Biomarker Insights

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ORIGINAL RESEARCH

Biomarkers of Disease and Treatment in Murine and Cynomolgus Models of Chronic Asthma

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Abstract

Background: Biomarkers facilitate early detection of disease and measurement of therapeutic efficacy, both at clinical and experimental levels. Recent advances in analytics and disease models allow comprehensive screening for biomarkers in complex diseases, such as asthma, that was previously not feasible.

Objective: Using murine and nonhuman primate (NHP) models of asthma, identify biomarkers associated with early and chronic stages of asthma and responses to steroid treatment.

Methods: The total protein content from thymic stromal lymphopoietin transgenic (TSLP Tg) mouse BAL fluid was ascertained by shotgun proteomics analysis. A subset of these potential markers was further analyzed in BAL fluid, BAL cell mRNA, and lung tissue mRNA during the stages of asthma and following corticosteroid treatment. Validation was conducted in murine and NHP models of allergic asthma.

Results: Over 40 proteins were increased in the BAL fluid of TSLP Tg mice that were also detected by qRT-PCR in lung tissue and BAL cells, as well as in OVA-sensitive mice and house dust mite-sensitive NHP. Previously undescribed as asthma biomarkers, KLK1, Reg3γ, ITLN2, and LTF were modulated in asthmatic mice, and *Clca3, Chi3l4* (YM2), and *Ear11* were the first lung biomarkers to increase during disease and the last biomarkers to decline in response to therapy. In contrast, GP-39, LCN2, sICAM-1, YM1, *Epx, Mmp12*, and *Klk1* were good indicators of early therapeutic intervention. In NHP, AMCase, sICAM-1, CLCA1, and GP-39 were reduced upon treatment with corticosteroids.

Conclusions and clinical relevance: These results significantly advance our understanding of the biomarkers present in various tissue compartments in animal models of asthma, including those induced early during asthma and modulated with therapeutic intervention, and show that BAL cells (or their surrogate, induced sputum cells) are a viable choice for biomarker examination.

Keywords: biomarkers, asthma, proximal fluid/tissue, corticosteroids, asthma treatment, nonhuman primate, murine

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Introduction

Asthma is a chronic disease of the airways that leads to eosinophilia, lung remodeling, excess mucus secretion, and bronchoconstriction to non-specific spasmogens.[1] A hallmark of the disease is the activation of Th2 cells that produce IL-4, IL-5, and IL-13, which in turn contribute to the development of the characteristic symptoms of asthma. Asthma affects over 130 million people worldwide, and as such, many pharmaceutical companies are attempting to develop efficacious therapeutics for the disease.

A "biomarker" is an objectively measured indicator that reflects the presence, progression, or successful treatment of a particular condition. Biomarkers have long been used in drug development, and the discovery and validation of new efficacy biomarkers is expected to reduce the time and cost associated with drug development and therefore increase the success rate of translating experimental drugs into clinical therapeutics.[2] They are valuable both in non-clinical and clinical studies for improving predictive disease models, monitoring potential drug candidate efficacy, and detecting early pathology and changes in disease status.

The collection of proteins expressed during a disease (ie, the disease proteome) is particularly useful for the discovery of disease and treatment biomarkers. Although plasma analysis is common due to the ease of accessibility, biological fluids or tissues from the local site of pathology, known as "proximal fluids," often represent a more accurate state of the condition. In this study, we examined proximal fluids and tissues from murine and nonhuman primate (NHP) models of chronic asthma to identify and qualify predictive markers of asthma progression and treatment efficacy. Thymic stromal lymphopoietin Transgenic (TSLP Tg) mice express TSLP under the lung-specific surfactant protein C promoter and thereby develop the hallmarks of human asthma over the course of 12 weeks.[3] BAL fluid, lung tissue mRNA, or BAL cell mRNA from TSLP Tg mice was used to identify biomarkers of chronic and developing asthma. Dexamethasone was administered to TSLP Tg mice with chronic disease to characterize which biomarkers are modulated with treatment. Finally, asthma disease and treatment biomarkers were validated in a house dust mite allergen (HDMA)-sensitive cynomolgus macaque model of chronic allergic asthma before and after corticosteroid treatment. Taken together, the results comprise a comprehensive examination of biomarkers expressed in proximal fluids and tissues across multiple established animal models of asthma and at different stages of progression and treatment.

Methods

Mice and in vivo protocols

SPC-TSLP Tg mice, on the BALB/c background, were obtained from the laboratory of Steven Ziegler.[3] Non-transgenic littermates served as control mice; male or female cohorts were used in individual studies and were always age- and sex-matched. BALB/c mice were purchased from the Jackson Laboratory for ovalbumin (OVA)-induced asthma experiments, as described below. In dexamethasone-treatment experiments, 12 week-old TSLP Tg mice were injected i.p. daily for 1 week with 2 mg/kg dexamethasone 21-phosphate disodium salt (Sigma) in sterile PBS, or with sterile PBS alone as a control. Colonies were maintained in a specific pathogenic-free environment. Handling of mice and experimental procedures were conducted in accordance with the guidelines of the AAALAC-accredited Schering-Plough Biopharma Animal Care and Use Committee.

Standard model of OVA-induced asthma in mice

For the standard model of OVA-induced asthma, mice were sensitized i.p. with 50 ug of ovalbumin from chicken egg white (Sigma-Aldrich) complexed to 2 mg of Imject Alum (Pierce) in 0.15 M saline (Sigma-Aldrich) on day 0 and day 14, and primed 2 times on Day 27 and 28 with saline or nebulized OVA (10 mg/mL) for 45 min per session. Mice were given a final challenge of saline or OVA (25 mg/mL) on day 29, and tissues were harvested 1 day following OVA challenge.

Harvest of murine BAL fluid, BAL cells, and lung tissue

BAL fluid was isolated by washing the lung (through the trachea) with 1 mL of PBS. Lavage fluid was kept on ice and centrifuged at 400 g for 5 min. The supernatant was frozen for cytokine analysis, and the cell pellet was resuspended in 1 mL of PBS for total viable cell count by Vicell (Perkin-Elmer) and cell

differentials by cytospin. Slides were air-dried, fixed with 95% ethanol, and stained with Wright-Giemsa (Sigma-Aldrich). A minimum of 200 cells were counted under the microscope per slide for cell differentials. The postcaval lung lobe and pooled BAL cells were collected and snap-frozen in liquid nitrogen for qRT-PCR analysis as described previously for tissue[4] and below for BAL cells. The single left lung lobe was excised for histology and clinical scoring as described below.

Pulmonary function (Plethysmography) of dexamethasone-treated mice

Murine pulmonary responses to the non-specific bronchoconstrictor methacholine chloride were measured using Whole Body Plethysmography (WBP) (Buxco Electronics). Unrestrained mice were placed in individual chambers and exposed to nebulized methacholine (5 mg/mL) for 1 minute, and responses were recorded for the following 3 minutes. Penh was calculated to quantify lung function.

Murine lung histology and clinical scoring

Murine lungs were perfused with 10 mL of PBS via the right ventricle of the heart. The single left lung lobe was excised, fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Lung tissue was scored for hypertrophy of the airway epithelium and peribronchiolar/perivascular cellular inflammation on a scale of 0–5 by a board-certified pathologist.

mRNA isolation from BAL cells and qRT-PCR

Total RNA was isolated from BAL cells using the RNeasy method (Qiagen) and reverse-transcribed using WT-Ovation™ Pico System (NuGen Technologies). Primers were designed using Primer Express software (Applied Biosystems) or obtained commercially from Applied Biosystems (ABI). qRT-PCR was performed on 10 ng of cDNA from each sample as described previously.[4]

LC-MS/MS analysis of BAL fluid proteins

An equal volume of murine or cynomolgus BAL fluid was separated on a preparative 4%–12% NuPage gel and stained with GelCode Coomassie Blue (Pierce). Each lane was sliced into an equal number of bands and digested with sequencing-grade modified trypsin using a Progest (Genomic Solutions). Mass spectrometry was performed as described below. LC-MS/MS raw files were searched using the Mascot v2.1.6 software package (Matrix Sciences) against the mouse subset of the National Center for Biotechnology Information (NCBI) non-redundant protein database (updated as of August 2006) for murine BAL fluid and against the entire database (including all species, updated as of December 2007) for monkey BAL fluid. Categorization of proteins by function was performed using Ingenuity Pathway Analysis (Ingenuity Systems). LC-MS/MS analyses were performed on BAL fluid from 5, 7, 9, and 12 week old TSLP Tg mice with overlapping results; results presented are from 9-week old mice.

Mass spectrometry

Mass spectrometry was performed using a LCQ Deca Ion Trap (ThermoElectron), a 48-well Paradigm AS1 autosampler (Michrom Bioresources), and a Paradigm MS4 HPLC system (Michrom Bioresources). The column was packed with Vydac C18 resin (5 micron beads, 300Å pores), 10 cm long with a 15 micron tip (New Objectives). The chromatographic separation was performed using a linear gradient elution. Search parameters included no restriction on molecular weight or pI, fixed modification of cysteine residues (carbamidomethylation), variable modification of methionine residues (oxidation), a peptide mass tolerance of +/– 1.5 Daltons, a fragment mass tolerance of +/– 0.8 Daltons, and one missed tryptic cleavage. Protein identification was based on at least two matching peptides. Protein hits with only one matching peptide were reviewed manually and included as positive identifications when a stretch of at least 4 b or y ions was present.

Western blot analysis of BAL fluid samples

Western blot analysis was performed on BAL fluid samples. 200 uL of each BAL fluid sample was acetone precipitated in 4 volumes of cold acetone overnight and resuspended in 1X NuPage LDS sample loading buffer. An equal amount was separated on a 4%–12% NuPage gels, and gels were electroblotted onto PVDF membranes overnight at 10V in NuPage transfer buffer. Membranes were blocked in 5% fat-free milk in TBS/T (10 mM Tris-HCl pH 7.5, 100 mM

NaCl, 0.1% Tween 20) for 1 hour and incubated with specific antibodies according to the manufacturer's instructions in 1% milk in TBS/T for another 2 hours. Blots were incubated with HRP-labeled secondary antibodies (chicken anti-goat IgG, R&D Systems, Minneapolis, MN or donkey anti-rabbit Ig-G, GE Healthcare, Piscataway, NJ) in 1% milk in TBS/T for another hour and detection was performed with ECL+ (GE Healthcare). Primary antibodies used included anti-mouse LCN2, anti-mouse sICAM-1, anti-human sICAM-1 (for monkey studies), anti-mouse GP-39, anti-mouse YM1, and anti-mouse pIgR (all from R&D Systems); and anti-mouse UG, anti-mouse AMCase, anti-human AMCase (for monkey studies), anti-mouse CLCA3, and anti-human CLCA1 (for monkey studies) (all from Santa Cruz Biotechnology). Polyclonal anti-mouse Reg3γ was generated at Schering-Plough Biopharma. After autoradiography visualization, membranes were dried and scanned on a Typhoon 9400 (GE Healthcare) for ECL+ (457 nm excitation and 520BP40 emission). Image analysis was conducted using ImageQuant v5.2 (GE Healthcare) and band intensity reported as sum of pixel values above background.

ELISAs

Mouse LCN2, GP-39, sICAM-1, and YM1 proteins were quantified using Quantikine or DuoSet ELISA kits (R&D Systems) using a Vmax spectrophotometer with SoftMax Pro software (Molecular Devices); mouse IL-4, IL-5, and IL-13 were measured using a Luminex 100 machine with Lincoplex multiplex kits (Millipore) and analyzed with MasterPlex software (Miraibio). Monkey YKL-40 was quantified via ELISA (Quidel), analyzed as above.

House dust mite allergen (HDMA)-Induced asthma in nonhuman primates

Juvenile macaques (*Cynomolgus fascicularis*, 30 to 42 months of age) were purchased from Alphagenesis. Studies were performed as described.[5] Briefly, animals were sensitized to HDMA over a 7.5 month period by intraperitoneal injection of 312 AU *Dermatophagoides pteronyssinus* extract (Greer Laboratories) absorbed to Imject Alum (Pierce) administered every two weeks until HDMA-specific IgE titers approached levels in control allergic serum, and then at 4-week intervals until aeroallergen challenge. At this time, animals were challenged with nebulized HDMA (1 to 2500 AU/mL for 4 minutes) at a concentration that induced an early asthmatic response, defined as a 100% increase in lung resistance, 40% decrease in dynamic compliance, or decline in arterial oxygen saturation to ≤70%. Airway inflammation and reactions to nebulized histamine and methacholine 24 hours after allergen challenge were measured periodically to confirm chronic asthmatic responses. Wardle-Fick methods were used to obtain BAL fluid, described in further detail below. BAL cells were then separated from the fluid phase. Mass spectrometry compared BAL fluid from sensitized animals before and after HDMA challenge. In corticosteroid treatments experiments, animals were challenged with HDMA and BAL fluid was collected 24 hours later ("Pre" steroid). Animals then received weekly doses of methylprednisolone acetate (4.5 mg/kg intramuscularly) for two weeks, followed by a single dose of methylprednisolone succinate (10 mg/kg i.v.) one week later at the time of allergen challenge. BAL fluid was collected 24 hours following another HDMA challenge ("Post" steroid). Animal husbandry was conducted under USDA guidelines. All protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

Wardle-Fick methods to obtain BAL fluid from cynomolgus monkeys

Each animal received a premedication and anesthesia (*vide supra*), and Cetacaine spray (Cetylite Industries, Inc., Pennsauken, NJ) was administered locally to the hypopharynx surrounding the endotracheal tube. A 2.5 mm or 2.7 mm fiberoptic bronchoscope (Five Star Medical or Pentax FB-8V, respectively) was inserted via the endotracheal tube with the assistance of lubrication. The bronchoscope was wedged in the posterior division of the right upper lobe segment, and 5–10 mL aliquots of sterile 0.9% saline, totaling 40 mL, were sequentially administered and withdrawn with minimal dwell time through the suction channel (gentle aspiration, optimally 140–150 mm Hg).

Statistical analyses

The unpaired or paired two-tailed t test was performed using GraphPad Prism version 4.02 (GraphPad Software) to determine average ± standard error

of the mean. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Results

Identification and validation of protein biomarkers in TSLP Tg mice

Biomarkers are valuable in the diagnosis of disease, as well as in determining the efficacy of a therapeutic against the disease, thereby increasing the success rate of translating experimental drugs into clinical therapeutics. To determine which biomarkers are modulated in the lung during asthmatic responses, TSLP Transgenic (Tg) mice were used as a murine model of asthma. These mice express thymic stromal lymphopoietin (TSLP) under the lung-specific surfactant protein C promoter and begin to develop the pathophysiological characteristics of asthma at 5 weeks of age. By 9 weeks of age, all the hallmarks of chronic human asthma, including pulmonary eosinophilia, production of Th2 cytokines, airway fibrosis, and hyperplasia of airway epithelium, are present (See Figure S1 in the Supplemental Information).[3] Bronchoalveolar lavage (BAL) was performed on the lungs of 9-week old control or TSLP Tg mice, the cellular fraction of the BAL was removed, and the BAL fluid phase was analyzed by mass spectrometry. Approximately 150 proteins were identified, of which forty-four were found to be upregulated in the BAL fluid, compared to non-Tg wild-type littermate controls (see Table S1 in the Supplemental Information). Identified proteins could generally be divided up into functional groups (Fig. 1). The majority of classified proteins identified in the BAL fluid were considered enzymes (30%), whereas a smaller proportion of proteins were classified as transporters (10%), peptidases (6%), cytokines (2%), or kinases (2%). Proteins with uncharacterized or unclassified functions that did not fall into a specific functional group were termed "other."

As mass spectrometry is semi-quantitative, a subset of 18 proteins was chosen for further analysis via western blot, ELISA, and quantitative reverse transcriptase-PCR (qRT-PCR). Selected proteins were chosen based upon availability of reagents and their previous association or lack of association with pulmonary diseases (see Discussion for further information). Proteins were verified using western blot or ELISA, if reagents were

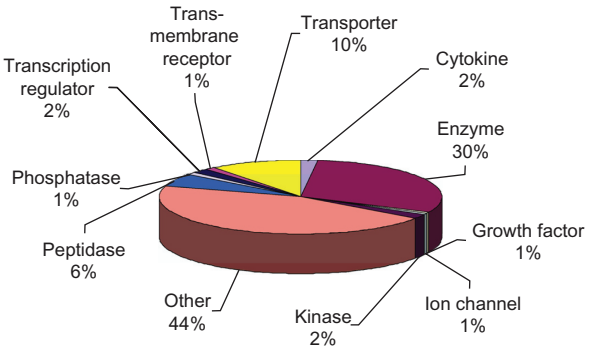


Figure 1. Functional groups of proteins identified in the BAL fluid of TSLP Tg mice and WT littermates by mass spectrometry.
Notes: Mass spectrometry was used to identify proteins present in the BAL fluid of 9-week old control and asthmatic TSLP Tg mice. Using Ingenuity Pathway Analysis, proteins were categorized by known function into enzymes (30%), transporters (10%), peptidases (6%), transcription regulators (2%), cytokines (2%), kinases (2%), phosphatases (1%), growth factors (1%), transmembrane receptors (1%), or ion channels (1%). Proteins that did not fall into a specific functional group were termed "other" (44%) and consisted of a variety of proteins with uncharacterized or unclassified functions. See Table S1 in the Supplemental Information for the list of identified proteins.

available. Acidic mammalian chitinase (AMCase; gene name *Chia*), YM1 (YM1; *Chi3l3*), chloride channel regulator 3 (CLCA3; *Clca3*), the polymeric immunoglobulin receptor (pIgR; *Pigr*), regenerating islet-derived 3 gamma (Reg3γ; *Reg3g*); lipocalin 2 (LCN2; *Lcn2*), and intercellular adhesion molecule 1 (ICAM-1; *Icam1*) were increased in western blots of BAL fluid; some proteins, such as AMCase or CLCA3, appeared as doublet bands that were further intensified in TSLP Tg mice (Fig. 2A). LCN2, cartilage glycoprotein 39 (GP-39; *Chi3l1*), and sICAM-1 were all highly upregulated as measured by ELISA (Fig. 2C).

qRT-PCR validation of biomarkers in murine lung tissue

The expression of 18 putative biomarkers was examined in the lung tissue of age- and sex-matched control versus TSLP Tg mice using qRT-PCR. Interestingly, qRT-PCR analysis of TSLP Tg mouse lung tissue for these 18 genes showed a large range of relative expression and fold change that could generally be divided into "High" (>6-fold increase) and "Intermediate" (1.8- to 6-fold change) expression groups (Table 1). Showing the highest upregulation at the mRNA level (fold-change > 100) were YM2 (YM2; *Chi3l4*), eosinophil cationic protein (ECP; *Ear11*), resistin-like beta (RETNLβ; *Retnlb*), and *Clca3*; also falling into the "High" category were eosinophil major basic

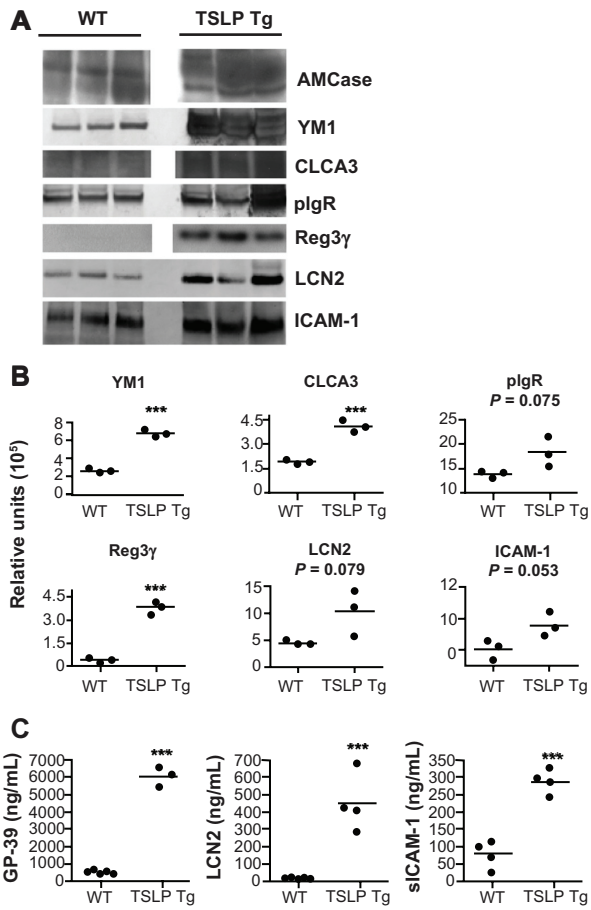


Figure 2. Verification of putative asthma biomarkers in TSLP Tg mice. BAL fluid and lung tissue were collected from control WT and asthmatic TSLP Tg mice for further biomarker evaluation. Western blot analysis was performed on the BAL fluid of WT or TSLP Tg mice for assessment of AMCase, YM1, CLCA3, pIgR, Reg3γ, LCN2, and ICAM-1 (**A**). Band intensity was determined using ImageQuant v5.2 and reported as the sum of the pixel values above background (**B**). ELISAs were used to quantify the amount of GP-39, LCN2, and ICAM-1 in BAL fluid (**C**).
Notes: Results are representative of 3–5 independent experiments. ***$P < 0.001$.

protein (EMBP; *Prg2*), eosinophil peroxidase (EPX; *Epx*), matrix metallopeptidase 12 (MMP12; *Mmp12*), *Chia, Chi3l3*, the Fc fragment of IgG binding protein (FCGBP; *Fcgbp*), and regenerating islet-derived 3 gamma (Reg3γ; *Reg3g*), whereas *Ltf, Pglyrp1, Pigr,* kallikrein 1 (KLK1; *Klk1*), *Lcn2*, and *Chi3l1* displayed "Intermediate" upregulation. It was surprising that *Chi3l1* (GP-39) and *Lcn2* (LCN2) were only 1.85- and 2.22-fold upregulated at the mRNA level, as average protein concentrations of GP-39 and LCN2 were 11- and 30-fold increased, respectively, in TSLP Tg mouse BAL fluid (Fig. 2C). Interestingly, although sICAM-1 was highly upregulated at the protein level via ELISA (Fig. 2C), *Icam1* was not modulated at the mRNA level (*data not shown*). These results were similar regardless of the sex of the control and TSLP Tg mice. Taken together, these results show that the 18 selected proteins all function as biomarkers of murine chronic asthma. They also indicate that qRT-PCR analysis of lung tissue mRNA is effective in assessing certain biomarkers such as *Chi3l4, Ear11, Retnlb,* and *Clca3*, but that protein assays may be more valuable in ascertaining differences in proteins such as GP-39, LCN2, or ICAM-1 that exhibit more discernable separations at the protein rather than mRNA level.

qRT-PCR validation of biomarkers in BAL cells

Lung tissue is difficult to procure from patients for biomarker analyses. However, induced sputum is easily obtained from individuals due to its relative noninvasiveness. It is also clinically relevant, as an ample number of cells can be acquired in this manner, so investigation of sputum cells may prove fruitful for effective biomarker analysis. In the mouse, BAL cells are considered analogous to human sputum cells, so we next analyzed the expression of biomarkers in BAL cells from TSLP Tg mice. In addition to the 18 biomarkers examined in murine lung tissue, we also examined 4 putative biomarkers derived from proteins found by mass spectrometry of TSLP Tg mouse BAL fluid but not highly upregulated in lung tissue by qRT-PCR: *Scgb1a1* (Secretoglobin family 1A member 1, ie, uteroglobin (UG) or Clara Cell-specific 10 kD protein (CC-10)), *Egfr* (Epidermal growth factor receptor, EGFR), *Itln2* (Intelectin-2, ITLN2), and *Ctsh* (Cathepsin H, CTSH). As shown in Table 2, qRT-PCR expression analysis of the 22 biomarkers gave a much different expression profile in BAL cells than in lung tissue from TSLP Tg mice. Eosinophil activation genes *Prg2* (EMBP), *Epx,* and *Ear11* (ECP) were all highly upregulated in BAL cells, not surprising as eosinophils constitute over 80% of the BAL cells of TSLP Tg mice. The 4 biomarkers with the highest upregulation in lung tissue—*Chi13l4* (YM2), *Clca3, Ear11* (ECP), and *Retnlb*—were also over 100-fold upregulated in BAL cell mRNA. *Mmp12, Fcgbp,* and *Reg3g* also remained in the "High" fold-change category. *Klk1*, present in the "Intermediate" lung biomarker group (Table 1), was highly expressed in BAL cells (Table 2). Conversely, *Chi3l3* (YM1), which was in the "High" lung biomarker category,

Table 1. qRT-PCR analysis of selected biomarker genes in 9-week old WT or TSLP Tg mouse lung tissue.

Gene name	WT*	TSLP Tg*	Fold change
High			
Chi3l4 (YM2)	2.39 ± 1.13	1453.11 ± 511.55	608.31
Ear11	12.19 ± 3.31	3860.82 ± 1173.10	316.71
Retnlb	0.32 ± 0.14	68.08 ± 34.40	212.57
Clca3	16.04 ± 8.13	1172.93 ± 335.42	152.85
Prg2 (EMBP)	19.09 ± 5.69	734.1 ± 181	38.45
Epx	6.25 ± 3.59	233.2 ± 64.89	37.31
Mmp12	193.27 ± 14.53	5182.02 ± 1774.17	26.81
Chia (AMCase)	395.56 ± 23.63	7358.75 ± 1636.71	18.6
Chi3l3 (YM1)	478.25 ± 39.94	8639.59 ± 437.79	18.06
Fcgbp	45.13 ± 0.95	644.46 ± 67.23	14.28
Reg3g	55.92 ± 16.52	743.43 ± 335.41	13.29
Intermediate			
Ltf	229.35 ± 62.47	1309.18 ± 297.88	5.71
Pglyrp1	58.59 ± 2.65	333.6 ± 28.62	5.66
Pigr	137.32 ± 21.13	445.02 ± 42.49	3.24
Klk1	32.80 ± 5.10	85.77 ± 12.95	2.61
Lcn2	1439.38 ± 161.21	3195.21 ± 203.18	2.22
Chi3l1 (GP-39)	2862.49 ± 195.24	5300.17 ± 442.57	1.85

Notes: *Relative expression units, normalized to ubiquitin, and the SEM. Expression units are representative of 3–6 mice per group. Results presented are representative of 5 independent experiments.

was only intermediately expressed in BAL cell mRNA. *Scgb1a1* (UG) exhibited high expression levels in WT mice, which was further increased 8.55-fold in TSLP Tg mice. The majority of the BAL cell biomarkers exhibited an intermediate level of expression. Although *Chia* (AMCase) was in the "High" lung expression group, the gene was not expressed in BAL cell mRNA.

As BAL cells are a representation of the cellular constituents of the asthmatic lung, we reasoned that further examination of the chemokine and chemokine receptor genes present in the BAL cells of TSLP Tg mice would help us identify a "cellular signature" that could prove valuable in characterizing the type of cellular inflammation present in the lung and thus the best course of treatment. Towards this goal, we examined chemokine and chemokine receptor genes that are known to participate in asthmatic responses. qRT-PCR expression analysis of BAL cell mRNA from WT and TSLP Tg mice revealed that *Ccr3* was over a thousand-fold upregulated in the asthmatic TSLP Tg mice (Table 2), depicting the large eosinophil population in the BAL. Although not increased as much as *Ccr3*, chemokine receptors *Ccr4* and *Ccr6* were also over 10-fold upregulated. Consistent with the expression of their receptors, chemokines *Ccl5, Ccl8, Ccl11, Ccl22*, and *Ccl24* were also highly upregulated (between 7- and several hundred-fold). In contrast, *Cxcr1* and *Cxcr2*, receptors for neutrophil-attracting chemokines, were not increased (*data not shown*). These results demonstrate that the chemokine and chemokine receptor profile of BAL cells accurately represents the cellular composition of an asthmatic lung.

Biomarker expression in OVA sensitized and challenged mice

As TSLP Tg mice have not previously been used as a model of chronic asthma to identify disease biomarkers, we verified our biomarker findings using the more established ovalbumin (OVA)-induced asthma model (Table 3, and Table S2 in the Supplemental Information), demonstrating that the chosen set of lung tissue asthma biomarkers is similarly upregulated in two different murine models of asthma.

Lung biomarker induction in early asthma

A criticism of using OVA-induced murine models of asthma is the difficulty in delineating biomarkers of developing versus chronic asthma. However, as TSLP Tg mice begin to develop asthma at 5 weeks of age and progress to a state of chronic asthma over

Table 2. qRT-PCR analysis of selected biomarker genes in BAL cells of 9-week old WT or TSLP Tg mice.

Gene name	WT*	TSLP Tg*	Fold change
High			
Prg2 (EMBP)	0.06	693.53	12002.03
Epx	0.01	158.61	10813.69
Chi3l4 (YM2)	4.42	5363.04	1212.53
Clca3	20.39	21345.26	1046.82
Retnlb	0.14	47.54	336.66
Klk1	0.20	40.09	203.08
Ear11	70.06	9318.96	133.00
Mmp12	212.80	8432.78	39.63
Scgb1a1 (UG)	465.03	5788.91	12.45
Fcgbp	259.87	2468.60	9.50
Reg3g	136.93	1046.53	7.64
Intermediate			
Itln2	83.58	352.78	4.22
Pglyrp1	1293.16	5300.36	4.10
Egfr	10.62	39.39	3.71
Pigr	1560.77	5523.00	3.54
Chi3l3 (YM1)	27002.91	95553.46	3.54
Chi3l1 (GP-39)	0.69	1.95	2.81
Ctsh	1665.02	3950.66	2.37
Lcn2	1198.03	2425.46	2.02
Ltf	591.75	1065.15	1.80
Chemokine genes			
Ccl5 (RANTES)	193.70	1363.41	7.04
Ccl8 (MCP-2)	0.34	231.05	689.65
Ccl11 (Eotaxin-1)	0.06	9.72	164.35
Ccl22 (MDC)	6.33	63.40	10.02
Ccl24 (Eotaxin-2)	0.05	28.51	609.57
Ccr3 (CD193)	0.05	175.28	3748.13
Ccr4 (CD194)	1.16	13.28	11.47
Ccr6 (CD196)	6.56	79.27	12.09

Notes: *Relative expression units, normalized to ubiquitin. qRT-PCR results presented are derived from RNA pooled from 3–6 mice per group. Results presented are representative of 5 independent experiments.

the subsequent 4 weeks, these mice can be used to examine our biomarker set during the development of asthma. At 5 weeks of age, TSLP Tg mice exhibit mucus production and minor cellular inflammation around airways and vasculature but lack the massive inflammation, tissue remodeling, airway hypertrophy, and eosinophilia present at 9 weeks. mRNA was isolated from the lung tissue of 5 week-old mice, and qRT-PCR was performed for the set of 18 lung biomarkers (see Table S3 in the Supplemental Information). BAL cell mRNA expression was not analyzed due to the scarcity of BAL cell infiltrates at this 5-week time point. Whereas the lung tissue of 9 week old mice had 11 biomarkers in the "High" category, only 5 biomarkers fell into the "High" category in 5 week old mice with developing asthma (Table 3): *Clca3, Chi3l4* (YM2), and *Ear11* (ECP) were over 100-fold increased, and the relative expression of *Retnlb* was much lower at 5 weeks (16-fold increased instead of 212-fold increased), whereas the upregulation of *Fcgbp* was similar in at both ages. These results indicate that CLCA3, YM2, ECP, RETNLβ, and FCGBP are expressed early in asthma pathogenesis and may be effective biomarkers of developing asthma.

Biomarkers modulated with dexamethasone treatment

Having identified a set of disease biomarkers in the BAL fluid, lung tissue mRNA, and BAL cell mRNA of asthmatic mice, we next sought to determine which biomarkers are reduced with treatment. As corticosteroid treatments have been proven highly effective as the standard of care for the disease, we administered systemic dexamethasone daily for one week to 12-week old TSLP Tg mice. Whereas 9-week old mice were used for biomarker analysis, 12-week old mice were used for dexamethasone treatment studies because their continued asthma pathogenesis, with extensive tissue remodeling and allergic inflammation, is a better analogy to severe chronic human asthma before treatment. When challenged with aerosolized saline, TSLP Tg mice exhibit worse basal lung function (Penh) than control mice, as measured by whole body plethysmography. They also have high Penh values when challenged with a very small dose (5 mg/mL) of methacholine chloride (Fig. 3A). Dexamethasone treatment of the TSLP Tg mice significantly improved both basal and challenged lung function. Correspondingly, dexamethasone treatment significantly reduced lung weight (Fig. 3B), lung pathology score (Fig. 3C), total number of BAL cells (Fig. 3D), and percentage of BAL eosinophils (Fig. 3E) in TSLP Tg mice. Although significantly decreased, these parameters were not absent with treatment, allowing us to discern which biomarkers are more quickly modulated with treatment.

Several biomarkers were downregulated in the BAL fluid of dexamethasone-treated TSLP Tg mice, including CLCA3, YM1, and pIgR (via western blot, Fig. 4A), as shown previously,[6] and GP-39, LCN2, sICAM-1, and YM1 (via ELISA, Fig. 4B). Interestingly, although *Chi3l4* (YM2), *Ear11, Retnlb,*

Table 3. Comparison of biomarker gene upregulation in 9-week old TSLP Tg mice, 5-week old TSLP Tg mice (with developing asthma), and OVA-challenged mice.

Gene name	Average fold change		
	TSLP Tg vs. WT (9 weeks)	TSLP Tg vs. WT (5 weeks)	OVA vs. saline
Chi3l4 (YM2)	**608.31**	**383.34**	**2377.87**
Ear11	**316.71**	**101.52**	**276.77**
Retnlb	**212.57**	**16.35**	**270.7**
Clca3	**152.85**	**427.13**	**526.22**
Prg2 (EMBP)	**38.45**	2.16*	**9.05**
Epx	**37.31**	1.09	**10.33**
Mmp12	**26.81**	3.04*	**37.67**
Chia (AMCase)	**18.6**	1.68*	**9.76**
Chi3l3 (YM1)	**18.06**	3.41*	**18.03**
Fcgbp	**14.28**	**11.09**	**13.33**
Reg3g	**13.29**	1.88*	2.83*
Ltf	5.71*	2.16*	−1.37
Pglyrp1	5.66*	1.6*	2.21*
Pigr	3.24*	2.32*	3.97*
Klk1	2.61*	1.39	4.11*
Lcn2	2.22*	1.16	2.81*
Chi3l1 (GP-39)	1.85*	1.03	2.17*

Notes: Bold-faced text indicates a value that falls into the "High" expression category (>6-fold upregulated); asterisks indicate a value that falls into the "Intermediate" expression category (1.5- to 6-fold increased). Average relative expression units and SEM for 9-week TSLP Tg, 5-week TSLP Tg, and OVA-treated mice are found in Tables 1, S3, and S2, respectively.

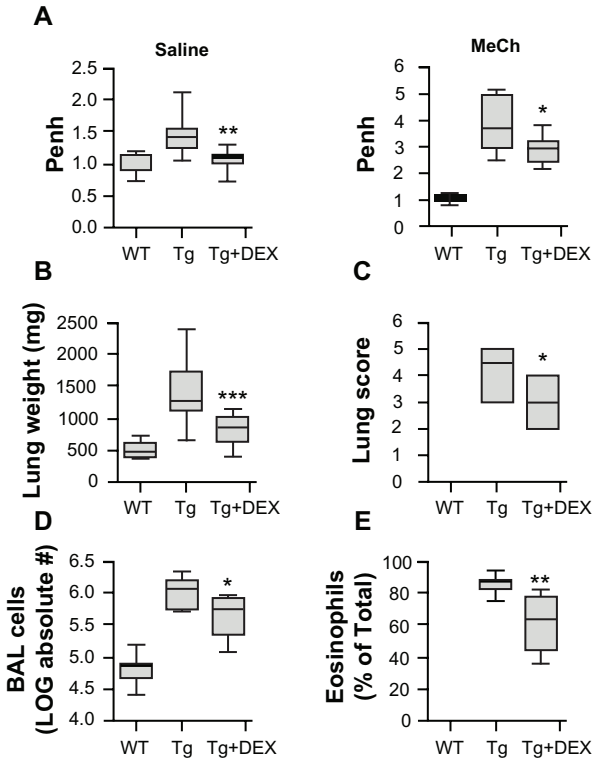


Figure 3. Reduction of asthma symptoms upon dexamethasone treatment in TSLP Tg mice. TSLP Tg mice were treated daily with 2 mg/kg dexamethasone (DEX) i.p. for 2 weeks. Lung function was measured in WT ("WT"), TSLP Tg ("Tg"), or DEX-treated TSLP Tg mice ("Tg+DEX") by whole body plethysmography upon saline or 5 mg/mL methacholine chloride challenge (**A**) Lung tissue was harvested and the weight of the superior lung lobe was measured (**B**) H&E stained lung tissue was scored for hypertrophy of the airway epithelium and peribronchiolar/perivascular cellular inflammation on a scale of 0–5 by a board-certified pathologist (**C**) The total number of BAL cells was determined using trypan blue exclusion criteria on a Vicell counter (**D**), and the proportion of eosinophils in the BAL was enumerated by Wright-Giemsa staining of cytospun cells (**E**) A minimum of 200 cells were counted.
Notes: Results presented are the combined data points of 3 independent experiments totaling 12 mice per group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

and *Clca3* showed the highest fold-upregulation of any of the biomarkers in lung tissue from TSLP Tg mice (Table 1), none of these were modulated with dexamethasone treatment at the mRNA level (data not shown). In contrast, several of the other biomarkers in the "High" lung biomarker category were reduced with treatment: *Prg2* (EMBP), *Epx, Mmp12, Chia* (AMCase), and *Chi3l3* (Ym1) were downregulated (Fig. 4C), although expression of *Fcgbp* and *Reg3g*—also in the "High" lung biomarker category—was unaffected (*data not shown*). In the "Intermediate" lung biomarker group, *Ltf, Pigr, Klk1*, and *Chi3l1* (GP-39) were all reduced with dexamethasone (Fig. 4C), whereas expression of *Pglyrp1* and *Lcn2* were not diminished at the mRNA level (*data not shown*), despite that LCN2 protein levels were significantly lowered (Fig. 4B).

In BAL cell mRNA from dexamethasone-treated mice, the biomarkers with the largest increase (*Prg2, Epx, Chi3l4, Clca3, Retnlb,* and *Klk1*) were all downmodulated with treatment (Fig. 4D). *Ear11* was not reduced, which was surprising as *Prg2* and *Epx,* the other two eosinophil activation genes, were reduced in both lung tissue and BAL cell mRNA. *Scgb1a1 (UG), Fcgbp, Itln2, Pglyrp1*, and *Ctsh* were also reduced in BAL cell mRNA upon treatment (Fig. 4D). Together, these results show that corticosteroid treatment quickly reduces the highest upregulated biomarkers in BAL cells but not lung tissue, indicating that BAL cells may be more useful for monitoring asthma treatment biomarkers.

As the percentage of eosinophils and total number of BAL cells were reduced in TSLP Tg mice treated with dexamethasone (Fig. 3D and E), we next examined chemokine and chemokine receptor genes in BAL cell mRNA, pooled per group. Th2 cell chemokine *Ccl22* and eosinophil chemok-

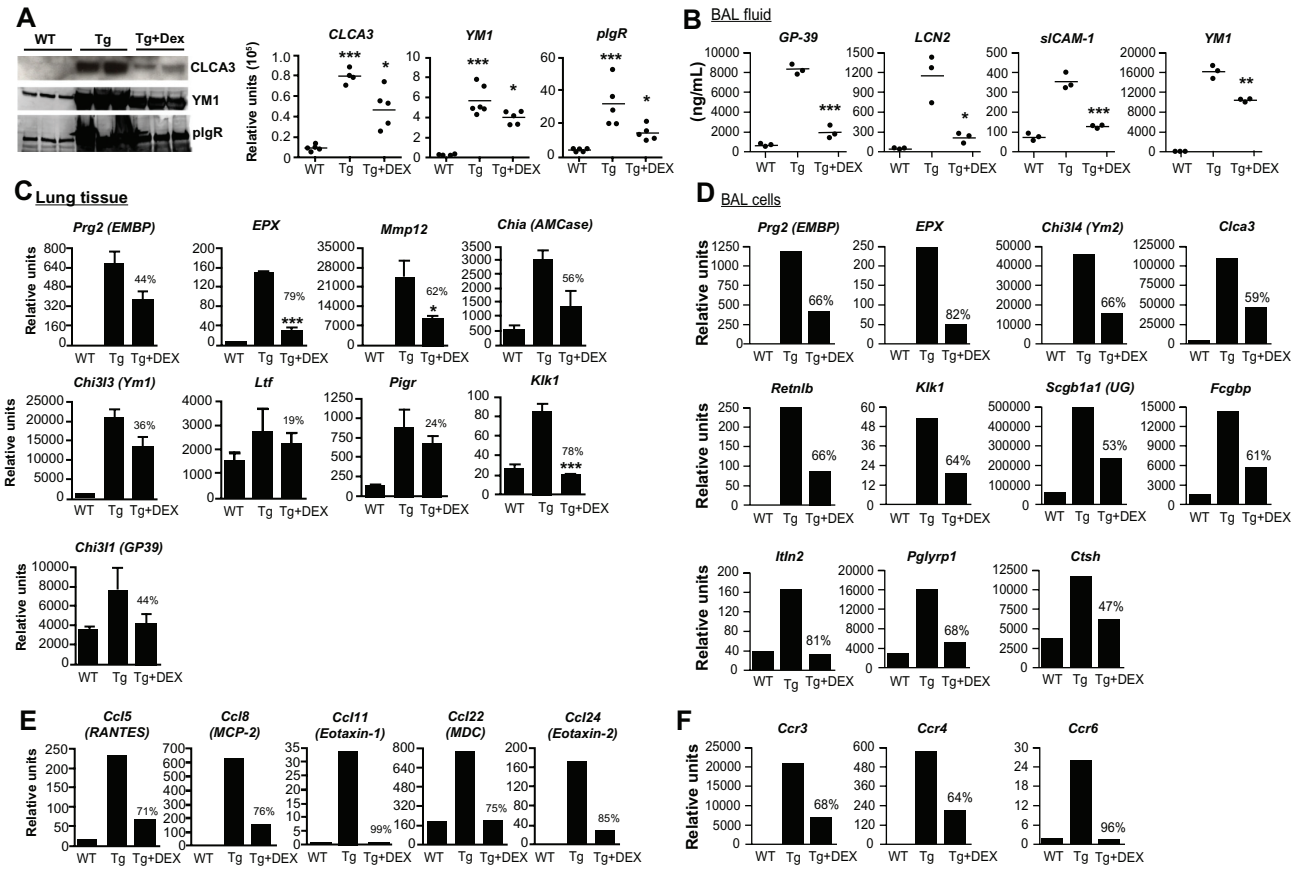


Figure 4. Downregulation of biomarkers in dexamethasone-treated TSLP Tg mice. TSLP Tg mice were treated with dexamethasone for 2 weeks, at which time BAL fluid was collected for western blot and band density analysis (**A**) of CLCA3, YM1, and pIgR or ELISA (**B**) of GP-39, LCN2, sICAM-1, and YM1. qRT-PCR of lung tissue (**C**) and pooled BAL cells (**D**) was performed for biomarker genes, and expression of chemokine (**E**) and chemokine receptor (**F**) genes was also assayed by qRT-PCR of pooled (per group) BAL cell mRNA.
Notes: Percentages indicated represent the % reduction of the respective biomarker in dexamethasone-treated TSLP Tg mice ("Tg+DEX"), compared to control-treated TSLP Tg mice ("Tg"). Results are representative of 3 independent experiments. *P* values cannot be determined for experiments using pooled mRNA. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ines *Ccl5, Ccl8, Ccl11*, and *Ccl24* were all reduced with dexamethasone treatment (Fig. 4E). Consistent with the reduction in chemokine genes, chemokine receptors *Ccr3, Ccr4*, and *Ccr6* were all downregulated with treatment (Fig. 4F). Thus, corticosteroid treatment reduces a subset of disease biomarkers in BAL fluid, lung tissue, and BAL cells and affects the expression of eosinophil and lymphocyte chemokine and chemokine receptor genes present in BAL cell mRNA of asthmatic TSLP Tg mice.

Verification of asthma biomarkers in a cynomolgus model of asthma

Although several of the biomarkers we verified have been individually documented to be involved in human asthma, we sought to further validate our set of biomarkers in a model of chronic asthma closer to human disease. Towards this goal, we used a house dust mite allergen (HDMA)-induced asthma model in cynomolgus macaques (*Macaca fascicularis*). As previously described, HDMA-sensitized animals present with a Th2 phenotype characterized by airway eosinophilia, bronchial hyperresponsiveness, and goblet cell metaplasia.[5,7] Mass spectrometry was performed on BAL fluid from HDMA-sensitized animals 24 hours before or after challenge with HDMA. Seventy identified proteins were upregulated in the BAL fluid of challenged NHP (Table S4), eight of which were also identified in the BAL fluid of asthmatic TSLP Tg mice: apoH, CLCA1/3, Factor H, Fibronectin 1, FCGBP, LCN2, pIgR, and S100A9. Although not identical, several upregulated proteins were identified from similar families in NHP and mouse, such as cyclophilin A and cyclophilin B/C or serpin A1 and serpin B6, respectively. It is important to note that several murine proteins are not expressed in

higher mammals (eg, lungkine, YM1, or YM2), and as less information is known at the protein level for *M. fascicularis* than for mouse or human, it is likely that monkey proteins with lesser homology to their human counterparts were not positively identified.

Although many reagents do not exist for the analysis of NHP proteins, antibodies have been made against several of the human orthologs of the murine biomarker proteins that exhibit cross-reactivity with NHP proteins. Present at very high amounts in the BAL fluid of HDMA-challenged animals were YKL40 and PAP (the human ortholog of GP-39 and Reg3γ, respectively) (by ELISA) and AMCase, ICAM-1, LCN2, UG, pIgR, and CLCA1 (the human ortholog of murine CLCA3) (by western blot) (*data not shown)*. As these results were consistent with the studies performed in TSLP Tg mice, we next examined which of these biomarkers were modulated with corticosteroid treatment. Towards this end, BAL fluid was collected from HDMA-challenged NHP either before ("Pre") or after ("Post") 2 weeks of high-dose corticosteroid therapy. As expected, corticosteroid-treated animals exhibited reduced asthma characteristics, such as improved lung function and reduced airway eosinophil recruitment (*data not shown*). Although trending downward, the expression of LCN2, UG, pIgR, PAP, and CLCA1 was not significantly reduced in BAL fluid following corticosteroid treatment (*data not shown*); however, AMCase, sICAM-1, CLCA1 (all by western blot) and GP-39 (by ELISA) were significantly reduced in corticosteroid-treated challenged animals (Fig. 5A and B), similar to results obtained in dexamethasone-treated TSLP Tg mice (see Fig. 4A and B). Taken together, these results indicate that the disease and treatment biomarkers characterized in TSLP Tg mice are also modulated in higher-order primate species.

Discussion

In this comprehensive study, we utilized TSLP Tg mice, which present with the hallmarks of human chronic asthma, as a murine model of the disease to study the expression and regulation of putative biomarkers. Proteome analysis identified over 40 proteins upregulated in the BAL fluid of TSLP Tg mice, and a subset of these was chosen for further examination by western blot, ELISAs, or qRT-PCR of lung tissue. Subsequently, we investigated which of these biomarkers are present in mice with developing asthma to identify early-onset biomarkers. As murine BAL cells, a surrogate for human induced-sputum cells, had not previously been examined for their utility in disease and treatment biomarker identification, we examined the gene expression profiles of BAL cell mRNA for biomarker and chemokine/chemokine receptor genes indicative of a chronic asthma phenotype. Finally, as corticosteroids are a standard, efficacious treatment for human asthma, we ascertained which biomarkers are reduced during treatment of TSLP Tg mice and validated these in a cynomolgus model of asthma.

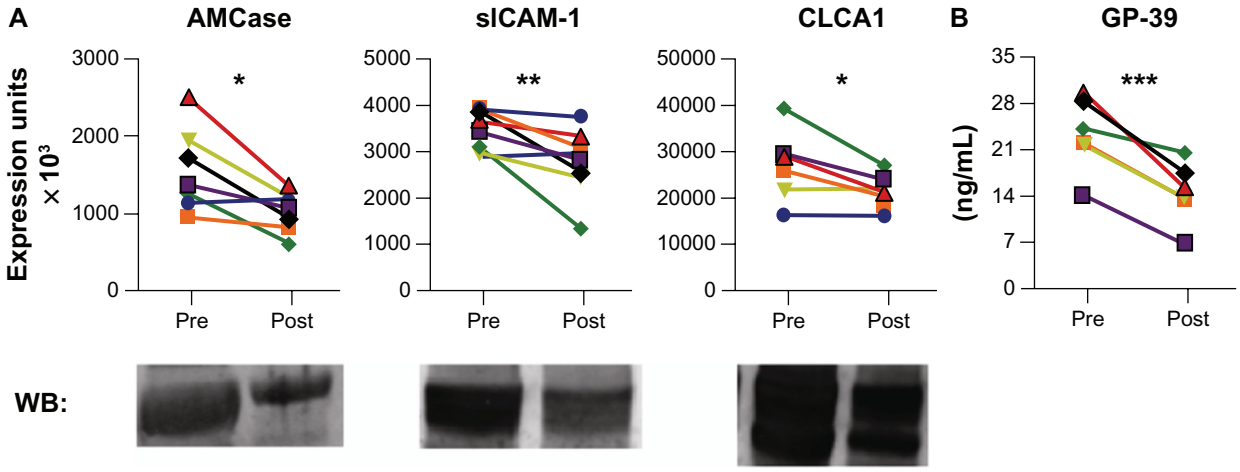


Figure 5. Reduction of disease biomarkers in HDMA-challenged NHP following corticosteroid treatment. BAL fluid was collected from HDMA-challenged NHP either before ("Pre") or after ("Post") 2 weeks of corticosteroid therapy (see *Methods*). Western blot ("WB") for AMCase, sICAM-1, and CLCA1 was performed, and band intensities were quantified using a Typhoon scanner (**A**). Representative western blot results are also shown. BAL fluid GP-39 was assayed via ELISA (**B**).
Notes: Each animal is represented by different colored symbol. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Previous studies identifying murine biomarkers of asthma have mainly relied upon the OVA-induced asthma model.[6,8–14] While very fruitful in deciphering the mechanisms of asthma, the model has been criticized for having a few potential shortcomings. The standard or acute model of OVA-induced asthma generally administers aerosolized OVA three times in a one-week period, which induces the cellular activation and goblet cell hypertrophy/activation but not the extensive tissue remodeling characteristic of human asthma.[15] Chronic models of OVA-induced asthma provoke collagen deposition and extensive tissue remodeling but can induce a state of partial tolerance rather than increased cellular inflammation.[10,15,16] Thus, we chose to utilize TSLP Tg mice, a different model of chronic asthma. These mice constitutively express TSLP under a lung-specific promoter,[3] and as TSLP sits at the top of a cascade that leads to production of IL-4, IL-5, and IL-13 and activation of eosinophils and Th2 cells,[17] TSLP Tg mice present with the characteristic properties of asthma (Fig. S1 in the Supplemental Information). As TSLP is constitutively expressed in the lung, these mice continue to exhibit progressively worse asthma pathology throughout their lives. Additionally, in contrast to previous studies using dexamethasone to prevent the *onset* of inflammation associated with OVA-induced asthma,[8,9] 12 week-old TSLP Tg mice have chronic, extensive asthma-associated inflammation and pathology present for ~3–5 weeks before treatment. As these mice express TSLP in the lung constitutively, the underlying cause of the disease continues during treatment. Thus, TSLP Tg mice can be considered a better surrogate model of human asthma, where therapy is started after symptoms and pathology exist, and the causal sources of the disease continue during treatment. Using this mouse model, we characterized proteins in BAL fluid that could function as biomarkers of asthma towards the goal of determining which are modulated during treatment.

The majority of proteins identified by mass spectrometry of asthmatic BAL fluid were enzymes, and several of these and related proteins were selected for follow-up by ELISA and qRT-PCR. As several of the identified proteins are general indications of inflammation and likely present during other non-allergic models of lung inflammation, a panel of biomarkers was selected based upon a logical association of the biomarkers to asthmatic processes. AMCase (gene name *Chia*), GP-39 (*Chi3l1*), YM1 (*Chi3l3*), and YM2 (*Chi3l4*) are all members of the chitinase or chitinase-like family and have previously been associated with asthma,[18–20] although YM1 and YM2 do not as yet have an identified human orthologue. MMP12 (*Mmp12*) is another enzyme with elastolytic activity involved in the local accumulation of cellular inflammation in the lung.[21,22] Mucus production and airway remodeling are two key characteristics of asthma, so proteins representing these processes were also chosen as putative biomarkers: FCGBP (*Fcgbp*), although not yet characterized in the lung, has a mucin-like structure,[23] and CLCA3 (*Clca3*), the murine homologue of human CLCA1, has been implicated in airway goblet cell hyperplasia and mucus production.[24,25] In addition, RETNLβ (*Retnlb*) is thought to play a role in airway fibrosis,[26,27] and KLK1 (*Klk1*) is a serine protease that may be involved in generating mediators of airway hyperresponsiveness.[28,29] The pIgR (*Pigr*) was also chosen due to its important role in delivering IgA, the major immunoglobulin at mucosal surfaces, across the respiratory epithelium.[30,31] Several proteins involved in the recognition of and defense against microbes were upregulated in the BAL fluid of the asthmatic TSLP Tg mice (see Table S1 in the Supplemental Information), and a subset of these was also chosen for further analysis: PGLYRP1 (*Pglyrp1*), Reg3γ (*Reg3g*), LTF (*Ltf*), and LCN2 (*Lcn2*).[32–35] Finally, as eosinophils are highly involved in the pathophysiology of asthma, a subset of eosinophil-associated genes was selected. Intercellular adhesion molecule 1 (ICAM-1; *Icam1*) is expressed on the vascular endothelium and is involved in the adhesion of chemotaxing leukocytes, while eosinophil cationic protein (ECP; *Ear11*), eosinophil peroxidase (EPX; *Epx*), and eosinophil major basic protein (EMBP; *Prg2*) are three eosinophil-associated activation proteins.[36,37] In BAL cell mRNA, 4 genes were added to the analysis: uteroglobin is an anti-inflammatory protein constitutively produced by airway epithelial cells, and EGFR is a receptor for members of the epithelial growth factor family also expressed by the airway epithelium, although the secreted form of EGFR is thought to act as an antagonist. CTSH is an amino peptidase produced by lung macrophages, and ITLN2 is a secreted protein of unknown function.

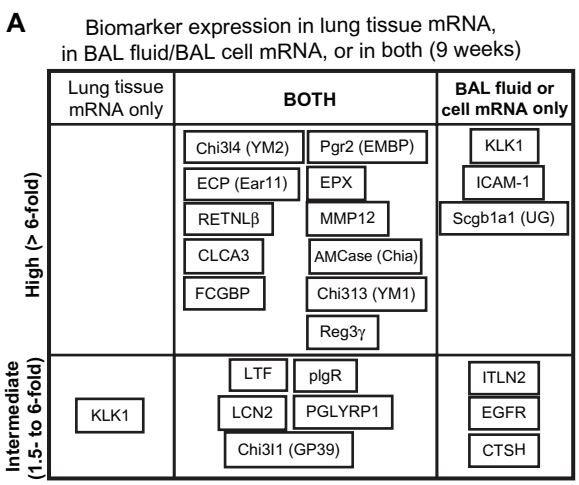
Some of the selected proteins have been described in association with asthma (eg, CLCA3, YM1, YM2, AMCase),[6,10–14,38] but several had not been characterized as being possible asthma biomarkers (eg, Reg3γ, KLK1, ITLN2, LTF). We verified these proteins with western blot assays and ELISAs, when reagents were available, and performed qRT-PCR of lung tissue for the entire set of biomarkers, allowing us to examine mRNA versus protein expression. Interestingly, mRNA upregulation did not always mirror results obtained at the protein level: ICAM-1, for instance, was significantly increased at the protein level but not modulated at the mRNA level, while other biomarkers, such as LCN2 or GP-39, were highly upregulated at the protein level but only ~2-fold increased via qRT-PCR analysis (Fig. 2 and Table 1). As such, biomarkers in the "Intermediate" expression category, although only upregulated between 1.8- and 7-fold, should not be overlooked as they could give more discernable differences at the protein rather than mRNA level.

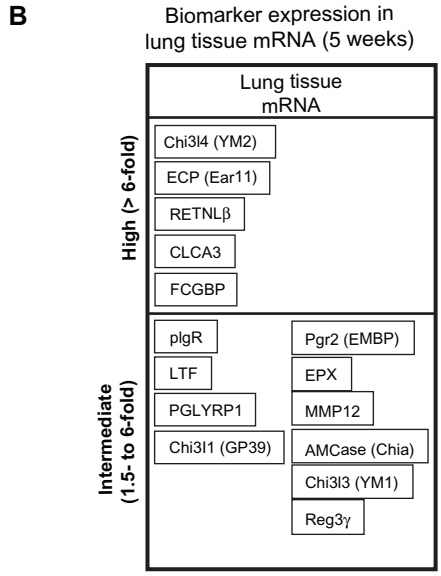
As TSLP Tg mice begin to develop the hallmarks of asthma around 5 weeks of age and progress to a state of chronic asthma over the subsequent month, we were able to utilize 5 week-old TSLP Tg mice to identify biomarkers present in the early stages of the disease. Not surprisingly, *Clca3, Chi3l4*, and *Ear11*, the biomarkers that exhibited the highest-fold upregulation at the mRNA level in 9 week-old TSLP Tg (chronic) asthmatic mice, were also the most highly increased during early development of asthma. However, the upregulation of the other biomarkers was not as great in 5 week-old mice, as biomarkers in the "High" lung category, such as *Chi3l3* (YM1) and *Mmp12*, fell to the "Intermediate" category, and *Chia* (AMCase), *Pglyrp1, Klk1, Lcn2, Epx,* and *Chi3l1* (GP-39) were no longer > 1.8-fold increased in 5 week-old TSLP Tg mice (Table 3). These differences should be appreciated when designing biomarker panels of chronic versus early asthma. Because TSLP Tg mice develop asthma as they age, this study was unable to determine if any of the above biomarkers are differentially modulated in younger versus older mice with similar disease. No differences were appreciated in male versus female mice, however.

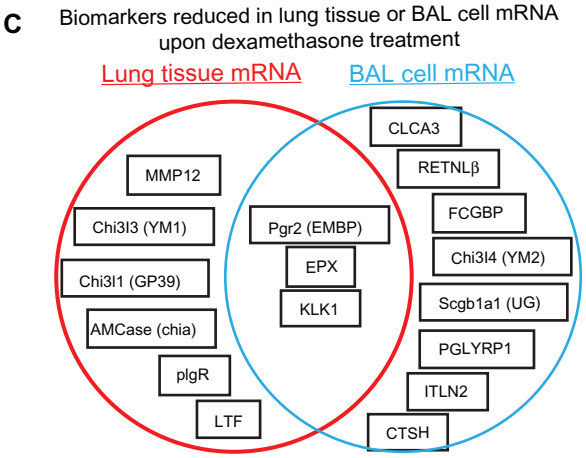
Whereas most studies pertaining to asthma biomarker identification and validation have used BAL fluid and lung tissue as proximal fluids/tissues,[3,8,11,13,14,38] we thought that the examination of BAL cell mRNA from TSLP Tg mice could be particularly fruitful in following the expression of asthma biomarkers. As continued patient participation in clinical trials or therapies is closely associated with the invasiveness of the protocol, we reasoned that BAL cells—considered a murine surrogate for human induced-sputum cells—were worth testing for biomarkers because human sputum cells are more easily acquired than lung biopsies. Being easily acquired, BAL cells could be useful not only in disease diagnosis but for determination of disease stage (such as early versus late or mild versus severe), which could aid clinicians in selecting the most effective treatment regimens. In addition, BAL cell biomarkers could be valuable in monitoring efficacy of treatment and making modifications when needed.

Interestingly, genes in the "High" lung expression category also fell into the same category of BAL cell mRNA expression (Tables 1 and 2), suggesting that the cellular infiltrate in the lung likely contributes to the expression of these biomarkers. As eosinophils constitute over 80% of the cells in the BAL of TSLP Tg mice, it was not surprising that eosinophil activation genes *Prg2* (EMBP), *Epx,* and *Ear11* were very highly increased in BAL cell mRNA. However, epithelial-associated genes (such as *Clca3* or *Retnlb)* were also in this category, suggesting possible epithelial cell contamination, although *Chia*, the gene coding for AMCase, was not expressed in BAL cell mRNA (Table 2), despite that AMCase is highly expressed in epithelial cells.[39,40] Taken together, our results suggest that BAL cell analysis may be useful in monitoring disease and treatment biomarkers, and that subsets of BAL cells may be a non-traditional source of several proteins during asthma.

We also show that BAL cell mRNA can be used to generate a "cellular signature" representative of the asthmatic airway constituents through examination of chemokine and chemokine receptor genes. CCR3 (CD193) is highly expressed on eosinophils and also detectable on Th2 cells, binding chemokines CCL5 (RANTES), CCL8 (monocyte chemotactic protein-2), CCL11 (Eotaxin-1), CCL22 (macrophage-derived chemokine), and CCL24 (Eotaxin-2).[36,37,41–48] CCR4 is found on memory Th2 cells and binds CCL22,[48–50] whereas CCR6 is found on eosinophils and memory







D

Potential biomarker	Up in chronic asthma (Lung tissue)	Up in chronic asthma (BAL fluid or cell mRNA)	Up in developing asthma (Lung tissue)	Down with steroids (Lung tissue)	Down with steroids (BAL fluid or cell mRNA)
Chi3l4 (YM2)	✓	✓	✓	–	✓
ECP (Ear11)	✓	✓	✓	–	–
RETNLβ	✓	✓	✓	–	✓
CLCA3	✓	✓	✓	–	✓
Prg2 (EMBP)	✓	✓	✓	✓	✓
EPX	✓	✓	–	✓	✓
MMP12	✓	✓	✓	✓	–
AMCase (Chia)	✓	✓	–	✓	–
Chi3l3 (YM1)	✓	✓	✓	✓	✓
FCGBP	✓	✓	✓	–	✓
Reg3γ	✓	✓	✓	–	–
LTF	✓	✓	✓	✓	–
PGLYRP1	✓	✓	–	–	✓
plgR	✓	✓	✓	✓	✓
KLK1	✓	✓	–	✓	✓
LCN2	✓	✓	–	–	✓
GP-39 (Chi3l1)	✓	✓	–	✓	✓
ICAM-1	–	✓	–	–	✓

Figure 6. Comparisons and summaries of biomarker expression. (**A**) Comparison of biomarkers upregulated in lung tissue mRNA, in BAL fluid/BAL cell mRNA, or in both compartments, of 9-week old TSLP Tg mice. (**B**) Biomarkers upregulated in lung tissue mRNA of 5-week old TSLP Tg mice. qRT-PCR of BAL cell mRNA was not performed due to low numbers of BAL cells at this time point. (**C**) Comparison of biomarkers with reduced expression in lung tissue mRNA and/or BAL cell mRNA following 1 week of steroid treatment in TSLP Tg mice. Chi3l3 (YM1), Chi3l1 (GP39), and plgR proteins were also reduced in BAL fluid, although not reduced at the mRNA level. (**D**) Summary of biomarkers of asthma found in lung tissue mRNA, BAL fluid, or BAL cell mRNA during chronic asthma; in lung tissue mRNA during developing asthma; and those biomarkers in lung tissue mRNA, BAL fluid, or BAL cell mRNA that are reduced with corticosteroid treatment.
Notes: For asthma biomarker categories, large checkmarks indicate a biomarker classified in the "High" expression category (>6-fold increased), small checkmarks indicate a biomarker categorized in the "Intermediate" expression category (1.5- to 6-fold increased), and dashes indicate biomarkers not upregulated in asthmatic mice. In treatment biomarker categories, a checkmark indicates the biomarker was significantly reduced upon corticosteroid treatment; a dash indicates a biomarker not reduced upon treatment.

Th2 cells.[36,37] Consistent with their receptor expression, CCL5, CCL8, CCL11, and CCL24 are important in eosinophil trafficking and activation, whereas CCL22 functions to recruit Th2 cells to the lung.[36,37,41,42,45–51] In TSLP Tg mice, neutrophils are quite rare and generally represent < 3% of the BAL cell subsets. Correspondingly, *Cxcr1* and *Cxcr2*, receptors present on neutrophils, were not expressed in BAL cell mRNA. *Ccr3* and *Ccr6*, receptors on eosinophils, and *Ccr4*, present on Th2 cells, were enhanced in asthmatic mice, however, and dexamethasone treatment reduced expression of these receptors. Thus, examination of BAL cell mRNA could function to both identify airway infiltrates and monitor treatment. Our model of chronic asthma is characterized by eosinophilia, but the identification of a BAL "cellular signature" can

also be extended to diagnose neutrophilic lung diseases or changes in the BAL cell constituents during the progression or treatment of the disease.

An interesting dichotomy appeared upon comparison of lung tissue and BAL cell mRNA expression in dexamethasone-treated TSLP Tg mice. Treatment reduced signs of asthma, but the most-highly upregulated lung biomarker genes were unmodulated. Taken together with the observation that these same genes are highly upregulated in 5 week-old TSLP Tg mice during the early development of asthma, it is likely that *Clca3, Chi3l4* (YM2), and *Ear11* are the first biomarkers to increase during disease and the last biomarkers to decline in response to therapy. Therefore, these are likely excellent disease biomarkers but poor biomarkers of early response to treatment in lung tissue. On the other hand, biomarkers such as GP-39, LCN2, sICAM-1, and YM1 or *Prg2 (EMBP), Epx,* and *Klk1*, which are significantly downregulated upon corticosteroid treatment in TSLP Tg mouse BAL fluid and lung tissue mRNA, respectively, are good indicators of early therapeutic intervention. Similarly, most of the biomarkers increased in BAL cell mRNA are lessened with treatment and could function in a similar capacity. Biomarkers of disease and treatment in the various proximal fluids and tissues are summarized in Figure 6. Examination of disease and treatment biomarkers in HDMA-sensitized and challenged monkeys (Fig. 5A and B) support these observations, and ongoing long-term studies are validating the set of biomarkers in proximal fluid and tissues from asthmatic humans. The homogeneity of the TSLP Tg mouse strain and subsequent coordinated biomarker expression prevented the identification of biomarker panels—groups of biomarkers that together are a better indicator of disease or treatment efficacy, compared to individual measurement—but panel identification and multi-variate analyses are being explored in ongoing human studies, allowing the fine-tuning of these biomarkers during human asthma.

Taken together, the studies presented herein significantly advance the field of asthma disease and treatment biomarkers by providing a comprehensive study of various proximal fluids and tissues in murine and cynomolgus models of chronic asthma and reveal BAL cells as a fruitful proximal tissue for biomarker analysis for diagnosis of disease stage and treatment efficacy.

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Competing Interests

At the time of the study, J.L., J.M., M.-C.M., Y.L., C.E., F.V., R.B.F., T.K.M., R.W.M., and M.B. were employees of Schering-Plough Biopharma (currently Merck Research Laboratories), which funded the work. CE is co-principal investigator on a phase 2 NIH SBIR grant.

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Abbreviations

AMCase, Acidic mammalian chitinase; BAL, bronchoalveolar lavage; Chia, Chitinase, acidic; Chi3l4, Chitinase 3-like 4; Chi3l3, Chitinase 3-like 3; Chi3l1, Chitinase 3-like 1; Clca, Chloride channel regulator; Ctsh, Cathepsin H; Ear11, Eosinophil-associated ribonuclease A family member 11; Egfr, Epidermal growth factor receptor; EMBP, eosinophil major basic protein; Epx, Eosinophil peroxidase; Fcgbp, Fc gamma binding protein; HMDA, house dust mite allergen; ICAM-1, Intercellular adhesion molecule 1; Itln2, Intelectin 2; Klk1, Kallikrein 1; Lcn2, Lipocalin 2; Ltf, Lactoferrin; Mmp12, matrix metalloproteinase 12; Pglyrp1, Peptidoglycan recognition protein 1; pIgR, polymeric immunoglobulin receptor; Prg2, Proteoglycan 2; Reg3 g, Regenerating islet-derived 3 gamma; Retnlb, Resistin-like beta; Scgb1a1, Secretoglobin family 1A member 1; Tg, transgenic; TSLP, thymic stromal lymphopoietin; UG, Uteroglobin.

Author Contributions

J.L. performed murine in vivo studies and tissue harvest with assistance from Y.L. in R.d.W.M.'s laboratory. J.D.M. performed qRT-PCR analyses, which T.K.M. supervised. M-C.M. and F.V. performed mass spectrometry. R.L.W., M.R.V.S., and R.B.F. performed in vivo monkey studies and harvest of BAL fluid. M.B. initiated the project, performed mass spectrometry

and western blot analyses, and supervised the entirety of the work.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Supplementary Information

Supplementary tables are available from 9776SupplementaryTables.zip

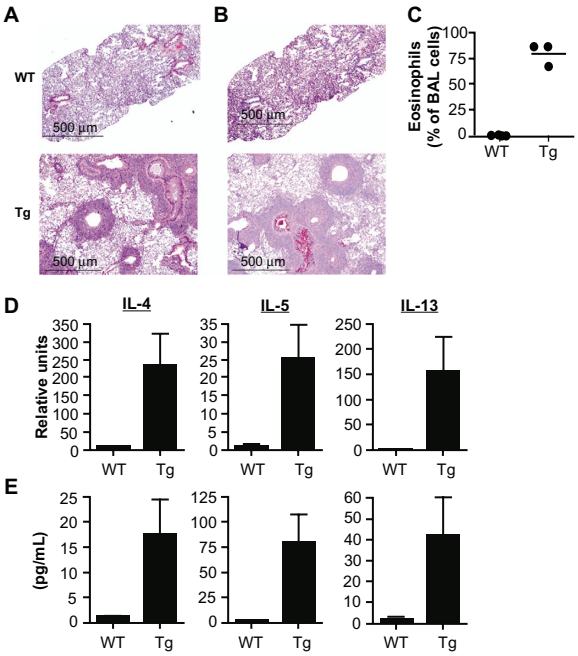

Figure S1. Characterization of TSLP Tg mice as a model of chronic asthma. Lung tissue was removed from 9-week old WT or TSLP Tg mice and examined for lung pathology by H&E staining (**A**) or for mucus production by PAS staining (**B**). Eosinophils in the BAL were enumerated by Wright-Giemsa staining of cytospun cells (**C**). The expression of IL-4, IL-5, or IL-13 in WT and TSLP Tg mice was assayed using qRT-PCR (**C**) or ELISA (**D**). Experiments are representative of at least 5 independent experiments.

Table S1: List of proteins identified by mass spectrometry in BAL fluid from 9-week old TSLP Tg mice and WT littermates. Proteins were classified as having greater (yellow), similar (gray), or decreased (green) expression in TSLP Tg mice compared to WT mice.

Protein	NCBI GI Protein Accession Number
14-3-3 Tau	38077229
adipsin	673431, 7304867
aldo-keto reductase family 1, member B3	1351911
annexin A1	113945, 6754570
apoH	231558
biliverdin reductase B	21450325
cathepsin H	13905172, 3929819, 7106279
C7	38077624
chloride channel calcium activated 3	8567336
cyclophilin B	2118329, 6755142
cyclophilin C	6679441
enolase 1	12963491, 13637776, 8101734
eosinophil-associated, ribonuclease A family, member 2	15420983, 6681251
epidermal growth factor receptor	10880776
factor H	19072788, 20071242
fatty acid binding protein 4	14149635
Fc gamma BP	26346705
fibronectin 1	28479106
GDI 2	6679987
GDI 3	12841706
glutathione peroxidase 1	27807638, 6680075
GP-39	13124008, 26341984
Fizz1	10048446, 12841171
hypothetical Glyoxalase/Bleomycin resistance protein	21313080, 12849397
intelectin 2	28932914
lactotransferrin	202291
L-carnitine dehydratase	34328352
leucine-rich alpha-2-glycoprotein	16418335
lipocalin 2	34328049
lungkine	6755436
Pigment epithelium-derived factor precursor	1747298, 31981504, 3355888, 46397639
peptidoglycan recognition protein 1	6679293
PIGR	31981570, 8099665
profilin 1	26344071, 6755040
regenerating islet-derived 3 gamma	6755310
S100A8	1200246, 7305453

S100A9	6677837
serpinB6	6678097
transglutaminase 2	6678329
tumor endothelial marker 7-related precursor	15987503
ubiquitin-conjugating enzyme E2	42741690
YM1	11140877, 6753416
YM2	18254403, 21553079
YNL	6934190
14-3-3 protein beta	3065925, 31543974, 7106546
14-3-3 epsilon	31981925
14-3-3 zeta	1526539, 1841387, 26341272, 6756041
abhydrolase domain containing 14b	27753960
actin	12697526, 12852068, 15076949, 15284015, 2724046, 29123076, 30425250, 37653273, 38079974, 38089227, 38089775, 46401542, 49864, 6752952, 71620, 728792, 728796, 728798, 809561, 8850209, 90263
afamin	20072386, 21553101
aldehyde dehydrogenase II	191804
aldo-keto reductase family 1, member A4	10946870
annexin A3	26354887, 7304887, 71766
annexin A5	13277612, 3318923, 4139939, 4139951, 6753060
antithrombin III	18252782
apo A4	29477189, 6680702, 12836356
beta-2 microglobulin	114775
C3	23956044, 28175786
C5	13278268, 26348393, 6754164
calmodulin	12851157
carbonic anhydrase 2	12832236, 26344335, 31981657, 33243954
carbonic anhydrase 3	31982861
carboxylesterase	14331135, 17512488, 2921308, 38089545
ceruloplasmin	38614350, 6680997
coagulation factor II (prothrombin)	6753798
cofilin 1	12861068, 6680924
cyclophilin A	38074758, 38077290, 38078688, 38080301, 6679439
cysteine and glycine-rich protein 1	6681069, 6681069
cystatin B	6681071
delta-aminolevulinic acid dehydratase	3642647
destrin	7441446, 9790219
Down syndrome cell adhesion molecule-like protein	19852058
dnaK-type molecular chaperone Hsc70	42542422, 476850
esterase 1	10946842, 16716505, 18088156, 22135640, 38089547, 6679689, 92052
factor B	6996919
fatty acid binding protein 3	51267, 6753810
ferritin heavy chain	111625
ferritin light chain	12832085
fetuin A	112459, 2546995, 7304875

fetuin Beta	112459, 10947006
four and a half LIM domains 1	22137390, 25091271, 6753864
FK506 binding protein 1a	6679803
gelsolin	18606238, 26354755
glutathione peroxidase 3	15011841
glutathione S-transferase, alpha 4	12859131, 12859396, 20141353, 6754082
glutathione S-transferase, mu 1	6754084
glutathione S-transferase, mu 2	6680121
glutathione S-transferase, mu 6	7110613
glutathione S-transferase, M7-7	28494710
glutathione S-transferase omega 1	6754090
haptoglobin	6016254, 8248739, 8850219
heat shock protein 1	6754254
hemoglobin	122639, 12845853, 12846616, 12852164, 2118940, 92361
hemopexin	1881768, 22022646, 23956086
kininogen	12963497, 40715898, 40715900, 4071590097, 41235784, 92436, 92471
lysozyme	7305247, 8393739
malate dehydrogenase 1	31982178, 37589957
nucleoside diphosphate kinase 1 or 2	127984, 19852058
murinoglobin 1	1168250, 31982171, 38174651
orosomucoid 1 or 2	6679182, 6754950
p25 alpha (CGI-38 protein)	13385968, 38079976
peroxiredoxin 1	6754976, 7948999
peroxiredoxin 5	26345188, 6746357, 6755114
Peroxiredoxin 6	3219774, 3789944, 4139186, 6671549
plasminogen	31982113
pzp	34785996, 6680608
SAP	134198, 91294
secretoglobin	6755947
serpinaA3K	34980957
serpin A6	15489028, 6680856
serpin C1	18252782
serpin F1	1747298, 31981504, 3355888, 46397639
superoxide dismutase 1	45597447
surfactant associated protein B	22296601
surfactant associated protein D	6677921
thyroglobulin	2055388, 3319332, 6678335, 69232
transferrin	18204720
Transgelin 2	9910901
triosephosphate isomerase	68423, 1864018, 6678413
Vitamin D binding protein	30802101, 38080263, 476569, 111243
14-3-3 gamma	3065929
Acyl-CoA-binding protein	6681137
aldehyde dehydrogenase A1	1083586, 28386049, 42560536, 7304881, 9755362
aldolase 1	27695278
aldose reductase	1351911, 31981909
Apo A1	6753096, 1245804
Rho GDP dissociation inhibitor (GDI)	13435747, 26344461, 31982030

alpha	
C9	755764, 15375312
carbonyl reductase 2	6671688
cytochrome C, somatic	6681095
D-dopachrome tautomerase	6753618
fructose-bisphosphate aldolase	42490830, 6671539, 68186, 7548322
glucose phosphate isomerase 1	6680067
Phosphatidylethanolamine-binding protein	12841975
Phosphoglycerate mutase 1 or 2	12844989, 20178035
prothrombin	6753798
RAGE	6671525 , 7441748
RNase 4	10946868
selenium binding protein 1 or 2	18848341, 9507079, 22164798
serpin D1	6680183
serpin F2	6679383
serpin G1	1772998
SH3-binding domain glutamic acid-rich protein like	9910548
thioether S-methyltransferase	12850108, 6678281
thioredoxin 1	6755911, 12841560
similar to SEC14-like protein 3	38091283
TI-225	1167510, 38079256, 38083720, 38089459, 38089953
ZAG	7304911

Table S2: qRT-PCR analysis of selected biomarker genes in Saline or OVA-challenge mouse lung tissue.

Gene Name	**Saline***	**OVA***	**Fold Change**
High			
Chi3l4 (YM2)	1.5 ± 0.70	3566.68 ± 820.67	2377.87
Clca3	6.52 ± 0.93	3428.78 ± 179.39	526.22
Ear11	20.78 ± 6.36	5751.26 ± 303.18	276.77
Retnlb	0.54 ± 0.17	144.94 ± 8.95	270.7
Mmp12	196.34 ± 35.63	7396.15 ± 635.09	37.67
Chi3l3 (YM1)	752.3 ± 42.83	13566.21 ± 572.54	18.03
Fcgbp	53.65 ± 2.68	715.14 ± 41.25	13.33
Epx	2.87 ± 0.32	29.66 ± 4.46	10.33
Chia (AMCase)	395.20 ± 42.82	3857.7 ± 422.35	9.76
Prg2 (EMBP)	19.11 ± 1.49	172.9 ± 26.36	9.05
Intermediate			
Klk1	12.89 ± 0.84	52.96 ± 3.95	4.11
Pigr	206.56 ± 36.52	820.66 ± 69.28	3.97
Reg3g	585.09 ± 152.69	1654.29 ± 151.29	2.83
Lcn2	2440.28 ± 257.78	6864.99 ± 365.70	2.81
Pglyrp1	46.55 ± 3.08	103.0 ± 4.413	2.21
Chi3l1 (GP-39)	3324.99 ± 144.23	7207.64 ± 221.13	2.17
No or Negative Change			
Icam1	3234.65 ± 424.99	4096.66 ± 211.15	1.27
Ltf	1631.02 ± 264.83	1185.75 ± 113.07	-1.37

* *relative expression units, normalized to ubiquitin*

Table S3: qRT-PCR analysis of selected biomarker genes in the lung tissue of 5-week old WT or TSLP Tg mice, which exhibit developing asthma.

Gene Name	**WT***	**TSLP Tg***	**Fold Change**
High			
Clca3	4.42 ± 2.17	1888.01 ± 712.69	427.13
Chi3l4 (YM2)	0.75 ± 0.22	288.25 ± 73.22	383.34
Ear11	5.14 ± 1.51	521.59 ± 128.66	101.52
Retnlb	0.24 ± 0.12	3.99 ± 1.50	16.35
Fcgbp	41.83 ± 3.70	463.94 ± 138.61	11.09
Intermediate			
Chi3l3 (YM1)	554.47 ± 46.98	1889.16 ± 832.03	3.41
Mmp12	151.9 ± 7.57	461.72 ± 229.31	3.04
Pigr	53.56 ± 11.82	124.02 ± 28.81	2.32
Ltf	200.49 ± 49.34	433.96 ± 143.91	2.16
Prg2 (EMBP)	13.27 ± 4.48	28.67 ± 7.16	2.16
Reg3g	268.07 ± 134.64	504.58 ± 135.54	1.88
Chia (AMCase)	617.32 ± 47.49	1038.85 ± 272.26	1.68
Pglyrp1	54.57 ± 4.06	87.08 ± 6.61	1.60
No or Negative Change			
Klk1	76.84 ± 11.24	107.17 ± 24.30	1.39
Lcn2	1684.04 ± 55.07	1947.97 ± 273.07	1.16
Epx	4.77 ± 0.98	5.20 ± 0.68	1.09
Chi3l1 (GP-39)	2751.35 ± 185.36	2824.22 ± 473.45	1.03
Icam1	2826.16 ± 198.63	1994.37 ± 123.1	-1.42

* *relative expression units, normalized to ubiquitin*

Table S4: List of proteins identified by mass spectrometry in BAL fluid from HDMA-sensitized NHP before or after challenge with HDMA. Proteins were classified as being upregulated (yellow), similarly expressed (gray), or downregulated (green) in the BAL fluid of HDMA-challenged animals, compared to HDMA-sensitized, unchallenged animals.

Protein	**NCBI GI Protein Accession Number**
14-3-3 beta	3065925, 4507949, 90076742, 1585294
alcohol dehydrogenase	114595327
ALDH1A1	2183299, 21361176
ALDH3A2	124004853, 1666529
alpha-1-microglobulin/bikunin precursor	579676
apoA1	109108768, 178775, 23379764, 23379768, 2914175, 342075, 3915607, 4557321, 73955106
apoA4	109108832, 28762, 461521
apoH	109116937, 109116939, 11513724, 28810, 543826
C2	34628, 14550407
C8	109109736, 21730336
C9	179726, 2258128
calcyphosine	4757908
calmodulin	4502549, 640285, 71664
Carbonic anhydrase 1	109086845, 4502517, 461679, 515084
carbonic anhydrase 2	30466252
Carbonyl reductase 1	51830180
Charcot-Leyden crystal protein	109124714
CLCA1	114557513
clusterin	32891795, 55846712, 90077304
coagulation factor II	109106394
cofilin 1	5031635, 90075040
copper containing amine oxidase 3	109115435, 4502119
cyclophilin A	114634944, 118098, 13937981, 1431788
cystatin B	4503117, 109065176
cystatin C	192912, 74136407
DC48	12006209
dopachrome tautomerase	109094852, 4503291
factor H	109018998, 109019002, 4504375
factor I	116133, 182607, 67967767
Fc Gamma BP	109124740, 4503681, 5080756, 109124740
fibronectin 1	109100908, 126337927, 1421281, 149710153, 30722344, 31397, 34364617
GAPDH	31645, 37730278, 41147378, 7669492
gelsolin	109110365, 4504165
GSTm5	37748321
haptoglobin	109129090, 1212947, 34785974, 466455
haptoglobin-related protein (HPR)	123510
HLA-E single chain trimer	33637489
HRG	109042262, 4504489
HSP90	18605741, 55730837
IGF binding protein	109127203, 2498123, 4826772

IgK-rh	4105843
ITIH1 subunit 3	2851501, 33989
ITIH2	109088171, 125000
ITIH4	1082547, 109039135, 7770149
kininogen	4504893, 386852
lactate dehydrogenase B	4557032
leukocyte protease inhibitor	109091820, 4507065
lipocalin 2	109112565, 55632379, 631308
lysozyme C	126608, 229157, 229916, 2497771, 48428997, 67408
mannose BP A	1449042
NPC2	5453678, 62896507
NUDC	5729953
ovostatin 2	109095618
peroxiredoxin 5	6166493, 6912238, 90078122
peroxiredoxin 6	109019544, 3219774, 3318841
phosphoglycerate mutase 1	4505753
PIGR	109018572, 31377806
prosaposin	1565257
prostaglandin D synthase	190444
proteasome subunit 7	12314029
retinol BP	73998292
S100A9	109016333, 4506773
S100P	114593120
SAP	109017517
selenium BP1	16306550, 73981582
serpinA1	6855601, 28637
sex hormone-binding globulin	67969615
SH3 domain binding glutamic acid-rich protein like	4506925
SOD3	4507151
tetranectin	267108, 4507557
transketolase	12018252, 1729977, 31417921, 346399
triosephosphate isomerase 1	136062, 16877874, 41058276, 4507645, 515257
tubulin polymerization-promoting protein	13385968
UBA-52 (TI-225)	13786827, 2627129, 4506713
ubiquitin-conjugating enzyme E2	1066080
Uteroglobin-related protein 2 precursor	109080162
X-prolyl aminopeptidase 3-like	46309521
14-3-3 zeta	109087133, 114621209, 30354619, 4507953, 71897035
A2M	112911, 1304084, 157954061, 4557225, 73997689, 75054706
actin	1070613, 113218, 113307, 139001520, 148806547, 32186898, 3219772, 47551039, 49868, 55732773, 58258865, 61676567, 71611, 7546413, 90811719, 14278147
actin beta	14250401, 15825662, 28336, 5107933, 6716561, 71625, 71629
afamin	109074517, 114594313, 27229290, 4501987
ALDH3A1	109113630, 126314265, 178375, 33871063, 399365
alpha globin	109129589, 122366, 122407, 122427, 13195586, 22671717, 319896, 75914658
alpha-1-B glycoprotein	109126335, 114679419, 23503038, 69990

alpha-2 HS glycoprotein	2116653, 109042277
angiotensinogen	109020030, 90075392, 4557287, 532198
annexinA1	109111790, 113942, 148229927, 4502101, 73946797
beta globin	1066765, 13273496, 13549112, 1431650, 183857, 223012, 22874, 229149, 29446, 40886941, 4929993, 66473265, 86611
beta-2 microglobulin	34616, 547299
C3	109123141, 114674922, 116597, 125804742, 179665, 284052, 40786791, 4557385, 544053, 557597, 78101267, 78101268
C4a or b	13936421, 24987346, 4063691, 40737319, 40737466, 40737478, 40737486, 4502501
C5	109110418
ceruloplasmin	109048806, 110347564, 126031006, 126338232, 126338232, 149408772, 149729967, 1620909, 180249, 1942284, 2493322, 42658910, 6970046
CRB1	41327708, 6912322, 34364816
factor B	109070536, 291922, 4502397, 7145102, 758090
factor D	109122706, 42544239, 77735465
fetuin B	109042265
GSTa1	109071513, 442977
GSTp1	111185949, 494066, 75070646, 87564
hemopexin	109107500, 11321561, 1335098
HSP8	13938297, 16041102, 5729877
orosomucoid 1	109110478
peroxiredoxin 1	109003875, 4505591, 55824562
peroxiredoxin 2	109123565, 32189392, 438069, 90076926, 33188452, 60654143
plasminogen	112807252, 2737906, 38051823, 4505881
S100A6	1173337, 20664042, 6755392, 7657532
serpinA3	109084779
serpinC1	114565551, 149708147, 179161, 4502261, 52695711, 576554, 179152
serpinF1	189778, 15559258
serpin G1	179617
SOD1	74136167
SP-D	18490171, 93352568
thioredoxin	1065111, 109002483
transthyretin	109121862, 119621670, 219978, 230651, 23574795, 443297, 443297, 57089193, 999643
vitD BP	109074554, 139641, 18655424, 455970
adiponectin	4757760, 74136307
ADP-ribosylation factor GTPase activating protein 1	8922652
aldo-keto reductase family 1, member B1	442618, 90075192
aldo-keto reductase family 1, member B10	3493209
aldolase A	75061505, 16740581, 28614
annexin A2	109081460, 18645167, 34364597, 4757756
annexin A5	3212603, 4502107, 75075702, 809185
CD44	109106764, 29799, 30268334, 3832518, 422780
Chloride intracellular channel protein 1	895845

FLJ31659	23308541
gamma globin	15988413, 31725
galectin 3 binding protein	5031863
HSP70	213804
HSPA1L	2119712, 87626
lymphocyte cytosolic protein 1	4504965
profilin 1	114665902, 149724221, 157833469, 999511
quiescin 6	13325075
SP-A	109089169, 109089171, 60390965, 71969
transaldolase 1	1082840, 109104910