Published in final edited form as:
J Proteome Res. 2009 April ; 8(4): 1631–1638.

Oxidative Stress and Asthma: Proteome Analysis of Chitinase-like Proteins and FIZZ1 in Lung Tissue and Bronchoalveolar Lavage Fluid

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Abstract

Oxidative stress plays an important role in the development of airway inflammation and hyperreactivity in asthma. The identification of oxidative stress markers in bronchoalveolar lavage fluid (BALF) and lung tissue from ovalbumin (OVA) sensitized mice could provide new insight into disease pathogenesis and possible use of antioxidants to alleviate disease severity. We used two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the impact of the thiol antioxidant, N-acetylcysteine (NAC), on protein expression in a murine OVA model. At least six proteins or protein families were found to be significantly increased in BALF from OVA-challenged mice compared to a control group: chitinase 3-like protein 3 (Ym1), chitinase 3-like protein 4 (Ym2), acidic mammalian chitinase (AMCase), pulmonary surfactant-associated protein D (SP-D), resistin-like molecule α (RELMα) or "found in inflammatory 1" (FIZZ1), and haptoglobin α-subunit. A total of 9 proteins were significantly increased in lung tissue from the murine asthma model, including Ym1, Ym2, FIZZ1, and other lung remodeling-related proteins. Western blotting confirmed increased Ym1/Ym2, SP-D, and FIZZ1 expression measured from BAL fluid and lung tissue from OVA-challenged mice. Intraperitoneal NAC administration prior to the final OVA challenge inhibited Ym1/Ym2, SP-D, and FIZZ1 expression in BALF and lung tissue. The oxidative stress proteins, Ym1/Ym2, FIZZ1 and SP-D, could play an important role in the pathogenesis of asthma and may be useful oxidative stress markers.

Keywords

proteomics; *asthma pathogensis*; *N-acetylcysteine*; *antioxidant*; *diesel exhaust particle*; *oxidative stress*

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Supporting Information Available: Figures showing the overall sample preparation flowchart, the protein concentration and cell counts from BALF from the different treatment of the animal groups, the measurements of serum OVA-specific IgG1 and IgE, and the H & E staining of lung tissues showing OVA-induced lung inflammation, and the 2D gels of lung tissue are in the supplemental information. This material is available free of charge via the Internet at http://pubs.acs.org

Introduction

Allergic asthma is a chronic inflammatory disease characterized by eosinophilic airway inflammation, mucus hypersecretion, and bronchial hyperresponsiveness.[1] While the pathogenesis of asthma is complex, it is well recognized that this disease is a Th2 cytokine-mediated disorder in which skewing of the T-cell response is promoted by a number of different factors, including a contribution by antigen presenting cells that process environmental allergens.[2] In particular, we have shown that ambient air pollution particles assist Th2 skewing of the immune response by exerting oxidant stress effects on the immune system, including modification of dendritic cell function.[3] Interference in oxidative stress could therefore be a useful adjuvant therapy for asthma. Blesa *et al* found that changes in oxidant status, transcription factor activation, and inflammatory cytokine and gene expression by antigen exposure were reduced by N-acetylcysteine (NAC).[4] NAC also blocks the inducible form of nitric oxide (NO) synthetase from inducing NF-κB activation[5] and has been used as an effective treatment of airway inflammation and reactivity in experimental animal models.[6]

We have previously shown that 2D-PAGE and mass spectrometry measurements can be used to probe particulate matter (PM)-induced oxidant stress responses in macrophages[7-9] and epithelial cells.[10] Specifically, we have reported previously that a diesel exhaust particle (DEP) extract can induce a linear increase in new protein expression and that greater than 50% of newly expressed proteins can be subtracted by the inclusion of NAC, which functions as an antioxidant as well as a direct neutralizing agent for redox cycling DEP chemicals.[11, 12] The overall proteome profile as well as the associated cellular responses allowed us to formulate a hierarchical oxidative stress hypothesis, which posits that low levels of oxidative stress induce antioxidant and cytoprotective responses while higher levels of oxidative stress could lead to the induction of inflammation or cellular apoptosis.[7, 13]

We were interested to see if a similar proteomic approach can be used to identify oxidative stress markers in biofluids and tissue samples from a murine model of ovalbumin (OVA)-induced allergic airway disease (AAD).[14, 15] Several research groups have explored the use of a proteomics-based research approach, using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry (MS)-based protein identification, to identify global protein expression changes in this asthma model.[2, 16, 17] For example, lungkine, a recently described chemokine, a family of chitinases including Ym1, Ym2, and acidic mammalian chitinase (AMCase), gob-5, a protein that mediates mucus secretion, and surfactant protein-D, a C-type lectin capable of modulating inflammatory responses, were undetectable or at very low levels in bronchoalveolar lavage fluid (BALF) of normal mice, but were significantly increased in abundance in airway inflammation.[17] Fajardo *et al* identified a number of marker proteins associated with the pathogenesis of allergic lung inflammation including glycolytic enzymes, a glucose-regulated 78 kDa protein, prolyl-4-hydroxylase, peroxiredoxin 1, and arginase from mouse lung tissue and BALF.[18] Among the proteins showing increased levels in the murine asthma models, Ym2 displayed the clearest increase, present at high levels in animals with lung eosinophilia, while being undetectable in control subjects. Furthermore, the levels of cathepsin S were markedly increased in inflamed tissue. Jeong *et al* identified 15 proteins that were differentially expressed in the lungs of mice with allergic asthma compared to normal mice; 9 proteins including Ym1, Ym2 and peroxiredoxin could be linked to asthma-related symptoms, oxidant injury and tissue remodeling.[16]

In the present study, we have adopted a similar proteomic approach to identify BAL fluid and lung proteins that are affected by NAC treatment of OVA-sensitized and challenged Balb/c mice. Ym1, Ym2, FIZZ1, and SP-D were identified as proteins that were affected by NAC treatment in this animal model. Our study identifies these proteins for the first time as potential oxidative stress markers that can be used to follow the role of oxidant injury in asthma.

Materials and Methods

Animals

Female BALB/c mice (7 to 8 week old) were obtained from Charles River Laboratories (Hollister, CA, USA). Mice were housed in filter-topped cages under standard laboratory conditions and maintained on autoclaved food and acidified water.

Sensitization and Aerosol Challenge

Three groups of animals were used; each group included 6 animals (Figure 1). All animals were sensitized intraperitoneally with 20 μg of OVA plus 2 mg of aluminum hydroxide (alum) in 500 μL of PBS on days 1 and 7. All mice from group one (PBS group) were exposed on days 15, 16 and 17 to nebulized saline. All mice from group two (OVA group) were exposed on days 15, 16 and 17 to nebulized saline for an hour followed by 1% aerosolized OVA 30 minutes using a nose-only Battelle exposure chamber. All mice from group three (NAC group) were treated with 320 mg/kg NAC intraperitoneally on days 15, 16 and 17 for 1 hr before the 1% OVA inhalation exposure. OVA (chicken egg albumin grade V, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in saline. Aerosols were generated by a compressed-air nebulizer (Collision 6-jet) at an airflow of 7 L/min using a nebulizer concentration of 1% OVA dissolved in saline.

Determination of OVA-specific IgE and IgG1 Antibody Titers

Serum OVA-specific IgE and IgG1 levels were measured by means of ELISA, as previously described.[19]

Measurement of Eosinophils in BALF

All mice were sacrificed by administering pentobarbital intraperitoneally 18 hr after the last aerosol challenge, and their tracheas were cannulated with polyethylene tubing. Bronchoalveolar lavage (BAL) was performed after exposing the trachea with a midline neck incision and doing a tracheostomy with a 14-gauge needle. Ice-cold sterile PBS was introduced (1 mL, 3 times); about 80% of the volume was recovered each time. The BAL fluid was centrifuged (400 × *g*, 10 min, 4°C) and the supernatants were collected for the 2D gel electrophoresis analysis. The lungs were removed after washing with PBS through the right ventricle; the left lobe was kept in liquid nitrogen, and the right part was stored in 4% formaldehyde (Sigma-Aldrich). The cells were resuspended in 0.4 mL PBS, and differential cell counts were performed on cytospin preparations and stained with HEMA3 for cytological examination, scoring at least 200 cells/sample.

Histology Staining with Anti-FIZZ1

Slides were baked and deparaffinized. Endogenous peroxidase was quenched with H_2O_2/70% methanol for 10 min and incubated with primary antibodies at a concentration of 1/50 for 2 hrs. Dako Envision (anti-rabbit) Horseradish peroxidase labeled secondary antibody was used. Sections were stained with diaminobenzidine (DAB) and followed by hematoxylin-eosin (H&E) counterstaining. Digital images of sections were obtained using a ScanScope XT System (Aperio Technologies Inc, Vista, CA) at 200X magnification courtesy of the UCLA Translational Pathology Core Laboratory.

Real-time PCR

Total RNA was isolated from lung tissue using Qiagen's RNeasy kit. Reverse transcription was carried out with the iScript cDNA Synthesis kit (BioRad) for RT-PCR. One μg RNA from each sample was used for the 20 μL cDNA reaction. For the real-time PCR, a BioRad iQ™ SYBR® Green Supermix and a myiQ™ machine were used. The PCR primer set is as follows: 5'-

AGGAACTTCTTGCCAATCCA-3' and 5'-CAGTAGCAGTCATCCCAGCA-3'. FIZZ1 and reference (actin) samples were triplicated and the standard curves were duplicated on a single 96-well PCR plate. PCR data was analyzed using the Pfaffl method.[20, 21]

Sample Preparation for Proteomic Analysis

The overall protocol for sample preparation for the proteomics experiments is depicted in Supplementary Figure 1. Aliquots of BALF supernatants corresponding to six different animals (six controls or six sensitized and challenged animals or six NAC-treated) were pooled and precipitated with cold acetone/10% TCA. Protein concentrations were quantified using the 2D Quant kit (Amersham Biosciences, Uppsala, Sweden) after samples were dissolved in rehydration buffer (7 M urea, 2 M thiourea, 20 mM DTT, 1.2% CHAPS, 5% glycerol, 10% isopropanol, 0.4 ASB-14, 0.25% ampholytes). Lungs were frozen in liquid nitrogen and stored at -80°C. For protein extraction, lungs were homogenized at 4°C in 300 μL of lysis buffer (4% Triton-X-100, 7 M urea, 2 M thiourea, 25 mM DTT, 1% protease inhibitor cocktail, and 0.5% ampholyte) with a Polytron PT 1200 homogenizer (Kinematica, Littau-Lucerne, Switzerland). Homogenates were sonicated in an ice bath for 10 sec × 6 times and were subsequently centrifuged at 12,000*g* for 20 min at 4°C. Supernatants were recovered and immediately stored at -80°C, after saving aliquots for the quantification of protein concentrations. Aliquots of lung protein from each mouse were then pooled and precipitated with the 2D clean up kit (Amersham Biosciences), and protein concentrations were quantified using the 2D Quant kit.

2D Gel Electrophoresis

All reagents and materials used for 2D gel electrophoresis were of electrophoresis grade purity and were provided by Amersham Biosciences, Sigma, or Merck Eurolab (Darmstadt, Germany). A total of 75 μg of protein from BALF samples or 150 μg protein from the lung extract was dissolved in rehydration buffer. A sample volume of 350 μL was then applied to 17-cm pH 4-7 immobilized pH gradient strips (Bio-Rad, Hercules, CA), which were subsequently allowed to rehydrate for 12 hr at 50 V (22°C). Subsequently, isoelectric focusing using a Protean IEF Cell (Bio-Rad) was performed at 22°C for 1 hr with a linear ramp to 500 V, followed by 3 hr at 500 V, and a 3 hr linear ramp to 10,000 V, and holding at 10,000 V until the V-hrs reached to 99,999. The strips were then equilibrated at room temperature for 15 min in SDS-equilibration buffer (0.375 mM Tris/HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 60 mM DTT) and for another 15 min with SDS-equilibration buffer supplemented with 135 mM iodoacetamide. After equilibration, the IEF strips were applied to 8-16% SDS-PAGE gels (Protean II ready gel, Bio-Rad). Molecular weight standards (Mark 12™ MW Standard, Invitrogen, Carlsbad, CA) were applied to filter paper beside the strip. Electrophoresis was carried out at 10 mA per gel during the first 30 min followed by 18 mA per gel until complete. Gels were stained using Sypro Ruby (Bio-Rad). For gel-image analysis, gels were scanned at high resolution with an FX Molecular Imager FX (Bio-Rad), and PDQuest software version 7.2 (Bio-Rad) was used for detection of qualitative and quantitative alterations in the protein spots. Gel images were normalized to the total grey intensity. Only protein spots with a minimum of 4-fold difference in intensities between OVA-treatment and control groups were selected. Gels were run in triplicate for each sample group.

Protein Identification by LC-MS/MS

Spots of interest were excised from the gels by the ProteomeWorks spot cutter (Bio-Rad). The in-gel reduction, alkylation, trypsin digestion, and peptide extraction was accomplished manually using standard protocols.[7] Data for protein identifications was acquired with an LC-quadrupole time-of-flight (QTOF) system (Dionex/LC Packings nano-LC and Applied Biosystems/Sciex QSTAR Pulsar XL mass spectrometer). The reverse-phase LC pre-column (75 μm × 10 mm) and column (75 μm × 150 mm) were packed with Jupiter 4 μm Proteo 90 Å

C12 resin (Phenomenex, Torrance, CA, USA). The eluent for the LC binary gradient was comprised of water containing 0.1% formic acid (buffer A) and 95% acetonitrile, 0.1% formic acid (buffer B). The flow rate was 200 nL/min and the gradient profile was 3% buffer B to 21% B in 36 min, 21% B to 35% B in 14 min, 35% B to 80% B in 6 min and 80% B constant for 8 min. Electrospray ionization was performed using a 30 μm internal diameter nano-bore stainless steel online emitter (Proxeon Biosystems, Odense, Denmark) and a voltage of 1900 V.

Peptide product ion tandem mass spectra were recorded during LC-MS/MS by information-dependent analysis (IDA) on the QSTAR XL mass spectrometer. Argon was employed as the collision gas. Collision energies for maximum fragmentation were automatically calculated using empirical parameters based on the charge and mass-to-charge ratio of the precursor peptide. The MS/MS spectra were searched against the Swiss-Prot protein sequence database (release date 01-09-2007) using the Mascot search program (Matrix Science, London, UK). For searching the sequence database, the following variable modifications were set: carbamidomethylation of cysteines, oxidization of methionines, conversion of N-terminal glutamate and aspartate to pyro-Glu, and cyclization of N-terminal cysteine. One missed tryptic cleavage was tolerated and the peptide and MS/MS mass tolerance was set as ±0.3 Da. Positive protein identification was based on standard Mascot criteria for statistical analysis of the MS/MS data. A -10Log (P) score, where P is the probability that the observed match is a random event, of 72 was regarded as significant.

Western Blot Analysis

Ten μg of BAL fluid protein or 50 μg of lung protein from different mice was electrophoresed on 10-20% SDS polyacrylamide gels before transfer to PVDF membranes. Horseradish peroxidase-conjugated secondary antibodies (GE Healthcare/Amersham Biosciences, Uppsala, Sweden) were used followed by ECL reaction to develop the blots according to the manufacturer's instructions. Primary antibodies were used to detect the expression of the following proteins: Ym1/Ym2 from Dr. Shioko Kimura (National Cancer Institute, Bethesda, MD, USA), β,actin (Sigma-Aldrich), SP-D (Chemicon International, Temecula, CA, USA), and FIZZ1 (Alpha Diagnostic International, Inc. San Antonio, TX, USA). Band intensities from film were analyzed by IMAGEQUANT 5.2 software (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical Analysis

Data were expressed as the mean ± SME and analyzed by Student's t-test. All differences of $p < 0.05$ were considered significant and showed as *. A $p < 0.01$ is represented as **.

Results

Suppressive Effect of NAC on Allergic Airway Inflammation

The protein concentration of BAL fluid increased significantly from 200 μg/mL to greater than 325 μg/mL in OVA-challenged animals sensitized with intraperitoneal OVA plus alum ($p < 0.01$). NAC administration to the OVA-challenged animals did not significantly affect BALF protein concentration levels (Supplemental Figure 2A). Total cell count and eosinophil count significantly increased ($p < 0.01$) in the BAL fluid from OVA-challenged mice (OVA group) compared to the saline control mice (PBS group). Macrophage and lymphocyte cell counts showed no significant difference among these three groups (Supplemental Figure 2B). However, injection of NAC inhibited significantly the increase of both total cell count and eosinophil count (Supplemental Figure 2B). Eosinophils are oxidant sensitive cells considered relevant in allergic inflammation. Serum levels of total OVA-specific IgE and OVA-specific IgG1 were substantially elevated in allergic airway inflammation (OVA group and NAC group)

(Supplemental Figures 3A, B). The application of NAC had a significant inhibitory effect on OVA-specific IgG1 and IgE expression (Supplemental Figure 3A). Histology of lung tissue showed that OVA aerosol challenge induced marked infiltration of inflammatory cells as compared to the saline challenge. NAC attenuated the OVA-induced lung inflammation to the level comparable to that of the saline control (Supplemental Figure 4). Taken together, these data suggest that oxidative stress plays an important role in specific aspects of OVA-induced allergic airway inflammation.

2D-PAGE of BALF and Lung Tissue from OVA-challenged Mice Showed Significant Changes in Protein Levels

2D gel electrophoresis was used to monitor protein expression in BAL fluid and lung tissue from OVA-challenged mice and to determine the potential effect of NAC on protein expression. Reproducible protein profiles were observed on triplicate gels (pI 4-7) that were carried out for each of the three groups of BALF and lung samples. Loading all of the 2D gels with 75-150 μg of protein resolved approximately 450 protein spots from BAL fluid and 870 spots from lung tissue that could be used for matching. Differential protein expression analysis was based on a criterion of at least a four-fold change in spot intensity. A total of 33 gel spots in the 2D gel of BAL fluid from the murine asthma model showed a significant increase in intensity compared to those of the control group (Figure 2 and Supplemental Figure 5). Many of the protein spots represent the same family of proteins or gene product with multiple potential modifications. From these 33 protein gel spots, 8 unique proteins were identified (Table 1), including chitinase 3-like protein 3 (Ym1), chitinase 3-like protein 4 (Ym2), acidic mammalian chitinase (AMCase), resistin,like molecule α (also known as FIZZ1, “found in inflammatory zone 1”), haptoglobin α, and pulmonary surfactant-associated protein D (SP,D). Similarly, a total of 11 2D gel spots in lungs from the murine asthma model showed significant increase in intensity compared to those of the control group (Supplemental Figures 6, 7); proteins that were identified from these spots include Ym1, Ym2, AMCase, and FIZZ1 (Table 2). Several of the BALF proteins showing increased abundance after OVA challenge showed partial suppression after NAC administration, including Ym1, Ym2, AMCase, FIZZ1, and SP-D (Table 1). The same NAC effect was observed for the lung proteins, AMCase, Ym1, and Ym2 (Table 2), confirming their potential role in oxidative stress.

Immunoblotting and Immunohistology Confirm Protein Level Changes and Subtractive Effect of NAC

To verify the 2D-PAGE data, Western blotting of Ym1/Ym2 and SP-D were conducted. Western blot analysis of Ym1/Ym2 of both the BAL fluid and lung tissue from OVA-challenged animals showed significant increased expression of Ym1/Ym2 compared to the control group ($p < 0.01$ for both samples) (Figures 3, 4). Also, there were significantly increased SP-D levels in BALF and lung samples from the OVA group compared to that of the control group (both $p < 0.01$). Introduction of NAC before the OVA challenge partially inhibited this increased protein expression for Ym1/Ym2 and SP-D in both lung and BALF samples (Figures 3, 4). Immunohistology staining (data not shown) with Ym1/Ym2 antibodies also showed significantly increased Ym1/Ym2 expression in the lungs of the OVA-sensitized and challenged mice.

In addition, FIZZ1 is more significantly increased in lung tissue from OVA sensitized mice than measured from control (PBS) mice as measured by Western blotting; NAC also had a significant inhibitory effect on FIZZ1 levels in the lung tissue (Figure 5A, B). RT-PCR analysis of FIZZ1 mRNA levels in lung tissue shows the same behavior profiled by Western analysis: FIZZ1 message levels are dramatically increased in the OVA group and are significantly reduced with co-introduction of antioxidant NAC measured from both pooled tissue samples (Figure 5C) and from individual tissue samples (data not shown). Immunohistology staining

with FIZZ1 antibodies also showed significantly increased FIZZ1 levels in lungs of OVA-sensitized and challenged mice (Figure 6). Positive staining was found in macrophages and epithelial cells from OVA-treated mice, and the OVA-induced FIZZ1 protein expression was significantly inhibited by antioxidant NAC.

In general, there is good consistency between the data obtained by several biochemical methods, including 2D gel electrophoresis/mass spectrometry, Western blot analysis, immunohistology, and real-time PCR. Proteins Ym1/Ym2, SP-D, and FIZZ1 are increased upon OVA sensitization, and the thiol antioxidant, NAC, partially inhibits OVA-induced responses.

Discussion

Asthma is characterized by airway eosinophilia, edema, mucus hypersecretion, bronchial epithelial injury, and hyperreactivity. The disease pathogenesis include a contribution from several cell types that can lead to the pathology as well as changes in the BALF, including airway epithelial cells, eosinophils, macrophages, dendritic cells, T-helper type 2 (Th2) cells, IgE secreting B cells and mast cells.[22]

BALF is a biofluid that mirrors the expression of secreted pulmonary proteins and the products of activated cells and destructive processes.[23, 24] BALF is among the most studied biomedia for sampling the components of the epithelial lining fluid and it can provide perhaps the most faithful reflection of the protein composition of the pulmonary airways.[25, 26] The characterization of the BALF proteome also provides a potential opportunity to establish temporal and prognostic indicators of airway disease.[27] Changes measured in the BALF proteome profile may reflect the asthmatic condition. The surfactant-associated proteins (SP), including SP-A, SP-B and SP-D, are among the low abundant BALF proteins that are produced locally by resident cells. Pulmonary surfactant is composed of specific phospholipids and several SP, that together with Clara cell and mucin-associated proteins, are secreted into BALF by different lung epithelial cell types. Detection of SP in BALF provides potential information about the changes in lung epithelial cell function or clearance during pulmonary disease states.

In the present study, we attempted to identify oxidative stress markers in the BALF and corresponding lung tissue by NAC administration in a murine asthma model. There is a growing body of literature linking the induction of oxidative stress and reactive oxygen species (ROS) production to increased risk of asthma, and more specifically with particulate matter (PM)-induced oxidative stress.[7, 11, 28, 29] PM contain redox-active chemicals and transition metals that can generate ROS. The balance between ROS/reactive nitrogen species (RNS) and antioxidants in asthmatic airways has been suggested to be in disequilibrium due to an excess production of ROS/RNS by accumulation of inflammatory and immune cells, such as macrophages, neutrophils, and eosinophils.[30] The production of large and small molecular weight antioxidants plays an important role in the *in vivo* and *in situ* defense of the lung against oxidative stress. Antioxidant depletion or excessive ROS production can induce oxidative stress, which proceeds in a hierarchical fashion to generate cellular responses. The most sensitive cellular response to mild oxidative stress is the activation of antioxidant and phase II enzymes (Tier 1), which include heme oxygenase (HO-1), glutathione-S-transferase (GST), superoxide dismutase (SOD), and glutathione peroxidase. Tier 1 antioxidant defenses are critical for protecting against airway inflammation and asthma. If this protection fails, further increase of oxidative stress can induce inflammation (Tier 2) and ultimately cell death (Tier 3).

Haptoglobin was found to be elevated in BALF in the OVA-model in our study (Table 1). Previously, Signor *et al* also found elevated haptoglobin levels in BALF from OVA-challenged

rats.[31] Haptoglobin is an acute-phase glycoprotein considered to be involved in tissue repair and is produced by fibroblasts and inflammatory cells. An elevated level of haptoglobin expression in BALF was reported from patients with mild asthma as compared to the other healthy subjects.[30] One of the major functions of haptoglobin is to bind to hemoglobin and thereby prevent the oxidative tissue damage mediated by free hemoglobin.[32]

However, the most significant set of proteins that were increased in the BAL fluid and lung tissue of our OVA-sensitized animals and sensitive to NAC treatment was proteins of the chitinase family, SP-D, and FIZZ1. SP-D is a member of the collectin family of innate defense proteins. SP-D plays critical roles in maintaining normal surfactant structure and metabolism. It also plays important roles in regulation of pulmonary macrophage activation, airspace remodeling, and surfactant lipid homeostasis.[33, 34] SP-D directly protects surfactant phospholipids and macrophages from oxidative damage.[35] SP-D also influences NFκB activity through oxidant sensitive pathways.

Fourteen isoforms of Ym1, displayed as separate spots on the 2D-gel profiles, were found from BAL fluid. These do not appear to be due to protein phosphorylation because a phosphoprotein-specific ProQ diamond stain[9] did not show any reactivity to the Ym1 gel spots (data not shown); however, this does not preclude other types of post-translational modifications or separate isoforms of Ym1. AMCase, SP-D, and other proteins that increased in the OVA-model samples displayed multiple 2D gel spots; 8 spots representing acidic mammalian chitinase (AMCase) were detected, and 5 2D-gel spots for SP-D were also detected.

Ym1 is a member of a family of mammalian proteins that share homology to chitinases of lower organisms,[36, 37] although Ym1 was originally described as an eosinophil chemotactic factor. Zhu *et al* have shown that AMCase and Ym1 are specifically upregulated in response to Th2 inflammation, but not in Th1 inflammation in the lung.[37] Ym1 and AMCase expression is mutually exclusive in proximal and distal airway epithelia, respectively; however, both are expressed in alveolar macrophages.[38] Ym1 and Ym2 are highly inducible STAT6-dependent transcripts in Th2-biased inflammation.[39] Although the molecular mechanisms of chitinase in asthma are largely unknown, they can specifically bind to carbohydrate moieties and other glycosaminoglycans such as heparin and heparan sulfate to regulate eosinophil chemotaxis, inflammation and tissue remodeling.[36, 37]

FIZZ1 was also significantly increased in the OVA-sensitized mouse group from both lung tissue and BAL fluid. FIZZ1 is a protein that was found to be induced in murine lung in an OVA-induced asthma model.[40] BAL fluid from mice with allergic pulmonary inflammation contains the 9.4 kDa cysteine,rich secreted FIZZ1 protein. Later, FIZZ1 was renamed as resistin-like molecule α (RELMα)[41] and also referred to as hypoxia-induced mitogenic factor (HIMF).[42] FIZZ1 has been found in macrophages,[43] and HIMF/FIZZ1 is a hypoxia-induced mitogenic factor in lung with potent angiogenic and pulmonary vasoconstrictive properties.[42] Recent findings suggest that FIZZ1 is involved in pulmonary fibrosis both through induction of myofibroblast differentiation and increased survival of myofibroblasts.[44] In addition, HIMF/FIZZ1 has been connected to signaling pathways that regulate lung inflammation by inducing VCAM-1 (vascular adhesion molecule-1) upregulation through the phosphatidylinositol-3-kinase (PI-3K)/Akt-NFκB signaling pathway.[45]

An observational link between Ym1/Ym2 and FIZZ1 has been established in several recent reports. Ym1 and FIZZ1 are secreted proteins that have been identified in a variety of Th2-mediated inflammatory settings.[46] Ym1 and FIZZ1 are induced strongly in *in vivo*- and *in vitro*-elicited, alternatively activated macrophages.[43] The expression of both genes is up-regulated during type II, cytokine-controlled, allergic pulmonary inflammation.[36, 40] A mouse model for idiopathic pulmonary fibrosis (IPF) from infection with murine γ-herpesvirus

demonstrated that recruited alveolar macrophages showed high levels of expression of the proteins Ym1/Ym2 and FIZZ1.[47] FIZZ1 levels of expression measured by RT-PCR and Western blotting showed early upregulation of FIZZ1 transcript and protein levels in BAL of infected animals. A progressive increase in the expression of Ym-proteins in infected animals was determined by Western blot analysis using lung lysates and BAL samples obtained at different time points after infection.

Concluding Remarks

A proteomics strategy was used to profile global protein expression differences between an asthma animal model and normal controls. Utilization of the 2D-PAGE method can be advantageous for relative quantification of changes to proteins that exhibit post-translational modifications, such as the proteins displayed in the present study. In our study, we found significantly increased levels of Ym1/Ym2, AMCase, SP-D, FIZZ1 in lung and BALF samples. Co-administration of NAC suppresses the abundances of these proteins, suggesting that these proteins could be associated with the pathogenesis of asthma and also establishes a possible link between oxidative stress-induced inflammation and asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Shioko Kimura (National Cancer Institute, Bethesda, MD, USA) for providing the Ym1/2 polyclonal antibody and for the associated Western blot data. The research was supported by United States Public Health service grants (NIH), R01 ES012053 and R01 ES013432, as well as the NIAID-funded UCLA AADCRC, U19 AI070453. JAL acknowledges also support from the W. M. Keck Foundation for the establishment of the UCLA Functional Proteomics Center.

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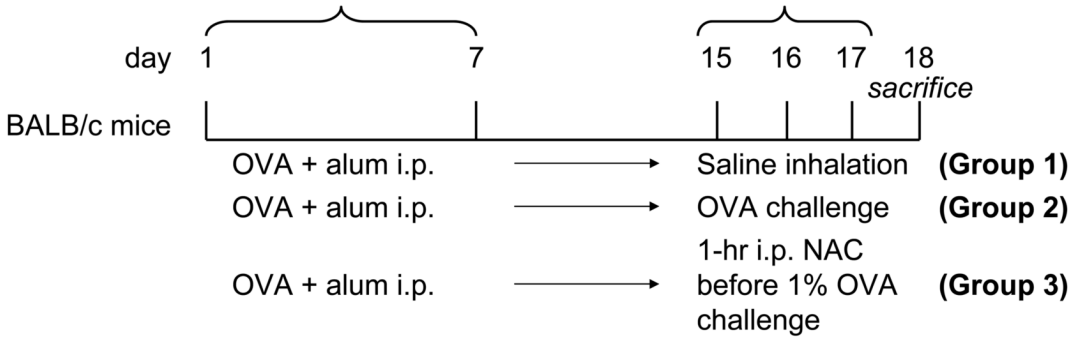

Figure 1.
Outline of protocol for murine model of allergic airway inflammation. OVA (20 μg) plus aluminum hydroxide (alum, 2 mg) in 500 μL PBS was introduced intraperitoneally to each group of animals (6 mice/group).

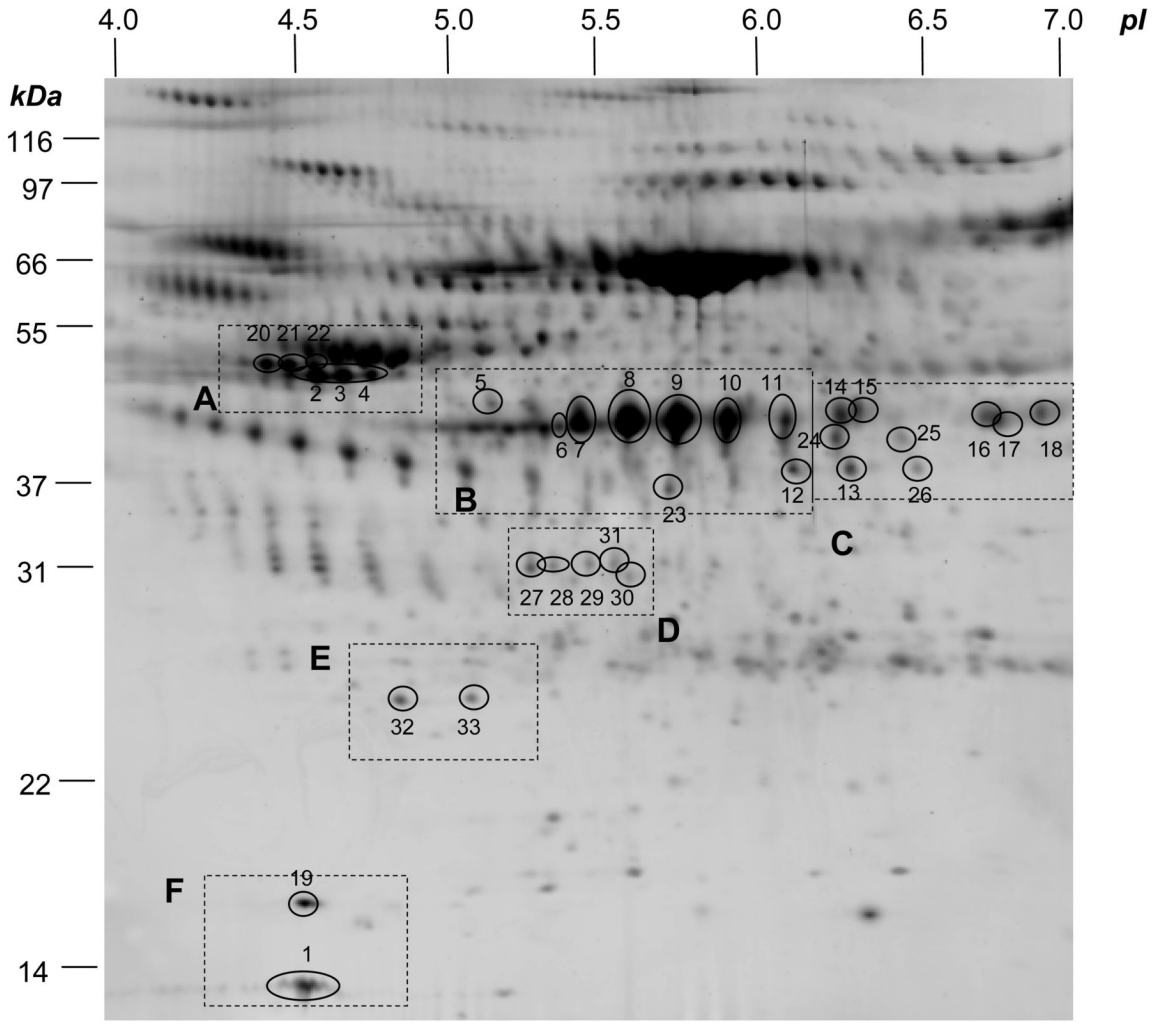

Figure 2.

Representative 2D gel of BAL fluid (from OVA group). 75 μg of murine BAL fluid protein was separated on 17-cm IPG (pH 4-7), followed by 8-16% SDS-PAGE. Proteins were stained with Sypro-Ruby. All spots labeled were increased at least 4 fold from OVA sensitized mouse compared to the PBS treated control.

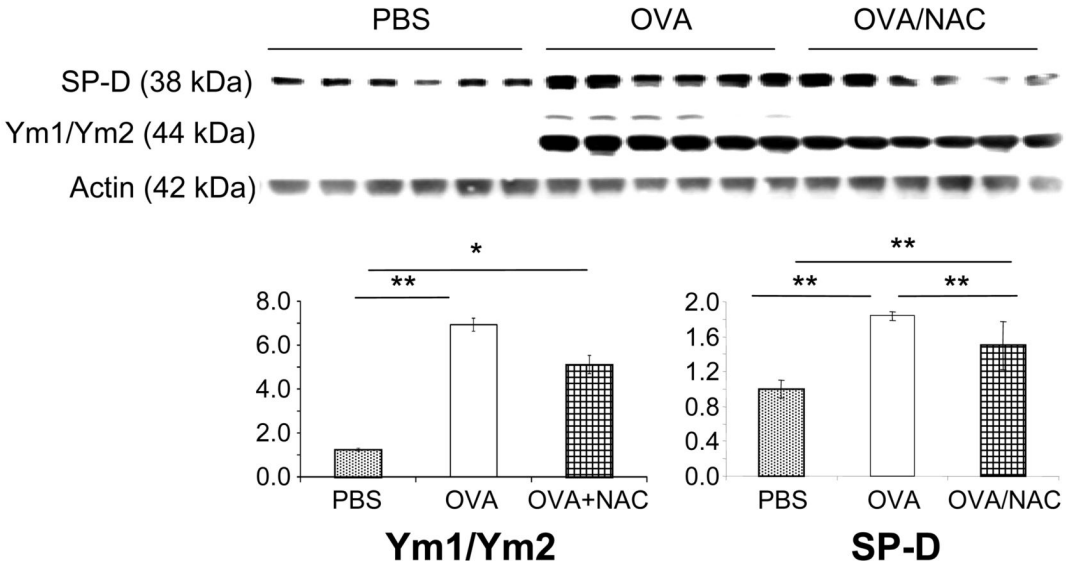

Figure 3.

Western blot analysis of SP,D and Ym1/Ym2 expression of BAL fluids from three different treated groups. 10 g BALF protein were separated by SDS,PAGE and probed with anti,SP,D and anti,Ym1/Ym2 antibodies. (* $p<0.05$, ** $p<0.01$)

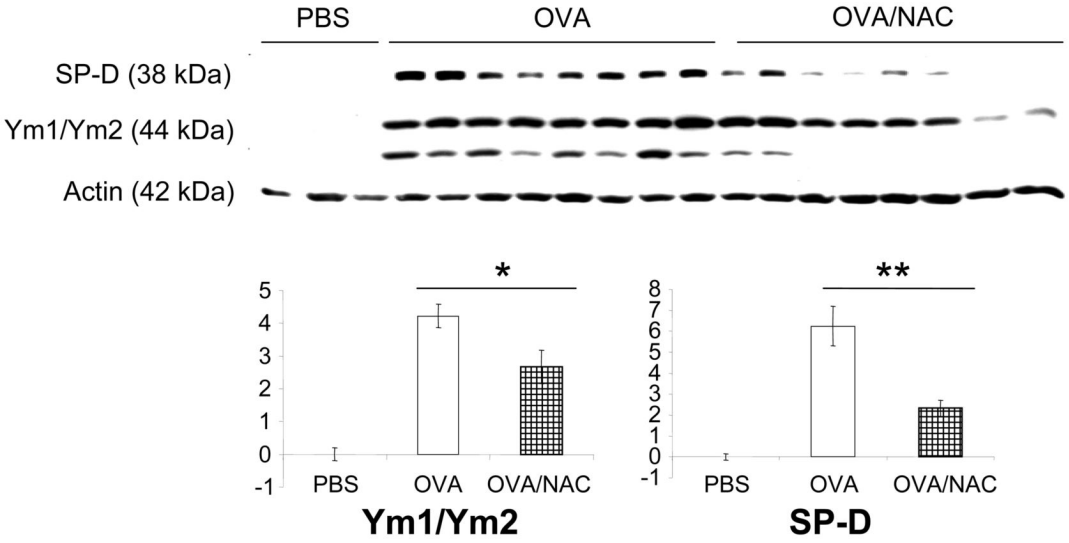

Figure 4.

Western blot analysis of SP,D and Ym1/Ym2 expression of lung from three different treated groups. 50 g lung protein were separated by SDS,PAGE and probed with anti,SP,D and anti,Ym1/Ym2 antibodies.

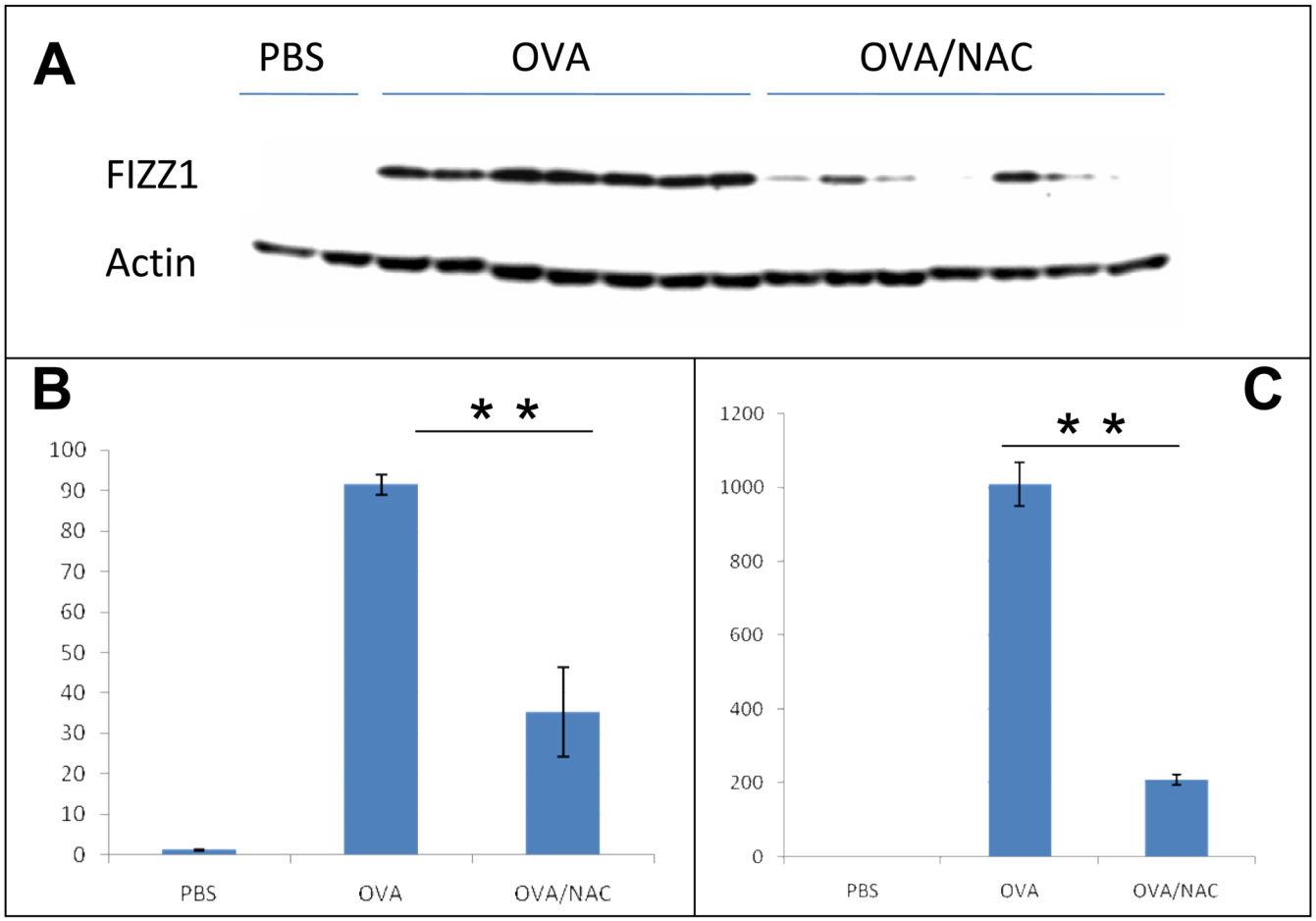

Figure 5.

(A, B) Western blot analysis of lung FIZZ1 expression from three different treated groups. 50 Ng total protein from lung tissue were separated by 10,20% SDS,PAGE and probed with anti,FIZZ1 antibodies. **p<0.01 (C) Real,time PCR analysis of FIZZ1 mRNA expression levels. Total RNA was isolated from pooled lung tissue. FIZZ1 mRNA was quantified using real,time polymerase chain reaction (PCR) based on SYBR Green I labeling (BioRad). **p<0.01

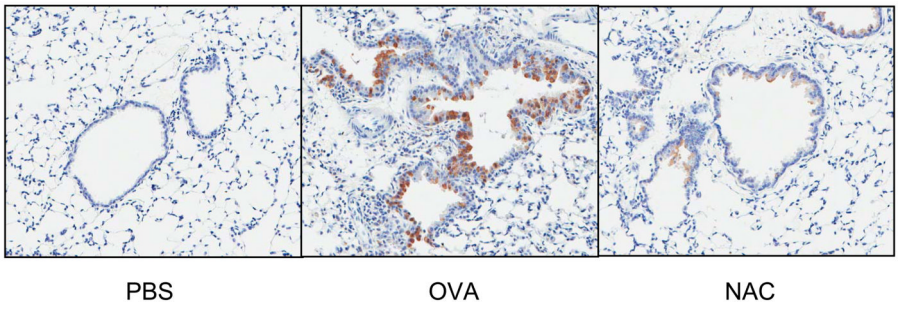

Figure 6.

Immunohistochemical staining of FIZZ1 protein in mouse lung tissue. Positive staining was found in macrophages and epithelial cells from OVA treat mice. OVA induced FIZZ1 protein expression was significantly inhibited by antioxidant NAC.

Table 1

Proteins with significant expression level changes in bronchoalveolar lavage fluid from mice with allergic airway inflammation.

Protein	Spot No.	Accession number[a]	MW (kDa)	Calculated pI	Sequence Coverage (%)	Score[b]	Peptides matched	NAC Suppressibility (%)[c]
Resistin-like molecule α (RELMα) (FIZZ1)	1	Q9EP95	11.9	5.18	20	70	2	24
Acidic mammalian chitinase (AMCase)	2	Q91XA9	52.0	4.91	20	421	12	53
Acidic mammalian chitinase (AMCase)	3	Q91XA9	52.0	4.91	20	421	12	40
Acidic mammalian chitinase (AMCase)	4	Q91XA9	52.0	4.91	17	364	9	28
Chitinase 3-like protein 3 (Ym1)	5	O35744	44.4	5.42	17	197	8	22
Chitinase 3-like protein 3 (Ym1)	6	O35744	44.4	5.42	28	441	11	31
Chitinase 3-like protein 3 (Ym1)	7	O35744	44.4	5.42	29	486	12	43
Chitinase 3-like protein 3 (Ym1)	8	O35744	44.4	5.42	43	683	18	10
Chitinase 3-like protein 3 (Ym1)	9	O35744	44.4	5.42	48	834	23	13
Chitinase 3-like protein 3 (Ym1)	10	O35744	44.4	5.42	44	768	22	20
Chitinase 3-like protein 3 (Ym1)	11	O35744	44.4	5.42	33	435	13	90
Chitinase 3-like protein 4 (Ym2)	12	Q91Z98	45.0	5.80	27	458	11	41
Chitinase 3-like protein 4 (Ym2)	13	Q91Z98	45.0	5.80	18	325	7	36
Pulmonary surfactant-associated protein D (SP-D)	14	P50404	37.7	7.55	18	233	7	26
Pulmonary surfactant-associated protein D (SP-D)	15	P50404	37.7	7.55	15	230	6	22
Pulmonary surfactant-associated protein D (SP-D)	16	P50404	37.7	7.55	10	167	3	8
Pulmonary surfactant-associated protein D (SP-D)	17	P50404	37.7	7.55	20	193	7	8
Pulmonary surfactant-associated protein D (SP-D)	18	P50404	37.7	7.55	8	120	3	0
Haptoglobin	19	Q61646	38.7	5.88	7	91	2	77
Acidic mammalian chitinase (AMCase)	20	Q91XA9	52.0	4.91	17	358	8	41
Acidic mammalian chitinase (AMCase)	21	Q91XA9	52.0	4.91	17	350	9	33
Acidic mammalian chitinase (AMCase)	22	Q91XA9	52.0	4.91	10	227	5	31
Complement C3 (HSE-MSF)	23	P01027	186.3	6.39	4	285	8	22
Chitinase 3-like protein 3 (Ym1)	24	O35744	44.4	5.42	15	309	8	32
Chitinase 3-like protein 3 (Ym1)	25	O35744	44.4	5.42	8	172	3	30
Chitinase 3-like protein 4 (Ym2)	26	Q91Z98	44.9	5.80	4	69	2	7
Chitinase 3-like protein 3 (Ym1)	27	O35744	44.4	5.42	11	223	4	4
Chitinase 3-like protein 3 (Ym1)	28	O35744	44.4	5.42	5	69	2	17
Chitinase 3-like protein 3 (Ym1)	29	O35744	44.4	5.42	3	73	1	19
Chitinase 3-like protein 3 (Ym1)	30	O35744	44.4	5.42	6	94	2	10
Chitinase 3-like protein 3 (Ym1)	31	O35744	44.4	5.42	9	157	3	33
Acidic mammalian chitinase (AMCase)	32	Q91XA9	52.0	4.91	3	43	2	22
Acidic mammalian chitinase (AMCase)	33	Q91XA9	52.0	4.91	1	52	1	11

[a] NCBI protein Accession number

[b] Mowse scoring algorithm

[c] (1- average NAC spot intensity/average OVA spot intensity) × 100

Table 2

Proteins with significant expression level changes from lung tissue of OVA-challenged mice

Protein	Spot No.	Accession number[a]	MW (kDa)	Calculated pI	Sequence Coverage (%)	Score[b]	Peptides matched	NAC suppressibility (%)
Myosin light chain 1, atrial/fetal isoform (MLC1A) (MLC1EMB)	1	P09541	21.0	4.96	37	248	7	38
Annexin A4 (Annexin IV)	2	P97429	35.8	5.43	23	238	8	30
Proteasome subunit alpha type 1 (Proteasome component C2)	3	Q9R1P4	29.5	6.00	29	244	9	39
Eukaryotic translation initiation factor 3 subunit 2 (elF-3 beta)	4	Q9QZD9	36.4	5.38	29	321	10	12
Eukaryotic translation initiation factor 3 subunit 2 (elF-3 beta)	5	Q9QZD9	36.4	5.38	29	402	10	44
Chitinase 3-like protein 3 (Ym1)	6	O35744	44.4	5.42	40	668	21	44
Acidic mammalian chitinase (AMCase)	7	Q91XA9	52.0	4.91	1	52	1	80
Resistin-like molecule α (RELMα) (FIZZ1)	8	Q9EP95	11.9	5.18	22	155	3	0
Isocitrate dehydrogenase [NAD] subunit alpha	9	Q9D6R2	39.6	6.27	20	359	8	53
Isocitrate dehydrogenase [NAD] subunit alpha	10	Q9D6R2	39.6	6.27	13	208	5	57
Chitinase 3-like protein 4 precursor (Ym2)	11	Q91Z98	44.9	5.80	9	206	4	53
Chitinase 3- like protein 3 (Ym1)	11	O35744	44.4	5.42	7	130	4	53

C(1- average NAC spot intensity/average OVA spot intensity) × 100

[a] NCBI protein Accession number

[b] Mowse scoring algorithm