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Rhinovirus-induced modulation of gene expression in bronchial epithelial cells from subjects with asthma

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Abstract

Rhinovirus (RV) infections trigger asthma exacerbations. Genome-wide expression analysis of RV1A-infected primary bronchial epithelial cells from normal and asthmatic donors was performed to determine whether asthma is associated with a unique pattern of RV-induced gene expression. Virus replication rates were similar in cells from normal and asthmatic donors. Overall, RV downregulated 975 and upregulated 69 genes. Comparisons of transcriptional profiles generated from microarrays and confirmed by quantitative reverse transcription PCR and cluster analysis showed some up- and downregulated genes in asthma cells involved in immune responses (*IL1B, IL1F9, IL24*, *IFI44*) and airway remodeling (*LOXL2*, *MMP10*, *FN1*). Notably, most of the asthma-related differences in RV-infected cells were also present in the cells before infection. These findings suggest that differences in RV-induced gene expression profiles of cells from normal and mild asthmatic subjects could affect the acute inflammatory response to RV and subsequent airway repair and remodeling.

INTRODUCTION

Human rhinoviruses (RVs) cause the common cold and are frequently detected in asthma exacerbations. RV typically induces neutrophilic inflammation in the upper airways of both asthmatic and non-asthmatic patients; however, in asthmatics these infections can lead to more severe lower respiratory symptoms, as well as reductions in lung function. Interestingly, the severity of asthma symptoms may not be related to viral load, prolonged viral shedding, or to differences in proinflammatory cytokines in the upper airway secretions.[1,2] Understanding of the mechanisms provoking RV-induced airway inflammation in asthma, as well as mechanisms linking RV infection to asthma exacerbations, may offer significant opportunities for improved disease management.

RV infection of airway epithelial cells induces the production of a wide range of mediators involved in inflammatory and immune processes.[3,4] Transcriptional profiling of differentiated cultures of human primary bronchial epithelial (PBE) cells from two normal subjects has shown that RV infection induces a number of genes in the interferon (IFN)-β-dependent pathway.[5]

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SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

DISCLOSURE
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Furthermore, cultured epithelial cells from the airways of subjects with asthma have been found to have deficient innate immune responses to RV16 infection, characterized by increased viral replication, impaired early induction of apoptosis and reduced type I and type III IFN production.[6,7] Collectively, these studies suggest that RV-induced airway disease could be due to asthma-related changes in gene expression in airway epithelium. The aim of this study is to compare RV-induced genome-wide gene expression profiles of cultured airway epithelial cells obtained from subjects with and without asthma to identify genes that may play a role in virus-induced asthma exacerbations.

RESULTS

RV1A infection of PBE cells and viral RNA quantification

Primary bronchial epithelial cells (samples 7–18) from six normal subjects and six subjects with asthma (Table 1) were inoculated with RV1A (MOI (multiplicity of infection) of 10 PFU (plaque-forming units) per cell); for one subject with asthma (sample 11), the RNA yield after infection was insufficient for further analysis. Total RNA isolated from adherent cells collected 16 h post infection (p.i.) was analyzed using microarrays. Viral RNA was measured by quantitative reverse transcription (qRT)-PCR in growth media from the PBE cell cultures. We then repeated the process of infecting cells from these donors so that the results of the microarray experiments could be retested in separate experiments and using a different technology (qRT-PCR) to measure changes in host gene expression and viral RNA. Virus-induced cytopathic effect, as determined by light microscopy (Figure 1a), was similar in cells from normal and asthmatic subjects. In addition, there were no group-specific differences in the amount of viral RNA released into the media (including floating cells), in adherent cells, or in the total amount of RNA per well (sum of RNA in media and floating and adherent cells) (Figure 1b, $P>0.05$).

Gene expression changes in response to infection

To determine the transcriptional response of normal and asthmatic PBE cells to RV1A infection, we started by comparing genome-wide gene expression profiles in infected cells and mock-infected controls in each group. We identified a total of 1,317 probe sets corresponding to 1,044 known human genes with at least a twofold change in expression in RV-infected PBE cells vs. mock-infected control cells, in both groups. Combined lists of the 40 most highly up- and downregulated transcripts found in normal and asthmatic cells are shown in Table 2, and complete lists of genes are provided in Supplementary Tables 1 and 2 online.

The majority of affected genes were down-regulated in infected cells compared to mock-infected control samples (Figure 2a). This finding is consistent with global host cell transcriptional shutoff due to RV-induced cleavages of multiple transcription factors and nuclear pore complex components.[8,9] Virus infection decreased the expression of genes related to antiviral defense (influenza virus NS1A binding protein), apoptosis (TIA1 cytotoxic granule-associated RNA binding protein) and regulation of cell-cycle (discs, large homolog 1 (Drosophila); ubiquitin-like, containing PHD and RING finger domains; cyclin-dependent kinase inhibitor 2B) as well as multiple proteins participating in cell metabolism (Table 2).

Genes exhibiting an increase in expression (greater than or equal to twofold) included 53 found in normal samples and 54 in the asthma group that together comprise 69 unique genes (Supplementary Table 1 online). Among the induced genes were those encoding chemoattractants for granulocytes, macrophages and T lymphocytes (chemokine (C-X-C motif) ligand (CXCL) 1, 2 and 3; interleukin (IL) 8 and chemokine (C-C motif) ligand (CCL) 20), cytokines (colony stimulating factors (CSF) 2 and 3; *IL1F9; IL6* and *IL24*), decoy cytokine receptors (IL1 receptor, type II (*IL1R2*); IL1 receptor antagonist (*IL1RN*); IL13 receptor, α 2)

and transcription factors and regulators such as early growth response 1 (*EGR1*), FOS-like antigen 1 (*FOSL1*), nuclear factor-kappa B (NFκB) inhibitors Z and A (*NFKBIZ* and *NFKBIA*) and zinc finger CCCH-type containing 12A (Table 2). Two cytokine genes (*IL1F9* and *IL24*) revealed more robust up-regulation after RV infection in the asthma group. We also observed an increase in expression of antiviral response genes (2′-5′-oligoadenylate synthetase-like (*OASL*), interferon-induced protein 44 (*IFI44*) and *IL28A* (IFN, λ 2)) in normal cells and of regulators of smooth muscle tone (adrenergic receptor, β 2 (*ADRB2*) and endothelin 1 (*EDN1*)) in both groups.

We then compared these gene expression changes with results from six additional PBE cell cultures (three normal donors and three donors with asthma, Table 1) that were similarly infected in preliminary studies and explored using the HG Focus chips (Affymetrix) with lower probe density (>8,700 probe sets). All of the probe sets from the smaller chip are also present in the higher-density HG U133 Plus 2.0 arrays to enable comparability. Although we observed some differences in magnitude of changes between two microarray data sets (Table 2), the overall core set of virus-induced genes was similar (Supplementary Table 3 online).

Asthma-specific gene expression profiles in PBE cells

We next compared RV-induced responses in the asthma vs. normal groups. In general, patterns of gene expression were quite similar in the two groups, with a few notable exceptions. Direct comparisons of transcriptional profiles after infection identified 9 genes with ≥ twofold up- or down-regulation in the asthma group compared to normal controls (Table 3). Genes with higher expression in the asthma group included those with functions related to inflammation (*IL1F9*), tumor suppressor activity (*C15orf48*) and airway repair and remodeling (inhibin, beta A (*INHBA*); lysyl oxidase-like 2 (*LOXL2*); matrix metallopeptidase 10 (*MMP10*)). In contrast, *IFI44*, an interferon response gene, and tumor suppressor gene microseminoprotein beta (*MSMB*) revealed lower expression in the asthma group. Six of these genes were also differentially expressed in mock-infected cells from asthma patients (Table 3).

Using a less stringent 1.5-fold criterion for group-specific differences in gene expression, a total of 42 genes were identified, including 32 with higher expression and 10 with lower expression in infected cells from the asthma vs. normal groups. (Supplementary Table 4 online). These genes separated the samples into four branches based on hierarchical clustering of gene expression patterns with or without viral infection (Figure 2b). Clusters of genes distinguished by this approach included those with higher expression in asthmatic cells after infection (Cluster I), those with higher expression in both mock-infected and RV-infected asthma samples (Cluster II), and those genes with increased expression in mock- or RV-infected cells from normal donors (Cluster III). Interestingly, one gene from this cluster (*MSMB*) revealed higher expression both at baseline (mock infection) and after RV infection in the samples from 3 normal female subjects.

Alternative hierarchical clustering of samples using gene expression ratios (fold differences) revealed groupings of up- and down-regulated genes, but did not reveal differences related to asthma (Supplementary Figure 1 online). These findings indicate that most of the asthma-related differences in HRV-induced patterns of gene expression were also present without infection.

Functional analysis of genes affected by RV infection

Functional analysis based on the Gene Ontology classifications (Database for Annotation, Visualization and Integrated Discovery (DAVID)) demonstrated that most genes induced by RV infection of both asthma and normal samples were related to inflammatory responses. Interestingly, there were nine RV-induced genes (*CCL5*, prostaglandin-endoperoxide synthase

2 (*PTGS2*), superoxide dismutase 2, *CSF2*, tumor necrosis factor (*TNF*), *IL1RN*, *EDN1*, *ADRB2* and suppressor of cytokine signaling 1 (*SOCS1*)) that have been associated with asthma in genetic studies (Genetic Association Database (http://geneticassociationdb.hih.gov/)). RV infection inhibited many important biological processes in the host cell including post-translational protein modification, ubiquitin cycle, intracellular transport and mRNA processing (Table 4). Of the 42 genes that were differentially expressed in the asthma vs. normal samples after RV infection, many were classified in the "defense response" and "cell-cell signaling" functional categories.

Analysis of the same data set with gene set enrichment analysis (GSEA, Broad Institute, Cambridge, MA) revealed that RV infection of both groups up-regulated genes classified in NFκB, TNF and double-stranded RNA (poly I:C) pathways. Down-regulated genes were related to metabolic pathways for pyruvate, propanoate and steroid biosynthesis, and the Krebs-TCA cycle. Group-specific differences in gene expression patterns after RV infection were found in "local acute inflammatory response" and "genes up-regulated by NFκB" categories. Overall, both functional classification approaches revealed similar findings.

PCR validation of microarray results

To test the validity of the microarray data, additional samples of cells from normal and asthmatic volunteers were grown, and host cell mRNA was analyzed by quantitative PCR. These additional experiments confirmed virus induction of 7 common up-regulated genes both in normal and asthmatic samples (Figure 3).

Of the mRNAs that appeared to be more highly expressed in asthma by microarray, similar patterns of expression were identified for several genes by qPCR (Figure 4). Notably, both *INHBA* and intercellular adhesion molecule 1 (*ICAM1*) tended to be expressed at higher levels in the uninfected asthma samples ($P<0.1$), and similar differences were present in infected samples. In contrast, *LOXL2* was expressed at higher levels in the asthma samples ($P<0.05$), and was downregulated by HRV in both groups. Similar nonsignificant patterns were observed for fibronectin 1 (*FN1*); ADAM metallopeptidase domain 19 (*ADAM19*), and secreted protein, acidic, cysteine-rich (*SPARC*). *IFI44* tended to be more up-regulated in normal samples after infection. Finally, *IL1F9* had similar baseline expression in the two groups, but was more highly induced by RV infection in the asthma group ($P<0.05$), and similar nonsignificant patterns were noted for *CSF3*, *IL6* and *IL24*.

The microarray analysis identified increased expression of IL28A but not IFNB1 mRNA after RV infection despite the availability of the corresponding probes in genechips. In the validation experiments using qPCR, both IFNB1 and IL28 mRNAs were up-regulated after infection of both normal (7.5-fold ($P=0.01$) and 6.3-fold ($P=0.04$), respectively) and asthmatic (8.2-fold ($P<0.01$) and 5.9-fold ($P=0.03$), respectively) cells. *IL29* gene was also up-regulated after infection, but its very low expression levels were not sufficient for reliable comparisons. There were no significant group-specific differences in RV-induced IFN mRNA expression.

Virus infection induces expression of inflammatory cytokines in vitro

Reagents were available for a subset of differentially expressed genes to test for group-specific differences in RV-induced protein expression. We quantified protein expression of three secreted pro-inflammatory cytokines IL1B, IL6 and IL8 in cell culture media of RV-infected and control samples. Virus infection increased protein levels of all three cytokines both in normal and asthmatic samples ($P<0.05$) 16 hrs p.i. (Figure 5). IL6 tended to be up-regulated in asthma samples with or without infection, but there were no significant differences between groups.

DISCUSSION

Genome-wide transcriptional analysis was employed to determine whether asthma is associated with a unique pattern of epithelial cell gene expression after RV infection. RV1A induced characteristic CPE and efficiently replicated in PBE cell monolayers producing similar amounts of viral RNA in cells from donors with vs. without asthma. The transcriptional response to RV infection, characterized by robust up-regulation of pro-inflammatory pathways, and down-regulation of cell metabolic processes, was also similar in normal and asthmatic cells. However, both paired (infected vs. mock) and unpaired (asthma vs. normal) comparisons revealed sets of differentially expressed genes related to inflammatory mechanisms and epithelial repair that clearly separated the asthma and normal groups by hierarchical clustering. Notably, most genes that were differentially expressed in the asthma group after RV infection were also differentially expressed in uninfected cells.

Different technical approaches can be used to study responses to viral infection in vitro. We chose to use the undifferentiated cell culture model that has the advantage of allowing analysis of cells that are fairly uniform in their susceptibility to infection. Air-liquid interface cultures of the well-differentiated cells are more resistant to RV infection with a relatively small proportion of infected cells (~5%).[5,10,11] Therefore, the transcriptional response to RV infection is measured primarily in uninfected cells. In our system, the much higher rate of cellular infection may account for the fact that RV infection inhibits global host transcription and induces relatively few antiviral genes.[12,13]

Recent studies using cultured PBE monolayers demonstrated increased (≥10-fold) RV replication in cell monolayers obtained from subjects with atopic asthma while cells from normal volunteers were more resistant to infection.[6,7] In contrast to these reports, but in a good agreement with the recent findings in differentiated PBE cell cultures[14], we found very similar amounts of viral RNA by qRT-PCR both in supernatants, adherent cells and in total virus yields in cells from normal and asthmatic subjects. Mechanisms that have been proposed to explain enhanced RV replication in asthmatic cells in previous studies are deficient production of type I and III IFN and impaired early induction of apoptosis. In our microarray analysis of RV1A infection, we detected a 2.1-fold induction of the type III IFN mRNA (IL28A) after RV infection in normal cells, and somewhat less (1.6-fold, P=0.115) in cells from the asthma group. Although IFNB1 expression was not detected by microarrays, we used more sensitive qPCR methodology to confirm induction of both IL28 and IFNB1 mRNAs. However, there were no significant differences in IFN expression related to asthma.

It is possible that the different findings were due to differences in either experimental technique, virus strain or subject selection. The subjects in our studies all had mild persistent atopic asthma, and we are currently conducting studies with cells obtained from donors with more severe disease. Interestingly, IFNB1 mRNA induction after RV16 infection was not detected in another study using similar Affymetrix arrays[5] indicating a possible problem with sensitivity of this probe set.

RV, like several other picornaviruses, induces gene expression shutoff in host cells via activities of two viral proteinases, $2A^{pro}$ and $3C^{pro}$ that cleave multiple translation and transcription factors and nuclear pore complex proteins.[15] Accordingly, more than 90% of differentially expressed genes in our study were reduced in expression, and many of these genes are involved in cell metabolism pathways.

The most highly up-regulated genes were enriched for inflammatory mechanisms, and many of the induced factors (e.g. CSF2, CSF3, IL6, IL8 and TNF) have been previously identified in experimental models and clinical infections[3,4,16,17] and shown to play roles in airway inflammation.[18–21] Additional inflammatory factors were also upregulated by infection,

including cytokines (IL1F9 and IL24) and transcription factors that regulate inflammatory responses in airways (EGR1 and FOSL1).[22–25] Notably, some of the up-regulated cytokines and their receptors (e.g. TNF, CCL5, CSF2 and IL1RN) and inflammatory factors (SOCS1, PTGS2, serpin peptidase inhibitor B2 (SERPINB2), and EDN1) have been linked to asthma in genetic and microarray studies and mouse models of asthma.[26–34]

The main goal of our study was to identify genes that were differentially-expressed with RV infection in the asthma vs. normal cell groups. Two categories of factors were identified by the analysis and hierarchical clustering: 1) different expression levels at baseline and after infection (most common), and 2) similar expression at baseline and different expression after infection. The first category included genes implicated in airway repair and remodeling (*INHBA*, *MMP10*, *LOXL2*, *FN1*, *SPARC*), and interestingly, *ICAM1*, which is used as a receptor by major group RV. These findings provide evidence that epithelial cells from individuals with asthma may be fundamentally different at baseline in the absence of infection. There were relatively few genes that were differentially expressed in asthmatic cells after infection but not at baseline (*IL1F9*, *CSF3*, *IFI44*, *IL24*). Notably, IL1F9 is up-regulated in PBE cells after microbial exposure,[22] and IL24 is the key cytokine to trigger the up-regulation of class I IFNs.[35] Additional clinical studies are required to determine whether these cytokines contribute to the increased morbidity of RV infections in patients with asthma.

One of the limitations of our study is that the differences in expression found between normal and asthmatic cells were not statistically significant after correcting for multiple comparisons, and we elected to test the validity of the microarray findings by conducting additional independent experiments that were analyzed by qRT-PCR. Overall, the two techniques demonstrated very good correlation both in terms of direction and magnitude of changes. Moreover, gene expression changes in six additional PBE cell cultures tested in preliminary studies using HG Focus GeneChips were consistent with those discussed in this paper. In addition to mRNA expression, we confirmed that protein expression of three secreted cytokines (IL1B, IL6 and IL8) was induced in cell culture media after RV infection, consistent with microarray and qRT-PCR results. Due to the limited number of replicates, it should be acknowledged that the chances of a type II statistical error are high, and there certainly could be small asthma-related differences in the RV-induced gene expression patterns that were not detected in our study.

In addition to confirmation of our findings by statistical and quantitative means, we compared them with those of other published studies involving microarray analysis. Gene expression profiles in nasal epithelial scrapings after experimental RV16 infection of normal volunteers have demonstrated up-regulation of chemokines, signaling molecules, interferon-responsive genes and antivirals, and a number of these factors (*CCL20*, *SOCS1*, *SOCS3* and *OASL*) were also identified in our study of isolated epithelial cells.[36] Microarrays have recently been used to analyze inflammatory responses in asthma after allergen challenge, neuropeptide stimulation, and corticosteroid resistance.[37–39] In spite of significant differences between cell types and/or stimulus we found overlap between RV-induced changes in gene expression, and those found in brushings of mild asthmatics after allergen challenge (*GOS2; IL1RN*, *IL1B*, *IL8*, *SERPINB2*, *MMP10* and *SPARC*),[37] after neuropeptide stimulation of epithelial cells (*INHBA*, *MMP10*, *EGR1*, *SERPINB2*, *FOSL1*, *CXCL2*, *IL8* and *PTGS2*),[38] and in bronchoalveolar lavage cells from subjects with corticosteroid-resistant asthma (*IL6*, *TNF*, *IL1B*, *CCL20*, *IL8*, *CXCL1*, *CXCL2*, *CXCL3*, *EGR1* and *TNFAIP3*).[39] Taken together, these similarities at transcriptional level could demonstrate the existence of some common mechanisms of asthma.

Overall, we demonstrated similar RV replication rates and transcriptional response to RV1A in normal and asthmatic PBE cells. These findings suggest that factors outside of the epithelial

cell, such as airway inflammation and abnormal airway structure and physiology, are important contributors to more severe clinical outcomes of common cold infections in asthma. Even so, our studies identified a subset of epithelial cell genes that were differentially expressed in asthma, compared to normal subjects with functions related to inflammatory pathways and regulation of airway repair and extracellular matrix. Further characterization of these potential asthma-related differences in the epithelial cell response to viral infection should provide a better understanding of molecular mechanisms of virus-induced asthma exacerbations.

METHODS

Cell culture and viral infection

Human PBE cells were obtained from the bronchial brushings of normal and asthmatic individuals (Table 1). Subjects in the asthma group were required to have doctor-diagnosed asthma, and either metacholine $PC_{20} \leq 8$ mg/ml, or at least 12% reversibility in FEV1 after administration of albuterol. Prick skin testing was performed using a panel of 15 common allergens, including grass and tree pollens, dust, dog and cat hair, and a positive response was defined as a wheal size greater than the histamine negative control. Cells were grown at 37°C (5% CO_2)in bronchial epithelial growth medium (BEGM, Lonza, Walkersville, MD). Purified and concentrated RV1A was diluted in BEGM with a reduced concentration of hydrocortisone (10^{-8} M) just before infection. One six-well plate of PBE cells from each patient was either infected with RV1A (10 PFU/cell), or mock-infected with medium alone. At collection (16 h p.i.), cell monolayers were washed three times with phosphate buffered saline and lysed by adding TRIzol Reagent (Invitrogen, Carlsbad, CA). Supernatant and cell lysate samples were stored in microcentrifuge tubes at −80°C until RNA isolation. Detailed information about the cell culture and infection procedures is provided in the Supplementary Materials online. Preliminary experiments to determine the optimal virus dose (MOI of 2, 10, and 50 PFU per cell) and time p.i. (8, 16 and 24 h) were conducted with PBE cells obtained by enzymatic digestion of bronchi from two lung transplants[40] and used at passages 2–3. Cells were grown in bronchial epithelial growth media and infected with RV1A as described above.

Optimization of rhinovirus infection procedure for microarray analysis

The minor group RV1A was chosen for this study because minor group viruses infect a much larger percentage of cultured epithelial cells compared to major group viruses,[40] and RV1A and RV16 strains have been shown to induce similar expression changes in host cells *in vitro*.[5] We carried out preliminary experiments to establish the optimal infectious dose of the virus and time p.i. that is the most informative for microarray analysis. The major criterion was to have a productive infection with clear CPE in host cells in parallel with sufficient total RNA yield and quality for use as starting material in GeneChip analysis. Previous studies from our laboratory using HG Focus array (Affymetrix, Santa Clara, CA) and cells from three normal subjects demonstrated that the maximal gene expression changes were observed 16 h p.i., and the vast majority of mRNAs up-regulated earlier (4 and 8 h p.i.) remained induced at later time points (unpublished data). We then used this time point to compare different virus doses of infection in PBE cells from two normal lung donors. Infection at MOI of 10 PFU per cell caused distinctive CPE with more than 50% of cells rounded and detached while producing sufficient amount of total RNA (≥10 μg) from adherent cells suitable for GeneChip hybridization (Supplementary Figure 2 online).

RNA extraction and microarray hybridization

Total RNA was isolated from the frozen TRIzol lysates according to manufacturer's protocol, and then purified by the RNeasy Mini Kit (Qiagen, Hilden, Germany). A total of 10 μg of purified total RNA samples were submitted to University of Wisconsin-Madison Gene Expression Center (Madison, WI) for labeling and hybridization. Following all appropriate

protocols and procedures for eukaryotic total RNA quality control, labeling and fragmentation, the biotin labeled cRNA samples were hybridized to either the Human Genome Focus GeneChip Array (samples 1–6) or Human Genome U133 Plus 2.0 GeneChip arrays (samples 7–18) (Affymetrix) according to the manufacturer's protocols.

Microarray data analysis

The CEL files extracted and processed with Affymetrix GeneChip Operating software (GCOS) were analyzed using Bioconductor[41] package "affy" based on R 2.4.1 statistical software (www.r-project.org). Log2-transformed expression values across all the chips were extracted using the Robust Multichip Average (RMA) method.[42] Processing with RMA involved background correction, probe level quantile normalization across all the chips and expression summarization. Statistical analysis for detecting differentially expressed genes in two sample comparisons involved either a paired t-test or a two independent sample *t*-test. We used the Benjamini-Hocberg false discovery rate controlling procedure to account for multiple testing.[43] Details are provided in the online supplement. Hierarchical clustering using centroid linkage method was performed based on the selected gene sets using Cluster 3.0[44] (Human Genome Center, Institute of Medical Science, University of Tokyo, Japan) and visualized using JavaTreeView 1.1.1[45] (http://jtreeview.sourceforge.net). The microarray data have been submitted to Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/projects/geo/) and assigned the accession number GSE13396.

Functional analysis of differentially expressed genes

Annotation and functional clustering of selected probe sets was performed using the DAVID (National Cancer Institute at Frederick, Frederick, MD) web-accessible program.[46] Genes with multiple corresponding probe sets were analyzed only when all probe sets demonstrated consistent changes in the same direction (up- or downregulation). The data were analyzed using the "Gene Functional Classification" tool using the "High" classification stringency setting.

Additionally, pathway analysis was performed using Gene Set Enrichment Analysis (GSEA) software[47] that determines whether an a priori defined set of genes shows statistically significant differences between two biological states (e.g. mock and virus infection). We have performed GSEA on our pre-ranked list of genes for each comparison of interest. The genes were ranked based on their t-test statistics and GSEA was run in the weighted mode. The main feature of this type of analysis is that it can detect subtle changes present in the data set.

Quantitative RT-PCR (qRT-PCR) validation of microarray results

First-strand cDNAsynthesis was performed using the RT^2 First Strand Kit (SuperArray, Frederick, MD). Human RT^2 RNA QC PCR Array (SuperArray) was used to assess the quality and integrity of purified RNAs. A total of 11 selected genes differentially expressed in asthma were targeted using custom-designed RT^2 Profiler™ PCR Array (SuperArray). The list of target genes, amplicon size and reference positions of SuperArray primers are shown in Supplementary Table S5 online. Expression of 11 additional genes was tested using primers shown in Supplementary Table S6 online. RT^2 Real-Time SYBR Green/ROX PCR master mix (SuperArray) was used to perform the reactions. Fold differences were determined by the $2^{-\Delta\Delta Ct}$ method. RV RNA was quantified in supernatants and adherent cells after infection using the two primers and probe described previously.[48] Additional details on qRT-PCR are provided in the online supplement.

Protein analysis

Supernatants from rhinovirus- and mock-infected cell cultures were assayed for IL8, IL1B and IL6 proteins. IL8 chemokine concentrations were determined by sandwich ELISA using anti-

human IL8 monoclonal antibody in combination with biotinylated polyclonal detection antibody and recombinant IL-8 protein as the standard (R&D systems, Minneapolis, MN). IL1B and IL6 cytokine levels were assessed using human IL1B and IL6 Beadmates™ assays (Millipore, Temecula, CA) according to the manufacturer's instructions. Luminex 100 (Luminex Corporation, Austin, TX) instrument was used to run plates and generate quantitative data. Sensitivity of the IL1B and IL6 assays for the protocol used was 8.2 $pgml^{-1}$.

Statistical analysis

Student's *t*-test was used to determine the statistical significance of the data. Significance was defined at $P<0.05$. Statistic calculations were carried out by SigmaPlot 11.0 software (Systat Software, San Jose, CA).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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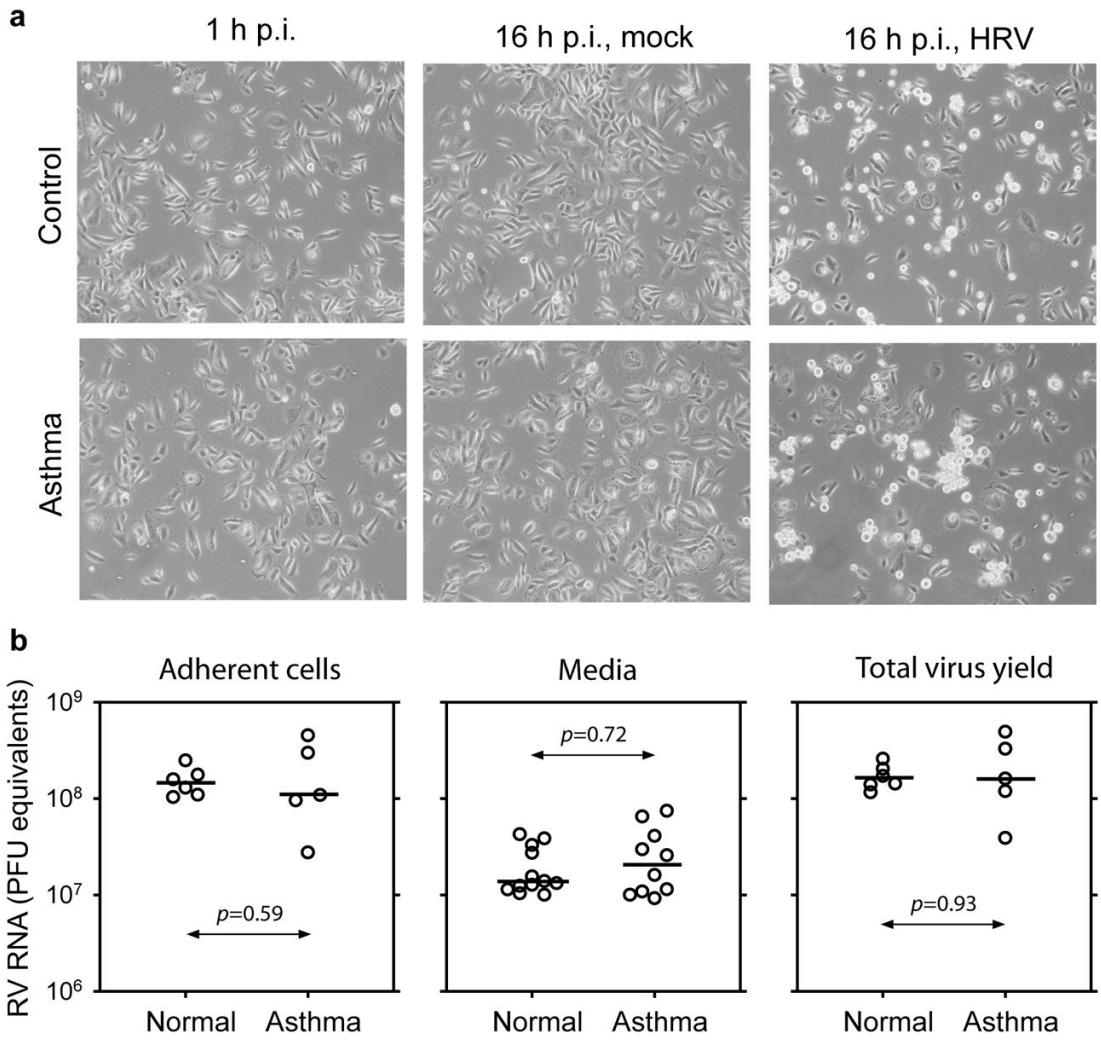

Figure 1.

RV1A infection induces similar cytopathic effect and viral RNA yield in cells obtained from donors with asthma and normal donors. (**a**) Representative microphotographs (magnification ×100) of primary bronchial epithelial (PBE) cells from one normal donor (no. 13) and one patient with asthma (no. 18) taken just after virus attachment period (left panel) and 16 hours post infection (p.i.) with medium alone (middle panel) or medium containing 10 plaque-forming units (PFU) per cell RV1A (right panel). (**b**) Quantification of viral RNA in adherent cells and growth media (including floating cells) collected 16 h p.i. of PBE cell cultures. Media samples both after microarray and quantitative PCR validation experiments were summarized. Viral RNA for each graph was calculated from each well of a six-well plate. Horizontal bars indicate medians. HRV, human RV.

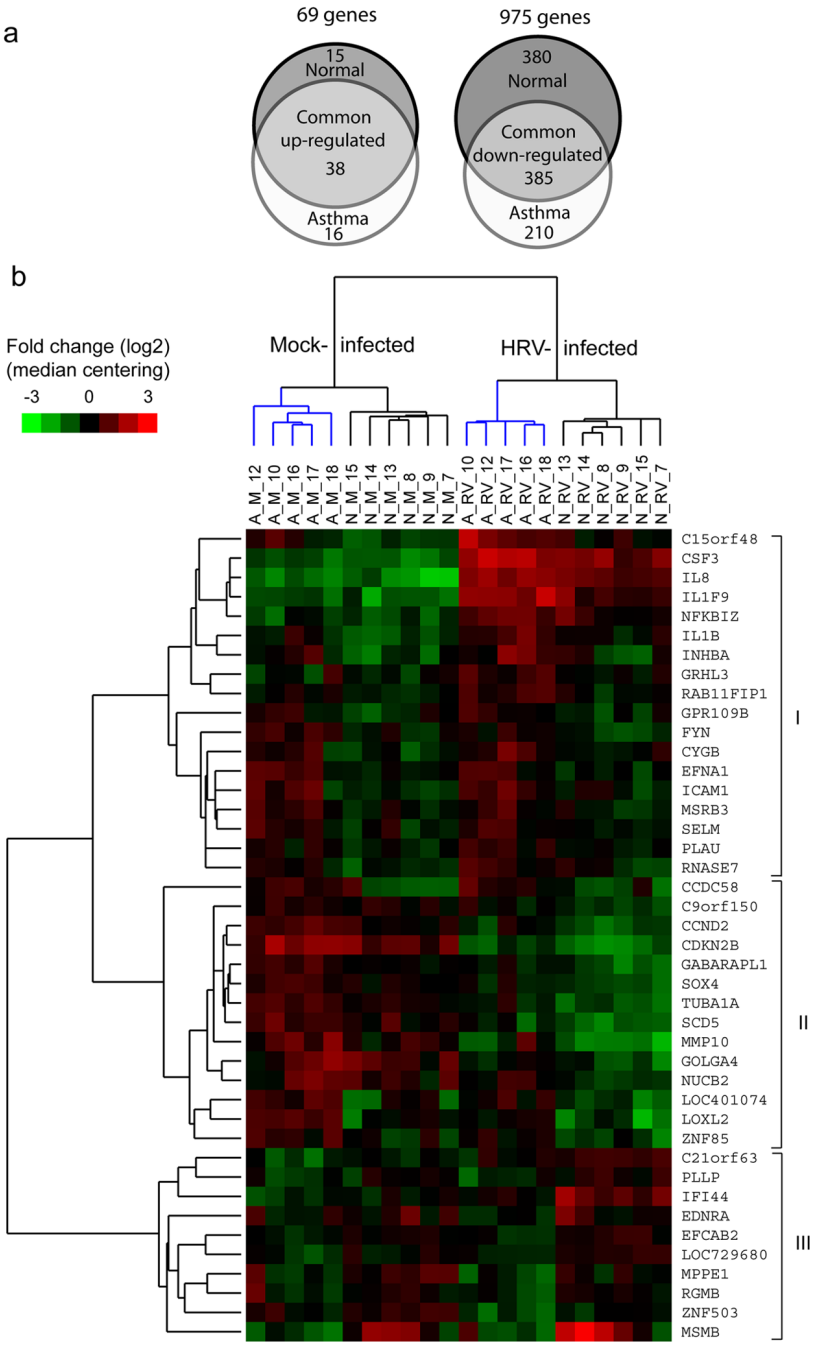

Figure 2.

Patterns of gene expression in normal and asthmatic cells. (**a**) Area-proportional Venn diagrams showing up- and downregulated genes determined in asthma and normal group samples after rhinovirus (RV) infection. Changes in gene expression (greater than or equal to twofold, adjusted $P<0.05$) common to both groups are shown by overlapping areas. The diagrams were generated with an online tool available at http://www.venndiagram.tk/. (**b**) Clustering analysis of gene expression patterns. Genes (n=42) were selected based on differential expression in infected cells from asthma vs. normal group, and then expression intensity values of mock- and RV-infected samples were analyzed by hierarchical clustering of samples and genes. The gene expression patterns of asthma (A) and normal (N) samples

clustered together for uninfected (M; mock), as well as RV-infected, cells. Clusters of asthma samples are shown in blue. Color bar represents fold changes (log_2 scale) in expression for each gene compared with median. HRV, human RV.

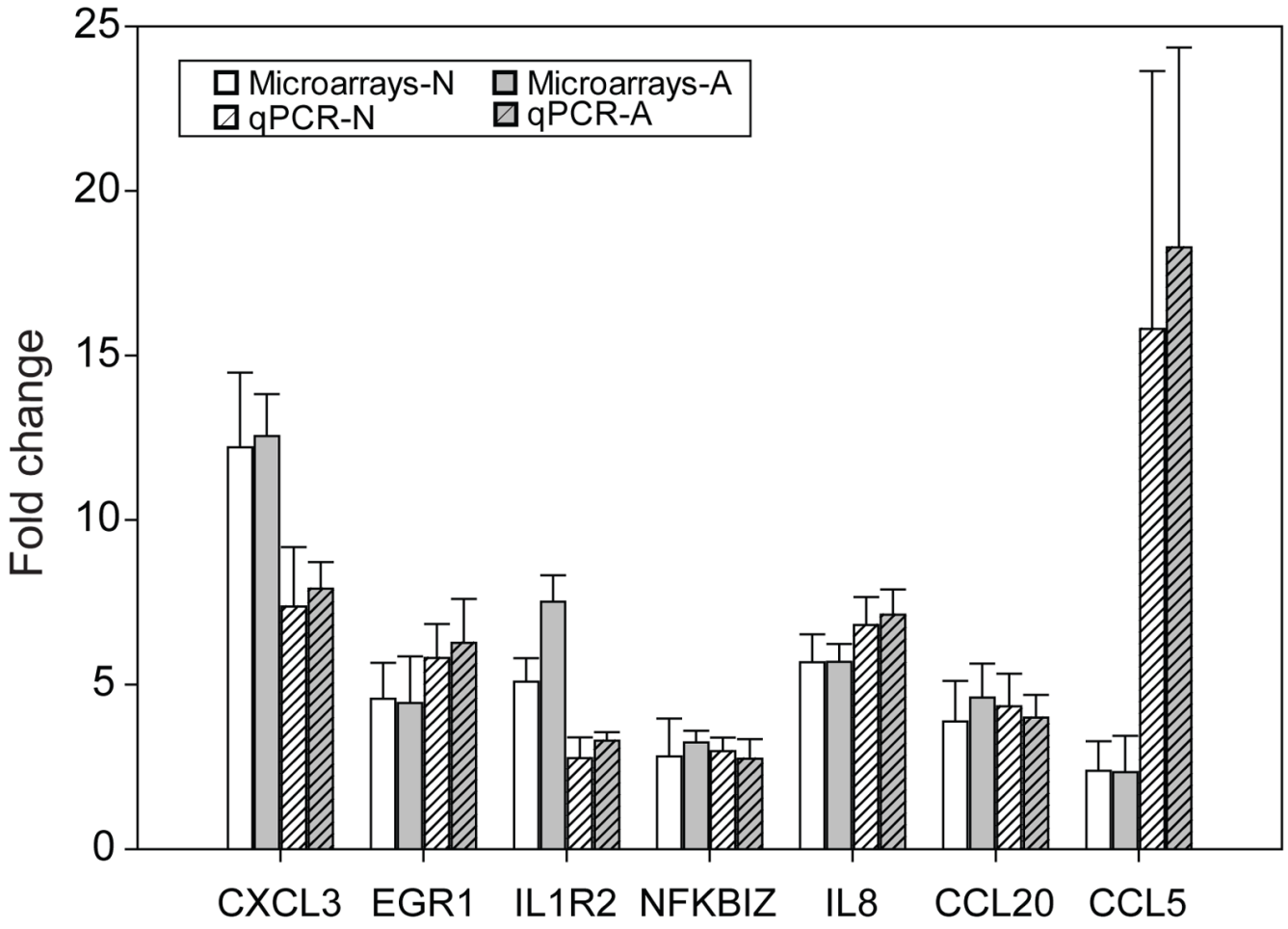

Figure 3.

Genes upregulated by rhinovirus (RV) infection: analysis by microarray vs. quantitative reverse transcription (qRT)-PCR. Seven target genes that were up-regulated in both RV-infected normal (N) and asthma (A) samples by microarray were analyzed in separate experiments using qRT-PCR. Expression levels in RV-infected cells were compared with those in mock-infected cells. Expression profiles were determined in six normal and five asthma samples.

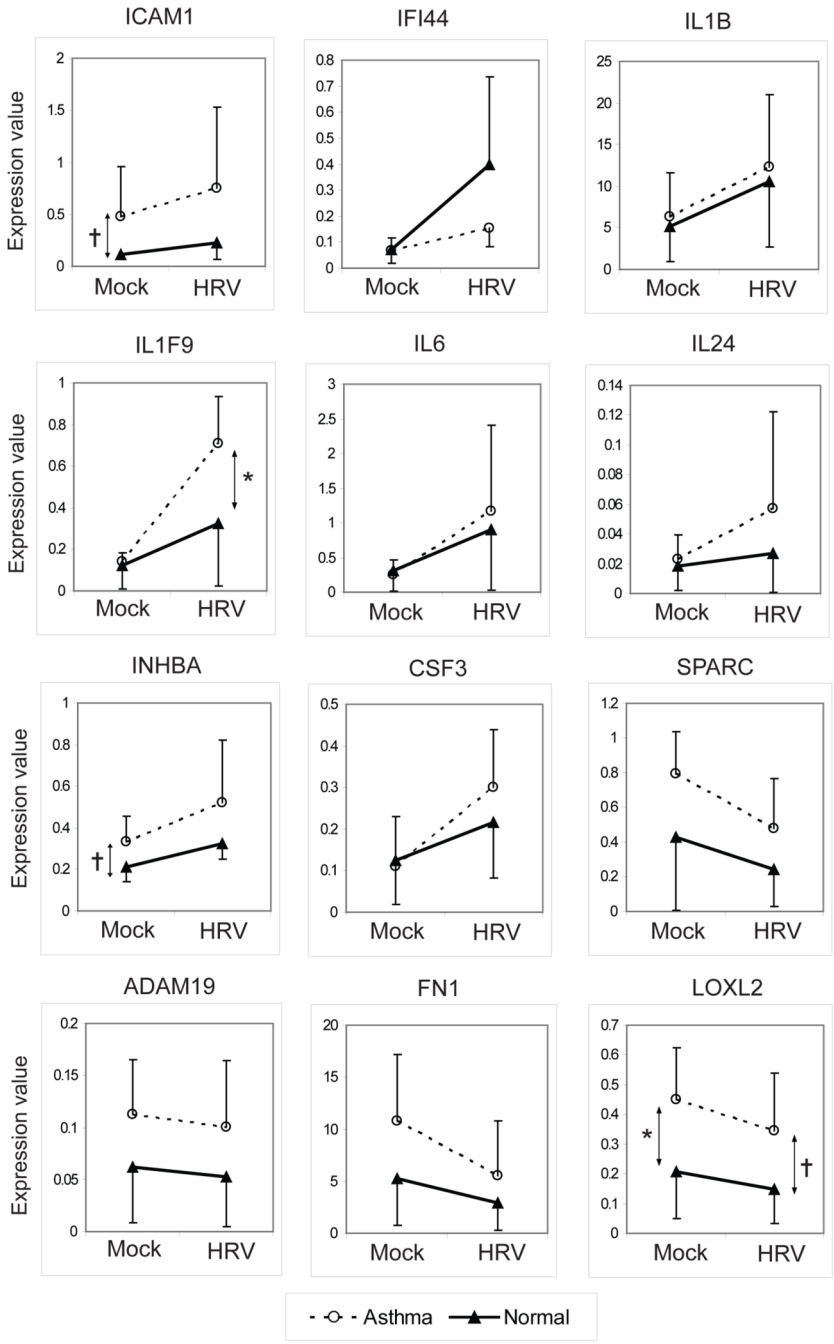

Figure 4.

Quantitative PCR analysis of genes differentially expressed in asthma. Genes that were induced ($n = 8$) or inhibited ($n = 4$) by rhinovirus (RV) infection, and also differentially expressed in asthma samples by microarray were analyzed in separate experiments using quantitative reverse transcription PCR. Expression values are $2^{-\Delta Ct}$ values determined by relative quantification method. Each line represents the mean and standard deviation. *$P<0.05$; †$P<0.1$. HRV, human RV.

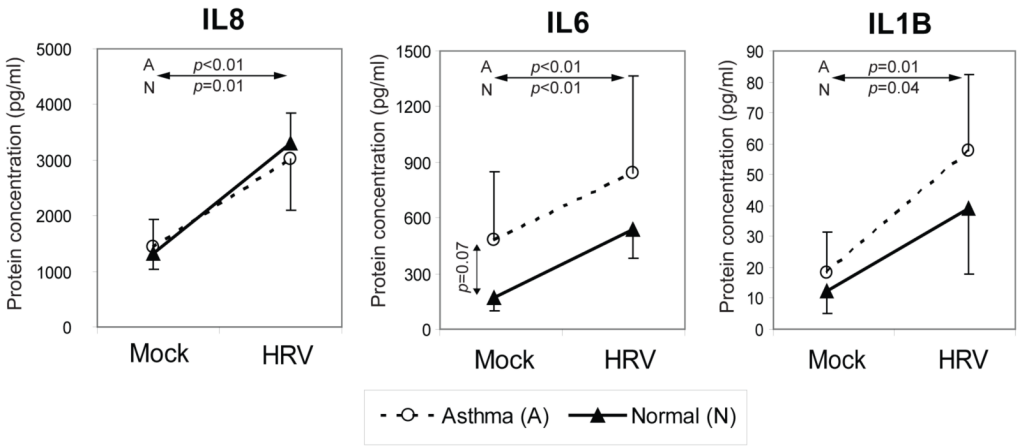

Figure 5.

Expression of three secreted pro-inflammatory cytokines in cell culture supernatants. Three tested cytokines were significantly induced at 16 h after rhinovirus (RV) infection, both in normal and asthmatic primary bronchial epithelial (PBE) cells ($P<0.05$); differences between asthma and normal groups were not significant ($P>0.05$). Each line represents the mean and standard deviation. HRV, human RV.

Table 1

Characteristics of subjects with asthma and controls

Subject[a]	Asthma	Gender	Age	PC_{20} mg/ml	FEV1 % predicted	Reversibility (%)	Allergen skin test
1	No	F	20	20	110	3	−
2	No	F	21	20	113	1	−
3	No	F	20	20	83	4	−
4	Yes	F	19	0.3	69	36	+
5	Yes	F	22	1.0	103	16	+
6	Yes	M	21	2.5	91	12	+
7[b]	No	M	24	20	86	7	−
8[b]	No	F	19	20	99	3	−
9[b]	No	M	21	20	99	5	−
10[b]	Yes	F	22	1.0	73	12	+
11[c]	Yes	M	25	2.2	80	16	+
12[b]	Yes	M	25	0.3	82	4	+
13[b]	No	F	41	20	91	5	−
14[b]	No	F	24	20	106	6	−
15[b]	No	M	37	20	110	1	−
16[b]	Yes	F	32	0.5	63	23	+
17[b]	Yes	M	35	4.6	90	7	+
18[b]	Yes	F	28	2.5	72	17	+

F, female; FEV1, forced expiratory volume in one sec; M, male; PC_{20}, provocative concentration of methacholine causing a 20% fall in FEV1;.

[a] Cells from subjects 1–6 were analyzed in preliminary experiments using HG Focus GeneChips; cells from subjects 7–18 were tested using HG U133 Plus 2.0 GeneChips.

[b] Samples were used for quantitative PCR validation and viral RNA quantification in independent experiments.

[c] Sample was not analyzed by GeneChip because of the insufficient total RNA yield.

Table 2

RV-induced changes in gene expression: combined asthma and normal groups

Gene symbol	Gene name	Biological function	Normal FD[a]	Asthma FD[a]
Upregulated genes				
CXCL3	chemokine (C-X-C motif) ligand 3	chemokine	12.2 / 36.7	12.6 / 5.6
CXCL2	chemokine (C-X-C motif) ligand 2	chemokine	6.7 / 9.4	7.3 / 6.6
IL8	interleukin 8	chemokine	5.7 / 20.7	5.7 / 4.4
CSF3	colony stimulating factor 3 (granulocyte)	cytokine	5.4	7.2
IL1R2	interleukin 1 receptor, type II	cytokine receptor	5.1	7.5
CXCL1	chemokine (C-X-C motif) ligand 1	chemokine	4.7 / 6.2	4.1 / 3.9
EGR1	early growth response 1	transcription factor	4.6	4.4
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	endopeptidase inhibitor	4.4	5.3
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	inhibitor of apoptosis	3.9 / 4.9	3.9 / 2.0
CCL20	chemokine (C-C motif) ligand 20	chemokine	3.8 / 5.7	4.5 / 3.8
IL13RA2	interleukin 13 receptor, alpha 2	cytokine receptor	3.7	4.7
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	cytokine	3.6 / 4.1	3.2
IL1F9	interleukin 1 family, member 9	cytokine	3.5	6.0
SPRR2B	small proline-rich protein 2B	structural molecule	3.1	4.5
ZC3H12A	zinc finger CCCH-type containing 12A	transcription regulator	3.1	3.2
IL6	interleukin 6	cytokine	2.9 / 5.6	3.7
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	transcription regulator	2.8	3.2
1558048_x_at[b]	cDNA clone IMAGE:4523513	NA	2.7	3.1
S100A3	S100 calcium binding protein A3	calcium and zink binding	2.7	2.6 / 2.0
SOCS3	suppressor of cytokine signaling 3	JAK2 kinase inhibitor	2.7	3.3
Downregulated genes				
225123_at[b]	FLJ33813	NA	9.1	8.4
IVNS1ABP	influenza virus NS1A binding protein	response to virus	8.6	8.3
ITGB6	integrin, beta 6	receptor for fibronectin	7.0	8.9
DLG1	discs, large homolog 1 (Drosophila)	cell-cycle regulation	5.7	5.4
TIA1	TIA1 cytotoxic granule-associated RNA binding protein	inductor of apoptosis	5.6	4.4
FN1	fibronectin 1	ECM constituent	5.6	4.1
SLITRK6	SLIT and NTRK-like family, member 6	membrane protein	5.4	4.2
PCMTD1	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 1	protein modification	5.3	5.2
ACSL3	acyl-CoA synthetase long-chain family member 3	lipid biosynthesis	5.2	4.3
UHRF2	ubiquitin-like, containing PHD and RING finger domains, 2	cell-cycle regulation	5.0	4.4
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	cell-cycle regulation	5.0	4.5
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	hydroxylation of lysyl residues in collagen	5.0	4.7

Gene symbol	Gene name	Biological function	Normal FD[a]	Asthma FD[a]
FLRT2	fibronectin leucine rich transmembrane protein 2	cell adhesion and/or receptor signaling	4.9	5.2
MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog	transcription factor	4.8	5.4
GBP3	guanylate binding protein 3	GTP binding	4.8	3.9
SFRS12	splicing factor, arginine/serine-rich 12	splicing factor	4.8	3.4
KITLG	KIT ligand	ligand of the tyrosine- kinase receptor	4.8	3.6
SMC4	structural maintenance of chromosomes 4	chromosome organization	4.8	2.7
TTC37	tetratricopeptide repeat domain 37	binding	4.8	3.1
SLC39A10	solute carrier family 39 (zinc transporter), member 10	zink ion transport	4.8	4.4

ECM, extracellular matrix; FD, fold difference (in expression); NA, not available; RV, rhinovirus.

[a]Corresponding fold differences in expression (nominal $P<0.05$) generated by HG Focus microarray using cells from six additional donors (three normal and three asthmatic) are indicated after “slash”.

[b]Affymetrix probe set ID is used when gene symbol is not available.

Table 3

Differentially expressed genes: asthma vs. normal groups

Gene symbol	Gene name	Biological function	FD[a]	P-value[b]
Differentially expressed after RV infection				
LOXL2	lysyl oxidase-like 2	cross-linking of collagen and elastin	2.2	0.010
MMP10	matrix metallopeptidase 10 (stromelysin 2)	metalloproteinase	2.2	0.040
C15orf48	chromosome 15 open reading frame 48	tumor suppressor	2.2	0.008
LOC401074	hypothetical LOC401074	NA	2.1	0.004
CCDC58	coiled-coil domain containing 58	NA	2.1	0.010
INHBA	inhibin, beta A	growth/differentiation factor	2.1	0.045
IL1F9	interleukin 1 family, member 9	cytokine	2.0	0.004
MSMB	microseminoprotein, beta-	tumor suppressor	−3.6	0.031
IFI44	interferon-induced protein 44	response to virus, cell-cycle arrest	−2.1	0.002
Differentially expressed in mock-infected cells				
ADAM19	ADAM metallopeptidase domain 19	metalloproteinase, cell-matrix interactions	2.8	0.023
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	synthesis of ECM, changes in cell shape	2.6	0.034
MAP1B[c]	microtubule-associated protein 1B	microtubule assembly	2.6	0.014
LOC401074	NA	NA	2.3	0.014
227140_at[d]	NA	NA	2.3	0.038
FN1	fibronectin 1	cell adhesion and migration	2.3	0.050
MAN1A1	mannosidase, alpha, class 1A, member 1	glycosyl hydrolase	2.3	0.002
AKAP12	A kinase (PRKA) anchor protein 12	scaffold protein, binds to protein kinase A	2.3	0.040
CCDC58	coiled-coil domain containing 58	NA	2.2	0.014
LOXL2	lysyl oxidase-like 2	cross-linking of collagen and elastin	2.2	0.006
CXCL5	chemokine (C-X-C motif) ligand 5	neutrophil activation	2.2	0.046
SCG5	secretogranin V (7B2 protein)	calcium binding	2.1	0.039
TMSB15	thymosin beta 15a	metastasis of human prostate and breast cancer	2.1	0.015
COL4A1[c]	collagen, type IV, alpha 1	ECM structural protein	2.1	0.022
IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3	RNA synthesis and metabolism	2.0	0.035
SLC46A3	solute carrier family 46, member 3	integral to membrane	2.0	0.031
C15orf48	chromosome 15 open reading frame 48	tumor suppressor	2.0	0.019
SERPINE1[c]	serpin peptidase inhibitor, clade E	plasminogen activator inhibitor	2.0	0.035
INHBA	inhibin, beta A	growth/differentiation factor	1.9	0.028
PPL	periplakin	structural protein, desmosomes	−2.0	0.025
MSMB[c]	microseminoprotein, beta-	tumor suppressor	−2.6	0.014

ECM, extracellular matrix; FD, fold difference (in expression); NA, not available; RV, rhinovirus.

[a] Negative numbers indicate down-regulation in asthmatic cells compared with normal cells.

[b] Nominal *P*-values from unpaired t-test comparison of asthma vs. normal samples (either infected or mock-infected).

[c] These genes had more than one probe set that revealed similar expression changes; corresponding fold changes and *P*-values were averaged.

[d] Gene symbol is not available for the probe set.

Table 4

Functional groupings of genes that are down-regulated after RV infection or differentially expressed in asthma

DAVID functional group term	Gene number[a]	Genes[b](%)	P-value[c]
Down-regulated genes (975 genes)			
Biological process (GO database)			
post-translational protein modification	120	14%	3×10^{-8}
biopolymer metabolic process	310	35%	5×10^{-7}
intracellular transport	65	7%	2×10^{-6}
ubiquitin cycle	50	6%	1×10^{-5}
protein transport	58	7%	9×10^{-5}
mRNA processing	29	3%	1×10^{-4}
Differentially expressed in asthma compared with normal (42 genes)			
Biological process (GO database)			
defense response	6	14%	0.008
cell-cell signaling	7	16%	0.003
response to external stimulus	7	16%	0.002
cell surface receptor linked signal transduction	10	23%	0.004
immune response	7	16%	0.003

DAVID, Database for Annotation, Visualization and Integrated Discovery; GO; Gene Ontology; RV, rhinovirus.

[a] Genes involved in the term.

[b] Percentage of involved genes from total gene list.

[c] Modified Fisher exact *P*-value.