in understanding

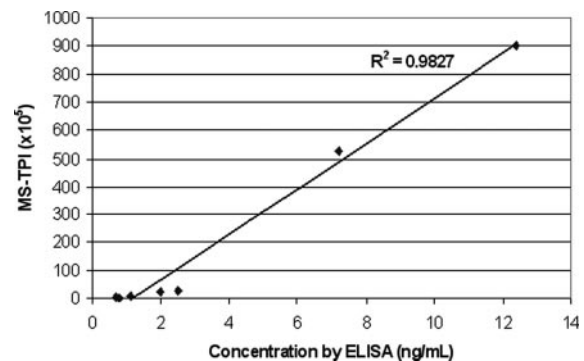


FIG. 2. Correlation of MMP-9 concentrations as measured by ELISA and MS total peptide ion intensities (TPI).

the molecular mechanisms underlying asthma.

The profile of abundant proteins in BALF mirrors that of plasma proteins largely due to the permeability of air-blood barrier. In agreement with the literature, the most abundant plasma proteins such as serum albumin, IgG, and transferrin were also found to be most abundant in BALF from both asthmatic patients and healthy controls. As shown in Fig. 1, HSA accounted for as much as 90% of the total proteins in this particular BALF sample. After SAC, the albumin level can further increase in BALF due to plasma leakage from pulmonary blood vessels into lung tissues as a result of inflammatory damage to the alveolar capillary barrier (8). The large dynamic range in the protein expression level presented a great challenge to the successful detection and relative quantitation of interesting BALF proteins that are usually present at very low abundance. As a critical step, we used an affinity column to remove the six most abundant proteins. The ratio of the mixed affinity resins in the column was originally optimized for the removal of such proteins from plasma, but it worked equally well in the case of BALF proteins. As shown in Table II, virtually quantitative depletion of all six proteins was achieved.

Quantitation of proteins directly based on MS signal intensities without internal standards has historically drawn limited attention primarily due to limited linearity of correlation between protein concentration and MS signal strength and variable ionization efficiency, especially for proteins present in a complex mixture. However, reports from several groups have shown that relative changes in total signal intensities of peptides correlated well with their concentration changes in one

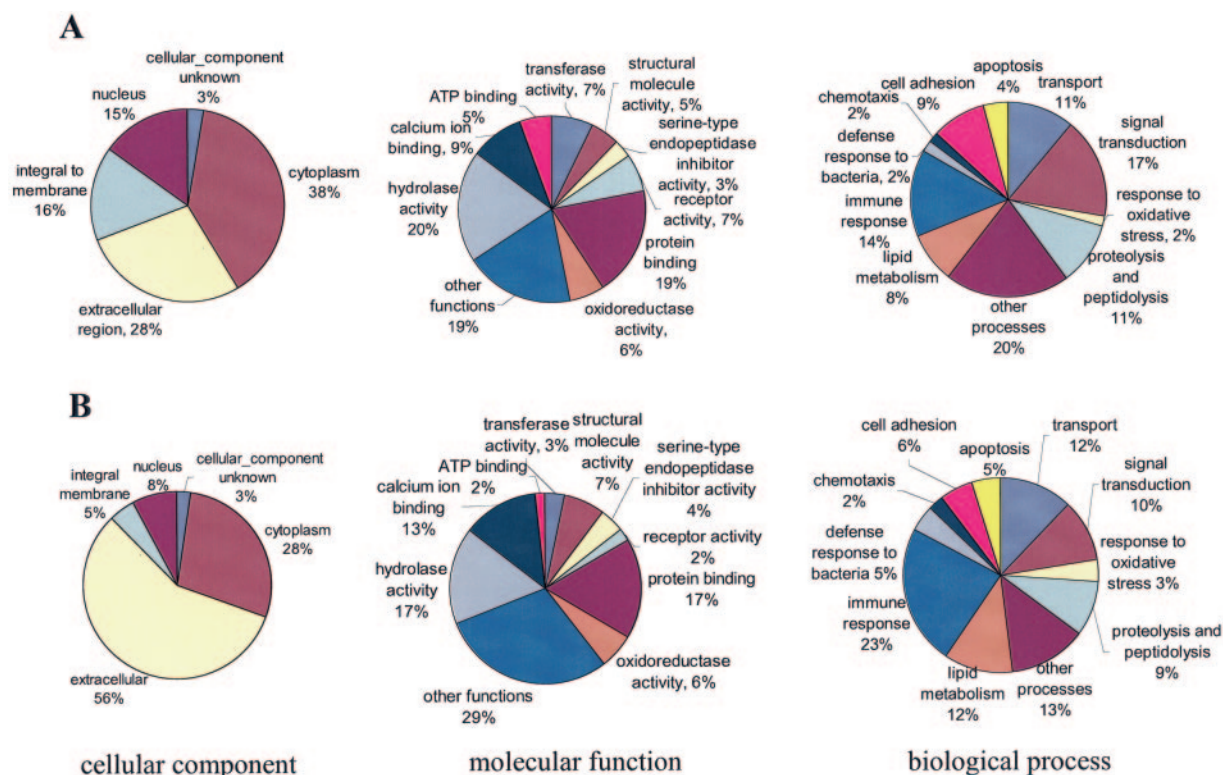


FIG. 3. **Molecular functions, cellular components, and biological processes of the BALF proteins using GO terminology.** A, GO of the entire BALF protein list. B, GO of the differentially expressed BALF proteins.

sample versus another (16, 19–21). In general, we also found our approach worked well for proteins identified from three or more peptides and proteins showing noticeable expression changes. For example, the relative quantity of MMP-9 in each sample measured by MS correlated very well with that obtained by ELISA (Fig. 2). However, it still should be noted that the method is semiquantitative and should be used with caution for comparison of proteins with low abundance and proteins with relatively small changes.

As shown by the gene ontology chart (Fig. 3, A and B), a large number of extracellular proteins and protein families were identified in BALF. These proteins are involved in numerous functions such as proteolysis, inflammatory responses, cell adhesion, cell mobility, cell proliferation, metabolism, and signal transduction. One interesting observation was that a variety of intracellular proteins were also found in the BALF from both asthmatics and controls with some of them clearly elevated after SAC (Table III). For example, seven proteins in annexin family (annexins A1, A2, A3, A5, A6, A8, and A11) were identified with high confidence of which A3 and A11 were elevated up to 116-fold following SAC (data not shown). It is not yet clear why these intracellular proteins were present in high abundance in the BALF. The possible causes may include: 1) cellular proteins shed into lung airway as a result of increased cell apoptosis and lysis, (2) increased leakage of those proteins circulating in the blood, and (3) protein secretions by pathways that remain largely unknown.

Because only a few ribosomal proteins, one of the most abundant intracellular protein families, were identified and with very low level, it is unlikely that cell lysis is a major reason for the identification of these intracellular proteins. On the other hand, it is more likely that these putative intracellular proteins play certain extracellular roles that are currently unknown because many proteins have pleiotropic cellular functions. One such example is annexin A1, which can bind to extracellular protein S100A11. The expression and secretion of annexin A1 is induced by glucocorticoids (38). Although the mechanism for the secretion of annexin A1 is unknown, the extracellular annexin A1 can interfere with granulocyte recruitment, migration, and/or activation at sites of inflammation (39). Another example is HMG-1. HMG-1 is a nuclear protein but can be secreted by activated monocytes and macrophages (40, 41). Outside the cell, it binds to receptor for advanced glycation end products (RAGE) and is a potent proinflammatory mediator that activates downstream cytokine release. It is well known to participate in the development of lethal sepsis (42, 43). The same scenario may apply to its other two family members, HMG-2 and high mobility group protein 1-like 2 (HMG-1L2) (see Supplemental Table I).

The pathophysiology of allergic asthma is complex and involves many cell types (1–3, 5, 44, 45). The initial allergic response is dominated by activation of resident mast cells. Twenty-four hours following allergen challenge, a variety of cell types including Th2 cells, eosinophils, macrophages, and

TABLE IV
Chemokines and cytokines identified in BALFs

Protein name	Accession no.	Molecular mass	No. unique peptides
		<i>kDa</i>	
CXCL7	4505981	13.9	4
CCL17	4506829	10.5	4
CXCL4	4505733	10.8	3
CCL14	4759070	10.7	2
CXCL1	4504153	11.3	2
CCL18	4506831	9.8	1
Megakaryocyte-stimulating factor	13559026	151.1	15
Macrophage-stimulatory protein	123114	80.4	13
Hepatitis-derived growth factor (HMG1L2)	4758516	26.8	6
HMG-1	4504425	24.9	5
Colony-stimulating factor 1 isoform b	27262663	47.8	4
Pre-B-cell colony-enhancing factor	33386695	41.9	3
Cytokine HIN-1	16418367	10.2	2
HMG-2	123375	24.1	2
Interferon γ -induced protein	4504701	10.9	2
Interferon α -inducible protein	14550514	17.9	1
Interleukin 18	14210477	22.3	1

neutrophils are recruited from the circulation and invade the airway epithelial lamina. Those immune effector cells together with the local fibroblasts, smooth muscle cells, and epithelial cells contribute to the persistent airway inflammation and remodeling through release of inflammatory mediators such as chemokines, cytokines, and proteases. Overall there is an increase in the cell numbers and expression levels of proteins within allergen-challenged subjects compared with those in unchallenged controls. As shown in Table III, most of the differentially expressed proteins found in our study can be correlated to the different aspects of the complex molecular events happening during an asthmatic attack. These molecules include chemokines and cytokines, proteases, complement factors, acute phase proteins, and monocyte-specific granule proteins that are proinflammatory; local matrix proteins that are associated with the increased mucus production and maintenance of airway function; and abundant plasma proteins that indicate the increased plasma infiltration. In addition, the overall level of protein expression changes also correlated well with the extent of the response of an individual asthmatic to SAC based on clinical observations with good responders (patients MM and HB) having much more protein abundance compared with those for poor responders (patients KG and JH) (Table I). Our observations in BALF protein expression changes are consistent with what is known about the pathophysiology of asthma.

Over a dozen chemokines and cytokines were identified in BALFs (Table IV). Some of the proteins (CCL17, megakaryocyte-stimulating factor, macrophage-stimulatory protein, and

high mobility group protein 1) were shown to be distinctly elevated after SAC according to our criteria. Others were present in much lower abundance with only three or fewer peptides identified, and thus the change in expression is not discernable. It is also interesting to note that some of the well known Th2-specific cytokines such as IL4, IL5, and IL13, and chemokines such as eotaxin and monocyte chemotactic protein were not detected. Possible reasons include the timing of the BALF harvest and tissue or cell type distribution profiles, and if present they were below the sensitivity limit of mass spectrometric detection.

Up-regulated proteins associated with potentiation of inflammation represented the largest group in Table III. Those include proteases, complement factors, acute phase proteins, and eosinophil and neutrophil granule proteins. It has been well documented that activation of eosinophils, once being recruited to the airway, leads to secretion of many eosinophil-specific granule proteins such as eosinophil cationic protein, major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, and Charcot-Leyden crystal protein, etc. (1, 3, 44). All but eosinophil peroxidase was identified in BALFs with highly elevated expression in asthmatics after SAC (Fig. 4). Asthmatics MM and HB who had stronger overall responses to SAC showed significant increases in the expression of these proteins. This observation was also supported by a previous report that the elevated level of eosinophil cell counts and their cationic protein release in BAL was related to the magnitude of the late phase response (5). Such observation on the response of the asthmatic patients to SAC is consistent with the phenomenon of eosinophilia during a typical allergic asthma exacerbation and thus further verifies the SAC as a clinical model for asthma exacerbation. In addition to the eosinophil-specific granule proteins, a number of neutrophil granule proteins, such as myeloperoxidase, C5a (neutrophil chemotactic factor), lipocalin 2, and cysteine-rich secretory protein-3 precursor (CRISP-3) were also significantly elevated in the airway of asthmatic patients with strong SAC responses, whereas the expression levels of those proteins in healthy controls are either unchanged or are elevated to a much lesser extent following SAC. From the molecular level, such changes in these proteins demonstrate the involvement of neutrophils in the late phase reaction after SAC.

MMPs are a large family of proteinases that play important roles in asthma by their influence on the function and migration of inflammatory cells and matrix deposition and degradation (46–50). MMP-7, MMP-8, MMP-9, and MMP-20 were identified with high confidence in BALFs among which MMP-9 was predominant with 21 unique peptides identified. MMP-9 plays a critical role in tissue repair and remodeling because of its function to degrade type IV collagen, which is an important constituent of basement membrane where airway remodeling occurs (46, 47, 50). A dramatic increase in MMP-9 expression was consistently observed in asthmatics after SAC. Interestingly TIMP-1, which regulates the activity of

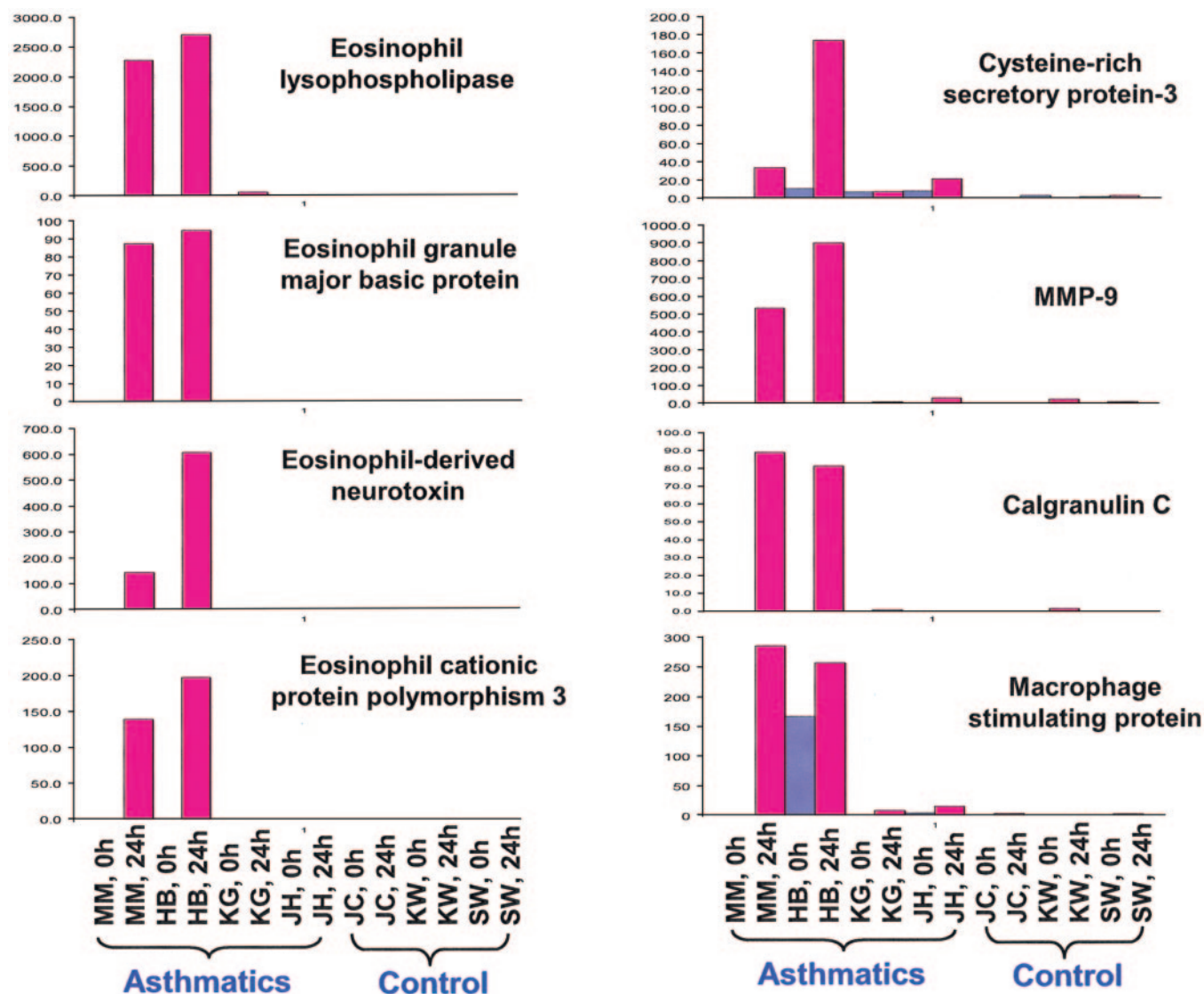


FIG. 4. Up-regulation of some signature proteins secreted from different inflammatory cells 24 h after SAC. The gray and purple lines correspond to protein levels before and after SAC, respectively. More significant protein up-regulation was observed for two asthmatic patients who had strong clinical responses to SAC.

MMP-9, was also elevated in challenged asthmatics, although the absolute concentration of TIMP-1 seemed to be lower than that of MMP-9.

A number of proteins secreted by local epithelial cells, smooth muscle cells, and fibroblasts in the airway were also highly up-regulated in BALF after SAC. Some of these proteins such as megakaryocyte-stimulating factor (also called lubricin) are possibly involved in the maintenance of normal airway function. Others including pulmonary surfactants, LPLUNC1, protein PLUNC, chitinase 3-like 2, chitinase 3-like 1, lipopolysaccharide-binding protein, mannose-binding lectin (MBL), ficolins 2 and 3, and intelectin are well known host defense proteins that are part of the innate immunity against pathogens (51–54). As one would expect, MBL-associated serine protease that is required for MBL and ficolins to trigger

the innate immunity through lectin-complement pathway upon recognition of infectious agents is also clearly up-regulated after SAC. Among these host defense proteins, pulmonary surfactant family contains four unique proteins, surfactant protein (SP)-A, SP-B, SP-C, and SP-D, which are all synthesized by lung epithelial cells. Both SP-A and SP-D are hydrophilic surfactant proteins and share similar roles by regulating immune cell functions and participate in first line defense within the lung against invading pathogens, whereas the hydrophobic surfactant proteins, SP-B and SP-C, are responsible for surface activities.

An interesting observation is the increased expression of two growth factors, insulin-like growth factor-I (IGF-I) and hepatocyte growth factor-like protein (HGFL, also named macrophage-stimulating protein), and their related proteins

(Table III). The increase of IGF-I and its binding proteins plays an important role in airway remodeling because there is a strong correlation between IGF-I expression and collagen thickening after inhaled corticosteroid treatment in asthmatics (55). On the other hand, HGFL is a multifunctional factor regulating cell adhesion and motility, growth, and survival. The increase of HGFL seems to play at least dual roles during the late phase inflammation reaction after SAC. It may contribute to the recruitment and activation of macrophages while also preventing the airway epithelial cells from apoptosis in an inflammatory environment (56, 57). More interestingly, the hepatocyte growth factor activator, which is a serine protease required for the activation of hepatocyte growth factor, was also up-regulated, whereas the level of hepatocyte growth factor activator inhibitor was decreased in the BALF after SAC. This observation clearly indicates a coordinated response at the molecular level to SAC in asthmatics.

CONCLUSIONS

Although the value of BALF analysis in determining the pathogenesis of lung disorders is well established, to our knowledge this study provides the most comprehensive database of the proteins present in BALFs of both healthy subjects and asthmatic patients. This is the first report of differential proteomic analysis of BALFs from both asthmatics and healthy subjects before and after SAC. The analysis of four asthmatics and three healthy individuals resulted in the identification of 1,592 proteins among which about 10% displayed altered expression for asthmatic patients at the peak of the late phase reaction to allergen challenge. Although the present findings are based on a limited number of asthmatic individuals, the results, from the protein level, clearly reflect the complex pathophysiology of asthma, such as inflammation, eosinophilia, airway remodeling, tissue damage and repair, mucus production, and plasma infiltration. This study provides new insights for uncovering novel inflammatory mediators that can be potential therapeutic targets or biomarkers for asthma.

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REFERENCES

- Cohn, L., Elias, J. A., and Chupp, G. L. (2004) Asthma: mechanisms of disease persistence and progression. *Annu. Rev. Immunol.* **22**, 789–815
- Fehniger, T. E., Sato-Folatre, J. G., Malmstrom, J., Berglund, M., Lindberg, C., Brange, C., Lindberg, H., and Marko-Varga, G. (2004) Exploring the context of the lung proteome within the airway mucosa following allergen challenge. *J. Proteome. Res.* **3**, 307–320
- Hamid, Q., Tulic, M. K., Liu, M. C., and Moqbel, R. (2003) Inflammatory cells in asthma: mechanisms and implications for therapy. *J. Allergy Clin. Immunol.* **111**, S5–S12
- Persson, C. G., Andersson, M., Greiff, L., Svensson, C., Erjefalt, J. S., Sundler, F., Wollmer, P., Alkner, U., Erjefalt, I., and Gustafsson, B. (1995) Airway permeability. *Clin. Exp. Allergy* **25**, 807–814
- Pradaliere, A. (1993) Late-phase reaction in asthma: basic mechanisms. *Int. Arch. Allergy Immunol.* **101**, 322–325
- Kavuru, M. S., Dweik, R. A., and Thomassen, M. J. (1999) Role of bronchoscopy in asthma research. *Clin. Chest Med.* **20**, 153–189
- Bowler, R. P., Duda, B., Chan, E. D., Enghild, J. J., Ware, L. B., Matthay, M. A., and Duncan, M. W. (2004) Proteomic analysis of pulmonary edema fluid and plasma in patients with acute lung injury. *Am. J. Physiol.* **286**, L1095–L1104
- Nocker, R. E., van der Zee, J. S., Weller, F. R., van Overveld, F. J., Jansen, H. M., and Out, T. A. (1999) Segmental allergen challenge induces plasma protein leakage into the airways of asthmatic subjects at 4 hours but not at 5 minutes after challenge. *J. Lab. Clin. Med.* **134**, 74–82
- Plymoth, A., Lofdahl, C. G., Ekberg-Jansson, A., Dahlback, M., Lindberg, H., Fehniger, T. E., and Marko-Varga, G. (2003) Human bronchoalveolar lavage: biofluid analysis with special emphasis on sample preparation. *Proteomics* **3**, 962–972
- Rennard, S. I., Basset, G., Lecossier, D., O'Donnell, K. M., Pinkston, P., Martin, P. G., and Crystal, R. G. (1986) Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. *J. Appl. Physiol.* **60**, 532–538
- Noel-Georis, I., Bernard, A., Falmagne, P., and Wattiez, R. (2002) Database of bronchoalveolar lavage fluid proteins. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **771**, 221–236
- Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat. Biotechnol.* **19**, 242–247
- Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M., and Yates, J. R., III (1999) Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **17**, 676–682
- Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., Springer, D. L., and Pounds, J. G. (2002) Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. *Mol. Cell. Proteomics* **1**, 947–955
- Pang, J. X., Ginanni, N., Dongre, A. R., Hefta, S. A., and Opitke, G. J. (2002) Biomarker discovery in urine by proteomics. *J. Proteome Res.* **1**, 161–169
- Liao, H., Wu, J., Kuhn, E., Chin, W., Chang, B., Jones, M. D., O'Neil, S., Clauser, K. R., Karl, J., Hasler, F., Roubenoff, R., Zolg, W., and Guild, B. C. (2004) Use of mass spectrometry to identify protein biomarkers of disease severity in the synovial fluid and serum of patients with rheumatoid arthritis. *Arthritis Rheum.* **50**, 3792–3803
- Anderson, N. L., Polanski, M., Pieper, R., Gatlin, T., Tirumalai, R. S., Conrads, T. P., Veenstra, T. D., Adkins, J. N., Pounds, J. G., Fagan, R., and Lobley, A. (2004) The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol. Cell. Proteomics* **3**, 311–326
- Gygi, S. P., Rist, B., Gerber, S. A., Turecek, F., Gelb, M. H., and Aebersold, R. (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* **17**, 994–999
- Chelius, D., Zhang, T., Wang, G., and Shen, R. F. (2003) Global protein identification and quantification technology using two-dimensional liquid chromatography nanospray mass spectrometry. *Anal. Chem.* **75**, 6658–6665
- Bondarenko, P. V., Chelius, D., and Shaler, T. A. (2002) Identification and relative quantitation of protein mixtures by enzymatic digestion followed by capillary reversed-phase liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **74**, 4741–4749
- Wang, W., Zhou, H., Lin, H., Roy, S., Shaler, T. A., Hill, L. R., Norton, S., Kumar, P., Anderle, M., and Becker, C. H. (2003) Quantification of proteins and metabolites by mass spectrometry without isotopic labeling or spiked standards. *Anal. Chem.* **75**, 4818–4826
- Drynda, S., Ringel, B., Kekow, M., Kuhne, C., Drynda, A., Glocker, M. O., Thiesen, H. J., and Kekow, J. (2004) Proteome analysis reveals disease-associated marker proteins to differentiate RA patients from other inflammatory joint diseases with the potential to monitor anti-TNF α ther-

- apy. *Pathol. Res. Pract.* **200**, 165–171
23. Wattiez, R., Hermans, C., Cruyt, C., Bernard, A., and Falmagne, P. (2000) Human bronchoalveolar lavage fluid protein two-dimensional database: study of interstitial lung diseases. *Electrophoresis* **21**, 2703–2712
24. Wattiez, R., Hermans, C., Bernard, A., Lesur, O., and Falmagne, P. (1999) Human bronchoalveolar lavage fluid: two-dimensional gel electrophoresis, amino acid microsequencing and identification of major proteins. *Electrophoresis* **20**, 1634–1645
25. Wang, J. Y., Shieh, C. C., Yu, C. K., and Lei, H. Y. (2001) Allergen-induced bronchial inflammation is associated with decreased levels of surfactant proteins A and D in a murine model of asthma. *Clin. Exp. Allergy* **31**, 652–662
26. Sabouchi-Schutt, F., Astrom, J., Hellman, U., Eklund, A., and Grunewald, J. (2003) Changes in bronchoalveolar lavage fluid proteins in sarcoidosis: a proteomics approach. *Eur. Respir. J.* **21**, 414–420
27. Noel-Georis, I., Bernard, A., Falmagne, P., and Wattiez, R. (2001) Proteomics as the tool to search for lung disease markers in bronchoalveolar lavage. *Dis. Markers* **17**, 271–284
28. Malmstrom, J., Larsen, K., Malmstrom, L., Tufvesson, E., Parker, K., Marchese, J., Williamson, B., Hattam, S., Patterson, D., Martin, S., Graber, A., Juhasz, H. P., Westergren-Thorsson, G., and Marko-Varga, G. (2004) Proteome annotations and identifications of the human pulmonary fibroblast. *J. Proteome Res.* **3**, 525–537
29. Houtman, R., Krijgsveld, J., Kool, M., Romijn, E. P., Redegeld, F. A., Nijkamp, F. P., Heck, A. J., and Humphrey-Smith, I. (2003) Lung proteome alterations in a mouse model for nonallergic asthma. *Proteomics* **3**, 2008–2018
30. Hirsch, J., Hansen, K. C., Burlingame, A. L., and Matthay, M. A. (2004) Proteomics: current techniques and potential applications to lung disease. *Am. J. Physiol.* **287**, L1–L23
31. Griesse, M., von Bredow, C., and Birrer, P. (2001) Reduced proteolysis of surfactant protein A and changes of the bronchoalveolar lavage fluid proteome by inhaled α 1-protease inhibitor in cystic fibrosis. *Electrophoresis* **22**, 165–171
32. Houtman, R., and van den Worm, E. (2005) Asthma, the ugly duckling of lung disease proteomics? *J. Chromatogr. B* **815**, 285–294
33. Wattiez, R., and Falmagne, P. (2005) Proteomics of bronchoalveolar lavage fluid. *J. Chromatogr. B* **815**, 169–178
34. National Heart, Lung, and Blood Institute (1997) *Expert Panel Report 2, Guidelines for the Diagnosis and Management of Asthma*, Publication 97-4051, National Institutes of Health, Bethesda, MD
35. Chai, H., Farr, R. S., Froehlich, L. A., Mathison, D. A., McLean, J. A., Rosenthal, R. R., Sheffer, A. L., Spector, S. L., and Townley, R. G. (1975) Standardization of bronchial inhalation challenge procedures. *J. Allergy Clin. Immunol.* **56**, 323–327
36. Thomassen, M. J., Buhrw, L. T., Connors, M. J., Kaneko, F. T., Erzurum, S. C., and Kavuru, M. S. (1997) Nitric oxide inhibits inflammatory cytokine production by human alveolar macrophages. *Am. J. Respir. Cell Mol. Biol.* **17**, 279–283
37. Ashburner, M., Ball, C. A., Blake, J. A., Botstein, D., Butler, H., Cherry, J. M., Davis, A. P., Dolinski, K., Dwight, S. S., Eppig, J. T., Harris, M. A., Hill, D. P., Issel-Tarver, L., Kasarskis, A., Lewis, S., Matese, J. C., Richardson, J. E., Ringwald, M., Rubin, G. M., and Sherlock, G. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat. Genet.* **25**, 25–29
38. Gerke, V., and Moss, S. E. (2002) Annexins: from structure to function. *Physiol. Rev.* **82**, 331–371
39. Perretti, M. (1998) Lipocortin 1 and chemokine modulation of granulocyte and monocyte accumulation in experimental inflammation. *Gen. Pharmacol.* **31**, 545–552
40. Degryse, B., Bonaldi, T., Scaffidi, P., Muller, S., Resnati, M., Sanvito, F., Arrigoni, G., and Bianchi, M. E. (2001) The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *J. Cell Biol.* **152**, 1197–1206
41. Erlandsson Harris, H., and Andersson, U. (2004) Mini-review: the nuclear protein HMGB1 as a proinflammatory mediator. *Eur. J. Immunol.* **34**, 1503–1512
42. Czura, C. J., Yang, H., Amella, C. A., and Tracey, K. J. (2004) HMGB1 in the immunology of sepsis (not septic shock) and arthritis. *Adv. Immunol.* **84**, 181–200
43. Sama, A. E., D'Amore, J., Ward, M. F., Chen, G., and Wang, H. (2004) Bench to bedside: HMGB1—a novel proinflammatory cytokine and potential therapeutic target for septic patients in the emergency department. *Acad. Emerg. Med.* **11**, 867–873
44. Lampinen, M., Carlson, M., Hakansson, L. D., and Venge, P. (2004) Cytokine-regulated accumulation of eosinophils in inflammatory disease. *Allergy* **59**, 793–805
45. Liu, L., Jarjour, N. N., Busse, W. W., and Kelly, E. A. (2004) Enhanced generation of helper T type 1 and 2 chemokines in allergen-induced asthma. *Am. J. Respir. Crit. Care Med.* **169**, 1118–1124
46. Vermaelen, K. Y., Cataldo, D., Tournoy, K., Maes, T., Dhulst, A., Louis, R., Foidart, J. M., Noel, A., and Pauwels, R. (2003) Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. *J. Immunol.* **171**, 1016–1022
47. Takafuji, S., Ishida, A., Miyakuni, Y., and Nakagawa, T. (2003) Matrix metalloproteinase-9 release from human leukocytes. *J. Investig. Allergol. Clin. Immunol.* **13**, 50–55
48. Suzuki, R., Miyazaki, Y., Takagi, K., Torii, K., and Taniguchi, H. (2004) Matrix metalloproteinases in the pathogenesis of asthma and COPD: implications for therapy. *Treat. Respir. Med.* **3**, 17–27
49. Parks, W. C., and Shapiro, S. D. (2001) Matrix metalloproteinases in lung biology. *Respir. Res.* **2**, 10–19
50. Maisi, P., Sorsa, T., Raulo, S. M., Prikki, K., Sepper, R., and McGorum, B. (2001) Increased matrix metalloproteinase (MMP)-9 in the airway after allergen challenge. *Am. J. Respir. Crit. Care Med.* **164**, 1740
51. Bingle, C. D., and Gorr, S. U. (2004) Host defense in oral and airway epithelia: chromosome 20 contributes a new protein family. *Int. J. Biochem. Cell Biol.* **36**, 2144–2152
52. Brass, D. M., Savov, J. D., Whitehead, G. S., Maxwell, A. B., and Schwartz, D. A. (2004) LPS binding protein is important in the airway response to inhaled endotoxin. *J. Allergy Clin. Immunol.* **114**, 586–592
53. Fujita, T., Matsushita, M., and Endo, Y. (2004) The lectin-complement pathway—its role in innate immunity and evolution. *Immunol. Rev.* **198**, 185–202
54. Tsuji, S., Uehori, J., Matsumoto, M., Suzuki, Y., Matsuhisa, A., Toyoshima, K., and Seya, T. (2001) Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. *J. Biol. Chem.* **276**, 23456–23463
55. Hoshino, M., Nakamura, Y., Sim, J. J., Yamashiro, Y., Uchida, K., Hosaka, K., and Isogai, S. (1998) Inhaled corticosteroid reduced lamina reticularis of the basement membrane by modulation of insulin-like growth factor (IGF)-I expression in bronchial asthma. *Clin. Exp. Allergy* **28**, 568–577
56. Danilovitch-Miagkova, A., and Leonard, E. J. (2001) Anti-apoptotic action of macrophage stimulating protein (MSP). *Apoptosis* **6**, 183–190
57. Wang, M. H., Zhou, Y. Q., and Chen, Y. Q. (2002) Macrophage-stimulating protein and RON receptor tyrosine kinase: potential regulators of macrophage inflammatory activities. *Scand. J. Immunol.* **56**, 545–553