In vitro allergen challenge of peripheral blood induces differential gene expression in mononuclear cells of asthmatic patients: inhibition of cytosolic phospholipase A2α overcomes the asthma-associated response

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Summary

Background Existing treatments for asthma are not effective in all patients and disease exacerbations are common, highlighting the need for increased understanding of disease mechanisms and novel treatment strategies. The leukotriene pathway including the enzyme responsible for arachidonic acid release from cellular phospholipids, cPLA2α, is a major contributor to asthmatic responses and an attractive target in asthma therapies.

Objective The study reported here investigates (a) the differential effects of in vitro exposure of peripheral blood mononuclear cells (PBMCs) to allergen between asthma and healthy subjects, and (b) the contribution of cPLA2α to these differences in gene expression.

Methods In vitro responses of asthma (N = 26) and healthy (N = 11) subject PBMC samples to allergen stimulation in the presence and absence of cPLA2α inhibition or 5-lipoxygenase inhibition were compared at the gene expression level using oligonucleotide arrays and at the protein level using ELISA.

Results Subject samples within both asthma and healthy groups showed allergen-dependent cytokine production and allergen-dependent gene expression changes, although transcriptional profiling identified 153 genes that were modulated significantly differently by allergen between asthma and healthy subjects. Among these were genes previously associated with asthma, but the majority (about 80%) have not previously been associated with asthma.

Conclusions Transcriptional profiling elucidated novel gene expression differences between the asthmatic and healthy subject samples. Although 5-lipoxygenase inhibition did not significantly affect allergen-modulated gene expression, the inhibition of cPLA2α activity affected many of the allergen-dependent, asthma-associated gene expression changes.

Keywords asthmatic response to allergen, cPLA2α inhibition, differential gene expression in asthma, transcriptional profiling

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Introduction

Asthma is a complex airway disease characterized by reversible airway obstruction, bronchial hyperresponsive-ness and airway inflammation. The prevalence of asthma has almost doubled in the past 20 years, with approximately 8–10% of the US population affected by the disease [1]. World-wide, over four billion dollars is spent on treating asthma patients each year [2]. Current asthma treatments target known disease mechanisms, but these treatments are not effective in all patients and suboptimal disease control among patients taking these medications is common. Additional insight into the pathogenesis and aetiology of asthma will facilitate development of more widely effective therapies.
Although the aetiology of asthma is multi-factorial, a common mechanism underlying the pathophysiology of asthma involves a dysregulated inflammatory response in the lung to environmental allergens. This ultimately results in increased vascular permeability, smooth muscle contraction, bronchoconstriction and inflammation. Asthma treatments currently available are designed to inhibit these physiologic processes. Corticosteroids and anti-allergy medicines, such as soluble IgE antibody (Xolair®) focus on inhibiting the dysregulated inflammatory response. A major characteristic of the inflammatory response is a shift in the balance of the immune response from a T-helper type 1 (Th1) to a Th2 cytokine profile [3]. Th2 cells mediate the inflammatory response through the release of cytokines including ILs, particularly IL-13 [4, 5] and IL-4 [6, 7] leading to IgE production and release [8] and therapies targeting these mediators are being developed. Other treatments counter bronchial airway narrowing, such as the bronchodilator salbutamol (Ventolin®), a short-acting β2-agonist [9, 10].

Reflecting the complexity of the disease, the therapies described above, and others currently available, do not consistently abrogate airway inflammation and airway remodelling in all patients [11]. Inter-individual variability in drug response [12–14] and frequent adverse drug reactions [15–17] to currently available drugs necessitate novel treatment strategies. Prior in vitro and in vivo studies have elucidated some critical mechanisms of asthma pathogenesis including the identification of some important mediators of allergen responsiveness. Previous studies on the differences between asthma and healthy subjects following in vivo allergen response have categorized asthma patients as mild persistent (N = 11, FEV1 75–80%), moderate persistent (N = 11, FEV1 65–74%) or severe persistent (N = 11, FEV1 < 65%) according to the 1997 NIH Guidelines [34]. Each clinical site’s Institutional Review Board or Ethics Committee approved this study, and the only study-specific procedure was sample collection. All asthma subjects were on standard of care treatment: 14 subjects were taking inhaled steroids, three were taking nasal steroids and nine both inhaled and nasal steroids. Samples collected included four (15%) from patients on oral steroids. Eleven healthy volunteers were recruited from the Allergy, Asthma and Dermatology Research Center in Lake Oswego, Oregon, and Bensch Research Associates in Stockton, California. Asthmatic status was confirmed by skin test in 15 of 20 subjects, and by clinical assessment in the remaining five subjects. Duration of disease ranged from 62 years to 1 year, with an average duration of 23 years. Ten of the subjects were diagnosed with asthma as children. Asthma subjects were categorized as mild persistent [N = 4, forced expiratory volume in 1 s (FEV1) 50–74%], moderate persistent [N = 11, FEV1 65–74%] or severe persistent [N = 11, FEV1 < 65%] according to the 1997 NIH Guidelines for the Diagnosis and Management of Asthma [34]. Each clinical site’s Institutional Review Board or Ethics Committee approved this study, and the only study-specific procedure was sample collection. All asthma subjects were on standard of care treatment: 14 subjects were taking inhaled steroids, three were taking nasal steroids and nine both inhaled and nasal steroids. Samples collected included four (15%) from patients on oral steroids. Eleven healthy volunteers were recruited from Wyeth Research in Cambridge, MA, and had no known history of asthma or seasonal allergies. Informed consent was obtained from all subjects. Demographic information for the subjects is shown in Table 1. Blood was drawn into (8 mL × 6 tubes) cell purification tubes (Becton Dickinson, Franklin Lakes, NJ, USA). All asthma samples where shipped at room temperature in a temperature-controlled box overnight from the clinical site in the western US and processed immediately upon receipt in Andover, MA (approximately 24 h after blood draw). Healthy subject...
samples, which did not require shipping, were stored overnight before processing to mimic the conditions of the asthma samples. Therefore, both groups of samples were treated similarly to ensure that any observed differences between the groups were not attributable to differences in sample processing. PBMCs were isolated from whole blood samples according to the manufacturer’s recommendations.

**In vitro cell stimulation**

The PBMCs were stimulated *in vitro* (2 × 10^5 cells/well, 96 well Costar-Corning CLS 3799) with a cocktail containing four different allergens from house dust mite, ragweed and cat. Recombinant allergens, Der p1, Der f2, Fel d1 (Indoor Biotech, Charlottesville, VA, USA) and natural ragweed allergen (Allergy Lab, Seattle, WA, USA) were screened for endotoxin levels (LAL Endotoxin Test, Catalog # HLT302, sensitivity, 0.0001 EU/mL; Cell Sciences, Canton, MA, USA), and allergen preparations were selected based on having the lowest endotoxin levels among available sources of these allergens. This was done to ensure endotoxin levels as low as could be achieved using purified natural allergens. The total level of endotoxin in allergen-stimulated cultures was 0.057 EU/mL, approximately 5.7 pg/mL, about 100-fold below the reported minimum required for endotoxin effects *in vitro* [35]. The final allergen cocktail concentrations in culture medium were as follows: Der p1 and Der f2 (dust mite), 1 μg/mL; Fel d1 (cat), 1.25 μg/mL; ragweed, 125 μg/mL. Antigen concentrations were chosen based on titration experiments (twofold dilutions starting at 10 μg/mL) performed using samples from four human donors before the initiation of work on the samples reported here. Optimal concentrations were chosen based on maximum cytokine production and antigen-dependent proliferation (data not shown). A time course was also performed (from 3 to 7 days) and 6 days was chosen as optimal (data not shown). The sensitivity of the subjects to the allergens used was unknown, but allergens were chosen based on the estimate that 80% of allergic individuals are believed to react to one or more of these allergens [36]. Culture medium contained RPMI-1640 (Sigma, St. Louis, MO, USA) with 10% heat-inactivated FCS (Sigma) and 100 unit/mL penicillin and 100 μg/mL streptomycin and 0.292 μg/mL glutamine (GIBCO RL Invitrogen, Carlsbad, CA, USA).

The cPLA_2_ inhibitor, WAY-196025, was used at a concentration of 0.3 μM, and zileuton, the 5-lipoxygenase inhibitor, at a concentration of 5 μM in the PBMC samples. The inhibitory activity of the batches of WAY-196025 and zileuton used in this study was verified in a human whole blood assay stimulated with calcium ionophore, A23187, as described previously [37]. WAY-196025, was confirmed to inhibit both the prostaglandin and leukotriene pathways with IC_{50} < 15 nM and zileuton was shown to inhibit the 5-lipoxygenase pathway with an IC_{50} of ~0.55 μM. The characterization of WAY-196025 has been described elsewhere [27]. In brief, WAY-196025 inhibits the production of arachidonate metabolites – TXB_2_, LTB_4_, PGE_2_ and PGF_2_ by 72%, 78%, 79% and 72%, respectively, at 0.039 μM in A23187 stimulated human whole blood. WAY-196025 is selective for cPLA_2_ in cell-based assays, does not inhibit COX-1 and COX-2 in microsomes, and is > 1000-fold selective against the closely related enzymes, cPLA_2_ and γ. The identity within the lipase domain between cPLA_2_ and cPLA_2_, and cPLA_2_ and cPLA_2_, are 37% and 32%, respectively [38]. Ghosh et al. [39] have reported the inhibition of cPLA_2_ using an analogue of WAY-196025 they called ‘Wyeth-1’, but the selectivity for WAY-196025 for cPLA_2_ was not tested in the work reported by Ghosh et al. Within 10 Å of the active site Ser-228, 25 of 51 residues are distinct between cPLA_2_ and cPLA_2_, and cPLA_2_ and cPLA_2_, are 37% and 32%, respectively [38]. Ghosh et al. [39] have reported the inhibition of cPLA_2_ using an analogue of WAY-196025 they called ‘Wyeth-1’, but the selectivity for WAY-196025 for cPLA_2_ was not tested in the work reported by Ghosh et al. Within 10 Å of the active site Ser-228, 25 of 51 residues are distinct between cPLA_2_ and cPLA_2_, and cPLA_2_ and cPLA_2_, are 37% and 32%, respectively [38].

### Table 1. Characteristics of study subjects

|                          | Healthy volunteers (N = 11) | Asthma subjects (N = 26) |
|--------------------------|-----------------------------|--------------------------|
| **Sex (M/F)**            | 7/4                         | 9/17                     |
| **Race (Caucasian/Hispanic)** | 11/0                      | 24/2                     |
| **Age (y)**              | 28–51                       | 21–73                    |
| **Asthma severity**      | NA                          | 4 mild                   |
|                          |                             | 11 moderate              |
|                          |                             | 11 severe                |

M, male; F, female; y, years; NA, not applicable. Of the 20 atopics, allergy assessments were done by skin test for 13, the remaining were characterized as atopic through clinical assessment.

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using an eight-channel pipettor and placed into a collection plate for cytokine ELISA. To the remaining cell pellet, 100 μL of RLT lysis buffer containing 1% β-mercaptoethanol was added and snap-frozen pending RNA purification (Qiagen, Valencia, CA, USA).

Cytokine assays

Levels of IFN-γ, IL-5 and IL-13 in supernatants were measured by ELISA. Supernatant was added to pre-coated IFN-γ, IL-5 and IL-13 ELISA plates (Pierce Endogen, Meridan, Rockford, IL, USA) according to the manufacturer’s instructions. The appropriate biotinylated antibody for each cytokine was used and streptavidin-HRP was added and developed using 3,3′, 5,5′-tetramethylbenzidine (TMB) substrate solution. Absorbance was measured by subtracting the 550 nm values from 450 nm values. Results were calculated using Softmax 4.7 software. The sensitivity of the assays was also within the limits of the manufacturer guidelines. The limit of detection was 2 pg/mL for IL-5, 7 pg/mL for IL-13 and 2 pg/mL for γ-IFN. Allergen-specific levels were determined by comparing levels in the presence and absence of allergen. For a subset of samples (N = 17 for asthma and N = 9 for healthy subjects) for which sufficient RNA was available following GeneChip analysis, levels of cytokine RNA expression were measured using a custom Taqman low density array (TLDA) described as follows.

RNA purification and microarray hybridization

Total RNA was isolated using the Rneasy mini kit (Qiagen). A phenol/chloroform extraction was performed, and the RNA was repurified using the Rneasy mini kit reagents. Eluted RNA was quantified using a Spectramax96 well plate UV reader (Molecular Devices, Sunnyvale, CA, USA) monitoring A260/280 OD values. The quality of each RNA sample was assessed by the integrity of the 28S and 18S peaks by capillary electrophoresis alongside an RNA molecular weight ladder on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). All procedures for assaying samples on GeneChips were performed by Expression Analysis (Expression Analysis, Inc. 2605 Meridian Parkway, Durham, NC, USA). Labelled targets for oligonucleotide arrays were prepared using a modification of the procedure described by Lockhart et al. [41]. Labelled targets were hybridized to the HG-U133A Affymetrix GeneChip Array as described in the Affymetrix technical manual. Eleven biotinylated control transcripts ranging in abundance from 3 parts per million (ppm) to 100 ppm were spiked into each sample to function as a standard curve [42]. GeneChip MAS 5.0 software was used to evaluate the hybridization intensity, compute the signal value for each probe set and make an absent/present call.

Taqman low density arrays

Both to confirm findings from GeneChip and to measure levels of cytokine RNA that were below GeneChip levels of detection, custom TLDA were designed using primers and probes as listed through the Applied Biosystems (Applied Biosystems, Foster City, CA, USA) website (www.appliedbiosystems.com). A 48-assay design was used and the individual assays are listed in supplementary Table S1. Briefly, for samples with sufficient RNA remaining following GeneChip measurements, 100 ng of total RNA was run for each sample per array and each sample was run in duplicate. The samples assayed by TLDA were the allergen-stimulated, allergen plus WAY-196025, and unstimulated sample sets from nine of the healthy and 17 asthma subjects. cDNA synthesis was carried out using a high capacity cDNA archive kit (Applied Biosystems, Product Number 4322171) according to the manufacturer’s protocols. Each cDNA sample (50 μL) was added to an equal volume of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems) and the resulting mixture was added to one of the eight channels on the array. The samples were loaded by centrifugation into the wells that contain the lyophilized primer probe sets. The card was then sealed and PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system according to the manufacturers protocols. (Thermal cycler conditions were as follows: 2 min at 50 °C, 10 min at 94.5 °C, 30 s at 97 °C, 1 min at 59.7 °C for 40 cycles. All steps were carried out according to the manufacturer’s protocol).

Taqman low density array analysis

Expression values were calculated using the comparative Ct method as described (User Bulletin No. 2, Applied Biosystems). Briefly, this technique uses the formula 2−ΔΔCT to calculate the expression of target genes normalized to a calibrator. The threshold cycle (Ct) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. The Ct values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal). The duplicate samples were averaged, then the 2−ΔΔCT value was determined for each gene for each donor using the total gene set average as the calibrator. The fold change was determined for each donor by the following formula: (2−ΔΔCT allergen treated − 2−ΔΔCT no treatment control) = log fold change. The average log fold change was then determined for the healthy volunteer group and for the asthma group by averaging the individual fold changes.

Data normalization and filtering of Affymetrix data

GeneChips were required to pass the pre-set quality control criteria that the RNA quality metric required an
average ratio of β-actin and GAPDH 5′–3′ probes sets >0.4. The signal value for each probe set was converted into a frequency value representative of the number of transcripts present in 10⁶ transcripts by reference to the standard curve [42]. Data for 10,280 probe sets that were called ‘present’ in at least five of the samples and with a frequency of 10 ppm or more in at least one of the samples were subject to the statistical analysis described as follows, while probe sets that did not meet these criteria were excluded.

Statistical analysis of Affymetrix data

The antigen-dependent fold change differences were calculated by determining the difference in the log-2 frequency in the presence and absence of antigen. ANOVA was performed using this metric to identify allergen-dependent differences, and also to identify significant differences between the asthma and healthy groups with respect to the response to allergen. Raw P-values were adjusted for multiplicity of testing according to the false discovery rate (FDR) procedure of Benjamini and Hochberg [43] using Spotfire (Somerville, MA, USA). Significant effects of WAY-196025 were identified by ANOVA comparing the log-2 differences in the groups treated with allergen to the groups treated with allergen and WAY-196025.

Hierarchical clustering

For hierarchical agglomerative clustering of probe sets and arrays, the log-2 scale MAS5 expression values from each probe set were first z-normalized so that each probe set had a mean expression level of zero and a standard deviation of 1 across all samples. Then, these normalized profiles were clustered hierarchically using UPGMA (un-weighted average link) and the Euclidean distance measure.

Ingenuity pathways analysis

Data were analysed through the use of Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, Mountain View, CA, USA (www.ingenuity.com). Asthma-associated gene identifiers and corresponding expression and P-values were uploaded into the application. Gene identifiers were mapped to the corresponding gene objects in the Ingenuity Pathways Knowledge Base. The Focus Genes were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Networks of these Focus Genes were then algorithmically generated based on their connectivity. Functional analysis, canonical pathways and annotations for these genes were also obtained using IPA.

Results

In vitro cytokine expression

We determined the allergen responsiveness of PBMC by measuring the levels of cytokines produced by the PBMC of asthma and healthy subjects following 6 days in vitro stimulation. A 6 day time-point was chosen because at both the mRNA [44] and protein levels [45], an effective response to allergen has been previously demonstrated and our pilot assays with protein and Taqman read-outs before the initiation of this study confirmed these results. ELISA analyses were carried out for IFN-γ, IL-5 and IL-13. As shown in Table 2, all healthy volunteers and approximately 80% of asthma subjects showed a cytokine response to allergen defined as a 1.5-fold or greater increase in the production of at least one cytokine compared with baseline levels. Although the highest levels observed for Th2 cytokines IL-5 and IL-13 were in the asthma group and the highest levels of Th1 cytokine IFN-γ were observed in the healthy group, the statistical power of this experiment was not sufficient to detect a significant difference between these groups with respect to allergen-specific cytokine production.

The samples were also treated with the cPLA₂ inhibitor WAY-196025 or zileuton in the presence of allergen and cytokine measurements were taken. Both drugs significantly reduced IL-13 secretion (P-value = 0.001 for the WAY-196025 treatment and P-value = 0.0005 for zileuton

| Cytokine | Healthy subjects | Asthma subjects |
|----------|------------------|-----------------|
|          | Responders/ total tested | Range without allergen (pg/mL) | Average without allergen (pg/mL) | Range with allergen (pg/mL) | Average with allergen (pg/mL) | Responders/ total tested | Range without allergen (pg/mL) | Average without allergen (pg/mL) | Range with allergen (pg/mL) | Average with allergen (pg/mL) |
| IFN-γ    | 11/11 (100%)     | 25–55           | 29              | 41–1080          | 343                     | 17/23 (74%)     | 25–92           | 29              | 25–863          | 183.3                  |
| IL-5     | 4/11 (36%)       | 6–110           | 35              | 6–148           | 42.6                    | 12/23 (52%)     | 6–284           | 44              | 6–243           | 60.3                   |
| IL-13    | 3/11 (27%)       | 25–699          | 149             | 24–305          | 118                     | 13/23 (57%)     | 25–188          | 51.5            | 25–510          | 118                    |

Production of cytokine was measured using ELISA assays on the supernatants from PBMC cultures after 6 day allergen stimulation as described in ‘Methods’. Subjects were classified as positive responders if, in the presence of allergen, cytokine production was increased at least 1.5-fold.

PBMC, peripheral blood mononuclear cell.
Peripheral blood mononuclear cell expression profile demonstrates response to allergen in both the healthy and asthma groups following in vitro stimulation with allergen

Gene expression levels from the asthma (n = 26) and healthy subjects (n = 11) were measured using Affymetrix GeneChip. From the 10 280 probe sets that met the inclusion criteria described in 'Methods', we identified 130 probe sets (representing 123 unique genes) that demonstrated a similar response to allergen in both the asthma and healthy groups. Similar response to allergen was defined as an average allergen-dependent fold change ≥1.5 in both groups, and no significant difference (FDR ≤0.051) between the asthma and healthy groups with respect to allergen-dependent changes. The complete list of these 130 probe sets and their descriptions are included in supplementary Table S2. Genes on this list include IL-9 [46–49], IL-17 [50, 51], IL-22 [52–55], IL-26 [56, 57] and genes such as CXCL1, CXCL2 and CXCL3 [58, 59], immune system genes that are involved in chemotaxis and activation of lymphoid cells, and MMP2 and MMP12 [60], implicated in degradation of the extra cellular matrix.

Peripheral blood mononuclear cell expression profile distinguishes asthma patients from healthy volunteers

Comparison of the expression levels of the 10 280 probe sets in the asthma and healthy subjects identified 167 probe sets (representing 153 unique genes) whose allergen-dependent changes differed significantly (FDR < 0.051) between asthma and healthy subjects. These genes also showed an allergen-dependent fold change > 1.5 in at least one group. The complete list of the 167 probe sets and, for each, the significance of the difference between the groups is shown in supplementary Table S3. A visualization of the differences between asthma and healthy subjects with respect to allergen-dependent changes in expression level of all 167 probe sets is shown in Fig. 1. The visualization was generated using an algorithm that groups subjects based on the similarities with respect to allergen-dependent gene expression changes. With one exception, all the healthy subjects were grouped together, and 22 of the 26 asthma subjects were grouped together. Table 3 shows 50 genes – a subset of genes that showed a significant difference between asthma and healthy subjects with respect to the response to allergen. The genes shown in Table 3 were associated with an allergen response of 1.5-fold or more increase or decrease in the asthma group, while having a <1.1-fold response to allergen in the healthy volunteer population. In this list are genes previously associated with the asthma phenotype including the Zap70 and LCK tyrosine kinases [61], the toll-like receptor 4 (TLR4) [62, 63], and complement component 3a receptor 1 (C3AR1) [64–68]. Allergen responsive genes not previously shown to be involved in the asthma phenotype included sialoadhesin (SN1-CD163) [69], and a disintegrin/metalloprotease, ADAM19 [70].

Confirmation of the Affymetrix results was carried out utilizing the custom TLDA described above using the subset samples used in the Affymetrix-based experiment that had sufficient RNA remaining (n = 19 for the healthy group and n = 9 for the asthma group. (See supplementary Table S1 for genes assayed by TLDA). Twenty-five genes were assayed both by GeneChip and by TLDA. The concordance between platforms was assessed by comparing the average allergen dependent fold change metric as measured by GeneChip and the average allergen-dependent ΔACt metric as measured by TLDA. As shown in Fig. 2, excellent correlation was observed (Pearson’s correlation = 0.910) indicating a tight concordance in the results obtained from the two platforms. While the power of the GeneChip and TLDA were not comparable due to the differences in sample size and therefore P-values cannot be directly compared, the trend of the change was consistent for all genes. (The benefit of the increased sensitivity of the TLDA can be seen in the results for IL-18, where the difference detected by GeneChip did not pass the significance filters but a significant difference was observed by TLDA.)

Because atopic subjects constituted the majority (77%) of the asthma group and none of those in the healthy group were atopic, we examined whether the differences observed between asthma and healthy groups could be attributable to atopy status rather than asthma. Comparisons between the healthy group and the asthma group exclusive of the non-atopic group reduced the significance of association with the asthma in all cases, indicating that the observed differences were not attributable to atopy status (data not shown). Moreover, comparison of the atopic and non-atopic profiles did not reveal genes expressed at significantly different levels between the groups.
A selective cPLA2α inhibitor alters the expression profile of allergen-responsive genes in asthma and healthy subjects

Treatment of allergen-stimulated samples with the 5-lipoxgenase inhibitor, zileuton, did not cause changes that passed the filters set for significant effect. In contrast, the asthma-specific gene expression of a majority of the 167 probe sets associated with the asthma response to allergen was altered significantly in the presence of the WAY-196025 cPLA2α inhibitor when compared with allergen treatment alone. Results, including fold change and significance metrics in the comparison of groups with and without cPLA2α inhibition, are shown in supplementary Table S4. The probe sets fall into three distinct categories. In the first category, probes that correspond to genes that were up-regulated in asthma samples in response to allergen, such as ZAP70, LCK and mini-chromosome maintenance protein (MCM2), are reduced to the levels seen in the allergen-treated healthy controls. In the second category, genes that were initially down-regulated in the asthma samples in the presence of allergen, such as sialoadhesin (SN), pleckstrin (PLEK) and PLAU are up-regulated in the presence of inhibition. Lastly, there is a group of genes that were down-regulated in the presence of allergen in the asthmatic samples that are not affected by cPLA2α inhibition, these genes include CD84 and tissue inhibitor of metalloproteinase 3 (TIMP3).

A hierarchical cluster analysis was performed to visualize the differences associated with cPLA2α inhibition for the 167 asthma-associated probe sets (Fig. 3). This visualization separated the asthma samples into the following three groups based on similarities in gene expression pattern: (1) asthma samples treated with allergen, (2) asthma samples treated with allergen and WAY-196025 and (3) a small population consisting of a mix of samples treated with allergen or allergen + WAY-196025. Interestingly, group 3 contains the same subjects who originally clustered with the healthy samples in response to allergen (see Fig. 1).

Functional annotation of gene expression

To explore the functional relatedness of the allergen-responsive genes and identify associated pathways, the asthma-associated allergen-responsive gene list (167 probe set) was functionally annotated using IPA. Of the 167 probes initially entered into the analysis, 127 met the criteria for inclusion in pathway analysis. The criteria are based on the ingenuity knowledge base and on our
Table 3. Gene expression differences between asthma and healthy subjects in response to allergen

| Symbol | Description | Function | AOS fold change | WHV fold change |
|--------|-------------|----------|----------------|----------------|
| A      |             |          |                |                |
| ZWINT  | ZW10 interactor | Kinetochore function | 1.78 | 1.08 |
| FLJ23311 | FLJ23311 protein | DNA binding and inhibits cell growth | 1.77 | 1.01 |
| PRC1   | Protein regulator of cytokinesis 1 | Cytokinesis | 1.74 | 1.09 |
| CD28   | CD28 antigen (T-p44) | Antigen processing | 1.74 | 1.09 |
| PCNA   | Proliferating cell nuclear antigen | DNA synthesis | 1.73 | 1.03 |
| RANBP5 | Karyopherin (importin) beta 3 | Nuclear/cytoplasmic transport | 1.72 | 1.06 |
| ZAP70  | Zeta-chain (TCR) associated protein kinase 70kDa | T cell function | 1.72 | 1.00 |
| CD3D   | CD3D antigen, delta polypeptide (Tf73 complex) | T cell function | 1.71 | 1.10 |
| MELK   | Maternal embryonic leucine zipper kinase | Stem cell renewal, cell cycle progression, and pre-mRNA splicing | 1.71 | 1.08 |

| B      |             |          |                |                |
|--------|-------------|----------|----------------|----------------|
| GM2A   | GM2 ganglioside activator | Glycolipid transport | −2.05 | −1.02 |
| SLC36A1 | Solute carrier family 36 (proton/amino acid symporter), member 1 | Small amino acid transporter | −1.90 | 1.01 |
| TM6SF1 | Transmembrane 6 superfamily member 1 | Unknown | −1.75 | −1.16 |
| LCK    | Lymphocyte-specific protein tyrosine kinase | T cell function/immune response | 1.53 | 1.09 |
| GPX7   | Glutathione peroxidase 7 | Oxidative stress response | 1.53 | 1.06 |
| RAD21  | RAD21 homolog (S. pombe) | DNA repair/mitosis | 1.53 | 1.03 |
| PTIT1  | Pituitary tumor-activating 1 | Tumorigenic/chromatid separation | 1.52 | 1.10 |
| C6orf149 | Chromosome 6 open reading frame 149 | Unknown | 1.52 | 1.06 |
| SNRD3  | Small nuclear ribonucleoprotein D3 polypeptide 18kDa | Pre-mRNA splicing and small nuclear ribonucleoprotein biogenesis | 1.52 | 1.03 |
| FYN    | FYN oncogene related to SRC, FGR, YES | Cell growth, immune cell signalling | 1.51 | 1.02 |

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Table 3. continued

| Symbol  | Description                                                                 | Function                                      | AOS fold change | WHV fold change |
|---------|------------------------------------------------------------------------------|-----------------------------------------------|-----------------|-----------------|
| HLA-DMB | Major histocompatibility complex, class II, DM beta                          | Antigen presentation                          | −1.56           | −1.01           |
| RNF13   | Ring finger protein 13                                                       | Unknown                                       | −1.56           | −1.08           |
| PRNP    | Prion protein (p27–30)                                                       | Prion diseases/oxidative stress               | −1.55           | −1.02           |
| GAS7    | Growth arrest-specific 7                                                     | Neuronal differentiation                      | −1.53           | −1.10           |
| ATP6V1A | ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A                      | Acidification of eukaryotic intracellular organelles | −1.52           | 1.02            |
| ATP6V0D1| ATPase, H+ transporting, lysosomal 38 kDa, V0 subunit d isoform 1            | Acidification of eukaryotic intracellular organelles | −1.51           | −1.09           |

Probes (genes) that changed 1.5-fold or greater in the asthma subjects (AOS) but changed less than 1.1-fold in the healthy volunteers (WHV), an FDR cutoff of \( \leq 0.051 \). (A) Genes up-regulated in asthma subjects 1.5-fold or higher compared with healthy volunteers. (B) Genes down-regulated by 1.5-fold or more in asthma subjects compared with healthy volunteers.

FDR, false discovery rate.

statistical filters as described above. Seven well-populated functional networks were generated based on this information. The top functions for the networks created using IPA include immune and lymphatic system development and function, immune response, DNA replication, recombination and repair. The top-scoring network (Network 1) consisted of 35 genes involved in immune response and cell cycle (Fig. 4a). Genes in this network that were up-regulated in the asthma compared with the healthy subjects included the T cell receptor signalling genes CD3D, CD28 and ZAP70 [71–73]. As expected, based on the selection criteria, the expression levels (depicted by the gene colour intensities) in Network 1 for the healthy volunteer population looked very different from the asthma subjects. However, in the healthy subjects, a few of the genes were down-regulated in response to allergen similarly to the asthma subjects, but to a significantly lesser extent. This set of genes includes cathepsin B (CTSB), TIMP3 and CD36 antigen (collagen type I receptor, thrombospondin receptor) (Fig. 4b).

The striking effect of cPLA2 inhibition on allergen-induced asthma-associated gene expression changes can be illustrated by utilizing IPA. In this analysis, the expression values obtained in the presence of the inhibitor were overlaid into the gene set created based on asthma-specific allergen gene changes. Every gene in Network 1 in the asthma group has an altered level of expression in the presence of the inhibitor (Fig. 4c). In the healthy population, the few genes that were down-regulated in response to allergen in Network 1 are brought back to the non-allergen-stimulated background levels in the presence of the inhibitor (data not shown).

Discussion

The study reported here was conducted to investigate (a) how effects of in vitro exposure to allergen differ between asthma and healthy subjects, and (b) the involvement of the cPLA2 pathway in the processes identified as different between the two groups. We approached these important objectives by seeking to identify differences between the healthy and asthmatic phenotypes at the molecular level. Transcriptional profiling methods have been employed in numerous previous studies as exploratory screening tools independent of pre-existing disease paradigms [74–76]. This gene expression profiling study was conducted to compare, at the gene expression level, the response of asthmatic and healthy subjects PBMC samples to in vitro stimulation with a cocktail of common allergens. Subject samples in both groups responded to
allergen as assessed by allergen-dependent cytokine stimulation both by ELISA, and, at the RNA level, by TLDA. In addition, using both Affymetrix GeneChip and TLDA platforms, we have identified many significant allergen-dependent gene expression differences between the asthma and healthy groups, and those differences are the focus of this study. We have extended this analysis further to include the effects of inhibition of the cPLA$_2$ pathway on gene expression patterns significantly associated with the asthma group.

The specific allergens used in this study are common environmental antigens and we report on many similarities in the in vitro responses to allergen among asthma and healthy subjects. Given the robust allergen responses that did not differ significantly between asthma and healthy subjects, our data indicate that the standard of care treatment that the asthma subjects were receiving did not prevent robust responses in this 6 day culture experimental system. Among genes that did not differ significantly between groups in the response to allergen are some chemokines and ILs. Some of these genes have previously been associated with the asthma phenotype including those involved in the T cell response such as IL-17 [77, 78] and IL-9 [49, 79] but in general, genes that have previously shown to be involved in the asthma subject response were modulated by allergen stimulation to a greater extent in the asthma group than in the healthy group. For example, the chemokine ligand 1 (CCL1) [80] and CCL18 [81] have recently been shown to be involved in the asthma phenotype and are up-regulated to a greater extent in the asthma group. Also contained within this gene set were genes not classically associated with the immune response, including those involved in protective stress responses such as the metallothionein (MT) gene family, MT2A and MT1X [82, 83] as well as those involved in glucose transport, GLUT-3 and GLUT-5 [84, 85]. Some of the genes with similarly increased expression in the presence of allergen in both the asthma and healthy volunteer groups included red blood cell specific genes, reflecting better 6 day in vitro survival of the small percentage of contaminating red blood cells under conditions of an in vitro immune response. This finding indicates that at least some of the allergen-dependent differential expression commonly observed in the two groups was due to differences in culture conditions in the presence and absence of an in vitro immune response to

Fig. 3. Gene expression profiling demonstrates differential modulation of 167 probes in the asthma subjects in response to allergen in the presence of cPLA$_2$ inhibitor. An unsupervised clustering algorithm, which determines similarities between subjects independent of group membership, was used to generate this visualization. Subjects are shown in columns, and genes in rows. Red indicates an allergen-dependent change higher than the mean. Green indicates an allergen-dependent change lower than the mean. Subjects are grouped according to the degree of similarity in expression pattern: A, asthmatic allergen-dependent fold change. $A^{+/-}$, effect of cPLA$_2$ inhibitor on allergen-dependent fold change. The numbers following the letters A or $A^{+}$ correspond to the individual donor ID.
allergen. Such differences were, however, very similar in the two groups, supporting the conclusion that response to allergen occurred in both groups and that cell survival was similar in both groups.

Although the asthma group contained a majority of atopic subjects and the healthy group contained no atopic subjects, our findings tend to support a conclusion that the observed differences are attributable to asthma and not to atopy status. If the findings were attributable to atopy status, the significance of the association for any given gene with the group containing the atopic samples would be predicted to increase when the non-atopic samples were excluded from analysis. The opposite was the case – the significance of association with the asthma group decreased for all genes reported in supplementary Table S3 when non-atopic asthmatics were removed from the analysis.

Fig. 4. Allergen-responsive genes specific to the asthma group are modulated in response to the cPLA₂α inhibitor. Network profile generated by Ingenuity Pathways Analysis (Ingenuity Systems). Genes are colour-coded according to whether they were up-regulated (red) or down-regulated (green) in the asthmatic samples compared with the healthy volunteer samples. (a) Asthma-specific allergen response, (b) healthy volunteer specific allergen response, (c) cPLA₂α inhibitor affect on the asthma-specific allergen response. The symbols represent the type of protein that is coded for by the above genes. Included are: Transporter, \( \text{\textbullet} \); transcription regulator, \( \text{\textcircled{\textcircled{\textbullet}}} \); phosphatase, \( \text{\textcircled{\textbullet}} \); enzyme/peptidase, \( \text{\textdiamond} \); other, \( \text{\textcircled{\textcircled{\textcircled{\textbullet}}}} \).
asthma group failed to reveal any significant differences. While failure to detect such differences could be due to the reduced power in this comparison, the results indicate that the differences observed in the asthma vs. healthy comparison are not attributable to atopy.

Comparison of the expression levels of the 10,280 probe sets in the asthma and healthy subjects identified 167 probe sets (representing 153 unique genes) whose allergen-dependent changes differed significantly (FDR ≤ 0.051) between asthma and healthy subjects following 6 days in vitro stimulation. In addition, TLDA analysis also identified IL-15, IL-18 and IL-13RA1 as significantly associated with the response to allergen in the asthma group. The identification of a relatively large subset of genes that distinguish between asthma and healthy subjects in this in vitro study with relatively few subjects underscores the power of the global profiling approach in elucidating differences between groups that had not been observed previously. In fact, despite the standard of care therapy that the asthma subjects were receiving, several genes were identified that were previously shown to be involved in the asthma phenotype. These include complements C3AR1 [64–68] and the TLR4 [62, 63]. C3AR1 [86, 87] is involved in Th2 inflammatory responses [64]. C3AR knockout mice challenged with allergens have a decrease in AHR, airway eosinophils and IL-4 producing cells relative to wild-type mice [64]. The TLRs are a family of proteins that enhance certain cytokine gene transcription levels in response to pathogenic ligands [88, 89], and TLR4 responds to endotoxin [90, 91]. Our data demonstrate that, under these in vitro conditions (6 days in culture), the TLR4 was differentially modulated in asthma subjects in the presence of allergen. Recent evidence suggests that TLR4 is important in the asthma phenotype, although the data is conflicting [62, 92], but discrepancies may be attributable to differences in experimental systems [93]. The results reported here implicate TLR4 as associated with the asthma subjects’ in vitro response to allergen. In a study comparing TLR4 expression levels in PBMC of asthma and healthy subjects before culture, and TLR4 expression levels show a highly significant association with asthma (manuscript in preparation). Also included in this list is the LCK tyrosine kinase. Two LCK GeneChip probe sets give discordant results. Such discordant results have been occasionally observed in other studies also, and Taqman analysis has indicated that discordance can be due to differences in expression of splice variants (data not shown), although in the case of LCK, the explanation of the discrepancy is unknown. TLDA analysis of LCK in the study reported here indicates down-regulated in asthma group.

The majority (approximately 80%) of the 167 differentially regulated probes have not been previously shown to be involved in the asthma phenotype. Among these are ATPase transporters, ATP6V0D1, ATP6V1A and ATP6AP1 and the CD antigens, CD163, CD169, CD84 and CD59, and PRNP, which is expressed on a variety of immune cell types. Macrophages obtained from mice that do not express PRNP have higher rates of phagocytosis than the wild-type cells in vitro [94]. Available data on the importance of macrophages in the asthmatic phenotype does not inform on the significance of macrophage PRNP in the asthma phenotype [95]. However, alveolar macrophages [96] play a role in innate immune responses and these responses have been shown to affect the severity of asthma [97] and bronchoconstriction in asthma [98]. Genes modulated in the allergen-treated PBMC of asthma subjects that have not previously been associated with asthma also include the following MCMs: MCM2, MCM5 and MCM7 along with polycomb group ring finger 4 protein, BMI1. BMI1 is involved in lymphoproliferation [99] and is implicated in T cell differentiation [100]. Therefore, the lymphoproliferative effect of BMI1 could be important in the asthmatic phenotype, perhaps playing a role in increasing the amount of CD4+ T cells in the lungs of asthma subjects [101].

Global profiling also revealed allergen-dependent, asthma-associated differential expression of several genes involved in regulating the oxidative state that had previously not been associated with the asthma phenotype. Reactive oxygen species are beneficial in host defence mechanisms. However, the production of these species can be pro-inflammatory in diseases such as asthma. Included among these are a glutathione peroxidase, glutathione peroxidase 7 (GPX7), the non-selenoperoxidase, peroxiredoxin 2 (PRDX2) and the microsomal glutathione S-transferase 3 (MGST3) gene. GPX proteins, such as GPX-1 have previously shown to be responsible for suppression of 5-LO activity in a monocytic cell line [102], but this is the first evidence that GPX7 may also be a factor in the asthmatic response to allergen. MGST3 is responsible for the conversion of LTA4 to LTC4, and both are important mediators of the arachidonic acid pathway. Lastly, the peroxiredoxin family of proteins are responsible for the reduction of a broad spectrum of peroxides [103] and have been shown to be up-regulated in lung carcinomas [104], but have not previously been associated with asthma.

cPLA2 catalysts the first step in the biosynthesis of arachidonic acid-derived leukotrienes and prostaglandins [24, 25] and is theoretically an attractive target for inhibition in the treatment of inflammatory diseases [105]. Anti-leukotriene therapy acting either at the level of 5-lipoxygenase or CysLT1 receptor has proven beneficial in the treatment of asthma [106]. Prostaglandin D2 and other prostaglandins may also mediate the hallmarks of asthma including airway inflammation AHR and bronchoconstriction [107]. cPLA2 knock-out mice are resistant in mouse model of asthma [105], and inhibitor WAY-196025 used in this study [27] and other cPLA2 inhibitors have shown efficacy in animal models of
asthma [108]. The *in vitro* allergen challenge model system was used in this study to evaluate the molecular effects of cPLA$_2$ inhibition in PBMCs. The inhibition of cPLA$_2$ had striking effects on the allergen-related, asthma-associated profile. The hierarchical clustering analysis revealed that cPLA$_2$ inhibition affected a shift in the asthma-associated allergen-dependent profile to a profile closely resembling the profile of healthy volunteers with approximately 80% of the probes changing significantly (FDR $\leq 0.051$) with treatment. As discussed in 'Results' and shown in Fig. 4c, all T cell responsive genes in the pathway depicted in Fig. 4a were significantly changed towards the levels in the healthy subject group by cPLA$_2$ inhibition. This result is noteworthy given that CD4$^+$ T cells are believed critical for the development and maintenance of the disease [109]. Other immune-related genes were also down-regulated by cPLA$_2$ inhibition including, CD28 and CD3D, IL-21R and the transcription factor, high-mobility group box 1 protein (HMGB1). The HMGB1 result is of particular interest as this protein has been shown to be a distal mediator of acute inflammation of the lung linked to an increased production of pro-inflammatory cytokines [110].

The effect of cPLA$_2$ inhibition on gene expression is more noteworthy in that it was not replicated by zileuton – suggesting that the effect seen with cPLA$_2$ inhibition is not strictly mediated by leukotrienes, but is at least partially due to the inhibition of a greater range of lipid mediators including the prostaglandins, platelet-activating factor and products of 12 and 15-lipoxygenases [27, 30]. As mentioned above, the effects of zileuton on gene expression did not pass the filters set for significance in the GeneChip portion of the study and are much more subtle. However, protein analysis revealed a significant effect of zileuton on IL-13 production at the 6 day time-point assayed, indicating that gene expression changes may have been detectable at earlier untested time-points.

Peripheral blood is easily accessible, and the transcriptome of PBMCs can be studied both directly upon collection and following *in vitro* stimulation. We have employed an *in vitro* model system to identify disease-related transcriptional profiles and to model the response of PBMCs in the clinical setting to drug exposure using an experimental inhibitor of cPLA$_2$. The results of this global profiling study have yielded broad ranging findings on the significant differences and similarities between asthma and healthy subjects as revealed by *in vitro* allergen responsiveness. The scope and size of the study have allowed us to confirm some previously reported asthma associations, and to discover novel association that were not predictable based on the pre-existing information. These results clearly demonstrate that global transcriptional profiling has utility as a sensitive exploratory tool to study molecular mechanisms of disease and pathways affected by candidate therapeutics.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.
Table S1. Genes assayed on TLDA.
Table S2. Allergen-responsive genes in healthy and asthmatic subjects.
Table S3. One hundred and fifty-three genes differentially regulated in asthmatics in response to allergen.
Table S4. WAY196025 – cPLA2 inhibitor.

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