

# Peripheral Blood Gene Expression Changes during Allergen Inhalation Challenge in Atopic Asthmatic Individuals

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**Objectives.** (1) To investigate the effects of globin mRNA depletion in detecting differential gene expression in peripheral blood and (2) to investigate changes in peripheral blood gene expression in atopic asthmatic individuals undergoing allergen inhalation challenge. **Methods.** Asthmatic subjects (20–60 years of age, with stable, mild allergic asthma,  $n = 9$ ) underwent allergen inhalation challenges. All had an early asthmatic response of  $\geq 20\%$  fall in forced expiratory volume in 1 second. Blood was collected immediately prior to and 2 hours after allergen challenge using PAXgene tubes ( $n = 4$ ) and EDTA tubes ( $n = 5$ ). Aliquots of the PAXgene blood samples were subjected to globin reduction (PAX-GR). Transcriptome analysis was performed using Affymetrix GeneChip® Human Gene 1.0 ST arrays. Data were preprocessed using factor analysis for robust microarray summarization and analyzed using linear models for microarrays. Pathway analyses were performed using Ingenuity Pathway Analysis. **Results.** Globin reduction uncovered probe sets of lower abundance. However, it significantly reduced the ability to detect differentially expressed genes (DEGs) when compared to non-globin-reduced PAXgene samples (PAX-NGR). Combined transcriptional analysis of four PAX-NGR and five EDTA sample pairs identified 1595 DEGs associated with allergen inhalation challenge (false discovery rate  $\leq 5\%$ ), with the top-ranked network of perturbed biological functions consisting of inflammatory response, cellular movement, and immune cell trafficking. **Conclusions.** While we have demonstrated a diminished ability to detect DEGs after globin reduction, we have nevertheless identified significant changes in the peripheral blood transcriptome of people with mild asthma 2 hours after allergen inhalation challenge.

**Keywords** factor analysis for robust microarray summarization, globin mRNA reduction, linear models for microarrays, microarray analysis

## INTRODUCTION

Genome-wide expression profiling (transcriptomics) has become one of the more powerful approaches to explore disease mechanisms, to assist diagnosis, and to determine better treatment strategies. Although specific target tissues and cells are ideal biological materials for gene expression studies, they are usually difficult to obtain. In recent years, peripheral blood mononuclear cells (PBMCs) have been widely used for gene expression studies in biomedical research as well as in clinical services. Since isolation of blood cell-type fractions is time consuming, expensive, and likely to introduce additional technical variation, peripheral whole-blood material has become increasingly popular among researchers conducting gene expression analyses. There are several advantages to using peripheral whole-blood cells in genome-wide expression profile analysis. They are very easy to obtain and are the most commonly used biological material in the clinical setting. In addition, peripheral whole blood contains various cell types, many of

which participate in the immune response and transport systems that contribute heavily to various biochemical conditions and disorders. Accumulating evidence has documented their usefulness over PBMCs in gene expression profile research in large-scale studies (1, 2).

However, there are some challenges in the use of peripheral whole-blood cells in genome-wide expression profile analysis. It was reported that globin mRNA accounts for 70% of total mRNA in whole blood, leading to decreased sensitivity due to increased variability in non-globin mRNA detection (3). Various methodologies have been developed to reduce the amount of globin mRNA in a sample, but globin mRNA reduction itself may have adverse effects on gene expression analysis, by increasing technical variability and hence reducing reproducibility (4). Inconsistent results indicate that it is necessary to optimize assays of different microarray platforms to check if globin mRNA reduction is beneficial (4).

Allergen inhalation challenge is a useful model to investigate the pathogenesis of allergic airway diseases (5). Nearly all atopic asthmatic individuals experience an early-phase asthmatic response that peaks at about 30 minutes after exposure to allergen. Many of these individuals also have a late-phase asthmatic response, which begins 3–7 hours after allergen exposure (5). Of particular

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interest is the 2-hour time point, which is when the early-phase response has ended, but before the late-phase response has begun (at least, as measured by forced expiratory volume in 1 second [FEV<sub>1</sub>]). For individuals who exhibit the latter response, it may be during this time that triggers for the late-phase response are being activated. Therefore, understanding the molecular mechanisms that contribute to these response patterns, especially at a 2-hour time point, may be helpful in identifying novel targets for therapeutic interventions. Transcriptomic analysis has been extensively used to investigate the mechanisms of allergen challenge in animal models of asthma (6–8). However, its application to the study of allergen exposure in human asthmatic individuals is not very common.

We therefore undertook a study of allergen inhalation challenges in human asthmatic subjects with the following aims: (1) to determine if globin mRNA reduction is more beneficial than non-globin mRNA reduction; and more importantly (2) to assess if genome-wide gene expression differences exist in the peripheral blood when comparing between pre- and 2 hours post-allergen inhalation challenge.

## METHODS

### *Subjects and Allergen Inhalation Challenge*

This study was approved by Institutional Review Boards with informed consents obtained in compliance with the Research Ethics Board of each recruiting center (UBC-PHC—H09-02114; McMaster—08-225). Nine subjects (20–60 years) with stable, mild atopic asthma were recruited from McMaster University (six subjects) and Vancouver General Hospital—UBC (three subjects). Subject demographics are listed in Table 1. Diagnosis of asthma was based on GINA criteria. All subjects were non-smokers, free of other lung diseases, and not pregnant. All had a baseline FEV<sub>1</sub> ≥ 70% of predicted, and the provocative concentration of methacholine required to produce a 20% decrease in FEV<sub>1</sub> (PC<sub>20</sub>) was ≤ 16 mg/mL (9). The methacholine inhalation challenge as described by Cockcroft (10, 11) and the skin prick test were used to determine the dose of allergen for inhalation (12). One day

after methacholine challenge, all subjects underwent allergen inhalation challenges as described by O'Byrne et al. (13), using extracts of house dust mite (*Dermatophagoides pteronyssinus*), cat dander, and ragweed and grass (Timothy or Orchard) pollen (14). All subjects developed an early fall in FEV<sub>1</sub> of ≥ 20% from baseline between 0 and 3 hours, and six subjects also developed a late-phase response (FEV<sub>1</sub> drop ≥ 15%) between 3 and 7 hours. Blood was drawn immediately before allergen inhalation challenge and approximately 2 hours after allergen inhalation.

### *Blood Sample Collection and RNA Extraction*

Peripheral venous blood samples from four subjects were collected into PAXgene Blood RNA tubes (PreAnalytiX—Qiagen/BD, Valencia, CA, USA) (subjects 1–4, Table 1). For the remaining five subjects, peripheral blood samples were collected into K2 EDTA Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) (subjects 5–9, Table 1). The samples collected pre- and post-challenge were frozen and transported to the laboratory on dry ice, where they were stored at –80°C until RNA extraction. From thawed PAXgene tube samples, total intracellular RNA was purified from 2.5 mL of whole blood according to manufacturer's protocols using the RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA). Total RNA was isolated from EDTA tube samples (3.0 mL of whole blood) following a modified TRIzol-based extraction method. The yield and quality of RNA were assessed by NanoDrop 8000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

### *Reduction of Globin mRNA*

Aliquots of RNA isolated from PAXgene tube samples taken from the four individuals were subjected to globin transcript reduction processes using the GLOBINclear™—Human kit (Ambion—AB, Austin, TX, USA). This was performed by Capture Oligo Mix and Streptavidin Magnetic Beads, following manufacturer-recommended protocols. Thus, these four subjects have provided RNA samples that have undergone globin reduction (PAX-GR)

TABLE 1.—Subject demographics.

| Cohort                               | Subject | Site | Age (years) | Sex | MCh PC <sub>20</sub><br>(mg/mL) | Allergen      | Percentage of fall<br>in FEV <sub>1</sub> (early) (%) | Percentage of fall<br>in FEV <sub>1</sub> (late) (%) |
|--------------------------------------|---------|------|-------------|-----|---------------------------------|---------------|-------------------------------------------------------|------------------------------------------------------|
| PAXgene (NGR and GR) (four subjects) | 1       | Van  | 36          | M   | 3.2                             | Timothy grass | –61                                                   | –38                                                  |
|                                      | 2       | Van  | 35          | F   | 0.64                            | Timothy grass | –27                                                   | –5                                                   |
|                                      | 3       | Van  | 47          | M   | 0.28                            | Orchard grass | –23                                                   | –17                                                  |
|                                      | 4       | McM  | 21          | F   | 1.09                            | HDMDP         | –32.1                                                 | –12.5                                                |
| EDTA (five subjects)                 | 5       | McM  | 20          | F   | 9.62                            | Cat           | –37.7                                                 | –17.3                                                |
|                                      | 6       | McM  | 27          | F   | 1.45                            | HDMDP         | –43.3                                                 | –16.7                                                |
|                                      | 7       | McM  | 23          | M   | 10.9                            | Cat           | –31.4                                                 | –15.1                                                |
|                                      | 8       | McM  | 60          | F   | 2.19                            | Cat           | –25.5                                                 | –6.7                                                 |
|                                      | 9       | McM  | 22          | M   | 4.28                            | HDMDP         | –22.7                                                 | –19.9                                                |

Note: FEV<sub>1</sub>, forced expiratory volume in 1 second; HDMDP, house dust mite (*Dermatophagoides pteronyssinus*); NGR, non-globin reduced; GR, globin reduced; MCh PC<sub>20</sub>, methacholine PC<sub>20</sub> (provocative concentration of methacholine required to produce a 20% decrease in FEV<sub>1</sub>); Van, Vancouver General Hospital—UBC; McM, McMaster University; M, male; F, female.

as well as samples that have not undergone the procedure (PAX-NGR).

#### Microarray Procedure

For genome-wide expression profiling, labeling and array hybridization were performed by the Centre for Translational and Applied Genomics at the BC Cancer Agency (Vancouver, BC, Canada), an Affymetrix-certified service provider. Affymetrix Human Gene 1.0 ST arrays were used (Affymetrix, Santa Clara, CA, USA), which provide whole transcript coverage and cover only well-annotated exons (15). The pre- and post-challenge RNA samples from each subject were processed at the same time to avoid possible confounding batch effects. All microarray data have been deposited in the Gene Expression Omnibus (approved GEO Series GSE34172).

#### Statistical Analysis

The “affy” package in R (statistical computing program) was used to read in CEL files (raw probe intensity data) and to annotate the affyIDs. Preprocessing and filtering of probe intensity data were performed using factor analysis for robust microarray summarization (FARMS) and informative/non-informative (I/NI) calls (16, 17). FARMS carries out probe summarizations through quantile normalization following background correction and uses perfect match values only, making it applicable to Gene ST arrays (16). I/NI calls filters probe sets into categories of “informative” and “non-informative,” with “informative” probe sets being the ones advanced to the next step of data analysis. Probe sets are considered “informative” if many of their constituent probes consistently reflect the same increase or decrease in mRNA levels across arrays (highly correlated), whereas probe sets that do not demonstrate a consistent probe behavior are classified as “non-informative” (17). The use of NI calls for filtering of probe sets avoids having to set arbitrary thresholds such as for overall variance and overall mean, which may eventually affect downstream analyses (18). Both FARMS and I/NI calls were accessed through the “farms” package (version 1.4.0, Institute of Bioinformatics, Johannes Kepler University Linz, Linz, Austria) which carried out the preprocessing and filtering of non-informative probes in combination (19, 20).

Following this, moderated robust regression in the linear models for microarrays (limma) package (Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) (19) was used to determine differential changes in gene expression between pre- and post-challenge. All analyses were performed in R (version 2.13.0) (21), and a Benjamini–Hochberg false discovery rate (FDR) of 5% was used (22). Two statistical analyses were conducted. The focus of the first was to investigate the effects of globin reduction on the detection of differentially expressed probe sets and differentially expressed genes (DEGs), pre- to post-allergen challenge. To accomplish this, independent preprocessing (FARMS) and statistical analyses (limma) were performed using the eight PAX-NGR samples and the eight PAX-GR samples (paired analysis). Due to the limited sample size, covariates such as sex,

age, PC<sub>20</sub>, FEV<sub>1</sub>, and RNA integrity number (RIN) were not included in this analysis. The second analysis focused on the study of differential gene expression due to allergen inhalation challenge. Data from the five EDTA sample pairs were combined with those of the four PAX-NGR sample pairs into one analysis in order to assess differential gene expression between pre- and post-challenge. Covariates included tube type (PAXgene vs. EDTA), sex, age, PC<sub>20</sub> (provocative concentration of methacholine) at pre-challenge, FEV<sub>1</sub> after onset and 2 hours after challenge, and RIN.

Subsequently, pathway analyses were carried out using Ingenuity Pathway Analysis (IPA—Ingenuity Systems, Redwood City, CA, USA) (23). The list of significant genes (FDR ≤ 5%) identified by the combined limma analysis of EDTA and PAX-NGR samples was imported, and the pathway analysis parameters were restricted to consider genes relevant to human immune cells, lung tissue, and immune cell lines only. Genes that passed this filter were deemed to be eligible to be organized into networks and canonical pathways which were developed from information contained in the Ingenuity Knowledge Base.

## RESULTS

#### RNA Quality and Quantity after Globin mRNA Reduction

We compared the quality of total RNA samples isolated from PAXgene blood tubes without globin reduction (PAX-NGR, four sample pairs) with that of total RNA isolated from EDTA whole-blood samples (five sample pairs). RNA from PAXgene whole blood was of excellent quality with a mean RIN of  $8.85 \pm 0.18$ , while RNA from EDTA whole blood was of moderate quality with a mean RIN of  $6.21 \pm 0.34$ . We performed globin reduction using PAXgene whole-blood samples only (PAX-GR, four sample pairs). The quality of PAX-GR sample RNA was slightly reduced compared with that of PAX-NGR sample RNA in terms of RIN ( $8.68 \pm 0.24$  for PAX-GR and  $8.85 \pm 0.18$  for PAX-NGR) and 260/280 ratio ( $1.89 \pm 0.02$  for PAX-GR and  $2.12 \pm 0.01$  for PAX-NGR). The average total RNA recovery after globin reduction was  $77.4 \pm 2.7\%$ , similar to the manufacturer’s expected yield as well as literature reports (24, 25). mRNA levels of five hemoglobin genes, *HBA1*, *HBB*, *HBD*, *HBE1*, and *HBG1*, were compared between PAX-GR and PAX-NGR samples. The transcript levels of all hemoglobin genes were significantly reduced following globin reduction ( $p < .0001$ ), except for *HBE1* ( $p = .252$ ). There was roughly a 50–70% reduction for the gene transcripts of *HBA1*, *HBB*, *HBD*, and *HBG1* (results not shown).

#### Effects of Globin Reduction on Detectable Probe Sets

Independent preprocessing of the PAX-NGR and PAX-GR CEL files using the “farms” package resulted in a total of 3349 and 3795 filtered probe sets, respectively, from an initial 32,321 probe sets. Figure 1A displays the density plots for the median expression values of probe sets for the PAX-NGR and PAX-GR data sets. The PAX-NGR data set consisted of probe sets with higher intensity signals

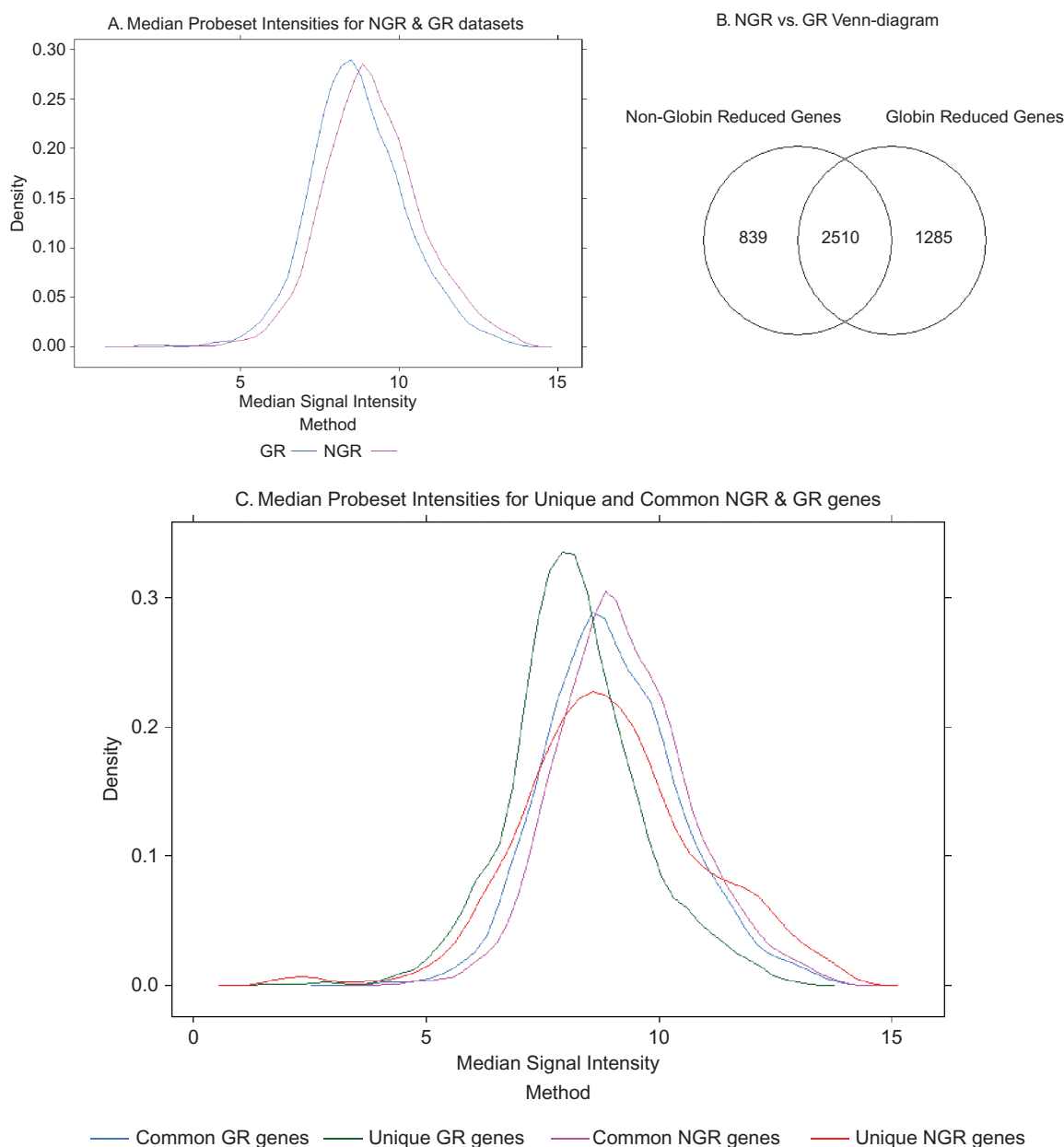


FIGURE 1.—Density plot and Venn diagram of PAX-NGR and PAX-GR filtered gene lists. (A) Density plot of the 3349 PAX-NGR and 3795 PAX-GR filtered probe sets. The PAX-GR probe sets have significantly lower median signal intensities than the PAX-NGR probe sets ( $p = 2.2 \times 10^{-16}$ ). (B) Venn diagram of common and unique PAX-NGR and PAX-GR probe sets. (C) Density plots of the common and unique probe sets in the PAX-NGR and PAX-GR data sets. Note: Green—median signal intensities of probe sets unique to the PAX-GR data set; blue—median signal intensities of common probe sets as filtered in the PAX-GR data set; red—median signal intensities of probe sets unique to the PAX-NGR data set; pink—median signal intensities of common probe sets as filtered in the PAX-NGR data set.

(higher abundance), whereas the PAX-GR data set consisted of probe sets of lower abundance. Figure 1B represents the common and unique probe sets between the 3349 PAX-NGR and 3795 PAX-GR filtered probe set lists. There are 839 probe sets unique to the PAX-NGR list and 1285 probe sets unique to the PAX-GR list; however, the majority—2510 probe sets—were common to both lists. Lastly, Figure 1C shows the breakdown of Figure 1A, illustrating the median signal intensities of the probe sets in accordance with whether they were unique or common in the PAX-NGR and PAX-GR groups. Unique PAX-GR probe sets (green) were found to be in lower abundance than either the unique PAX-NGR probe sets

(red) or the common probe sets as identified in PAX-NGR (pink) or PAX-GR (blue) samples.

#### *Effects of Globin Reduction on Detection of Differential Gene Expression*

Robust limma revealed a total of 674 (401 up- and 273 downregulated) DEGs between pre- and post-allergen inhalation challenge in the PAX-NGR data set at an FDR of 5%. At the same FDR, no genes were found to be differentially expressed between pre- and post-challenge in the PAX-GR data set. However, when the FDR threshold was relaxed to 10%, 550 (391 up- and 159

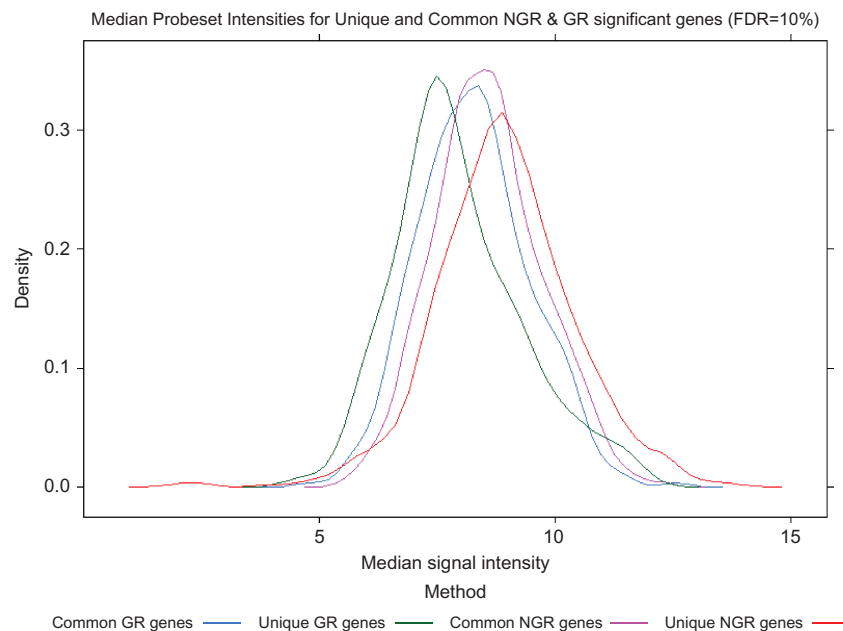


FIGURE 2.—Median probe set intensities for unique and common PAX-NGR and PAX-GR differentially expressed genes (FDR  $\leq 10\%$ ).

Notes: Density plot of the median probe set intensities for the unique and common differentially expressed PAX-NGR and PAX-GR probe sets at an FDR of 10%. Green—median signal intensities of probe sets unique to the PAX-GR data set; blue—median signal intensities of common probe sets as filtered in the PAX-GR data set; red—median signal intensities of probe sets unique to the PAX-NGR data set; pink—median signal intensities of common probe sets as filtered in the PAX-NGR data set. Significant probe sets unique to the PAX-NGR data set are found in greater abundance than significant probe sets unique to the PAX-GR data set.

downregulated) genes were found to be differentially expressed in the PAX-GR data set, while the corresponding PAX-NGR data set identified 1471 (910 up- and 561 downregulated) DEGs.

In order to compare the gene lists from both PAX-NGR and PAX-GR data sets, an FDR of 10% was used and the overlap between the gene lists was determined. We found that 1138 DEGs were unique to the PAX-NGR data set and 217 were unique to the PAX-GR data set. There were a total of 333 DEGs common to both PAX-NGR and PAX-GR data sets. In addition, the significant genes unique or common to the data sets differed in their intensity values (Figure 2). In highest abundance were the genes unique to the PAX-NGR data set (red), followed by common genes as identified in PAX-NGR and PAX-GR samples, respectively, and finally the genes unique to the PAX-GR data set (green) were in lowest abundance. This pattern is similar to that seen in Figure 1, in which filtered probe sets (before statistical analysis using limma) were observed to be higher in intensity for the PAX-NGR group than for the PAX-GR group.

#### *Effects of Allergen Inhalation Challenge on Gene Expression*

Following our finding that PAX-NGR samples identified more DEGs than PAX-GR samples, the 8 PAX-NGR samples and 10 EDTA samples were combined into one analysis to determine differential gene expression. Incorporating the tube type, RIN, site, sex, age, methacholine PC<sub>20</sub> at pre-challenge, and FEV<sub>1</sub> at early and late response as covariates, a total of 1595 (1126 up- and 469 downregulated) DEGs were identified post-allergen challenge at an FDR of 5%.

#### *Pathway Analysis of DEGs*

We performed pathway analysis using IPA in order to reduce the false-positive findings that are often associated with single gene level analysis. Keeping the FDR  $\leq 5\%$  threshold, 1595 genes were entered into the analysis, of which 303 met the criteria for inclusion into networks and canonical pathways (Supplementary Table). The top network identified was that involving inflammatory response, cellular movement, and immune cell trafficking (Figure 3). The top three canonical pathways were eukaryotic initiation factor 2 signaling ( $p = 1.98 \times 10^{-4}$ ), interferon signaling ( $p = 7.83 \times 10^{-4}$ ), and phosphatidylinositol 3-kinases/protein kinase B (PI3K/AKT) signaling ( $p = 1.82 \times 10^{-3}$ ).

#### DISCUSSION

Whole-blood mRNA in global gene expression profiling has relatively low detection sensitivity, partly attributable to the abundant globin mRNA (26). Since the first report of the use of globin mRNA reduction as part of the methodology for gene expression profiling (26), several additional studies have been published describing the effectiveness of such techniques. While most of these suggest that globin mRNA reduction can increase sensitivity by unmasking additional genes and increasing call rates (24, 25, 27, 28), some studies have also suggested that globin reduction does not always increase call rates (4) or that it does so from a non-beneficial standpoint (28). Therefore, it is useful to test if globin mRNA reduction is beneficial for a particular study design before any large-scale study is underway.

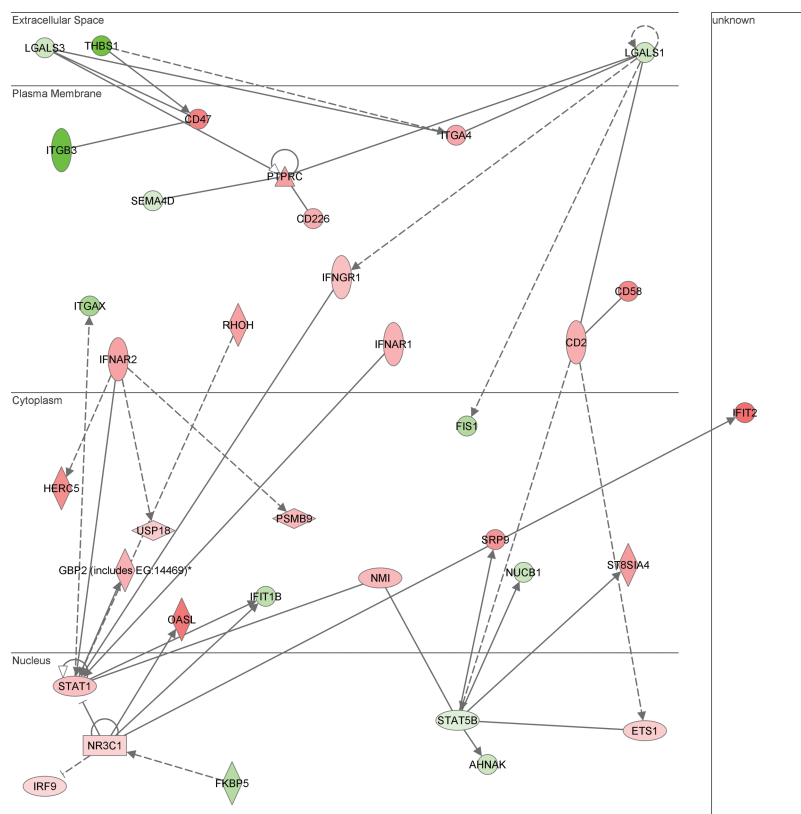


FIGURE 3.—Network of inflammatory response, cellular movement, and immune cell trafficking.

Notes: The top-ranked biological network as identified by IPA using DEGs at FDR  $\leq 5\%$  was that involving inflammatory response, cellular movement, and immune cell trafficking. Red denotes genes that are upregulated post-challenge, while green denotes genes that are downregulated post-challenge.

In accordance with the literature (24), our results showed that more probe sets of lower signal intensities can be uncovered from samples that have undergone the globin reduction process, as compared to non-globin-reduced samples. However, after statistical analysis, it was evident that samples without globin reduction demonstrated more DEGs (most of which are in the range of higher abundance) as opposed to globin-reduced samples at the same FDR.

These findings demonstrate that although globin reduction is indeed beneficial to uncover lower abundance probe sets, this benefit has its limitations. First, the globin reduction procedure introduces technical variability, as evidenced by the decreased ability to detect differential gene expression in globin-reduced samples compared to non-globin-reduced samples. Within our small sample size, this effect prevented any declaration of DEGs at an FDR of 5%. Second, globin reduction increases the cost of the experimental analysis in general. Performing globin reduction is more expensive in terms of the price of the globin reduction kit, as well as the time required for a technician to carry out the procedure. In our study, we have identified DEGs exclusive to the globin-reduced group and exclusive to the non-globin-reduced group, suggesting that it may be scientifically beneficial to carry out analyses on both types of samples in order to reveal the maximum number of DEGs. At an FDR of 10%, the majority of DEGs identified using globin-reduced samples can also be observed from the non-

globin-reduced samples, leaving only 217 DEGs unique to the globin reduction group. Considering this minimal gain in DEGs through globin reduction, and comparing it against the much larger number of DEGs identified using non-globin-reduced samples (both unique and in total), it is evident that the extra costs associated with carrying out globin reduction and the subsequent microarray analysis may not be justifiable given limited budgets.

Having demonstrated that non-globin-reduced samples are more effective in detecting DEGs, we proceeded to explore the genome-wide gene expression differences between pre- and 2 hours post-allergen inhalation challenge using a larger set of nine non-depleted sample pairs (four PAX-NGR plus five EDTA sample pairs). Inflammatory response, cellular movement, and immune cell trafficking comprised the top network formed by the DEGs identified (Figure 3). This is highly relevant to asthma, a disease driven by inflammation of the airways. Furthermore, cellular movement and immune cell trafficking are consistent with the characteristic observations of the late-phase response, in which there is heavy infiltration of immune cells to the airways (5). Further analysis (beyond the scope of this article) on additional asthmatic individuals undergoing allergen inhalation challenge is required to validate our findings.

A limitation in our study design is that we did not measure complete blood cell counts and differentials. Therefore, we were unable to take into account possible

confounding effects on gene expression due to changes in relative cell-type frequencies. In addition, although we have demonstrated that 2 hours post-allergen inhalation challenge is a reasonable time point to observe changes in the peripheral blood gene expression profile, future investigations could include additional time point sampling.

### CONCLUSIONS

We have identified differential changes in the gene expression of peripheral blood leukocytes in atopic asthmatic individuals 2 hours following allergen inhalation challenge. Furthermore, we have demonstrated that the use of globin mRNA reduction technology is not required to determine changes in gene expression.

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### DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

### REFERENCES

1. Debey S, Zander T, Brors B, Popov A, Eils R, Schultze JL. A highly standardized, robust, and cost-effective method for genome-wide transcriptome analysis of peripheral blood applicable to large-scale clinical trials. *Genomics* 2006; 87(5):653–664.
2. Wang Z, Neuburg D, Li C, Su L, Kim JY, Chen JC, Christiani DC. Global gene expression profiling in whole-blood samples from individuals exposed to metal fumes. *Environ Health Perspect* 2005; 113(2): 233–241.
3. McPhail S, Goralski TJ. Overcoming challenges of using blood samples with gene expression microarrays to advance patient stratification in clinical trials. *Drug Discov Today* 2005; 10(22):1485–1487.
4. Dumeaux V, Lund E, Børresen-Dale A-L. Comparison of globin RNA processing methods for genome-wide transcriptome analysis from whole blood. *Biomark Med* 2008; 2(1):11–21.
5. Gauvreau GM, Evans MY. Allergen inhalation challenge: a human model of asthma exacerbation. *Contrib Microbiol* 2007; 14:21–32.
6. Dharajiya N, Vaidya S, Sinha M, Luxon B, Boldogh I, Sur S. Allergen challenge induces Ifng dependent GTPases in the lungs as part of a Th1 transcriptome response in a murine model of allergic asthma. *PLoS One* 2009; 4(12):e8172.
7. Bailey MT, Kierstein S, Sharma S, Spaitis M, Kinsey SG, Tliba O, Amrani Y, Sheridan JF, Panettieri RA, Haczku A. Social stress enhances allergen-induced airway inflammation in mice and inhibits corticosteroid responsiveness of cytokine production. *J Immunol* 2009; 182(12): 7888–7896.
8. Zou J, Young S, Zhu F, Gheys F, Skeans S, Wan Y, Wang L, Ding W, Billah M, McClanahan T, Coffman RL, Egan R, Umland S. Microarray profile of differentially expressed genes in a monkey model of allergic asthma. *Genome Biol* 2002; 3(5):research0020.
9. Gauvreau GM, Hessel EM, Boulet L-P, Coffman RL, O'Byrne PM. Immunostimulatory sequences regulate interferon-inducible genes but not allergic airway responses. *Am J Respir Crit Care Med* 2006; 174(1):15–20.
10. Cockcroft DW, Killian DN, Mellon JJ, Hargreave FE. Bronchial reactivity to inhaled histamine: a method and clinical survey. *Clin Allergy* 1977; 7(3):235–243.
11. Cockcroft DW. Measure of airway responsiveness to inhaled histamine or methacholine; method of continuous aerosol generation and tidal breathing inhalation. In: Hargreave FE, Woolcock AJ, eds. *Airway Responsiveness: Measurement and Interpretation*. Mississauga: Astra Pharmaceuticals, 1996:22.
12. Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987; 135(1):264–267.
13. O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic responses. *Am Rev Respir Dis* 1987; 136(3):740–751.
14. Cockcroft DW, Davis BE, Boulet L-P, Deschesnes F, Gauvreau GM, O'Byrne PM, Watson RM. The links between allergen skin test sensitivity, airway responsiveness and airway response to allergen. *Allergy* 2005; 60(1):56–59.
15. Pradervand S, Paillusson A, Thomas J, Weber J, Wirapati P, Hagenbüchle O, Harshman K. Affymetrix Whole-Transcript Human Gene 1.0 ST array is highly concordant with standard 3' expression arrays. *Biotechniques* 2008; 44(6):759–762.
16. Hochreiter S, Clevert D-A, Obermayer K. A new summarization method for Affymetrix probe level data. *Bioinformatics* 2006; 22(8):943–949.
17. Talloen W, Clevert D-A, Hochreiter S, Amaratunga D, Bijnsens L, Kass S, Göhlmann HW. I/NI-calls for the exclusion of non-informative genes: a highly effective filtering tool for microarray data. *Bioinformatics* 2007; 23(21):2897–2902.
18. Bourgon R, Gentleman R, Huber W. Independent filtering increases detection power for high-throughput experiments. *Proc Natl Acad Sci U S A* 2010; 107(21):9546–9551.
19. Smyth GK. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; 3:Article3.
20. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy—analysis of Affymetrix GeneChip data at the probe level. *Bioinforma* 2004; 20(3): 307–315.
21. Team RDC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2006.
22. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Series B* 1995; 57(1):289–300.
23. Ingenuity Pathways Analysis software. Available at: <http://www.ingenuity.com/> [Accessed on: November 23, 2011]
24. Field LA, Jordan RM, Hadix JA, Dunn MA, Shriver CD, Ellsworth RE, Ellsworth DL. Functional identity of genes detectable in expression profiling assays following globin mRNA reduction of peripheral blood samples. *Clin Biochem* 2007; 40(7):499–502.
25. Raghavachari N, Xu X, Munson PJ, Gladwin MT. Characterization of whole blood gene expression profiles as a sequel to globin mRNA

- reduction in patients with sickle cell disease. *PLoS One* 2009; 4(8): e6484.
26. Wu K, Miyada G, Martin J, Finkelstein D. Globin reduction protocol: a method for processing whole blood RNA samples for improved array results. Affymetrix technical note. 2003. Available at: [http://media.affymetrix.com:80/support/technical/technotes/blood2\\_technote.pdf](http://media.affymetrix.com:80/support/technical/technotes/blood2_technote.pdf). [Accessed on: September 3, 2010]
27. Liu J, Walter E, Stenger D, Thach D. Effects of globin mRNA reduction methods on gene expression profiles from whole blood. *J Mol Diagn* 2006; 8(5):551–558.
28. Li L, Ying L, Naesens M, Xiao W, Sigdel T, Hsieh S, Martin J, Chen R, Liu K, Mindrinos M, Davis R, Sarwal M. Interference of globin genes with biomarker discovery for allograft rejection in peripheral blood samples. *Physiol Genomics* 2008; 32(2):190–197.