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Gene expression profiling and network analysis of peripheral blood monocytes in a chronic model of allergic asthma

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Abstract

The *Aspergillus fumigatus* mouse model of asthma mimics the characteristics of human fungal asthma, including local and systemic inflammation. Monocyte/macrophage lineage cells direct innate immune responses and guide adaptive responses. To identify gene expression changes in peripheral blood monocytes in the context of fungal allergy, mice were exposed to systemic and intranasal inoculations of fungal antigen (sensitized), and naïve and sensitized animals were challenged intratracheally with live *A. fumigatus* conidia. Microarray analysis of blood monocytes from allergic versus non-allergic mice showed $\geq$ twofold modulation of 45 genes. Ingenuity pathway analysis revealed a network of these genes involved in antigen presentation, inflammation, and immune cell trafficking. These data show that allergen sensitization and challenge affects gene expression in peripheral monocytes.

Keywords

allergy; *Aspergillus fumigatus*; microarray; monocytes

Environmental exposure to *Aspergillus* fungal species may elicit a number of pulmonary diseases in humans, including allergic asthma. Mold sensitivities often complicate clinical asthma presentation, increasing the need for medication in asthmatic children and the number of hospital stays in patients 16–60 years of age (1–5). Allergic asthma is a multifaceted syndrome consisting of airway hyperresponsiveness, immune cell trafficking, airway obstruction, and tissue remodeling. Fungal sensitization is linked to asthma severity, although the basis for this increased pathology remains ambiguous. The *Aspergillus fumigatus* mouse model of fungal allergic asthma is a useful tool for studying pulmonary immune responses *in vivo*, as it has been previously demonstrated to mimic many of the pulmonary immune responses typical of human allergic asthmatics, including local and systemic inflammation, chronic pulmonary eosinophilia, elevated IgE levels, goblet cell hyperplasia, and peribronchial fibrosis (6, 7). This model uses live fungal spores to

challenge an animal that has been sensitized to the soluble fungal antigen, allowing a protracted course of study.

Previous research of gene expression changes in monocytes and macrophages in response to *Aspergillus* conidia has focused on time points immediately after direct, acute exposure, examining the innate response to the fungus. These studies have been carried out using primary human monocytes, human monocyte cell lines, or mouse AM cultured *in vitro* with *A. fumigatus* (8–12). Other groups have carried out gene expression profiling on whole lung tissue from an ovalbumin model for acute and chronic asthma (13–15). While providing insight into the gene expression changes occurring after *A. fumigatus* exposure *in vitro* or in the whole lung after ovalbumin *in vivo*, none of these studies carried out transcriptional profiling of monocytes in an *in vivo* chronic allergic model in which the *Aspergillus* antigen is seen multiple times. As cells of the monocyte/macrophage cell lineage not only phagocytose fungal spores in the innate response, but also inform the inflammatory and adaptive immune response, we tested the hypothesis that systemic sensitization affects gene regulation in peripheral blood monocytes that may impact the pathogenesis of subsequent allergic pulmonary responses. We used the *A. fumigatus* model of allergic asthma to obtain an immunologically relevant gene expression profile from peripheral blood monocytes and compared them to cells from normal mice that had been challenged with the allergen without sensitization.

Figure 1 depicts the experimental protocol used to isolate the peripheral blood monocytes from allergic and non-allergic mice used in the present study. All animal studies were conducted with prior approval from the North Dakota State University Institutional Animal Care and Use Committee, which sets best practice standards for the university under the guidance of the Office of Laboratory Animal Welfare. BALB/c mice were used for the chronic allergic asthma model and the treatment protocols were carried out as previously described (6). Briefly, mice treated with the allergy protocol were given intraperitoneal (IP) and subcutaneous (SQ) injections of 10 µg soluble *A. fumigatus* antigens in incomplete Freund's adjuvant (Sigma, St Louis, MO) for systemic sensitization. In weeks 3, 4, and 5, a 20 µl intranasal sensitization of 20 µg of the same soluble antigen dissolved in normal saline was given to localize the reaction to the airways. In week 6, a challenge with 5×10^6 live *A. fumigatus* conidia was given intratracheally in 30 µl PBS containing 0.1% Tween 80. Control mice received the live spore challenge only. Three days post-challenge, peripheral blood was collected, and red blood cells were lysed. As previous experiments had shown that F4/80⁺ cells from the blood of mice consisted predominantly of monocytes and eosinophils, the resulting cells were Fc blocked and stained with FITC anti-CCR3 (R&D Systems, Minneapolis, MN). Magnetic bead-conjugated anti-FITC antibodies (Miltenyi Biotec, Gladbach, Germany) were used to deplete CCR3⁺ eosinophils from the populations by MACS. F4/80⁺ cells were purified by FACS from the resulting CCR3[−] population using an anti-F4/80-PE antibody (eBioscience, San Diego, CA). The CCR3[−], F4/80⁺ cells were analyzed and sorted on a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA). An aliquot of these cells was cytospun onto a glass slide and stained with Quik-Dip differential stain (Mercedes Medical, Sarasota, FL) to assess purity. Morphology and staining verified the cell population to be >95% monocytes.

Frozen cell pellets containing 30 000 CCR3[−], F4/80⁺ peripheral blood monocytes from allergic mice or 30 000 CCR3[−], F4/80⁺ peripheral blood monocytes from non-allergic mice were sent to Miltenyi Biotec for microarray analysis. Cell pellets were lysed using SuperAmp Lysis buffer and SuperAmp RNA amplification was carried out according to Miltenyi Biotec's proprietary procedure. Approximately 2 µg cDNA was generated from each sample, and the integrity of the samples was analyzed via the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Two hundred and fifty nanograms of each cDNA

sample was used for Cy3 or Cy5 labeling and both were hybridized to a mouse PIQOR Immunology Microarray containing 1076 genes representing pathways involved in the areas of apoptosis, signal transduction, stress, CD antigens, cell cycle, DNA repair, chemokines, chemokine receptors, complement system, cytokines, cytokine receptors, cytokine signaling and regulation, extracellular matrix proteins, and inflammation. Fluorescence signals were detected using an Agilent Microarray Scanner system (Agilent Technologies). Ratios of gene expression changes obtained and the CV among the quadruplicate features are reported in Table 1 and are divided into functional groups. Forty-five gene expression changes were detected that showed at least a twofold change. Ten genes were upregulated and 35 genes were downregulated in allergic blood monocytes versus non-allergic blood monocytes.

An Excel file containing gene names and fold changes was uploaded to the Ingenuity Pathway Analysis (IPA) website as previously described (16). Briefly, the Ingenuity Knowledge Base contains information from scientific publications regarding direct and indirect relationships between genes and proteins (17). A network was constructed that contains 17 focus genes (those identified by our microarray analysis) and 17 non-focus genes, which was optimized for high degrees of interconnectivity. Figure 2 shows the network of genes modulated in peripheral blood monocytes in a chronic allergic asthma model. IPA also categorizes modulated genes into biological process categories. Genes involved in major functional areas were identified and included: immune cell trafficking/cell movement: *Foxo4*, *Ccl4*, *Gadd45a* (a member of the GADD45 stress sensor gene family), *Cx3cr1*, *Itgb4*, *Bax*, *Spock1*, *Ptpn12*, *Nrp1*, *Cd83*, *Cd63*; antigen presentation: *Il1r2*, *Ccl4*, *Map3k7*, *Cd83*, *Cx3cr1*, *Bax*, *Tap1*, *Cd63*; cell-mediated immune response: *Foxo4*, *Cd83*, *Bax*, *Cx3cr1*, *Tap1*, *Cd63*, *Ptpn12*, *Il1r2*, *Ccl4*, *Znf287*, *Gadd45a*, *Map3k7*, *Prkch*, *Itgb4*, *Spock1*, *Nrp1*; and the inflammatory response: *Il1r2*, *Ccl4*, *Map3k7*, *Cd83*, *Cx3cr1*, *Bax*, *Tap1*, and *Cd63*.

Fungal spores are ubiquitous in the environment. When they are inhaled into the airway of a normal, non-sensitized individual, they are eliminated by AM without an exaggerated inflammatory response. However, in an individual who has been sensitized to these allergens, the inhalation of fungal spores elicits a number of immediate and ongoing immune responses, including a frank inflammatory response. To better understand the effect of sensitization on monocyte function in the allergic immune response, we analyzed the gene expression of blood monocytes from allergic animals that had been challenged with spores from the allergenic fungus *A. fumigatus* and compared them with non-allergic animals that had also been exposed to fungal spores.

Several genes that were modulated in these experiments have been identified as important to the interaction of the host cell and the fungus in other similar microarray studies, although the direction of the modulation was not always consistent between our chronic *in vivo* model and the *in vitro* examinations. For example, although the upregulation of *Egr1*, *Ifng*, *CD83* and *Icam1* that we observed was not tested or did not reach significance in similar microarray studies, there was overlap with at least one of the other studies in the upregulation of these genes. In contrast, however, members of the *Gadd45* family, *Cflar* and *Icam1* were modestly upregulated in murine AMs after co-culture with conidia, whereas our results showed a decrease in their transcription (12) and our study of murine blood monocytes from allergic animals did not show many of the changes revealed by direct co-incubation of the host cell and fungi (8, 9, 12). We believe this to be a reflection of the activation state of the allergen-sensitized mice and of the fact that the monocytes do not encounter the particulate fungal conidia until reaching the lung, thus different parameters are being measured.

We have previously highlighted the importance of various chemokines and their receptors in this disease state and have shown that both an inflammatory and an allergic, Th2-mediated response are observed after exposure to fungus (18–22). Classically activated macrophages respond to interferon (IFN)- γ to produce a number of inflammatory mediators and chemokines, including CCL3 and CCL4. Indeed, after direct exposure to fungus in primary human monocytes from healthy subjects, a human monocytic cell line and murine AM, these chemokine genes were upregulated, reflecting an inflammatory response against the fungus (8, 9, 11, 12). The present study marks the first time that the genetic expression patterns of peripheral blood monocytes have been explored in an allergic animal. Refuting the hypothesis that monocytes may enter the allergic lung from the blood already expressing an alternative activation pattern, we found that allergic sensitization did not skew the monocyte lineage to the pro-allergy alternative activation pattern, but did exacerbate the inflammatory capacity of the cells in the blood with *Ifng* and *Il1r2* expression being upregulated in conjunction with the chemokines *Ccl3* and *Ccl4*. Interestingly, these same four genes (*Ifng*, *Il1r2*, *Ccl3*, and *Ccl4*) that were upregulated in monocytes from allergic animals were expressed prominently in a human monocyte cell line that was co-cultured with *A. fumigatus* hyphae and treated with voriconazole, an anti-fungal medication (11). Again, this represents a strong, protective inflammatory response in these antigen-exposed animals, which contradicts the idea that allergic sensitization is an aberrant immune response.

The IPA investigation of the data revealed several interactions between focus genes, including *Cd63-Itgb4-Bax*, *CD83-Prkch* and *Foxo4-Gadd45*. All of these genes have been associated with cellular proliferation. Regarding the *Cd63-Itgb4-Bax* relationship, Cd63 and integrin beta 4 proteins have been shown to bind to each other on the plasma membrane (23), and integrin beta 4 signaling has been shown to upregulate *Bax* (24). CD83 and protein kinase C ϵ are related, as the addition of a PKC inhibitor blocked phosphatidic acid and tumor necrosis factor- α induced CD83 surface expression (25). Last, the Foxo4 and Gadd45 relationship has been established in that Foxo4, a forkhead transcription factor, has been shown to bind to the *Gadd45* promoter and modulate its transcription to influence G2/M cell cycle arrest (26). These interactions, demonstrated in the IPA network, enhance our knowledge regarding the relationships of gene expression changes in peripheral blood monocytes in allergic fungal asthma.

In conclusion, the present study presents significant findings confirming *in vivo* changes in the expression of several genes in peripheral monocytes including *Egr1*, *Ccl3*, *Ccl4*, *Gadd45*, *Cflar* and *Icam1*, which were observed in previous acute exposure *A. fumigatus*-induced allergic asthma studies. These data also support the supposition that sensitization has global systemic effects on innate immune cells which affect local inflammatory responses and may change the host's response to environmental exposures. The novel targets we discovered and the relationships highlighted by IPA will provide direction for additional studies with the ultimate hope of providing more useful drug targets to aid the amelioration of allergic asthma.

List of Abbreviations

AM	alveolar macrophage
CV	coefficient of variation
FITC	fluorescein isothiocyanate
Ig	immunoglobulin
MACS	magnetic cell sorting

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Fig. 1. Schematic of the experimental design

Five mice in the naïve/challenged (N/C, control protocol) or sensitized/challenged (S/C, allergy protocol) group were treated following the time course shown above. Live *A. fumigatus* conidia were delivered intranasally or intratracheally in the allergy protocol, whereas the control group received the challenge only. Whole blood was collected, CCR3⁺ cells were removed by MACS, and 30 000 F4/80⁺ FACS purified cells from each group were sent to Miltenyi Biotec (Auburn, CA) for microarray analysis. Flow cytometry and cytopsin analysis of the CCR3⁺, F4/80⁺ population using anti-F4/80-PE and anti-CCR3-FITC is shown. RNA was isolated from both N/C and S/C blood monocyte samples and amplified using Miltenyi's proprietary SuperAmp amplification protocol, and was labeled with Cy5 and Cy3, respectively. Samples were then hybridized to a PIQOR Immunology Microarray (Miltenyi Biotec) containing 1076 genes in quadruplicate.

Fig. 2. IPA network analysis of molecule interactions

The microarray expression value changes were uploaded to the Ingenuity Pathway Analysis website and were analyzed by their proprietary software. The IPA Network Generation Algorithm created a network containing 17 ‘focus genes’ that were shown to be differentially expressed between the naïve and sensitized groups and are indicated by asterisks. An additional 17 intermediate molecules called ‘non-focus genes’ were placed in the network by the Ingenuity software. Whereas the gene expression levels of the non-focus genes were not shown to be changed by the microarray analysis, they have been previously shown to interact with the focus genes identified in this study. Symbols representing functional categories of the molecules are listed in the legend.

Table 1**Genes modulated in allergic vs non-allergic PBM**

	Fold change/CV
Adhesion	
ICAM1: intercellular adhesion molecule 1	-4.12/29%
CLEC4A2: C-type lectin domain family 4 member A, CLEC4A	-2.05/4%
ITGA4: integrin alpha-4, VLA-4	-2.35/6%
ITGB4: integrin beta-4	-4.66/102%
LAMA3: laminin alpha-3 chain	-2.10/73%
Apoptosis, intracellular proteins, signal transduction and stress	
ATF5: activating transcription factor 5, ATRF	2.12/35%
BAX: apoptosis regulator BAX	2.17/4%
CFLAR: CASP8 and FADD-like apoptosis regulator precursor, CLARP, MRIT, CASH	-6.83/46%
EGR1: early growth response protein 1, ZNF225	2.5/16%
FOXO4: forkhead box protein O4, AFX1, AFX, MLLT7	-2.06/46%
GADD45: growth arrest and DNA-damage-inducible protein, DDIT1, GADD45	-2.61/96%
IGFBP1: insulin-like growth factor binding protein 1	-2.07/36%
MADD: map kinase-activating death domain protein	-4.40/46%
MAP3K4: mitogen-activated protein kinase kinase kinase 4, MTK1	-2.30/2%
MAP3K7: mitogen-activated protein kinase kinase kinase 7, TAK1	-2.46/48%
MAP3K8: mitogen-activated protein kinase kinase kinase 9, COT, TPL2	-5.42/9%
MAPK8IP1: C-jun-amino-terminal kinase interacting protein 1, JIP1, IB1	-2.37/13%
MAPK9: mitogen-activated protein kinase 9, PRKMP, JNK2	-2.15/0%
MLL3: myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog, HALR	-2.48/41%
PREPL: prolyl endopeptidase-like	2.17/8%
PRKCH: protein kinase C, ETA, PKCL	-2.18/27%
PTPN12: protein-tyrosine phosphatase G1	-2.82/18%
SKAT2: zinc finger protein SKAT2	-2.06/47%
TAP1: antigen peptide transporter 1, ABCB2, PSF1, RING4, Y3, MTP1, HAM-1	-2.03/58%
CD antigens and cell surface receptors	
CD45: leukocyte common antigen, PTPTC, LY-5	-2.03/4%
CD63: CD63 antigen, LAMP-3, granulophysin	3.22/7%
CD83: CD83 antigen, HB15, BL11	-3.05/78%
CD94: CD94 antigen, KLRD1	-2.68/46%
NRP1: neuropilin-1, VEGF165R	-2.21/6%
Cell cycle and DNA repair	
TOPBP1: DNA topoisomerase II binding protein 1	-4.16/45%
Chemokines and chemokine receptors	
CCL3: macrophage inflammatory protein 1-ALPHA, MIP1A, SCYA4	2.17/8%
CCL4: macrophage inflammatory protein 1-BETA, MIP1B, SCYA4	3.03/8%
CX3CR1: CX3C chemokine receptor 1, GPR13	-4.96/3%
Cytokines and cytokine receptors	

	Fold change/CV
ACVR1: activin receptor type I	-3.98/69%
IFNG: interferon gamma	2.56/8%
IL1R2: interleukin-1 receptor, type II, IL1RB	2.04/4%
IL1RAPL2: interleukin-1 receptor accessory protein-like 2, IL1R9	2.22/17%
TNFRSF21: tumor necrosis factor receptor superfamily member 21, DR6	-3.35/21%
Cytokine signaling, secretion and regulation	
AMSH: STAM-associated protein, STAMP	-2.16/48%
CBP: cytohesin binding protein HE, PSCDBP	-2.29/11%
IL18BP: interleukin-18 binding protein	-11.12/102%
NFATX: nuclear factor of activated T-cells, cytoplasmic 3, NFAT3C, NFAT4	-2.25/23%
SOCS6: suppressor of cytokine signaling 6, SOCS4, CIS4	-2.04/5%
STX3A: syntaxin 3	-2.26/65%
Extracellular matrix proteins	
SPOCK1: TESTICAN-1, TIC1, TICN1	-5.23/61%