Comparative Analysis of Genes Induced by Respiratory Syncytial Virus and DsRNA in Human Epithelial Cells

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Abstract

Epithelial cells are the primary target of respiratory viral infections and play a pivotal role in virus-induced lung inflammation and in anti viral immune response. A common signal for the presence of viral infections and induction of inflammation is recognition of double stranded RNA (dsRNA). Thus far, there has not been a high-throughput transcrptome analysis of RSV- or dsRNA-induced genes in primary human bronchial epithelial cells (PHBE), nor there has been a comparison between dsRNA- and RSV-induced genes. To establish the transcriptome profiles and to determine the contribution of dsRNA in the induction of inflammation during respiratory virus infection, we compared the gene expression profiles of PHBE cells that were infected with Respiratory Syncytial Virus (RSV) or were treated with dsRNA. Our transcriptome analysis showed that RSV infection and and dsRNA treatment induced up-regulation of 2024 and 159 genes in PHBE respectively. Comparison of genes revealed that RSV and dsRNA commonly induced 75 genes in PHBE cells. The common up-regulated genes were functionally grouped in multiple response pathways involved in inflammation and immune responses. Interestingly, there were several previously unreported genes that were up-regulated in primary human epithelial cells that are relevant to a TH2 allergic phenotype. This comparison of a high-throughput gene expression study offers a comprehensive view of transcriptional changes induced by dsRNA and RSV, and importantly compares dsRNA-induced genes with RSV-induced genes in PHBE cells.

Keywords: RSV, dsRNA, transcriptome, immune response, inflammation

Introduction

RSV is the most common cause of childhood respiratory viral infections, and can lead to severe bronchiolitis, which is associated with an increase in the induction of asthma (Singh *et al*., 2007). Previous transcriptomic studies have reported gene expression analysis of RSV-infected cell lines (Zhang *et al*., 2001), RSV-infected mice (Janssen *et al*., 2007) and RSV-infected infant whole and cord blood (Fjaerli *et al*., 2006; Fjaerli *et al*., 2007). Thus far, there has not been a transciptome analysis of genes that are induced during RSV infection in primary human bronchial epithelial cells, which are the primary target of RSV and other respiratory viruses.

During viral infections, including infections with respiratory viruses, dsRNA can be formed, which acts as a potent inducer of inflammation. Experimentally, dsRNA, both intracellular and extracellular, has been widely used to mimic viral-induced inflammation. Previous high-throughput gene analysis using dsRNA in the form of poly I:C have reported the transcriptomeic changes in human glioma GRE cells (Geiss, 2001), peripheral blood mononuclear cells (PBMC's) (Huang *et al*., 2006) and rat pancreatic beta cells (Rasschaert *et al*., 2003). However, to date there has been no characterization or comparison of RSV- and

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dsRNA- induced genes in primary human airway epithelial cells.

In this study, the comprehensive gene expression profiles of PHBE treated with dsRNA or infected with RSV were analyzed using total cellular RNA, hybridized onto high-density oligonucleotide arrays (Agilant Human Array 1A version 2), which contain 20,000 probes. Interestingly, ours results reveal novel RSV- and dsRNA-induced genes. Some of the novel RSV-induced genes identified in our result including ADAM8 and IL13RA2. ADAM8 has been reported to be induced in experimental asthma in mice, and in patients with severe and moderate asthma (Foley *et al*., 2007; King *et al*., 2004; Matsuno *et al*., 2006; Richens *et al*., 2007) and IL-13RA2 is important for IL-13 signaling and TGF-μ1- mediated lung fibrosis (Fichtner-Feigl *et al*., 2007; Fichtner-Feigl *et al*., 2006).

We also identified novel dsRNA induced genes such as SOCS3 and PRDM1 (BLIMP-1). A report by Seki *et al*. (2003) showed that SOCS3 was important in the induction and maintenance of allergic TH2 reactions. BLIMP-1 is required for epidermal cell c-myc regulation and for the development of the effector function of B and T lymphocytes (Lin, 2000; Lin, 1997; Martins, 2008; Messika *et al*., 1998; Zhou *et al*., 2007).

Importantly, study showed that 75 genes were commonly induced by RSV infection or dsRNA treatment in PHBE cells. These genes were functionally grouped in multiple response pathways such as inflammation, apoptosis, transcription regulation, cell signaling, and cell differentiation. The induction of gene expression was confirmed by determination of changes in expression of select proteins.

This is, to our knowledge, is the first study that has analyzed global gene expression in PHBE cells after dsRNA treatment and RSV infection. This characterization of the common genes validates the use of dsRNA as viral mimetic to detect a subset of pro-inflammatory signal during RSV infection, and may likely contribute to developing specific anti-inflammatory agents.

The aim of this study is to unravel the gene expression profile of human epithelial cell induced by RSV infection and DsRNA treatment.

Materials and Methods

Cells, tissue culture conditions, antibodies and reagents.

The primary human bronchial epithelial (PHBE) were grown as a monolayer at 37°C in a 5% CO2 humidified chamber in a serum-free epithelial cell growth medium from Cambrex (Lonza, Walkersville, MD). The synthetic dsRNA, poly I:C, was purchased from Sigma Chemicals (St. Louis, MO), dissolved in phosphate-buffered saline, and was used at concentrations indicated in each figure legend. The dsRNA structural fidelity of poly I:C was determined by the ability of each dsRNA batch to activate the dsRNA-specific protein kinase PKR in in vitro kinase assays (Imani, 1988).

RNA extraction and RT-PCR

For RNA extraction, cells were left untreated, were treated with 1 μg/ml poly I:C, or were infected with RSV at multiplicity of infection (MOI) of 2.5 plaque forming unit (pfu)/cell. After 2 hr for dsRNA and 24 hr for RSV, total cellular RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. The quality of RNA was evaluated by electrophoresis and only high quality RNA was used in the microarray and RT-PCR experiments.

For real-time PCR, first strand cDNA was synthesized using superscript reverse transcriptase (Invitrogen, Carlsbad, CA) using 1 μg total RNA. Following reverse transcription, 1 μl of cDNA was amplified by real-time PCR. Each experiment was performed in duplicate in 96 well plates by using 1x Sybr Green master mix (Bio-Rad, Hercules, CA) in a final volume of 25 μl. Amplifications were performed with the following protocol: 95°C for 3 minutes followed by 50 cycles of 94°C for 10 sec and 60°C for 30 sec.

Microarray, linear amplification, labeling Protocol and feature extraction

Gene expression analysis was conducted using Agilent Human1Av2 arrays (Agilent Technologies, Palo Alto, CA). Total RNA was amplified using the Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol. Starting with 500ng of total RNA, Cy3 or Cy5 labeled cRNA was produced according to manufacturer's protocol. For each two color comparison, 750 ng of each Cy3 and Cy5 labeled cRNAs were mixed and fragmented using the Agilent In Situ Hybridization Kit protocol. Hybridizations were performed for 17 hours in a rotating hybridization oven using the Agilent 60-mer oligo microarray processing protocol. Slides were washed as indicated in this protocol and then scanned with an Agilent Scanner. Data was obtained using the Agilent Feature Extraction software (v7.5), using defaults for all parameters.

Rosetta Resolver, Genespring GX, and Ingenuity pathways analysis.

Images and GEML files, including error and p-values, were exported from the Agilent Feature Extraction software and deposited into Rosetta Resolver (version 4.0, build 4.0.1.0.7. RSPLIT) (Rosetta Biosoftware, Kirkland, WA). The resultant ratio profiles were combined into ratio experiments as described (Dai *et al*., 2002). Intensity plots were generated for each ratio experiment and genes were considered "signature genes" if the p value was < 0.001.

Data from Agilent Feature Extraction software were also loaded to genespring (version 7.3.1) and filtered for markers and flagged spots. The data were then adjusted for dye swap experiment, normalized and filtered based on fold change.

Genes that differentially regulated by dsRNA and RSV infection in primary epithelial cell, BEAS-2B and A549 were analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

The Functional Analysis identified the biological functions and/or diseases that were most significant to the data set. Genes from the dataset that met the normalized cut off of 1.5 fold and were associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone.

The Functional Analysis of a network identified the biological functions and/or diseases that were most significant to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

Cytokine and chemokine protein determination.

The amount of cytokines and chemokines present in media and cell extract was determined using a multiplexed cytokines assay system (Bio-plex 200) (Biorad, Hercules, CA). In brief, premixed antibody-coupled beads were reconstituted in assay buffer and loaded onto a pre-wetted 96 well plate. The beads were washed once with the provided wash buffer and then diluted standard or samples were added, mixed by vortexing and incubated at room temperature for 60 min. After washing 3 times to remove unbound proteins, a second specific biotinylated detection antibody was added to the beads, mixed and incubated for an additional 30 min. For final detection, after washing, streptavidin-phycoerythrin (streptavidin-PE) was added to the mixture and incubated for 10 min. The beads were then washed, resuspended in assay buffer and analyzed using Bio-

Table 1. List of PCR primers used to validate microarray data

1	BBC3-FWD	tgtgaccactggcattcatt
	BBC3-REV	tcctccctcttccgagattt
2	BF-FWD	atgctgaccccaatacttgc
	BF-REV	tccactactccccagctgat
3	BIRC3FWD	tgagcccaggagtttgaatc
	BIRC3REV	acaccatgtccctaaaatgtca
4	CCL20-FWD	GTGTGCGCAAATCCAAA AC
	CCL20-REV	CAAGTCCAGTGAGGCAC AAA
5	CSF2-FWD	cttcctgtgcaacccagatt
	CSF2-REV	ccatcctgagtttctagctcttg
6	CXCL-1-FWD	cccttggacattttatgtctttc
	CXCL-1-REV	aacatcaataattaagccccttg
7	CXCL-10-FWD	cctctcccatcacttccctac
	CXCL-10-REV	gctgatttggtgaccatcatt
8	CXCL-11-FWD	gggtgaaaggaccaaaaaca
	CXCL-11-REV	acaacaagtaagaacgtgaaagca
9	CXCL2-FWD	gaggctgaggaatccaagaa
	CXCL2-REV	tgttgaccacacactgtgaaa
10	CXCL-3-FWD	caacatttttatgctgaagtttcc
	CXCL-3-REV	ttccaagggaaagagaaacg
11	DUSP1-FWD	AGT GCT GAA AAC AAA CCT GCT
	DUSP-1-REV	CCA GTA TTA TTC CCG ACG ACA
12	EFNA1-FWD	gggatggagaaagaagtgga
	EFNA1-REV	cagctcccactgtgcctta
13	EGR1-FWD	TGTTTGGCTTATAAACA CATTGAA
	EGR1-REV	CCCAATCGCAGCTACTTT ATTT
14	G1P2-FWD	caccgtgttcatgaatctgc
	G1P2-REV	ctttatttccggcccttgat
15	IFIT1-FWD	cctccttgggttcgtctaca
	IFIT1-REV	ggctgatatctgggtgccta
16	IFIT2-FWD	tgcttggaacgattgagattt
	IFIT2-REV	caggcaaacattttccaacc
17	IFIT4-FWD	gtggttgtgacgggtaggag
	IFIT4-REV	ctcccaaagtgctgggatta
18	IFNB-FWD	CAG CAG TTC CAG AAG GAG GA
	IFNB-REV	AGC CAG TGC TCG ATG AAT CT
19	IL1A-FWD	caccctatatttgcatagtaccaa
	IL1A-REV	tcctaggtctgtggcttcttg
20	IL-6-FWD	ATT CTG CGC AGC TTT AAG GA
	IL-6-REV	ATC TGA GGT GCC CAT GCT AC
21	IL8-FWD	tctggcaaccctagtctgct
	IL8-REV	gcttccacatgtcctcacaa
22	INHBA-FWD	accaagctgagacccatgtc
	INHBA-REV	ttgccactgtcttctctgga
23	NFKBIA-FWD	ttcatggaaggaccacatttt
	NFKBIA-REV	caccatttacaggagggtaaca
24	NFKBIZ-FWD	ctaagtctttgtagataaagcagatgg
	NFKBIA-REV	ttcaatgcatgagaaaaagca
25	RIG-I-FWD	tagtttgcggtttcctggac
	RIG-I-REV	cactgctcaccagattgcat
26	TNFAIP3-FWD	taccctggtattgggacagc
	TNFAIP3-REV	acagcaaccacaaagcacac
27	ZFP36-FWD	CAC TGC CCC TCC AGT TTT TA
	ZFP36-REV	TGA TTT ATG GGT GGG GTT TG
28	ADAM8-FWD	GCAACCAC AAGCAGGA GTG
	ADAM8-REV	CAGA ACCACCACCACGA AG
29	IL13RA2-FWD	TTGCGTAAGCCAAACAC CTA
	IL13RA2-REV	TGAACATTTGGCCATGA CTG
30	RND3- FWD	AATAGAGTTGAGCCTGT GGG
	RND3-REV	CTAATGTACTAACATCT GTCCGC
31	BMP2-FWD	TGCACCAAGATGAACA CAG
	BMP2-REV	GCTGTTTGTGTTTGGCTTG
32	CCNA1-FWD	GTTGTGCTGGCTACAGT GG
	CCNA1-REV	CCTGCTCTAGTTCATCCA TGTA
33	ADRA2C-FWD	GTGGTGATCGCCGTGCTG AC
	ADRA2C-REV	CGTTTTCGGTAGTCGGGG AC

Plex 200 system. The concentration of each cytokine was then determined by measuring magnitude of the fluorescently labeled reporter molecules associated with individual target proteins.

Results

Identification of RSV-induced genes in PHBE cells

To identify Genes that induced by double stranded RNA (Poly I:C), global gene expression profiling were performed

by using labeled cRNA derived from total RNA extracted from mock-infected or RSV-infected (2.5 pfu/cell) PHBE cells after 24 hr. The cRNA was hybridized onto Agilent Human1Av2 arrays. Each comparison (mock vs infected) was done in duplicate with dye swap experiments. Data were then vigorously filtered to remove the biases. The genes that have more than 1.5 relative fold inductions compare to untreated control were considered differentially regulated genes.

Our result shows that RSV infection induced up-regulation of 2024 genes in PHBE cells. Genes that were highly induced by RSV included, cytokines and chemokines involved in innate and adaptive immunity, matrix metalloproteinases, cell signaling molecules, TGF family members, cell cycle regulatory molecules, and heat shock proteins (see supplement for complete list). We next compared our data with the previously published RSV-induced genes in other cells. The comparison revealed several novel RSV-induced genes that are listed in Table 2. The novel RSV-induced genes that are shown in Table 1 included ADAM8, ADRAC2C, and IL13RA2. This genes are associated with induction of experimental asthma in mice, and are up-regulated in patients with severe and moderate asthma (Foley *et al*., 2007; King *et al*., 2004; Matsuno *et al*., 2006)

Identification of dsRNA induced genes in PHBE cells

To determine the dsRNA induced gene profiles, PHBE cell were either left untreated or were treated with dsRNA at 1μg/ml for 2 hr prior to RNA extraction. Gene expression profiling was carried out as above. The results showed that expression of 159 genes in primary epithelial cells was induced by dsRNA treatment (see supplement for complete list). DsRNA induced genes included inflammatory and regulatory cytokines and chemokines, signal transduction molecules, cell migration molecules, and apoptosis pathways. Some of the highly induced gene included interferon

Table 2. List of significant diseases and cellular function with related genes identified by IPA .

Function	-log (p-value)	Molecules
Inflammatory Response	9.73E-15-4.59E-06	IL1A, PTGER4, CXCL10, PLAU, ICAM1, TNFAIP3, CCL5, MMP13, IL8, IL23A, CXCL2, ZFP36, NFKBIA, DUSP1, TNF, SOCS3, SOD2, CXCL3, CSF2, EGR1, IL6
Viral Function	1.95E-10-9.13E-06	IFIH1, NFKBIA, CXCL10, TNF, CXCL3, CCL5, RIPK2, TNIP1, IL6, IL8
Immunological Disease	2.28E-09-6.12E-06	IER3, PTGER4, CXCL10, IL7R, ICAM1, CCL5, PRDM1, IL23A, IL8, EFNA1, NFKBIA, DUSP1, KLF2, TNF, SOCS3, SOD2, BIRC3, CSF2, BMP2, EGR1, IL6
Respiratory Disease	3.34E-07-8.08E-07	TNF, CXCL3, ICAM1, CSF2, EGR1, IL6
Cell Death	3.27E-16-1.28E-05	IL1A, IER3, PMAIP1, NEDD9, PTGER4, MMP13, SDC4, KLF6, IL8, ZFP36, EFNA1, NFKBIA, PPP1R15A (includes EG:23645), CXCL3, RIPK2, BTG1, INHBA, PLAUR, BMP2, EGR1, TNFAIP8, IL6, ATF3, PLK3, HSPA1A, PLAU, ICAM1, IL7R, TNFAIP3, CCL5, PRDM1, IFIH1, CXCL1, HBEGF, ELF3, DUSP1, KLF2, SOCS3, TNF, SOD2, BIRC3, CSF2
Cellular Growth and Proliferation	1.27E-15-1.14E-05	IER3, IL1A, PMAIP1, PTGER4, IFIT3, KLF6, ISG15, IL8, IL28B, EFNA1, CXCL2, NFKBIA, PPP1R15A (includes EG:23645), CXCL3, RIPK2, INHBA, PLAUR, BTG1, BMP2, EGR1, TNFAIP8, IL6, ATF3, PLK3, HSPA1A, CXCL10, PLAU, IL29, ICAM1, IL7R, PIM3, CCL5, PRDM1, CD83, IL23A, IFIH1, CXCL1, HBEGF, DUSP1, ELF3, KLF2, TNF, SOCS3, SOD2, CSF2, ISG20
Cell Cycle	1.8E-07-1.21E-05	PLK3, IER3, IL1A, NEDD9, PLAU, KLF6, IL8, NFKBIA, HBEGF, DUSP1, PPP1R15A (includes EG:23645), TNF, SOD2, CSF2, PLAUR, INHBA, BMP2, EGR1, IL6, ATF3

Table 3. Novel RSV induced genes

	Gene ID	Gene Name	Fold Change	Description	Location
1	NM_018661	DEFB103A	37.91	defensin, beta 103A	Extracellular Space
2	NM_007036	ESM1	14.71	endothelial cell-specific molecule 1	Extracellular Space
3	NM_001458	FLNC	13.72	filamin C, gamma (actin binding protein 280)	Cytoplasm
4	NM_001109	ADAM8	12.09	ADAM metallopeptidase domain 8	Plasma Membrane
5	NM_000683	ADRA2C	10.74	adrenergic, alpha-2C-, receptor	Plasma Membrane
6	NM_001323	CST6	9.20	cystatin E/M	Extracellular Space
7	NM_004633	IL1R2	7.69	interleukin 1 receptor, type II	Plasma Membrane
8	NM_025245	PBX4	6.75	pre-B-cell leukemia homeobox 4	Nucleus
9	NM_174873	P2RX2	6.71	purinergic receptor P2X	Plasma Membrane
10	BC008283	CCK	6.30	cholecystokinin	Extracellular Space
11	NM_004636	SEMA3B	6.28	semaphorin 3B	Extracellular Space
12	NM_001305	CLDN4	6.14	claudin 4	Plasma Membrane
13	BC015503	AGR2	5.94	anterior gradient homolog 2	Extracellular Space
14	NM_003033	ST3GAL1	5.80	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	Cytoplasm
15	NM_000952	PTAFR	5.72	platelet-activating factor receptor	Plasma Membrane
16	NM_015277	NEDD4L	5.35	neural precursor cell expressed, developmentally down-regulated 4	Cytoplasm
17	NM_001874	CPM	5.30	carboxypeptidase M	Plasma Membrane
18	NM_016354	SLCO4A1	5.26	solute carrier organic anion transporter family, member 4A1	Plasma Membrane
19	AK000158	CA12	5.25	carbonic anhydrase XII	Plasma Membrane
20	NM_003914	CCNA1	5.23	cyclin A1	Nucleus
21	NM_032957	RTEL1	5.12	regulator of telomere elongation helicase 1	Nucleus
22	NM_006043	HS3ST2	5.07	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	Cytoplasm
23	NM_013259	TAGLN3	5.02	transgelin 3	Unknown
24	NM_000640	IL13RA2	5.01	interleukin 13 receptor, alpha 2	Plasma Membrane

induced genes (IFIT1, IFIT2, IFIT4), TNF-α induced gene (TNFAIP2), apoptosis inhibitor (BIRC3), eph receptor (EFNA1) and several cytokines and chemokines (IL6, IL-8, CXCL1, CXCL2, CXCL3, PTX3).

We also identified several novel dsRNA induced genes in PHBE cells. The list of novel dsRNA induced genes is presented in Table 3. Some of these genes such as BMP2, GBP, and RND3 have been implicated in inflammation.

BMP2 has been shown to be induced and may play a role in BMP/SMAD signaling during airway inflammation (Rosendahl *et al.*, 2002), and RND3/RhoE was reported to be involved in inflammatory response by regulating IRAK/ERK/NF-κB pathway and cytoskeleton organization (Guasch *et al.*, 2007).

Comparison of differentially expressed genes in dsRNA treated cells and RSV infected cells

Since dsRNA is commonly used as viral mimetic, we compared the gene expression profiles of RSV-infected and dsRNA-treated PHBE cells. Data from Venn diagram comparing RSV- and dsRNA- induced genes (Figure 1) revealed that 75 genes were induced in common. The genes that were commonly up-regulated were listed in Table 4. Several genes that are involved in innate immune responses that were highly induced by both RSV and dsRNA in PHBE cells included apoptosis gene (BIRC3), chemokines (IL-8, CXCL1, CXCL11), and interferon induced protein (IFIT1, IFIT2, IFIT4, ISG15). This data indicate that dsRNA can mimic an innate

Table 4. Novel dsRNA induced genes

	Gene ID	Gene Name	Fold Change	Description	Location
1	NM_019618	IL1F9	3.03	interleukin 1 family, member 9	Extracellular Space
2	NM_018724	IL20	2.02	interleukin 20	Extracellular Space
3	NM_052941	GBP4	3.89	guanylate binding protein 4	Cytoplasm
4	NM_018284	GBP3	2.93	guanylate binding protein 3	Cytoplasm
5	NM_004120	GBP2	2.57	guanylate binding protein 2, interferon-inducible	Cytoplasm
6	NM_005168	RND3	2.14	Rho family GTPase 3	Cytoplasm
7	NM_006018	GPR109B	4.99	G protein-coupled receptor 109B	Plasma Membrane
8	NM_001200	BMP2	2.46	bone morphogenetic protein 2	Extracellular Space
9	NM_152439	BEST3	3.76	bestrophin 3	Unknown
10	NM_000891	KCNJ2	3.44	potassium inwardly-rectifying channel J2	Plasma Membrane
11	NM_173200	NR4A3	2.08	nuclear receptor subfamily 4A3	Nucleus
12	NM_004484	GPC3	4.62	glypican 3	Plasma Membrane
13	NM_019016	KRT24	3.88	keratin 24	Cytoplasm
14	NM_025047	ARL14	3.41	ADP-ribosylation factor-like 14	Unknown
15	NM_006813	PNRC1	2.49	proline-rich nuclear receptor coactivator 1	Nucleus
16	NM_014350	TNFAIP8	2.45	tumor necrosis factor, alpha-induced protein 8	Cytoplasm
17	NM_020652	ZNF286A	2.28	zinc finger protein 286A	Nucleus
18	NM_001198	PRDM1	2.90	PR domain containing 1	Nucleus

inflammatory subset of genes that are up-regulated during RSV infection.

Validation of microarray result with real time PCR

To validate the results of the gene array experiments, we used RT-PCR and Bio-Plex assay. Initially the novel RSV-induced genes (ADAM8, IL13RA2, CCNA1 and ADRA2C) novel dsRNA-induced genes (RAND3, BMP2 and SOCS3), and several commonly induced genes were verified by RT-PCR (Figure 2). We also verified 28 genes from the 75 genes that were found to be up-regulated by RSV and dsRNA in PHBE cells and all of the 27 genes that were found to be up-regulated by RSV and dsRNA in PHBE cells and all of the 27 genes that were shown to beup-regulated by microarray analysis were proven to be up-regulated by Sybr Green RT-PCR (Table 5).

We next confirmed up-regulation of several cytokines and chemokines by protein determination using Bio-Plex assay, this

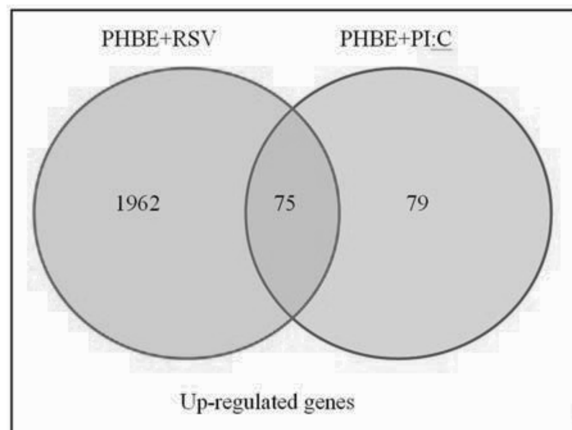

Figure 1. Venn diagram of differentially expressed genes. Comparison of genes induced by RSV infection and dsRNA treatment in PHBE cells.

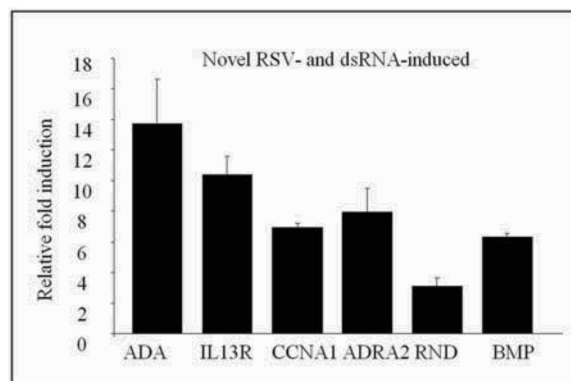

Figure 2. RT-PCR validation of novel RSV- and dsRNA-induced genes.

Table 5. Genes that were commonly induced by DsRNA and RSV in PHBE.

	Gene ID	Gene Name	PHBE+PI:C	t-test p-value	PHBE+RSV	t-test p-value
1	NM_001547	IFIT2	57.11	0.00914	11.01	6.12E-05
2	NM_001548	IFIT1	21.33	0.0133	11.21	8.72E-06
3	NM_000584	IL8	15.47	0.00791	12.52	7.49E-05
4	NM_005409	CXCL11	13.22	0.00879	12.83	6.99E-05
5	NM_000594	TNF	11.73	0.000855	4.491	0.000318
6	NM_006291	TNFAIP2	11.36	0.055	4.016	0.00237
7	NM_001565	CXCL10	11.32	0.0179	3.228	0.00515
8	NM_002090	CXCL3	10.61	0.0252	4.565	0.00157
9	NM_006290	TNFAIP3	10.51	0.0417	2.476	0.0145
10	NM_001549	IFIT4	10.05	0.00175	4.968	0.00245
11	BC032663	PMAIP1	8.979	0.0161	3.882	5.13E-21
12	NM_000600	IL6	8.817	0.0232	1.911	0.0445
13	NM_001511	CXCL1	8.533	0.0139	6.731	4.37E-05
14	NM_020529	NFKBIA	7.578	0.0217	3.455	0.00402
15	NM_138397	LINCR	7.363	0.0378	1.966	0.0392
16	NM_001511	CXCL1	5.994	0.0169	3.951	0.00253
17	NM_002089	CXCL2	5.693	0.0786	2.471	0.0146
18	NM_005101	G1P2	5.686	0.0273	9.094	0.000185
19	NM_001165	BIRC3	5.581	0.0613	28.13	1.59E-07
20	BC009507	ISG15	5.525	0.000307	7.542	0.000322
21	BC006322	ATF3	5.231	0.000144	3.306	0.00473
22	NM_004428	EFNA1	5.136	0.0208	2.228	0.0224
23	NM_006018	GPR109B	4.987	0.0471	11.96	0.000135
24	NM_000758	CSF2	4.964	0.00017	11.11	0.0392
25	NM_002192	INHBA	4.622	0.0117	8.515	0.000224
26	NM_172139	IL28B	4.577	0.0205	2.067	0.0229
27	NM_052941	GBP4	3.887	0.22	2.004	0.0488
28	NM_003733	OASL	3.695	0.000456	7.352	0.00035
29	NM_004417	DUSP1	3.522	0.00383	3.681	0.00321
30	NM_004428	EFNA1	3.5	0.0642	1.686	0.0825
31	NM_000636	SOD2	3.45	0.000578	5.162	0.00103
32	NM_000958	PTGER4	3.337	0.152	1.945	0.0416
33	NM_000575	IL1A	3.146	0.000829	2.027	0.0343
34	NM_006417	IFI44	2.967	0.0522	9.982	0.000142
35	AF038963	RIG-I	2.959	0.004	11.36	0.00603
36	NM_001198	PRDM1	2.896	0.00794	2.906	0.00762
37	NM_016584	IL23A	2.665	0.0845	79.9	0.0125
38	NM_012118	CCRN4L	2.53	0.126	1.703	0.0786
39	NM_006813	PNRC1	2.486	0.016	3.293	0.00485
40	NM_001200	BMP2	2.458	0.00221	3.743	0.00303
41	NM_014350	TNFAIP8	2.45	0.0312	2.104	0.029
42	NM_000201	ICAM1	2.44	0.021	15.9	0.00286
43	NM_003407	ZFP36	2.377	0.00455	3.605	0.00345
44	NM_003955	SOCS3	2.263	0.0103	3.328	0.014
45	NM_004233	CD83	2.194	0.00374	1.685	0.0828
46	NM_001945	DTR	2.133	0.00142	3.551	0.00365

47	NM_016270	KLF2	2.066	0.00492	1.872	0.0493
48	NM_172138	IL28A	2.061	0.108	1.817	0.0568
49	NM_002999	SDC4	2.005	0.349	2.051	0.0293
50	NM_002659	PLAUR	1.985	0.128	5.033	0.00338
51	NM_014330	PPP1R15A	1.973	0.187	2.139	0.0266
52	NM_004556	NFKBIE	1.968	0.0296	4.053	0.0023
53	BC017083	PIM3	1.891	0.00494	2.462	0.0148
54	AK056293	IFIH1	1.88	0.1	4.157	0.00211
55	NM_020651	PELI1	1.837	0.00837	1.674	0.0854
56	NM_001964	EGR1	1.77	0.045	1.793	0.0608
57	NM_001731	BTG1	1.761	0.0419	3.277	0.00486
58	NM_006403	NEDD9	1.735	0.0177	1.82	0.0575
59	NM_001300	COPEB	1.733	0.011	1.998	0.0362
60	A_23_P57836	A_23_P57836	1.71	0.0122	2.27	0.0206
61	NM_153341	IBRDC3	1.701	0.247	2.564	0.00519
62	NM_006403	NEDD9	1.662	0.212	2.989	0.00694
63	NM_004433	ELF3	1.656	0.102	2.674	0.0105
64	NM_004073	PLK3	1.655	0.0168	3.122	0.00582
65	AB014515	N4BP1	1.651	0.0432	1.625	0.0996
66	NM_002985	CCL5	1.648	0.0382	1.98	0.0379
67	NM_002185	IL7R	1.648	0.0155	2.14	0.0269
68	NM_006058	TNIP1	1.634	0.0121	3.308	0.00471
69	NM_031866	FZD8	1.621	0.307	2.645	0.0111
70	NM_003897	IER3	1.614	0.00857	2.826	0.00847
71	NM_130781	RAB24	1.595	0.0196	2.43	0.0156
72	NM_172140	IL29	1.584	0.0205	2.269	0.0206
73	NM_002658	PLAU	1.582	0.02	6.92	0.00042
74	NM_002201	ISG20	1.579	0.0109	9.915	0.0196
75	AF099011	EHD1	1.577	0.0109	3.771	0.00296

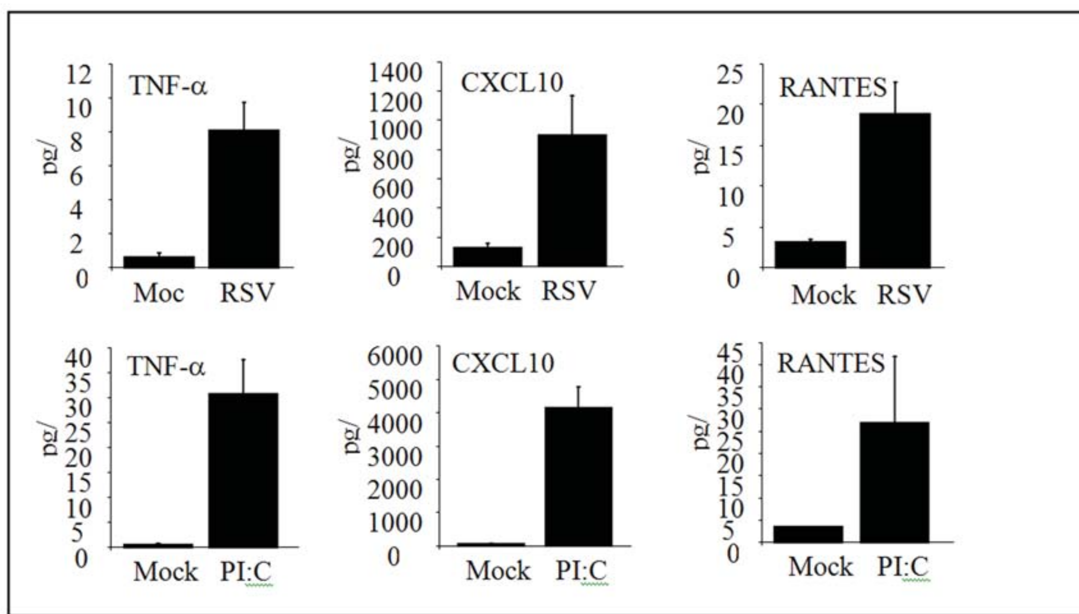

Figure 3. Analysis of protein expression.

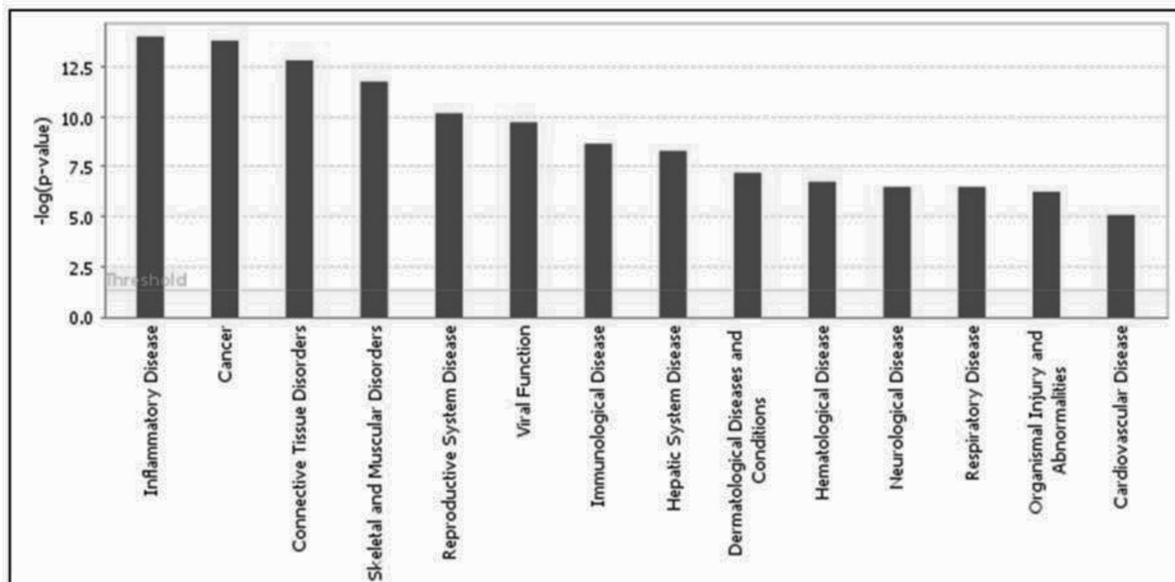

Figure 4A. Functional analysis of differentially expressed genes.

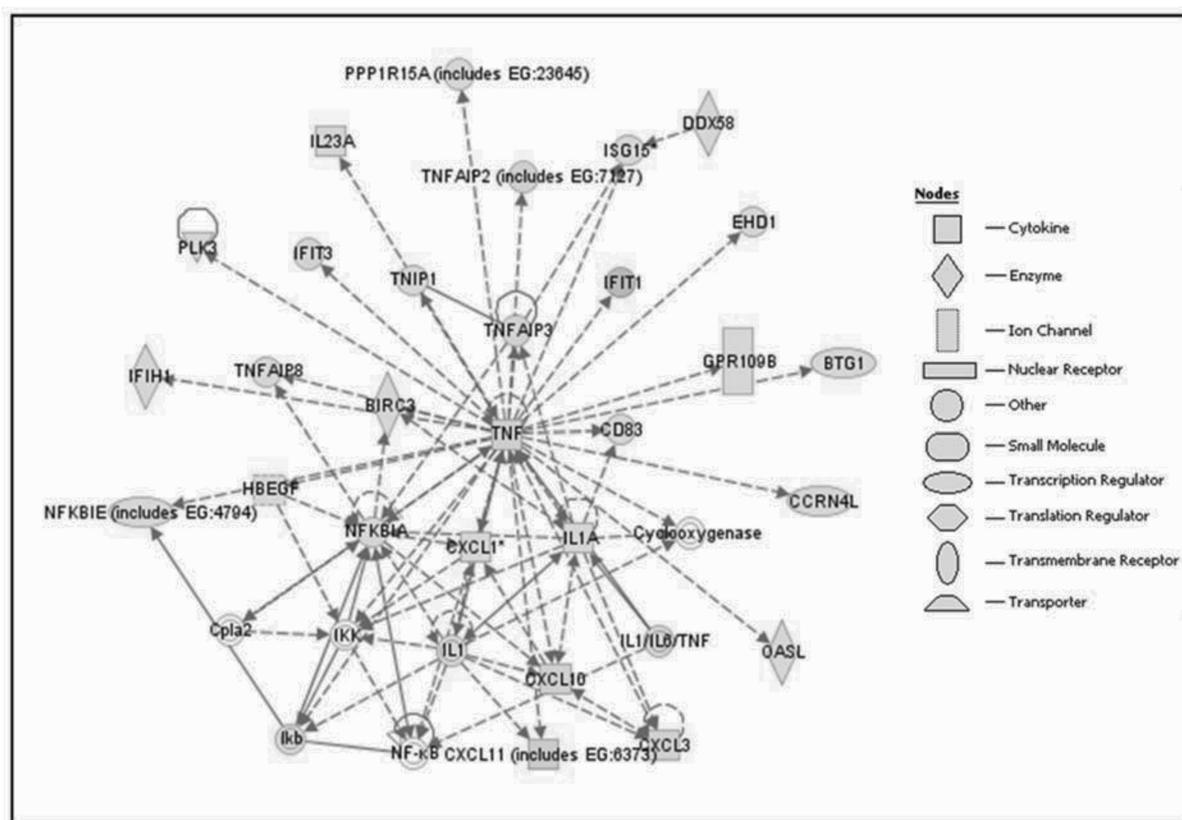

Figure 4B. Functional network analysis of the genes induced by dsRNA and RSV using IPA. Functional network of genes that was commonly induced by RSV infection and dsRNA treatment. The top-scoring network consists of nodes, where each node represents a gene. The shape of the node is indicative of the functional class of the gene product. Multiple connections indicate the biological relationships between the nodes.

included TNF-α, IP10/CXCL10 and RANTES/CCL5. Data in Figure 3 showed that cytokine protein expression was significantly increased in infected or treated cells, which validated the data obtained from microarray experiments.

Functional analysis of differentially expressed genes

To investigate the relation and interaction of genes/molecules that were differentially regulated by dsRNA and RSV, we analyzed the up-regulated genes with Ingenuity Pathways Analysis (IPA, Ingenuity Systems). Functional analysis of 75 genes that were commonly induced by RSV and dsRNA in PHBE showed that the up-regulated genes are involved in various diseases and cellular activation pathways. The highest significant diseases and molecular pathways corresponded to these genes are inflammatory disease Figure 4A. From 75 commonly upregulated genes, 20 genes were determined by IPA to be related to inflammatory disease (Table 1).

TNF-α has been reported to be induced by dsRNA in vitro and in vivo, and is among the most important cytokines that play a role in viral induced inflammation. Therefore, particularly important nodes in the network are the genes that are known to interact or induced by TNF-α (Figure 4B).

Discussion

During viral infections, a large repertoire of genes is induced to prevent viral replication and spread. The initial site of infection for respiratory viruses is the airway epithelium; therefore, determining the viral-induced transcriptional events in these cells can shed lights on the initial steps leading to anti viral immune response. The establishment of the anti viral state requires coordinate transcriptional activation of many genes of innate and adaptive immunity. Previous reports have shown that viral infection of various cell types or treatment with poly I:C induced several genes, including type I IFN's. TNF-α RANTES, IL-1, IL-4, IL-6 and IL-8.

DsRNA is recognized as signal to the presence of viral infection. Experimentally, dsRNA in the form of poly I:C has been widely used as a viral mimetic to induce an anti-viral state. Thus far, there have been several reports on the transcriptomic profiling of virus infections and dsRNA treatment in cells. What has been lacking is a global expression profiling in PHBE cells, which are the natural target of respiratory viral infections. In addition, there has not been a comparison between RSV infection and dsrNA treatment to assess the contribution of dsRNA-induced events during virus infections in these cells.

Our global transcriptomic analysis identified 2024 genes that were upregulated by RSV and 159 genes that were induced by dsRNA treatment. A comparison of these genes revealed that 75 genes were commonly up-regulated in PHBE cells after treatment with dsRNA or infection with RSV. Interestingly, dsRNA induced similar pattern of inflammatory cytokines and chemokines to RSV infection. These included type I IFN's, TNF-α, IL-1, IL-6, IL-8, CCL-5, CXCl1, CXCL2, CXCL3, CXCL10, and CXCL11. However there were a number of genes that were specifically induced only by dsRNA or RSV (see table 3). Our results also revealed several dsRNA- and RSV-induced genes that have not been reported to be induced in in epithelial cells. These novel genes that are induced by RSV and or dsRNA included ADAMs, ILR13R2, CCNA1, ADRA2C, and BMP2, RND3, SOCS3 and BLMP-1.

ADAMs such as ADAM8 and ADAM10 are disintegrins metalloproteinases that play an important role in releasing soluble CD23 membrane bound precursor (Fourie *et al*., 2003). The release of the soluble form of CD23 has been reported to increase IgE production (Christie *et al*., 1997; Kilmon *et al*., 2001; Saxon *et al*., 1990), which is the key component of allergic reactions. In as much IgE is critical for allergic reactions, this observation is consistent with previous reports showing that RSV and dsRNA are

Table 6. RT-PCR validation of gene expression.

	Gene ID	Gene Name	PHBE + PI:C	PHBE +RSV	Family
1	NM_014417	BBC3	374.81	2.26	Other
2	NM_001710	BF	6.82	1.48	peptidase
3	NM_001165	BIRC3	4.10	59.51	Other
4	NM_004591	CCL20	492.13	1.89	cytokine
5	NM_000758	CSF2	22.78	1518.56	cytokine
6	NM_001511	CXCL1	6.17	11.66	cytokine
7	NM_001565	CXCL10	46.05	84.37	cytokine
8	NM_005409	CXCL11	5.62	47.73	cytokine
9	NM_002089	CXCL2	2.32	2.82	cytokine
10	NM_002090	CXCL3	9.32	21.33	cytokine
11	NM_004417	DUSP1	8.52	5.02	phosphatase
12	NM_004428	EFNA1	3.67	1.71	Other
14	NM_001964	EGR1	5.01	1.48	transcription regulator
15	NM_005101	G1P2	35.27	8.73	Other
16	NM_001548	IFIT1	5.05	6.99	Other
17	NM_001547	IFIT2	12.60	6.30	Other
18	NM_001549	IFIT4	4.68	5.35	Other
19	NM_002176	IFNB1	58.00	3.76	cytokine
20	NM_000575	IL1A	2.78	2.38	cytokine
21	NM_000600	IL6	8.00	16.58	cytokine
22	NM_000584	IL8	30.59	46.88	cytokine
23	NM_002192	INHBA	3.92	17.10	growth factor
24	NM_020529	NFKBIA	5.74	3.61	transcription regulator
25	NM_031419	NFKBIZ	3.29	1.22	transcription regulator
26	AF038963	RIG-I	1.87	4.73	enzyme
27	NM_006290	TNFAIP3	12.73	4.17	Other
28	NM_003407	ZFP36	4.48	9.59	transcription regulator

associated with induction of allergic immune responses (Akira, 2006; Graham, 2000; von Herrath, 2003). Based on epidemiologic and experimental evidence, expression of ADAM8 has been shown to be up-regulated in a murine model of asthma, and in patients with severe and moderate asthma (Foley *et al*., 2007; King *et al*., 2004; Matsuno *et al*., 2006). Taken together, these data may suggest a novel mechanism for the association of virus infections with induction and exacerbations of allergic diseases such as asthma.

Another interesting gene that has not been reported previously that was up-regulated by RSV in ours transcriptomic analysis was the alpha 2 subunit of IL-13 receptor (IL-13RA2). Recent reports have shown that IL-13RA2 maybe decoy receptor for IL-13 which can abrogate a stimulatory signal generated from IL-13 and and IL-13RA1 interaction (Wu, 2003). IL-13 is a pleiotrophic cytokine with potent immune and epithelial cell-regulatory functions such as suppression of inflammation, epithelial growth, and mucus secretion (Hershey, 2003; Holgate, 2003; Kasaian, 2008). Based on the accumulated data, it is tempting to speculate that up-regulation of the decoy IL13RA2 during RSV infection maybe underlying factor for the immuno-deviation and severe bronchiolitis associated with this infection. Furthermore, IL-13RA has been suggested to play important role in TGF-β1 mediated fibrosis and could be considered for new therapeutic target (Fichtner-Feigl *et al*., 2007; Fichtner-Feigl *et al*., 2006).

A pathway analysis or our data using IPA revealed that TNF-α was a central player in the virus- and dsRNA-induced events (see figure 4B). TNF-α is an early and potent inducer of inflammation, which can regulate innate and adaptive immune responses, monocyte and lymphocyte migration, and tissue damage. We previously reported that induction of TNF-α during RSV infection or dsRNA treatment was regulated by dsRNA-activated protein kinase (PKR) and p28 MAPK activation (Meusel, 2003). In addition, several of the intracellular antiviral pathways are also up-regulated including, RIG-1, MDA5, TLR3 and MX-1. Predictably, our data suggest that a coordinated activation of several anti-viral pathways may be required for an effective response against respiratory viral infections

Collectively, our data showed that dsRNA could mimic several of the inflammatory and regulatory cytokines and chemokines that RSV induces. Our data further provide several novel genes that are induced by dsRNA and RSV, which may be associated with the induction of allergic conditions. Our future studies are aimed at further characterizing the regulation of these novel RSV- and dsRNA induced genes in airways epithelial cells.

The expression of the Novel genes that were up-regulated in primary human lung epithelial cell after RSV infection or dsRNA treatment by microarray analysis was confirmed by RT-PCR. Y axis represents the relative fold increase of expression. X axis represents genes.

The protein expression level of selected genes were analyzed using bioplex assay. Consistent with the increase in RNA expression, protein expression levels of the selected genes also significantly increase.

Shown are selected Ingenuity Pathway Analysis (IPA) assigned functional categories for the genes induced by dsRNA and RSV. The –log (significance) indicates an increase in confidence for each category.

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