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RESEARCH ARTICLE

Identification of gene biomarkers for respiratory syncytial virus infection in a bronchial epithelial cell line

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Abstract Respiratory syncytial virus (RSV) infection involves complex virus-host interplay. In this study, we analyzed gene expression in RSV-infected BEAS-2B cells to discover novel signaling pathways and biomarkers. We hybridized RNAs from RSV- or vehicle-treated BEAS-2B to Affymetrix HU133 plus 2.0 microarrays ($n = 4$). At 4 and 24 h post-infection, 277 and 900 genes (RSV/control ratio $\geq$2.0 or $\leq$0.5), and 1 and 12 pathways respectively were significantly altered. Twenty-three and 92 genes at 4 and 24 h respectively matched respiratory disease biomarkers with ARG2 flagged at 24 h and SCNN1G, EPB41L4B, CSF1, PTEN, TUBB1 and ESR2 at both time points. Hierachical clustering showed a cluster containing ARG2 and IL8. In human bronchial epithelial cells, RSV upregulated arginase II protein. Knockdown of ARG2 increased RSV-induced IL-8, LDH and histone release. With microarray, we identified novel proximal airway epithelial cell genes that may be tested in the sputum samples as biomarkers of RSV infection.

Keywords Arginase · Microarray · Interleukin-8

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Introduction

Respiratory syncytial virus (RSV), a negative-stranded RNA virus, is a common viral pathogen capable of causing respiratory tract infection and acute lung injury. In children, RSV bronchiolitis is estimated to cause 91,000 hospital admissions per year in the United States (Openshaw 2005). RSV is the pathogen in up to 50% of children hospitalized with bronchiolitis and 25% of children with pneumonia (Hall and McCarthy 1995). In adults, RSV may cause severe pneumonia with high mortality, especially in patients with underlying cardiopulmonary illnesses, the elderly and the immunocompromised (Falsey and Walsh 2000; La Montagne 1997). RSV infection also predisposes to the development of acute lung injury (Hammer et al. 1997; Hertz et al. 1989; Zaroukian et al. 1988).

The bronchial epithelial cell is the primary target of RSV. When RSV enters the respiratory tract, the surface proteins G (attachment) and F (fusion) of the virus interact with a heparan sulfate located on the bronchial epithelial cell surface (Feldman et al. 2000) (Levine et al. 1987). Once inside the cells, RSV replicates and assembles using the actin cytoskeleton (Ulloa et al. 1998) (Burke et al. 1998; Burke et al. 2000) (Werling et al. 1999) (Parton et al. 1994). Although the mechanisms by which RSV causes epithelial infection and injury are not entirely clear, there is increasing evidence that much of the pathology caused by

RSV is due to the immune response to the infection (Openshaw 1995, 2005). Replication and proliferation of RSV inside the cells induce Th1/Th2 immune responses (Peebles and Graham 2005) (Welliver et al. 2002). The infected epithelial cells and immune cells release proinflammatory cytokines and chemokines that may result in acute lung injury (Krishnan et al. 2004). Inappropriate activation of the Th2 cellular immune response and deposition of antigen–antibody immune complexes may trigger complement activation and modulate the severity of RSV-induced cellular injury (Graham 1995; Openshaw 1995, 2005; Polack et al. 2002).

Our knowledge of the pathogenesis of RSV-induced lung infection is incomplete in part due to the complex virus-host cell interplay following the infection. Many previous studies have focused on specific aspects of the host response against RSV. Several microarray studies also have been reported (Kong et al. 2003; Martinez et al. 2007; Zhang et al. 2001). These studies provided a global view of gene expression during the complex virus-host cell interactions, but they were performed in transformed human alveolar epithelial cells using smaller arrays (A549 cells). It is unclear whether or not these events in distal lung cells could be extrapolated to the epithelial cells of proximal airways, which are early targets of RSV infection. In addition, clinical diagnosis of RSV infection still relies on the demonstration of the virus in the biological specimens. It is unclear how these gene biomarkers obtained in distal lung cells may help clinical diagnosis since these cells are less available. Thus in this study, we examined gene expression profile in human bronchial epithelial cells using microarray technology. The main goals were to explore signaling pathways and genes that may be used as biomarkers of infection in sputum specimens, which contain proximal airway cells.

Methods

Cell culture

For microarray experiments, human bronchial epithelial cell line (BEAS-2B, subclone S6) was obtained from the laboratory of Dr. Curtis C. Harris and maintained in serum-free growth medium (KGM, Clonetics, San Diego, CA) in T75 tissue culture flasks. The cells were used in experiments in their 62nd to 73rd passage. Cells were plated at 100 mm-diameter tissue culture plates (Costar, Cambridge, MA) in KGM and incubated at 37°C and 5% CO_2 for 72 h before they were used for microarray experiments.

Primary human bronchial epithelial cells (HBEC) were also used for all non-microarray experiments (including PCR confirmation and siRNA).These primary cells were obtained from normal volunteers through bronchoscopic bronchial brushings (Ghio et al. 2000; Huang et al. 2003). Subjects were informed of the procedures and potential risks and each signed an informed consent. The protocol was approved by the University of North Carolina School of Medicine Committee on Protection of the Rights of Human Subjects and US Environmental Protection Agency. All brushings were processed by a single experienced technician following the established standard of procedures in our laboratory. The cells (passage 2 or 3) were maintained in bronchial epithelial growth medium (BEGM) (Clonetics, San Diego, CA), supplemented with bovine pituitary extract, insulin 5 μg/ml, hydrocortisone 0.5 μg/ml, gentamicin 50 μg/ml, retinoic acid 0.1 ng/ml, transferrin 10 μg/ml, triiodothyrodine 6.5 ng/ml, epinephrine 0.5 μg/ml, human epidermal growth factor 0.5 ng/ml.

Preparation of RSV

RSV (Long strain/lot 15D) was obtained from American Type Culture Collection (Bethesda, MD) and was propagated in mycoplasma-free HEp2 cells (ATCC 23-CCL, Bethesda, MD) as previously described (Becker et al. 1997). HEp2 supernatants containing infectious RSV were collected and the virus was precipitated using 10% polyethylene glycol (Sigma, St. Louis, MO). The precipitate was dissolved in NTE (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA), pH 7.4, and overlaid on a discontinuous 60, 45, and 30% sucrose gradient made up in NTE. After centrifugation for 90 min at $85{,}000\times g$ in a Sorvall TH-641 rotor, the virus was collected from the 45–60% interface. This preparation contained $\sim 1 \times 10^7$ plaque-forming units (PFU)/ml of RSV when tested for syncytia formation on HEp2 cells. The virus was snap frozen in liquid nitrogen and stored in small aliquots at −70°C until use.

RSV infection

For microarray experiments, confluent monolayer cultures of BEAS-2B were infected with RSV at a multiplicity of infection (MOI) of 1.0 PFU/cell. Virus was added to the cells for 2 h and then removed by a gentle wash with culture medium, followed by addition of 1 ml/well fresh KGM for 4 or 24 h (Soukup and Becker 2003). The control cells were incubated with vehicle.

Purification and hybridization of RNA

Total cellular RNA was extracted from the cells with Trizol reagent (GIBCO BRL Life Technologies, Gaithersburg, MD) and further purified with phenol/chloroform. The RNA integrity was assessed with an Agilent 2100 bioanalyzer (Agilent Technologies, Inc, Pao Alto, CA). The

260 nm/280 nm ratios for all RNAs were >1.9. The RNAs were hybridized to Hu133 plus 2.0 gene chips (Affymetrix, Inc, Santa Clara CA). The chip contained probes for 38,500 human genes. The hybridization was performed by the Expression Analysis, Inc. (Durham, NC) according to the "Affymetrix Technical Manual". Briefly, total RNA (10 μg) was converted into cDNA using Reverse Transcriptase (Invitrogen Corp, Carlsbad, CA) and a modified oligo(dT)24 primer that contains T7 promoter sequences (GenSet Corp, San Diego, CA). After first strand synthesis, residual RNA was degraded by the addition of RNaseH and a double-stranded cDNA molecule was generated using DNA polymerase I and DNA ligase. The cDNA was then purified and concentrated using a phenol: chloroform extraction followed by ethanol precipitation. The cDNA products were incubated with T7 RNA Polymerase and biotinylated ribonucleotide using an In Vitro Transcription kit (Enzo Diagnostics, Inc, New York, NY). One-half of the cRNA products were purified using an RNeasy column (Qiagen Inc, Valencia, CA) and quantified with a spectrophotometer. The cRNA target (20 μg) was incubated at 94°C for 35 min in fragmentation buffer (Tris, magnesium acetate, potassium acetate). The fragmented cRNA was diluted in hybridization buffer (2-morpholinoethanesulfonic acid, NaCl, EDTA, Tween 20, herring sperm DNA, acetylated bovine serum albumin) containing biotin-labeled OligoB2 and Eukaryotic Hybridization Controls (Affymetrix). The hybridization cocktail was denatured at 99°C for 5 min, incubated at 45°C for 5 min and then injected into a GeneChip cartridge. The GeneChip array was incubated at 42°C for at least 16 h in a rotating oven at 60 rpm. GeneChips were washed with a series of non-stringent (25°C) and stringent (50°C) solutions containing variable amounts of 2-morpholinoethanesulfonic acid, Tween20 and SSPE (3 M NaCl, 0.2 M, NaH_2PO_4, 0.02 M EDTA). The microarrays were then stained with Streptavidin Phycoerythrin and the fluorescent signal was amplified using a biotinylated antibody solution. Fluorescent images were detected in a GeneChip® Scanner 3000 and expression data was extracted using the default settings in the MicroArray Suite 5.0 software (Affymetrix). All GeneChips were scaled to a median intensity setting of 500. A total of 9 microarrays were performed, including control and RSV (1.0 MOI) at 4 and 24 h ($n = 3$ biological replicates). The vehicle-treated cells were harvested at 24 h and used as the control for all RSV experiments.

Microarray data analysis

The microarray data were deposited in the Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/) (accession number GSE3397). Gene expression values were background-corrected and normalized globally using the default setting of the Affymetrix Microarray Suite version 5.0 and log2-transformed according to the "Affymetrix Statistical Algorithm Reference Guide". The log2 signal ratios of RSV over control for all probe sets were compared to zero using the one class *t*-test with a p of <0.05 using the Multi experiment Viewer (MeV version 3.0, The Institute of Genomic Research, Rockville, MD). Genes that showed statistically significant expression over control and a cut-off fold change of >2.0 or <0.5 were retained. If more than one probe set for the same gene were flagged, their ratios were averaged. Only data from three microarray experiments were analyzed because one of the control data sets was found to be very different from the other three controls during the clustering analysis.

Pathway and biomarkers analysis

The differentially expressed genes at 4 and 24 h post-infection were categorized based on the KEGG_Pathway using the Database for Annotation, Visualization and Integrated Discovery (DAVID, version 2006) (http://niaid.abcc.ncifcrf.gov/). Pathways with $p \leq 0.1$ were considered significant. We also used Venn diagram and Ingenuity Pathway Analysis (http://www.ingenuity.com) (Ingenuity System, Inc, Redwood City, CA) to identify potential biomarkers for respiratory diseases.

Quantitative polymerase chain reaction (Q-PCR)

Q-PCR was performed for selected genes. cDNAs were synthesized from 0.4 μg of total RNA in 100 μl of buffer containing 5 μM random hexaoligonucleotide primers (Pharmacia, Piscataway, NJ), 10 U/μl Moloney murine leukemia virus reverse transcriptase (GIBCO BRL Life Technologies), 1 U/μl RNase inhibitor (RNasin®, Promega, Madison, WI), 0.5 mM dNTP (Pharmacia), 50 mM KCl, 3 mM $MgCl_2$ and 10 mM Tris-HCl (pH 9.3) for 1 h at 39°C. Reverse transcriptase was heat-inactivated at 94°C for 4 min.

Q-PCR of specimen and standard cDNA was completed using *Taq*Man pre-developed assay reagents. Quantitative fluorogenic amplification of cDNA was performed using the ABI Prism 7500 Sequence Detection System, primers and probes of interest and *Taq*Man Universal PCR Master Mix (Applied Biosystems). The relative abundance of mRNA levels was determined from standard curves generated from a serially diluted standard pool of cDNA prepared from control HPAEC cultures. The relative abundance of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, Unigene accession #544577) mRNA was used to normalize levels of the mRNAs of interest. For Q-PCR verification, RNA from six additional experiments was collected. RNA samples for microarray and Q-PCR were

collected from different experiments and do not represent the same sample.

Cytokine/chemokine measurements

IL-8 and CSF2 (GMCSF) in the medium were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN) according to the manufacturer's directions. The detection limit for the kits was 10 pg/ml.

Western blot analysis

After the exposure, the cells were washed once with ice cold phosphate-buffered saline (PBS) and then lysed with radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 0.1 mM vandal sulfate and protease inhibitors (0.5 mg/ml aprotinin, 0.5 mg/ml E-64, 0.5 mg/ml pepstatin, 0.5 mg/ml bestatin, 10 mg/ml chymostatin, and 0.1 ng/ml leupeptin). The cell lysates were then centrifuged at 3,000*g* for 10 min at 4°C. Protein concentration of supernatant was measured with Bio-Rad protein assay reagent. Cellular proteins were separated by 10% SDS–PAGE and transferred to a polyvinylidene difluoride membrane. The blot was blocked with 5% milk in PBS with 0.05% Tween-20 for 1 h at room temperature, washed briefly, and then probed with a rabbit antibody against human arginase II (Catalog# sc-20151, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibodies. Bands were detected by using ECL and films.

Transfection of HBEC with arginase II siRNA

Cells were grown to ~60–70% confluency in 6-well plates. They were then transfected with Gene Silencer® transfecting agent plus (Gene Therapy System, Inc, San Diego, CA) with arginase II siRNA (100 nM) (Catalog# sc-29729, Santa Cruz Biotechnology, Santa Cruz, CA) in serum-free EGM-2 medium for 3 h according to the manufacturer's recommendation. Control cells were incubated with Gene Silencer® negative control #1 siRNA (Ambion, Inc, TX). Fresh EGM-2 medium with 2% fetal bovine serum was then added and cells were cultured for an additional 24 h. Cells were then treated with RSV or vehicle as described above. Cell lysates were collected at 24 and 48 h post-infection and arginase II protein expression was measured by western blotting.

Statistical analysis

All non-microarray data are expressed as mean ± standard errors (SE). Data from multiple groups were analyzed by one way analysis of variance (ANOVA) followed by the Tukey's subtest for between-group comparisons. The statistical analysis was performed using StatView (version 5.0.1, SAS, Inc, Cary NC). A p value of <0.05 was considered statistically significant.

Results

Gene expression profile induced by RSV

Using the statistical algorithm, we found 2,630 probes differentially expressed at 4 h post-infection. After eliminating duplicate and non-annotatable probes, there were 277 genes with a RSV/control ratio ≥ 2.0 or ≤ 0.5. Of these genes, 106 were upregulated and 171 were downregulated. KEGG pathway analysis using these genes showed the neuroactive ligand-receptor interaction as the only significant pathway. There were 11 genes in this pathway ($p = 0.012$) (Table 1). The expression of these genes at 24 h after RSV infection is also shown in Table 1. Compared to the 4-h time point, the expression of many of these genes remained altered, although the fold changes were smaller.

At 24 h post-infection, we identified 6,818 probes differentially expressed. After eliminating duplicate and non-annotatable probes, there were 912 genes with a RSV/control expression ratio ≥ 2.0 or ≤ 0.5. KEGG pathway analysis of the 912 genes identified 12 pathways (Table 2). These pathways include P52 signaling pathway, apoptosis, the JAK-STAT signaling pathway and the cytokine-cytokine receptor interaction known to be related to RSV infection. Genes in these pathways are shown in Tables 3, 4, 5 and 6 respectively. Several genes in the cytokine-cytokine receptor interaction pathway, e.g., CSF1, CSF2 and IL2, already had increased expression at 4 h after infection. Other pathways mapped with at least ten genes included ubiquitin mediated proteolysis (16 genes), prostate cancer (11 genes), small cell lung cancer (11 genes), cell cycle (13 genes), and hematopoietic cell lineage (10 genes).

We confirmed upregulation of 7 genes with Q-PCR in independent experiments (Fig. 1). In addition, we confirmed that RSV increased the release of IL-8 and CSF2 in a dose-dependent manner (Fig. 2). In microarray, IL-8 and CSF2 gene expression was increased by more than 3 and 20-fold respectively.

Pathways and biomarkers analysis

Using Venn diagram, we found 129 genes were upregulated and 192 genes were downregulated at both 4 and 24 h post-infection (Fig. 3). The top 10 up- and down-regulated genes are listed in Table 7. We also mapped the significant

Table 1 Genes in the neuroactive ligand-receptor interaction pathway that were differentially expressed at 4 h (4 H) after RSV infection. Fold changes at 24 h (24 H) are also shown

UniGene	Symbol	Fold		Gene name
		4 H	24 H	
Hs.388226	HCRTR1	5.37	2.26	Hypocretin (orexin) receptor 1
Hs.258580	P2RX2	5.14	3.59	Purinergic receptor p2x, ligand-gated ion channel, 2
Hs.530653	GRID1	2.92	1.47	Glutamate receptor, ionotropic, delta 1
Hs.200262	TACR1	2.41	1.15	Tachykinin receptor 1
Hs.405348	AGTR2	2.35	1.12	Angiotensin II receptor, type 2
Hs.302026	NPFFR1	−2.00	−2.09	Neuropeptide FF receptor 1
Hs.251412	GIPR	−2.27	−1.30	Gastric inhibitory polypeptide receptor
Hs.147361	GRM5	−2.27	−1.52	Glutamate receptor, metabotropic 5
Hs.7195	GABRG2	−2.50	−1.86	Gamma-aminobutyric acid (gaba) A receptor, gamma 2
Hs.348500	VIPR1	−2.56	1.08	Vasoactive intestinal peptide receptor 1
Hs.2131	AVPR1A	−3.23	−1.39	Arginine vasopressin receptor 1a

Table 2 Pathways associated with RSV infection at 24 h

Pathways	Gene count	*p* value
p53 signaling pathway	12	0.002
Circadian rhythm	5	0.0059
Ubiquitin mediated proteolysis	16	0.011
Jak-STAT signaling pathway	17	0.019
Cytokine–cytokine receptor interaction	24	0.032
Prostate cancer	11	0.034
Small cell lung cancer	11	0.034
Cell cycle	13	0.039
Terpenoid biosynthesis	3	0.046
Apoptosis	10	0.063
Hematopoietic cell lineage	10	0.067
Methionine metabolism	4	0.090

genes at 4 and 24 h post-infection to Biomarker Filter (Ingenuity Pathways Analysis) to identify potential biomarkers for respiratory diseases. We identified 23 and 92 genes at 4 and 24 h respectively, that were the biomarkers linked to respiratory diseases. Six genes were differentially expressed at both time points (Fig. 4). Four genes (SCNN1G, EPB41L4B, CSF1, PTEN) were upregulated and one gene (TUBB1) was down-regulated at both time points. ESR2 was upregulated at 4 h, but downregulated at 24 h post-infection.

Role of arginase II in RSV-induced epithelial injury

In examining the 24-h genes (92 genes) that were biomarkers for respiratory diseases, we noted ARG1 and ARG2 were among the upregulated genes (2.2 and 2.1-fold

Table 3 Genes in P53 signaling pathway that were differentially expressed at 24 h (24 H) after RSV infection. Fold changes at 4 h (4 H) are also shown

UniGene	Symbol	Fold		Gene name
		4 H	24 H	
Hs.446564	DDB2	1.39	7.50	Damage-specific DNA binding protein 2, 48 kDa
Hs.567303	MDM2	−1.07	4.74	MDM2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)
Hs.469543	SESN2	1.32	3.13	Sestrin 2
Hs.467020	BBC3	1.44	3.02	BCL2 binding component 3
Hs.110571	GADD45B	1.18	2.76	Growth arrest and DNA-damage-inducible, beta
Hs.96	PMAIP1	−1.04	2.61	Phorbol-12-myristate-13-acetate-induced protein 1
Hs.500466	PTEN	1.06	2.11	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
Hs.370771	CDKN1A	1.04	2.07	Cyclin-dependent kinase inhibitor 1A (p21, CIP1)
Hs.295923	SIAH1	−1.55	2.02	Seven in absentia homolog 1 (*Drosophila*)
Hs.558304	CASP8	−1.58	−2.63	Caspase 8, apoptosis-related cysteine peptidase
Hs.24529	CHEK1	−1.05	−2.86	CHK1 checkpoint homolog (s. Pombe)
Hs.567387	CCNE2	−1.39	−4.00	Cyclin E2

Table 4 Genes in the apoptosis pathway that were differentially expressed at 24 h (24 H) after RSV infection. Fold changes at 4 h (4 H) are also shown

UniGene	Symbol	Fold		Gene name
		4 H	24 H	
Hs.127799	BIRC3	1.53	5.88	Baculoviral IAP repeat-containing 3
Hs.81328	NFKBIA	1.41	4.58	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
Hs.278901	PIK3R5	−0.26	3.98	Phosphoinositide-3-kinase, regulatory subunit 5, p101
Hs.356076	XIAP	1.03	2.32	X-linked inhibitor of apoptosis
Hs.449207	IRAK2	1.14	2.22	Interleukin-1 receptor-associated kinase 2
Hs.516966	BCL2L1	1.11	2.05	BCL2-like 1
Hs.557403	IL1R1	−1.17	−2.27	Interleukin 1 receptor, type I
Hs.213467	TNFRSF10D	−1.26	−2.27	Tumor necrosis factor receptor superfamily, member 10D, decoy with truncated death domain
Hs.86131	FADD	−1.21	−2.33	Fas (TNFRSF6)-associated via death domain
Hs.558304	CASP8	−1.58	−2.63	Caspase 8, apoptosis-related cysteine peptidase

Table 5 Genes in JAK-STAT signaling pathway that were differentially expressed at 24 h (24 H) after RSV infection. Fold changes at 4 h (4 H) are also shown

UniGene	Symbol	Fold		Gene name
		4 H	24 H	
Hs.1349	CSF2	4.04	21.38	Colony stimulating factor 2 (granulocyte–macrophage)
Hs.411311	IL24	1.21	4.52	Interleukin 24
Hs.278901	PIK3R5	1.56	3.98	Phosphoinositide-3-kinase, regulatory subunit 5, p101
Hs.120658	OSMR	1.45	3.76	Oncostatin M receptor
Hs.389874	TSLP	1.44	3.52	Thymic stromal lymphopoietin
Hs.272373	IL20	1.09	3.26	Interleukin 20
Hs.430589	CBLB	1.20	2.73	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
Hs.18676	SPRY2	1.23	2.58	Sprouty homolog 2 (*Drosophila*)
Hs.2303	EPO	1.40	2.49	Erythropoietin
Hs.81170	PIM1	1.33	2.47	PIM-1 oncogene
Hs.89679	IL2	4.05	2.44	Interleukin 2
Hs.23581	LEPR	1.17	2.24	Leptin receptor
Hs.520414	IFNGR1	1.21	2.19	Interferon gamma receptor 1
Hs.516966	BCL2L1	1.11	2.05	BCL2-like 1
Hs.531081	SOCS4	−1.30	−2.27	Suppressor of cytokine signaling 4
Hs.181315	IFNAR1	1.01	−3.45	Interferon (alpha, beta and omega) receptor 1
Hs.567294	IL12RB1	−2.61	−5.56	Interleukin 12 receptor, beta 1

respectively). Since arginase II is the major arginase isoform in the bronchial epithelial cells (Que et al. 1998), we further investigated the role of arginase II in RSV-induced bronchial epithelial infection. We first performed the hierarchical clustering analysis using the 912 differentially expressed genes at 24 h post-infection (Fig. 5a). We located the smallest cluster that contained ARG2. There were 11 genes in this cluster, including IL8, a major RSV-induced chemokine gene (Fig. 5b).

In separate experiments, we confirm that RSV upregulated arginase II protein expression by fourfold (Fig. 6). We then incubated HBEC with arginase II siRNA for 24 h before treating the cells with RSV. Arginase II siRNA knocked down constitutive and RSV-induced arginase II protein expression by more than 75% (Fig. 7a). Knockdown of arginase II expression increased RSV-induced release of IL-8 (Fig. 7b), LDH (Fig. 7c) and histone (Fig. 7d), consistent with the putative role of arginase II as a repair enzyme (Curran et al. 2006; Satriano 2003; Witte and Barbul 2003).

Discussion

At 4 and 24 h after RSV infection of human bronchial epithelial cells, approximately 5 and 12.5% of the probe sets

Table 6 Genes in cytokine–cytokine receptor interaction pathway that were differentially expressed at 24 h (24 H) after RSV infection. Fold changes at 4 h (4 H) are also shown

UniGene	Symbol	Fold		Gene name
		4 H	24 H	
Hs.1349	CSF2	4.04	21.38	Colony stimulating factor 2 (granulocyte–macrophage)
Hs.415768	NGFR	1.51	12.34	Nerve growth factor receptor (TNFR superfamily, member 16)
Hs.411311	IL24	1.21	4.52	Interleukin 24
Hs.120658	OSMR	1.45	3.76	Oncostatin M receptor
Hs.376208	LTB	1.14	3.68	Lymphotoxin beta (TNF superfamily, member 3)
Hs.389874	TSLP	1.44	3.52	Thymic stromal lympho poietin
Hs.272373	IL20	1.09	3.26	Interleukin 20
Hs.624	IL8	1.11	3.06	Interleukin 8
Hs.173894	CSF1	2.69	2.88	Colony stimulating factor 1 (macrophage)
Hs.73793	VEGF	1.37	2.83	Vascular endothelial growth factor
Hs.583348	INHBA	1.36	2.81	Inhibin, beta A (activin A, activin AB alpha polypeptide)
Hs.169191	CCL23	1.36	2.55	Chemokine (c-c motif) ligand 23
Hs.507621	FLT1	−1.08	2.53	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
Hs.2303	EPO	1.40	2.49	Erythropoietin
Hs.89679	IL2	4.05	2.44	Interleukin 2
Hs.1048	KITLG	−1.19	2.29	KIT ligand
Hs.23581	LEPR	1.17	2.24	Leptin receptor
Hs.520414	IFNGR1	1.21	2.19	Interferon gamma receptor 1
Hs.75765	CXCL2	1.04	2.18	Chemokine (c-X-c motif) ligand 2
Hs.557403	IL1R1	−1.17	−2.27	Interleukin 1 receptor, type I
Hs.213467	TNFRSF10D	−1.26	−2.27	Tumor necrosis factor receptor superfamily, member 10D, decoy with truncated death domain
Hs.181315	IFNAR1	1.01	−3.45	Interferon (alpha, beta and omega) receptor 1
Hs.105407	EDA	−2.35	−5.00	Ectodysplasin A
Hs.567294	IL12RB1	−2.61	−5.56	Interleukin 12 receptor, beta 1

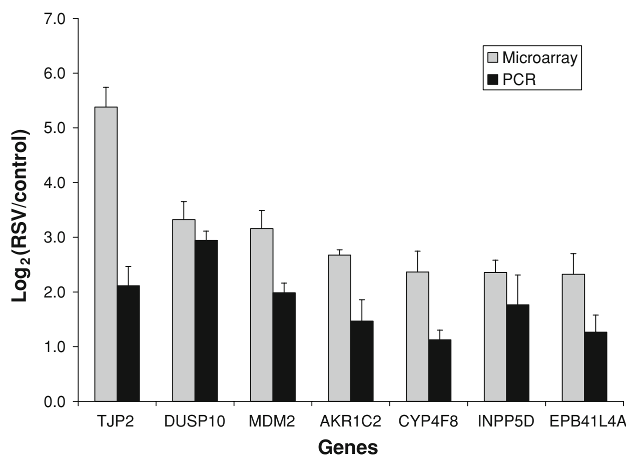


Fig. 1 Confirmation of expression of 7 upregulated genes by Q-PCR. $n = 4$ for microarray; $n = 4$ experiments in bronchial epithelial cells from 4 different donors for Q-PCR

were altered respectively. There were 277 unique genes at 4 h and 912 genes at 24 h with a RSV/control ratio of ≥2.0 or ≤0.5. Three previous microarray studies with a smaller number of probe sets and genes have reported gene expression profile associated with RSV infection in distal airway epithelial cells (A549 cells) (Kong et al. 2003; Martinez et al. 2007; Zhang et al. 2001). Our study characterized gene expression pattern in proximal airway cells, an early target of RSV. Compared to these previous studies, our results showed some similarity and differences. All studies showed significant induction of inflammation-related genes, including many cytokines and chemokines, and induction of genes involved in cell growth/proliferation, cellular protein metabolism and cytoskeleton organization. Some of the unique results from our study were early effects on genes involved in neuroactive ligand-receptor interactions and later effects on a repair enzyme, arginase II.

The neuroactive ligand-receptor interaction pathway contains 11 highly significant genes with a RSV/control ratio of ≥2.0 or ≤0.5 (Table 1). These genes included six that encode G protein-related receptors (HCRTR1, TACR1, AGTR2, NGFFR1, GRM5 and AVPR1A), three that encode neurotransmission-related receptors (P2RX2, GRID1 and GABRG2) and two that encode receptors with

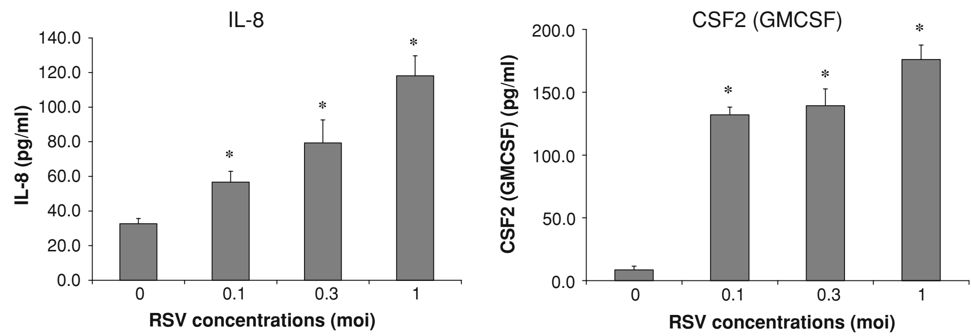


Fig. 2 Effects of RSV on the release of CSF2 (GMCSF) and interleukin-8 (IL-8). BEAS-2B cells were incubated with 0.1, 0.3 and 1.0 moi of RSV for 48 h. * $p < 0.05$ vs. no RSV. $n = 4–6$ each

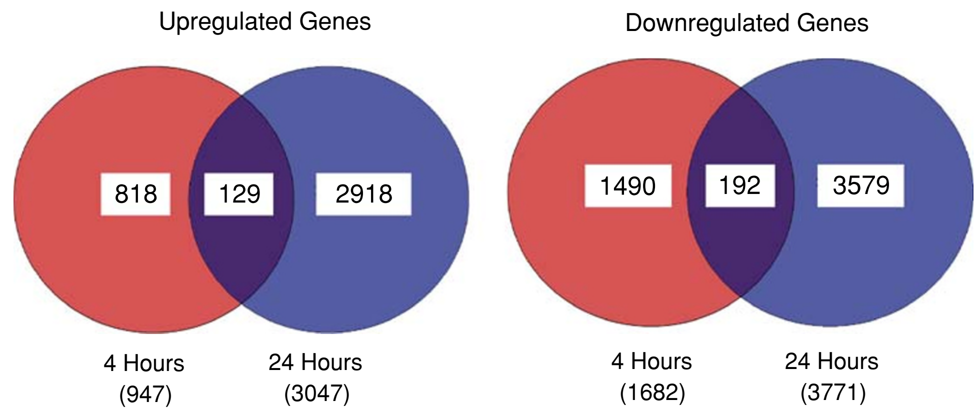


Fig. 3 Venn diagrams comparing probe sets differentially expressed at 4 and 24 h post-infection. *Numbers* in each area represent the numbers of probe sets

Table 7 Top ten genes that were up- and down-regulated at both 4 and 24 h after RSV infection

UniGene	Symbol	Gene name
Upregulated genes		
Hs.436360	ATP6V0D2	ATPase, H+ transporting, lysosomal 38 kDa, V0 subunit d isoform 2
Hs.408336	OTOA	Otoancorin
Hs.371727	SCNN1G	Sodium channel, non-voltage-gated 1, gamma
Hs.469116	SLC9A1	Solute carrier family 9 (sodium/hydrogen exchanger), isoform 1 (antiporter, Na+/H+, amiloride sensitive)
Hs.529793	SMA5	SMA5
Hs.268554	CYP4F8	Cytochrome P450, family 4, subfamily F, polypeptide 8
Hs.269180	EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B
Hs.8739	ELP2	Signal transducer and activator of transcription 3 interacting protein 1
Hs.97432	PRKCE	Protein kinase C, epsilon
Hs.162032	HBP1	HMG-box transcription factor 1
Downregulated genes		
Hs.1183	DUSP2	Dual specificity phosphatase 2
Hs.303023	TUBB1	Tubulin, beta 1
Hs.567495	TRNT1	tRNA nucleotidyl transferase, CCA-adding, 1
Hs.520189	ELOVL5	ELOVL family member 5, elongation of long chain fatty acids
Hs.146050	PTPRG	Protein tyrosine phosphatase, receptor type, G
Hs.531754	MAP2K7	Mitogen-activated protein kinase 7
Hs.120267	TSGA10	Testis specific, 10
Hs.66739	KRT12	Keratin 12 (Meesmann corneal dystrophy)
Hs.225936	SYN1	Synapsin I
Hs.134565	SIRPB1	Signal-regulatory protein beta 1

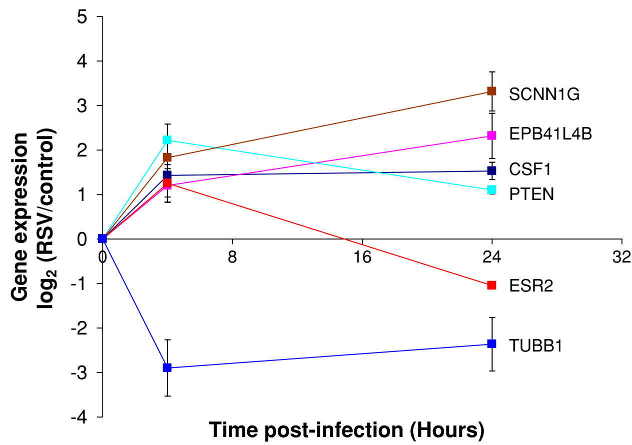
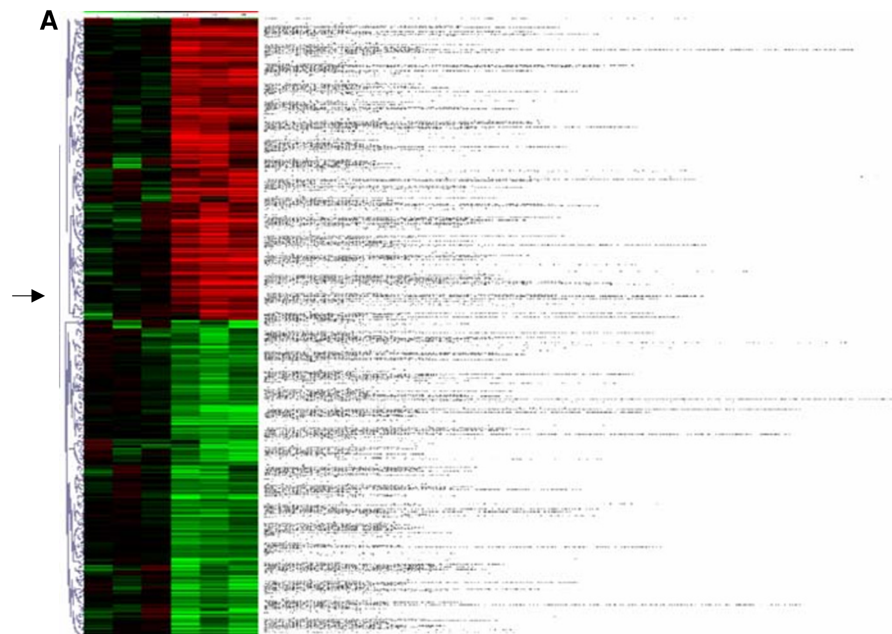


Fig. 4 Expression of six genes that were significantly altered at both 4 and 24 h post-infection and were also biomarkers for respiratory diseases based on the biomarker filter of ingenuity pathway analysis

endocrine and paracrine functions (GIPR and VIPR1). Two genes (TACR1, VIPR1) are known to be related to RSV infection. TACR1 belongs to a family of genes that function as receptors for tachykinin substance P (neurokinin 1). RSV infection increases the mRNA expression of TACR1 (Auais et al. 2003; King et al. 2001; Piedimonte 2003). Upregulation TACR1 would further enhance the substance P-mediated signaling during RSV-induced airway inflammation and dysfunction (Amadesi et al. 2001; Dakhama et al. 2005; Joos et al. 1994; Tan et al. 2008; Tripp et al. 2000). Persistent RSV infection in mice decreases VIP signaling (Tan et al. 2008). Downregulation of VIPR1 in our study is consistent with this observation. None of the other neuroactive ligand-receptor genes have been reported previously to be associated with RSV infection. These results showed a much more extensive network of



B

Unigene	Fold	Gene Symbol	Gene Description
Hs.590921	2.18	CXCL2	Chemokine (C-X-C motif) ligand 2
Hs.255664	2.28	CYLN2	Cytoplasmic linker 2
Hs.524692	2.54	NUAK1	AMP-activated protein kinase family member 5
Hs.580300	2.08	GFPT1	Glutamine-fructose-6-phosphate transaminase 1
Hs.624	3.06	IL8	Interleukin 8
Hs.58831	4.52	IL24	Interleukin 24
Hs.592312	2.06	ARG2	Arginase, type II
Hs.449942	2.19	INPP5B	Inositol polyphosphate-5-phosphatase, 75 kDa
Hs.154276	2.14	BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
Hs.125715	2.12	MBNL2	Muscleblind-like 2 (Drosophila)
Hs.445818	3.92	SPON1	Spondin 1, extracellular matrix protein

Fig. 5 Hierarchical clustering of the 912 differentially expressed genes at 24 h after RSV infection. **a** The heat map. The *arrow* indicates the location of the cluster that contains IL-8 and arginase II; **b** genes in this cluster

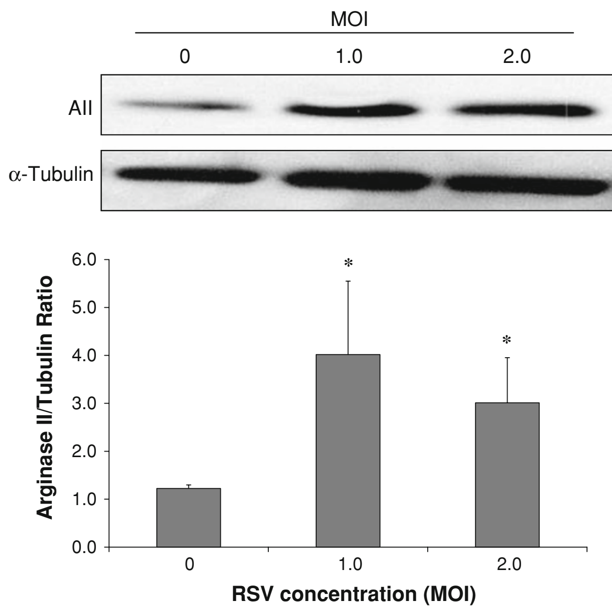


Fig. 6 Up-regulation of arginase II by RSV in primary human bronchial epithelial cells. Human bronchial epithelial cells were treated with RSV at multiplicity of infection (*MOI*) of 1.0 and 2.0. Arginase II protein expression was measured at 48 h post-infection. *Upper panel* shows a representive western blot. *Lower panel* is the densitometry result. $n = 3$–4 independent experiments. * $p < 0.05$ vs. time 0 or MOI = 0

neuroactive ligand-receptor interaction that can be activated early after RSV infection. These characteristic patterns underscore the unique localization of dendritic cells within bronchial epithelium and their critical role in respiratory viral infections (Grayson and Holtzman 2007). Early changes in these receptor genes are consistent with alterations in neuroimmune mechanisms that have been linked to airway inflammation and airway hyper responsiveness following RSV infection (Krishnan et al. 2004; Piedimonte 2003).

By 24 h after RSV infection, many more genes and signaling pathways were altered (Table 2). Alterations in some of these pathways were qualitatively similar to those shown in previous microarray studies. The p53 signaling pathway regulates cell growth and apoptosis induced by genotoxic and non-genotoxic stresses. Twelve genes were in the p53 pathway (Table 3), including upregulation of MDM2, a critical negative regulator of p53, that may contribute to the inhibition of p53 signaling and the anti-apoptotic effects in lung epithelial cells (Bitko et al. 2007; Groskreutz et al. 2007; Monick et al. 2005; Takeuchi et al. 1998; Thomas et al. 2002). Downregulation of pro-apoptotic genes (e.g., FADD, CASP 8) and upregulation of antiapoptotic genes (e.g., BIRC3, XIAP) may further inhibit apoptosis during RSV infection (Table 4). The JAK-STAT signaling pathway is another important pathway known to be activated by RSV in response to type I interferon’s (Kong et al. 2003) (Ramaswamy et al. 2004). There were 17 genes in this pathway (Table 5). Another DNA microarray study in A549 cells also identified genes containing STAT-binding elements (Kong et al. 2003). Activation of the JAK-STAT pathway also has been shown in previous non-gene profiling studies (Nguyen et al. 1997; Ramaswamy et al. 2004; Tarnawski and Jones 1998). The cytokine-cytokine receptor interaction pathway contained genes that are well known to be induced by RSV in lung epithelial cells (Table 6) (Arnold et al. 1994) (Becker et al. 1991) (Casola et al. 2001) (Fiedler et al. 1995) (Pazdrak et al. 2002) (Saito et al. 1997) (Soukup and Becker 2003) (Thomas et al. 1998) (Thomas et al. 2000) (Zhang et al.

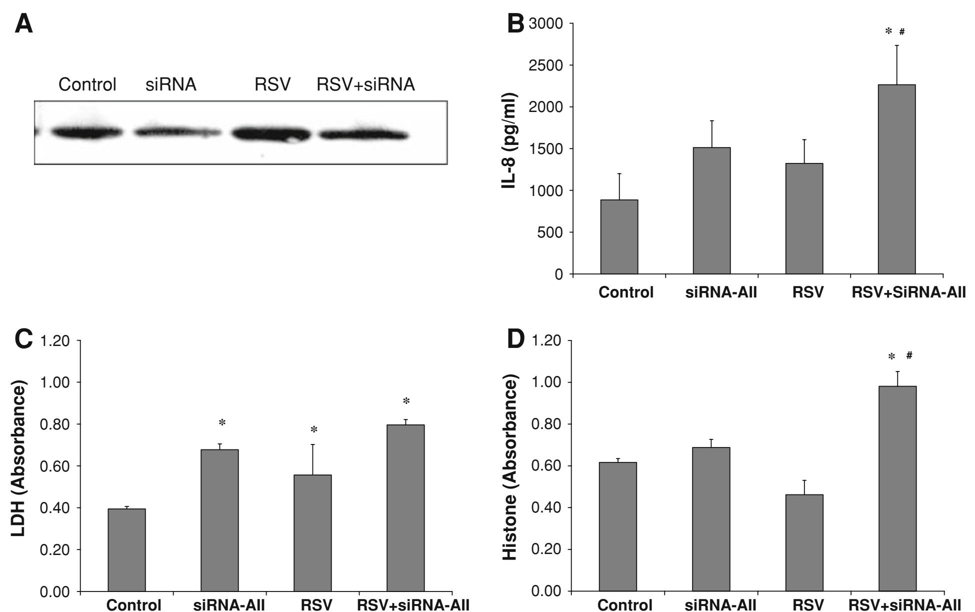


Fig. 7 Effects of arginase II knockdown on RSV-induced injury in primary human bronchial epithelial cells. **a** Western blot analysis of arginase II in cells with or without arginase II siRNA pretreatment. Knockdown of arginase II gene expression with siRNA increased the release of IL-8; **b** LDH; **c** and histone; **d** induced by RSV. $n = 4$ independent experiments. * $p < 0.05$ vs. control; # $p < 0.05$ vs. RSV

2001). Genes in the cytokine–cytokine receptor interaction pathways included three TNF/TNF receptor family genes (LTB, NGFR, TNFRSF10D), two IFN-related genes (IFNAR1, IFNGR1), six interleukin family genes (IL12RB1, IL1R1, IL2, IL20, IL24, IL8) and 4 cytokine/chemokine genes (CCL23, CSF1, CSF2, CXCL2). We also noted upregulation of VEGF and FLT1, a VEGF receptor gene. Upregulation of these two genes may explain the activation of VEGF signaling responsible for increased permeability of bronchial epithelial cells during RSV infection (Kilani et al. 2004; Lee et al. 2000).

We also matched the altered genes with respiratory disease biomarkers in the Ingenuity Pathway Analysis. Twenty-three and 92 genes were identified at 4 and 24 h post-infection. Six genes were present at both time points. Four genes (SCNN1G, EPB41L4B, CSF1, PTEN) were upregulated, one gene (TUBB1) was down-regulated at 4 and 24 h and one gene (ESR2) was upregulated at 4 h, but downregulated at 24 h post-infection. These six genes may not only be pathogenetically important in RSV infection (e.g., CSF2 and TUBB1) (Arnold et al. 1994; Huang et al. 2005; Noah and Becker 1993), but may also serve as biomarkers of RSV infection.

The 92 respiratory disease biomarker genes also contain arginase II gene (ARG2). Arginase II is the mitochondrial isoform of arginase that catalyzes the conversion of L-arginine to L-ornithine and urea (Morris 2006). Arginase II is expressed constitutively in a number of extra-hepatic tissues, including bronchial epithelial cells (Que et al. 1998). Hierarchical clustering linked ARG2 closely to IL8, a major RSV-induced chemokine gene. The same cluster also contains other cytokine genes that are important in inflammation, including IL-24 (4.52-fold) and CXCL2 (2.18-fold). Upregulation of ARG2 during RSV infection is important in limiting cell injury since knockdown of arginase II gene expression by siRNA increased the release of IL-8, histone and LDH induced by RSV. This is consistent with the putative role of arginase II in tissue repair (Morris 2005). The potential implication of upregulation of arginase II during RSV infection may also extend to the pathogenesis of human asthma. Increased arginase expression and activity has been linked to the pathogenesis of asthma (King et al. 2004) (Vercelli 2003) (Zimmermann and Rothenberg 2006). Using gene expression profiling, Zimmerman et al. found genes related to metabolism of arginine, including cationic amino acid transporter 2, arginase I and arginase II, prominently expressed in an experimental model of asthma (Zimmermann et al. 2003). Since RSV is an important exacerbating factor and trigger for asthma (Kalina and Gershwin 2004; Openshaw 2003; Stein et al. 1999), arginase II may be one of the important mechanistic links between RSV infection and asthma.

In conclusion, using DNA microarray, we provided the gene expression profile in human bronchial epithelial cells during RSV infection. The gene expression profile in proximal airway cells share some similarity with those shown in distal lung cells, primarily in inflammation and cell growth and cell death. Our results also reveal novel genes and pathways that may be important in the pathogenesis of RSV infection and the associated complications. More significantly, we identified a set of genes that may be used as biomarkers of RSV infection. Since these genes are expressed in proximal airway cells, the clinical utility of these biomarker genes can be readily tested in the sputum samples in the future.

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