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Virology 486 (2015) 146–157

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

A systems approach to understanding human rhinovirus and influenza virus infection

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ARTICLE INFO

Article history:
Received 8 July 2015
Returned to author for revisions
28 July 2015
Accepted 13 August 2015
Available online 6 October 2015

Keywords:
Rhinovirus
Influenza virus
Co-infection
Epithelial cell
Gene expression time-course

ABSTRACT

Human rhinovirus and influenza virus infections of the upper airway lead to colds and the flu and can trigger exacerbations of lower airway diseases including asthma and chronic obstructive pulmonary disease. Novel diagnostic and therapeutic targets are still needed to differentiate between the cold and the flu, since the clinical course of influenza can be severe while that of rhinovirus is usually more mild. In our investigation of influenza and rhinovirus infection of human respiratory epithelial cells, we used a systems approach to identify the temporally changing patterns of host gene expression from these viruses. After infection of human bronchial epithelial cells (BEAS-2B) with rhinovirus, influenza virus or co-infection with both viruses, we studied the time-course of host gene expression changes over three days. We modeled host responses to these viral infections with time and documented the qualitative and quantitative differences in innate immune activation and regulation.

Introduction

Rhinovirus and influenza virus are among the leading causes of infections of the upper respiratory tract and epithelial cells are usually the initial targets of these viral infections (Eccles, 2005; Nichols et al., 2008). The patterns of host responses evoked by each virus are beginning to emerge. Recent in vitro studies have identified overall changes as well as key host factors during influenza virus infection (Watanabe et al., 2010; Shapira et al., 2009). In vivo studies on subjects who were experimentally infected with rhinovirus (Proud et al., 2008) or influenza (Woods et al., 2013) have identified host-derived changes in gene expression and biological processes, and some of the findings may have diagnostic potential (Zaas et al., 2013). Smith et al. (2012) described 67 host biological pathways that were up-regulated in common by seven different respiratory viruses including rhinovirus and influenza by reviewing published data from a number of laboratories. Zaas et al. (2009) developed gene expression signatures from peripheral blood cells that distinguished individuals experimentally infected with either rhinovirus, influenza virus, or respiratory syncytial virus and more recently this group described a RT-PCR based gene expression signature from blood cells that could detect and discriminate between two types of influenza virus in experimentally infected subjects (Zaas et al., 2013).

While classifiers of single virus infections would be clinically valuable, especially if they are derived from multiple cell types and different stages of infection, a given individual may be infected with more than one virus. Indeed, Greer et al. (2009) and Casalegno et al. (2010) reported that detection of rhinovirus was associated with a reduced probability of detecting influenza virus in clinical samples. In another co-infection study, 30 samples (13%) from individuals infected with the influenza virus were positive by a PCR assay for 31 viral co-pathogens of which the most prominent was rhinovirus (61%) (Esper et al., 2011). While co-infection may be common, more recent studies found that disease severity and clinical course were essentially similar in patients with co-infections compared to patients with single infections (Choi et al., 2015; Asner et al., 2014; Blyth et al., 2013; Navarro-Mari et al., 2012).

Several groups have studied gene expression changes in epithelial cells after infection in vitro with rhinovirus [see Gene Expression Omnibus (accession GSE55271, Schuler et al., 2014; accession GSE28904, Naim et al. unpublished; accession GSE27973, Proud et al., 2012) or influenza virus (Li et al., 2011; Mitchell et al., 2013; Josset et al., 2014; see also accession GSE48466, Gerlach et al., 2013)], but co-infection with both viruses in a well-controlled in vitro system

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http://dx.doi.org/10.1016/j.virol.2015.08.014

has not been explored nor has an extensive time-course of host cell transcriptional changes following viral infection of a respiratory cell been investigated. Thus in order to better understand the host respiratory cellular transcriptional response to either rhinovirus, influenza virus and co-infection with both viruses, we performed a detailed time-course analysis of transcriptional changes following infection of the human bronchial epithelial cell line (BEAS-2B). From this analysis, we detail the changes in host cell biological processes and transcription that result from infection, including changes that occur with co-infection of influenza and rhinovirus.

Results and discussion

Virus infections and derivation of differentially expressed genes

We evaluated the productivity, timing, and specificity of virus infection by measuring two mRNAs specific for influenza virus, the influenza A matrix protein 2 (M2) and non-structural protein 1 (NS; accession Z21498), and an amplicon that was specific for the rhinovirus 16 genome (accession EU096003). As shown in Fig. 1A, viral specific gene expression was only observed in the appropriate samples and changes in the expression of the viral specific genes demonstrated that productive infections occurred. To confirm the expected host cellular response to rhinovirus and influenza virus infection, we evaluated the temporal transcriptional profiles of 3 genes that were previously found to be up-regulated during infection of epithelial cells by these viruses: ICAM1, the receptor for rhinovirus 16 (Papi and Johnson, 1999); CXCL10, a chemokine for monocytes and macrophages (Spurrell et al., 2005); and TLR3, an intracellular receptor for double-stranded viral RNA (Hewson et al., 2005; Guillot et al., 2005). Steady-state levels of these mRNAs were strongly induced by viral infection as expected, but with several virus specific differences as shown in Fig. 2. CXCL10 and TLR3 mRNA levels peaked at 24 h, earlier after influenza infection compared to rhinovirus infected cells, while ICAM1 mRNA levels were higher after rhinovirus infection, raising the possibility that subtle viral infection specific differences in regulation of these genes exist. As a final evaluation of the robustness of viral infection and the host cell response to viral infection, we compared the mRNA and protein changes for three cytokines that were previously shown to change during respiratory virus infection in vitro as well as in vivo. As shown in Supplementary File 3 steady-state mRNA levels for IL-6, CXCL10 and CCL5 peaked at 48–60 h post-infection (depending on the virus infection) while the levels of the corresponding proteins secreted into the medium peaked 12–48 h later as expected for simple transcriptional regulation. Finally, as a general validation of the microarray results we reconfirmed the expression of three host mRNAs that increased during the infection time-course (DDX60, IFI27, SCD) and three mRNAs that decreased during the time-course (CBX5, FBN2, EPCAM). Messenger RNA levels measured by RT-PCR corresponded closely with the microarray results for each of these mRNAs Supplementary File 4.

For our analysis of the microarray data, differentially expressed genes from the BEAS-2B cells infected with rhinovirus (RV), influenza virus (IV) or both viruses (RV+IV) were derived by comparison with mock-infected cells. Hierarchical cluster analysis (not shown) revealed that relatively few host cell genes were differentially expressed by 8 h post-infection. But by 24 h, the expression of hundreds of genes was changing. Slightly more genes were differentially expressed after IV infection compared to RV infection at 24–36 h post-infection, but this pattern was reversed by 48 h post-infection and at later times (Fig. 1B and C). There were more up-regulated than down-regulated genes, and down-regulated genes tended to lag up-regulated genes. Fig. 1D compares the total number of unique host genes for each virus infection. For example, 310 genes were up-regulated and 141 were down-regulated in all three virus infections, while RV, IV and RV+IV specific and commonly up- and down-regulated genes were also observed. Supplementary File 5 lists the differentially expressed genes shown in Fig. 1D. These differentially expressed genes were subsequently used for enrichment analysis and for the derivation of pathways and networks.

The changing pattern of host cell responses after infection

Next, using the differentially up-regulated host genes from each infection and at each time point, we derived a time-series map of the changing pattern of biological processes, shown in Fig. 3. This analysis demonstrated that some host cell pathways were common to both viruses, including the RIG-I-like receptor (Entrez id: DDX58) signaling pathway which senses cytoplasmic viral RNA, and other

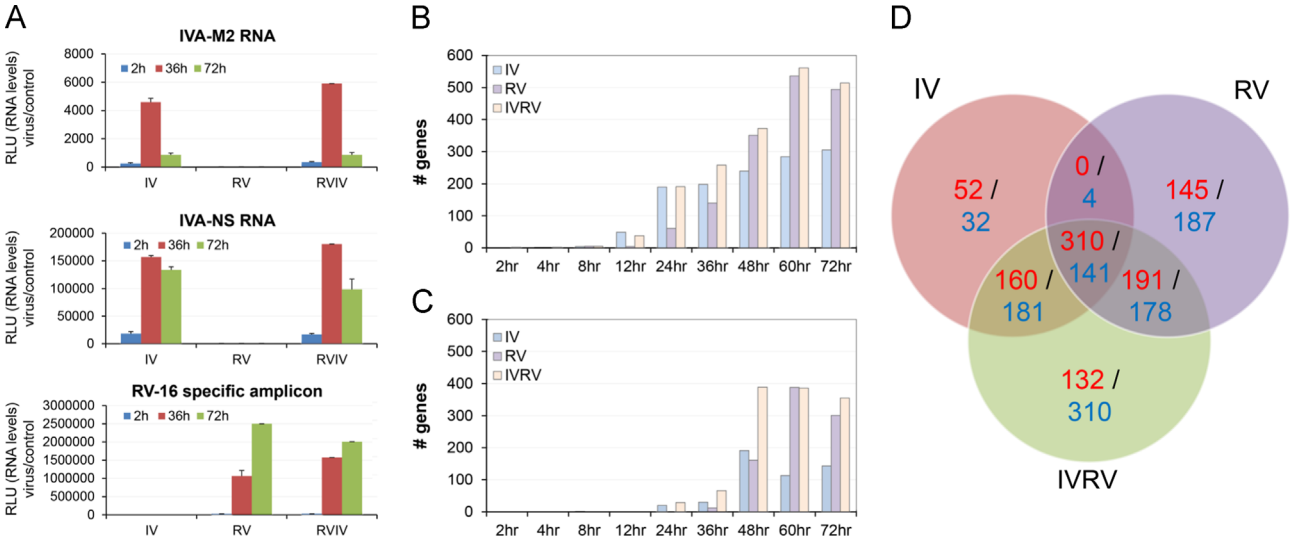

Fig. 1. Differentially expressed host genes (FDR < 0.01; log2 $\pm$ 1.5). (A) Assay of influenza virus or rhinovirus amplicons. The levels of influenza-a M2 mRNA, influenza-a NS mRNA, and an amplicon specific for the rhinovirus 16 genome were determined by qRT-PCR. (B) Up-regulated host genes relative to mock-infected cells; (C) down-regulated host genes relative to mock-infected cells; (D) Venn diagram showing how the differentially expressed host genes overlap among the three virus infections. Uniquely expressed up or down regulated genes were derived for each virus infection over all time points and compared. Red labels, up-regulated genes; blue labels, down-regulated genes.

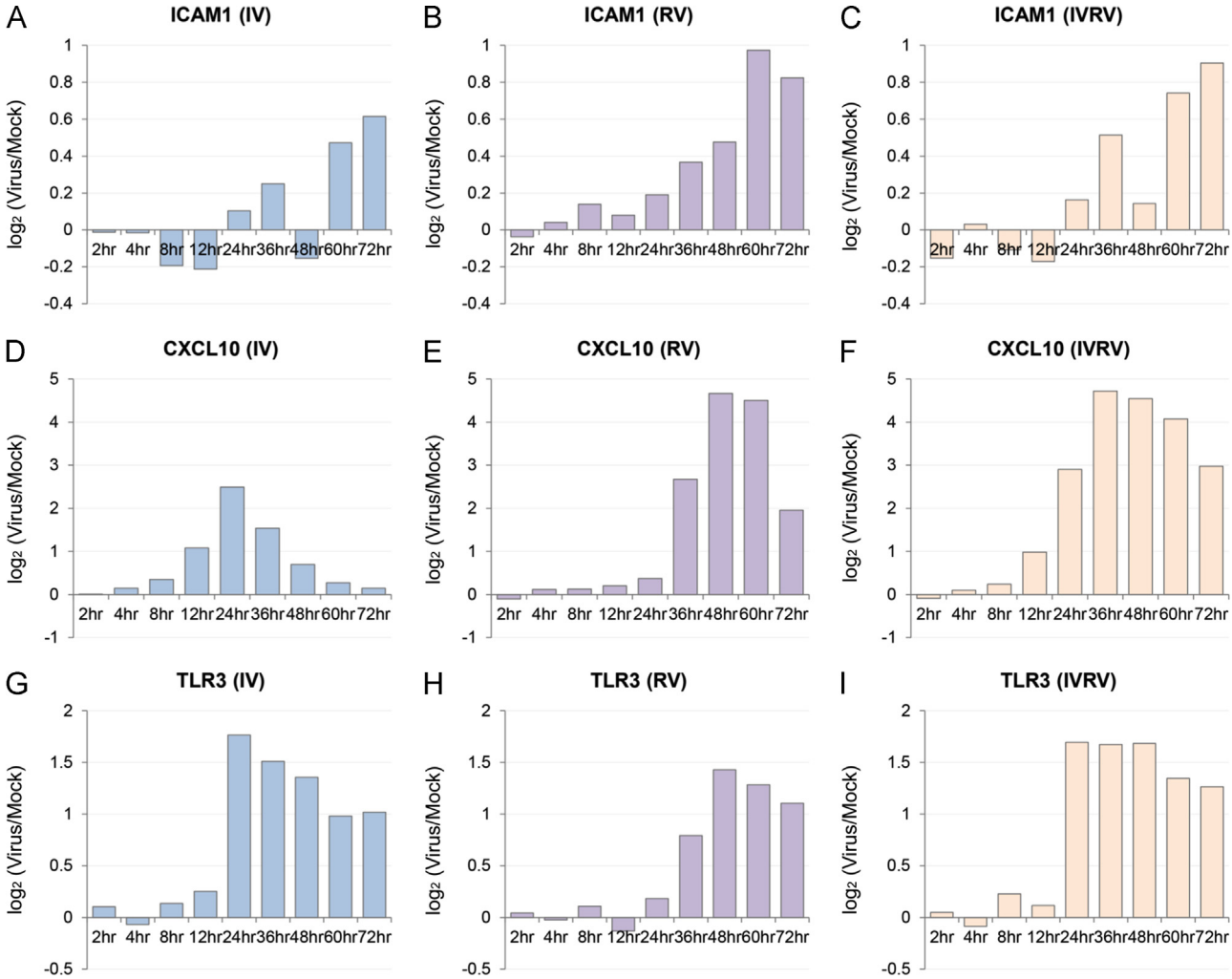

Fig. 2. Steady-state mRNA profiles for ICAM1, CXCL10, and TLR3. For all nine panels, the log2 ratio of the signal intensity of the indicated RNA for virus-infected cells relative to mock infected (control) cells is plotted against time.

innate immune sensing pathways including the NOD-like receptor signaling pathway and other members of the toll-like receptor signaling pathway. Influenza-specific pathways included steroid biosynthesis, terpenoid backbone biosynthesis, pathways for glutathione and purine biosynthesis and primary immunodeficiency. Rhinovirus-specific pathways included cytokine and chemokine signaling, p53 signaling, extracellular matrix-receptor interactions, small cell lung cancer, cell adhesion/focal adhesion and B cell receptor signaling. We next derived sub-sets of the differentially expressed genes that were enriched over multiple sampling times by the method of non-negative matrix factorization. Twenty patterns or sub-sets of genes with defined p-values (<0.01) and consistent expression patterns were recognized as shown in Supplementary File 6. This analysis revealed genes that were expressed for a short period (i.e., one or two time points: patterns 7 or 10) and genes expressed for a long period (i.e., four or five time points: patterns 1 or 2). The genes that comprised the former patterns (5, 7, 10–14, 16–20) with continuous expression over several time points (shown in Supplementary File 7) were adopted for a more detailed pathway analysis. These ten gene groups are presented in more detail in Fig. 4. For example pattern 1 was defined by 628 genes that were down-regulated after infection by both viruses from 24 h through 72 h while the 52 genes in pattern 15 (Fig. 4A) were up- regulated late after rhinovirus infection. The ten patterns illustrated in Fig. 4 encompassed over 1000 differentially expressed genes that were either virus-specific or common to both viruses. Expression information from these genes over the 72 h time course was used to illustrate diverse cellular processes and to compose a comprehensive network for up-regulated host genes in infected BEAS-2B cells as shown in Fig. 5. The group of virus-specific and common networks summarized in Fig. 5 are presented in detail at 2, 4, 8, 12, 24, 36, 48, 60, and 72 h post-infection in Supplementary Files 8–16. Interferon responses were of course induced by the RIG-1, NOD, and Toll receptor pathways (see below).

The interferon response of BEAS-2B cells to viral infection

One pathway of specific interest to viral infection of cells is the interferon response pathway. Thus we studied the expression of the type 1 and 2 interferons, the interferon regulatory factors, and fork-head family of transcription factors that have been implicated as regulators of the interferon response. We evaluated the simple normalized gene expression intensity values for members of these gene families, rather than their differentially expressed values, since low-level expression was important to document and would otherwise have been filtered out. IFNB1(interferon, beta-1) was identified as the predominant type I interferon produced in BEAS-2B cells following infection with either virus. Messenger RNAs for other type I interferons (IFNA1, IFNE) and the type 2 interferon IFNγ were not expressed above background (Fig. 6 and data not shown). Among the family of interferon regulatory factors, IRF7

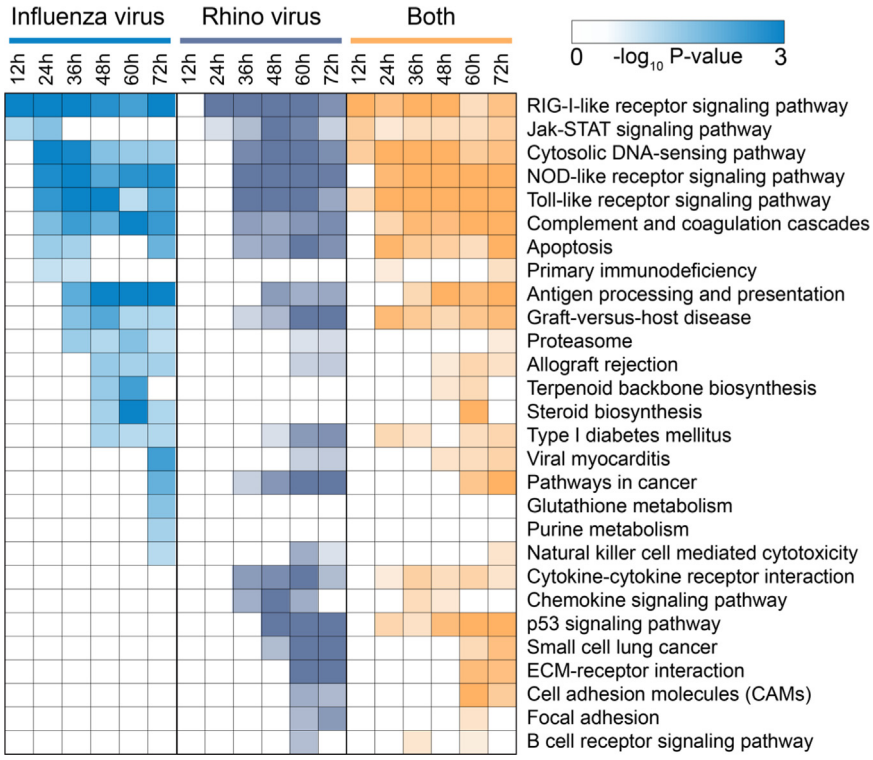

Fig. 3. Time-series map of the changing pattern of biological processes.

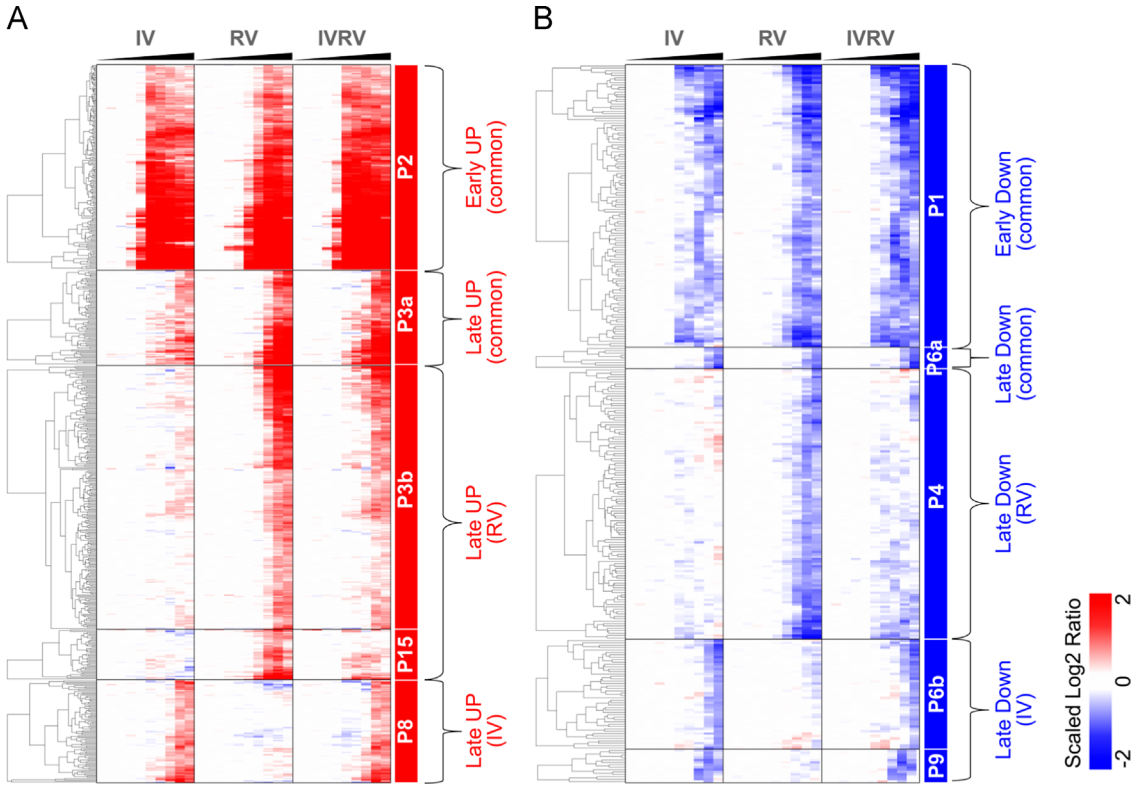

Fig. 4. Profiles of pattern genes: clustering analysis was performed by non-negative matrix factorization as described in *Methods*. (A) Early and late up-regulated differentially expressed genes from IV, RV, or IV+RV infected cells. (B) Down-regulated differentially expressed genes from IV, RV, or IV+RV infected cells.

and IRF9 were strongly up-regulated by both viruses. Both IRF7 and IRF9 mRNAs were induced 24-fold by influenza virus and 8-fold by rhinovirus, with a peak at 24–48 h post-infection. IRF1 mRNA was up-regulated after rhinovirus infection (about 8 fold) but only about 2-fold by influenza virus. Messenger RNAs for IRF3 and other IRFs were not expressed above background levels. We also profiled FoxO (fork-head box, subgroup O) family transcription factors since FoxO3 has been implicated in the regulation of IRF7 in murine macrophages (Litvak et al., 2012). Messenger RNAs for FoxO1, FoxO3, and FoxO4 were induced by rhinovirus (but not

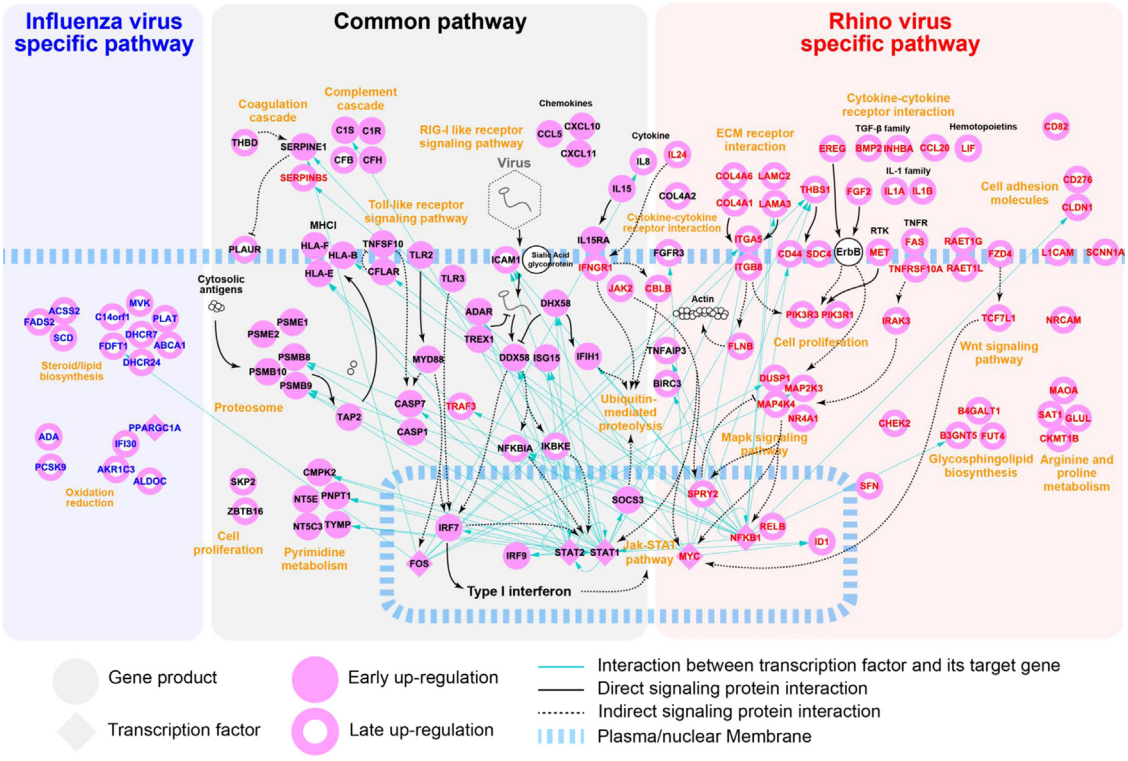

Fig. 5. Summary of RV, IV, combined pathways. Virus perturbed signaling network. Hypothetical signaling pathway commonly enriched by both virus infections or specifically enriched by influenza virus or rhinovirus infection are shown in middle/left/right side, respectively. Round node and diamond indicate gene product and transcription factor, respectively. Pink color in node or in border is early up-regulation or late up-regulation, respectively. Sky blue line denotes the interaction between transcription factor and its target gene. Solid or dashed black line is direct or indirect signaling protein interaction based on KEGG pathway. Dotted solid sky blue line shows cellular/nucleus membrane.

influenza virus) above background with FoxO1the strongest, showing a ~2.6 fold induction at 48–60 h post-infection while FoxO3 and FoxO4 were induced about 1.3 fold.

Host genes up-regulated by both viruses

Only a few differentially expressed mRNAs were detected as early as 2–4 h after infection with either rhinovirus, influenza virus or both viruses, but by 12 h post-infection many genes were up regulated and in particular we noted the expression of genes whose products comprise one of three initiating branches of the innate immune system. One key part of innate immune activation is based on detection of viral RNA in the cytosol (Hacker et al., 2011). By 8 h-post-infection DDX58 and IRF9 were activated (Supplementary File 10) and increased throughout the rest of the study. The DDX58 gene product (also known as RIG-I) is a RNA helicase which detects viral double strand RNA and thus infection by influenza virus (and certain others), leading to one of the earliest activation steps in innate immunity. After 12 h post-infection (Supplementary File 11), genes from other members of this family of viral receptors were detected including IFIH1 (interferon induced with helicase C domain 1; also known as melanoma differentiation associated gene 5 or MDA5) and the related gene DHX58 (also known as LGP2), whose product has been reported to be a feedback inhibitor for antiviral signaling by DDX58 and IFIH1 (Cui et al., 2001; Childs et al., 2012; Komuro and Horvath, 2007). IFIH1 has also been reported to recognize ds-RNA from replicating rhinovirus intermediates (Triantafilou et al., 2011). DDX58 and IFIH1 can interact with mitochondrial-antiviral signaling protein (MAVS; also known as VISA) which was also differentially expressed (data not shown), TRAF3, and IKBKE (both shown on Fig. 5) leading to the induction of IRF3 (Xu et al., 2005; Seth et al., 2005) and possibly contributing to induction of IRF7 and IRF9 which were much more strongly induced than IRF3 in this study. The net result of this early signaling is the activation of a group of interferon regulatory transcription factors IRF3, IRF7 and IRF 9, leading to production of interferon-β, the predominant type I interferon that was produced in these BEAS-2B cells. The viral RNA sensing pathway also activates production of pro-inflammatory cytokines via NF-κB, STAT1 and STAT2 resulting in a sustained anti-viral state for the cell. IRF7 and IRF9 are prominent in this data and likely contribute to the induction of IFN-β and CCL5, CXCL10, and CXCL11, which are all interferon inducible cytokines (Nakano et al., 2012).

By 24 h post-infection (Supplementary File 12) our data shows that TLR2 and TLR3 genes were active. A second innate immune activation pathway derives from Toll-like receptors that signal from the cell membrane. TLR2 is located in the plasma membrane, recognizes the human rhinovirus 16 capsid (Triantafilou et al., 2011) and signals via myeloid differentiation factor 88 (MyD88), and TRAF3 to activate proinflammatory cytokines via the NF-κB and the JNK-p38 kinase pathways (Hacker et al., 2011). A third branch of innate immune activation is based on TLR family members that signal from endosomes. In BEAS-2B cells, TLR3 (Fig. 5) recognizes double-stranded RNA, resides in endosomal vesicles, and it signals via TRIF, TRAF3, and IkBke to activate the type 1 interferon response, NF-κB, and the JNK, p38 kinase pathways (Hacker et al., 2011). This report confirms the study of Hewson et al. (2005) which also noted the up-regulation of TLR3 in BEAS-2B cells after infection with rhinovirus.

By 8 h post-infection, in response to transcription by NF-κB, the TNFAIP3 gene (tumor necrosis factor alpha induced protein 3) becomes active. TNFAIP3 has been proposed to limit NF-κB activation and to antagonize TNF-induced apoptosis by binding to TRAF family adaptor proteins (Song et al., 1996; De Valck et al., 1996; Heyninck et al., 1999). Also at 8 h post-infection, and in

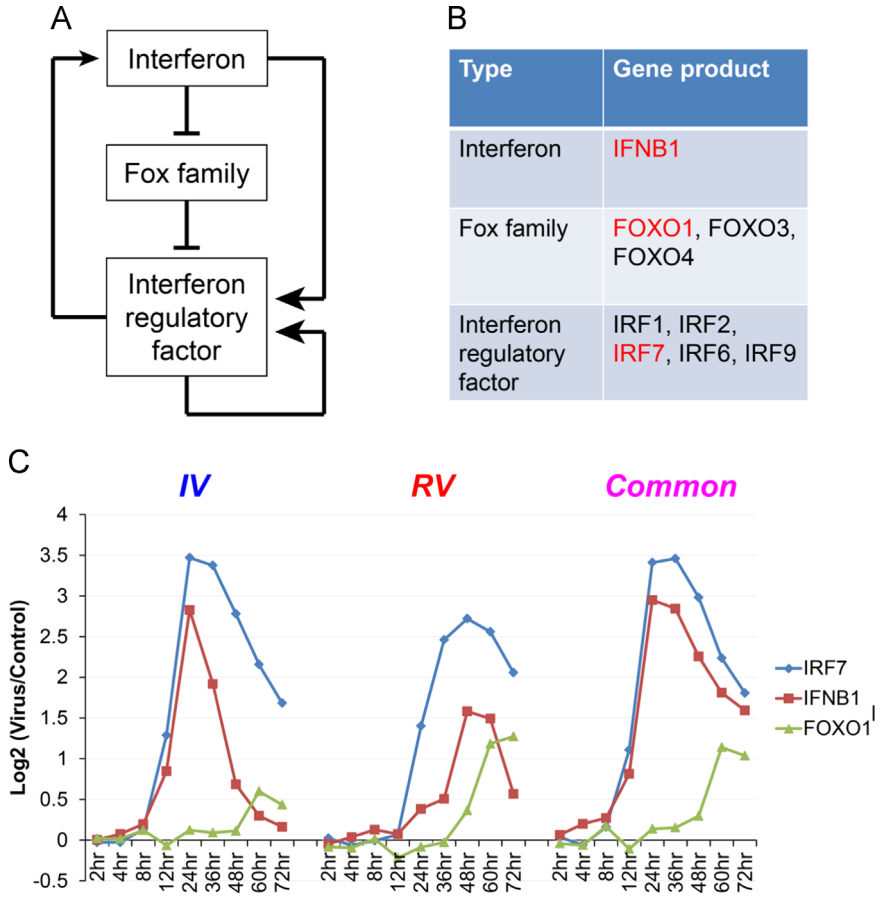

Fig. 6. Speculative feed forward loop for regulation of IFN. (A) Feed forward regulation of IFN-β can be mediated by a Fox family transcription factor that negatively regulates an IRF transcription factor. (B) IFNB1, FOXO1, IRF7 and IRF9 may comprise such a system in BEAS-2B cells. (C) Simple steady-state mRNA levels for FoxO1 rise late, after peak levels of IFNB1 and IRF7 in BEAS-2B cells, consistent with feed forward loop regulation.

response to interferon binding, IRF9 becomes active, forming a complex with STAT1 and STAT2 which specifically activates the interferon program of gene expression that regulates the cellular innate antiviral immune response and also influences adaptive immunity (Biron, 2001; Samuel, 2001; Lau et al., 2003). By 12 h post-infection, the chemokines CCL5, CXCL10 and CXCL11 become active. CXCL10 and CXCL11 are chemo-attractants for monocytes and macrophages and other immune cells. CCL5 (RANTES) is a chemoattractant for T cells, eosinophils, and basophils. Also at 12 h post-infection TNFSF10 (TRAIL) activity was induced by both viruses. This cytokine in the TNF family can signal apoptosis via its receptors DR4 (TRAIL-RI) and DR5 (TRAIL-RII) (Wiley et al., 1995; Pitti et al, 1996).

In addition by 24 h post-infection, increasing NF-κB and STAT1-STAT2-IRF9 activity may contribute to activation of antigen processing pathways ultimately leading to MHC I presentation of processed viral antigens and activation of cytotoxic T cells. Among the targets of these transcription complexes are genes whose products activate the immune-proteosome that generates MHC class 1 antigens and signal that the cell is infected. These genes include PSMB8 (proteasome subunit beta type-8), PSMB9, and PSMB10 that are integral parts of the immune-proteosome (Bodmer et al., 1992; Schmidt et al., 1999), or genes whose proteins contribute to its regulation: PSME1 and PSME2 (Honore et al., 1994). TAP2 (protein antigen transporter; Bahram et al., 1991), transports these peptides from the cytoplasm to the endoplasmic reticulum so that they can be presented by HLA-B, HLA-E and HLA-F molecules at the cell membrane. Recently, Josset et al. (2014) showed that more pathogenic serotypes of influenza A such as H7N9 do not induce the antigen presentation pathway to the same degree as the less pathogenic H1N1 strain used here.

Also up-regulated by IRF9-STAT1-STAT2 at 24 h post-infection, particularly after influenza infection, are genes whose products dephosphorylate purines (NT5E: 5′-nucleotidase) or pyrimidines (NT5C3: cytosolic 5′-nucleotidase 3), degrade thymidine (TYMP, thymidine phosphorylase), a 3′-to-5′ exoribonuclease (PNPT1 polyribonucleotide nucleotidyltransferase 1), or cytidine (CMPK2, cytidine monophosphate (UMP-CMP) kinase 2). Degradation of purines would commonly result in generation of free energy e.g. ATP for respiration and thus may be an indication of cell stress, starvation or autophagy. Adenosine, another product of purine metabolism is involved in suppression of the T effector response by inhibiting production of NF-KB dependent cytokines. Conceivably, catabolism of nucleic acids may permit the cell to prolong its survival in the face of lethal influenza infection, and to indirectly defer or suppress an overt T cell response.

Components of the complement cascade including C1S, C1R, and the regulator CFH are also increased after influenza infection by 24 h post-infection. Greater up-regulation of complement components could also be an indication of the robust, potentially lethal early innate response triggered by influenza. The eventual outcome of complement activation is likely activation of the membrane attack complex that kills cells through lysis resulting in extensive tissue damage and a massive amplification of the innate response.

Two negative regulators of TNFα and NF-κB-induced inflammatory responses also appeared at 24 h post-infection: TNFAIP3 and BIRC3. TNFAIP3 inhibits NF-κB activation as well as TNFα-mediated apoptosis by binding to TRAF family adaptor proteins (Heyninck

et al., 1999). BIRC3 (baculovirus IAP repeat-containing 3; Liston et al., 1996; Rothe et al., 1996) can also bind to TRAF proteins, but it could antagonize apoptosis via its E3 ubiquitin ligase, which is specific for certain caspases including CASP7, an effector of apoptosis which was also up-regulated at 24 h post-infection.

By 36 h post-infection, IL-8 was up-regulated by both viruses. IL-8 is a strong chemoattractant for neutrophils and is a key mediator of viral-induced bronchiolitis, or inflammation of respiratory tract tissues (Baggiolini and Clark-Lewis, 1992). By 48 h post-infection, SOCS3 was strongly up-regulated by both viruses. SOCS3 has been described as a cytokine-induced negative regulator of cytokine signaling, in large part because it can bind to JAK2 (Sasaki et al., 1999). JAK2 was up-regulated after rhinovirus infection, but not influenza infection.

The interferon response of BEAS-2B cells to viral infection

Signaling from the RIG-1, NOD-like, Toll-like and other early innate-immune pathways (Fig. 3) resulted in activation of interferon responses (Fig. 5). Thus we studied the expression of the type 1 and 2 interferons, the interferon regulatory factors, and some members of the fork-head family of transcription factors that have been implicated as interferon regulators. We evaluated the simple normalized gene expression intensity values for members of these gene families, in addition to their differentially expressed values, since low-level expression was important to document and would otherwise have been filtered out. IFNB1(interferon, β-1) was identified as the predominant type I interferon produced in BEAS-2B cells following infection with either virus. Messenger RNAs for other type I interferons (IFNA1, IFNE) and the type 2 interferon IFNγ were not expressed above background (Fig. 6 and data not shown). Among the family of interferon regulatory factors, IRF7 and IRF9 were strongly up-regulated by both viruses. Both IRF7 and IRF9 mRNAs were induced 24-fold by influenza virus and 8-fold by rhinovirus, with a peak at 24–48 h post-infection. IRF1 mRNA was up-regulated after rhinovirus infection (about 8 fold) but only about 2-fold by influenza virus. Messenger RNAs for IRF3 and other IRFs were not expressed above background levels. We also profiled FoxO (fork-head box, subgroup O) family transcription factors since FoxO3 has been implicated in the regulation of IRF7 in murine macrophages (Litvak et al, 2012). Messenger RNAs for FoxO1, FoxO3, and FoxO4 were induced by rhinovirus (but not influenza virus) above background with FoxO1the strongest, showing a ~2.6 fold induction at 48–60 h post-infection while FoxO3 and FoxO4 were induced about 1.3 fold. In BEAS-2B respiratory epithelial cells, the fork-head factors FoxO1, FoxO3, and FoxO4 were expressed late, at 60–72 h post-infection, consistent with a possible role in a coherent feed forward regulatory loop linking IFN-β with either IRF7 or IRF9. Testing whether any one or all three of these factors have an actual role in the regulation of IRF7 or IRF9 in BEAS-2B epithelial cells is the subject of a future study. In HEK293 and HeLa cells expressing IFN-β, FoxO1 was described as a negative regulator of IRF3 (Lei et al., 2013), while in murine macrophages FoxO3 was implicated as a negative regulator of IRF7 (Litvak et al., 2012). The beneficial results of the interferon response such as inhibiting viral replication, removal of infected cells, and induction of the adaptive arm of the immune system must be balanced by a mechanism to control interferon levels to prevent an excessive response which could lead to tissue damage.

Host genes up-regulated by rhinovirus

Fig. 2 shows that over 300 genes were specifically up-regulated by rhinovirus. At 36 h post-infection, and later, several diverse rhinovirus-specific genes were up-regulated including genes whose products are part of the MAP kinase pathway (DUSP1, MAP4K4, MR4A1 (60 h); the extracellular matrix-cellular receptor interactions (COL4A6, LAMC2, COL4A1, LAMA3, ITGA5, ITGB8); the Wnt-frizzled signaling pathway (FZD4, TCF7L1, MYC); the extended transforming growth factor signaling network (EREG, BMP2, INHBA); cytokines and growth factors (FGF2, IL1A, IL1B, CCL20, LIF, CD82); cell adhesion molecules (L1CAM, CD276, CLDN1 (60 h). COL4A2 (common pathway) and COL4A1 and COL4A6 (rhinovirus-specific) are all principal components of the extracellular basement membrane that separates epithelia from underlying tissues. LAMC2 and LAMA3 are key components of laminin 5, a complex glycoprotein that forms filaments that connect epithelial cells to the basement membrane (Mizushima et al., 1998; Spirito et al., 2001). LIF and Wnt signaling has been described as synergistic at times, making independent but similar contributions to cell fate, which signal a decision for self-renewal or to remain undifferentiated (Ombrato et al., 2012). Our data suggests that LIF was produced by the infected BEAS-2B cells, consistent with either autocrine or paracrine signaling. The role of FZD4 in this system is less clear since no wnt ligands were differentially expressed at any time. Wnt ligands could of course arise from paracrine sources. Wnt-frizzled signaling often occurs in conjunction with the β-catenin (CTNNB1) pathway and β-catenin can bind to and negatively regulate TCF7L1 which may have effects on cell cycle regulation and cell senescence or may inhibit differentiation of stem cells (Davidson, 2014). TCF7L1 may also act as a repressor in the absence of β-catenin. Wnt signaling typically also regulates cytoskeletal gene products, cell polarity, and proliferation that might represent a restorative wound healing signature triggered later by rhinovirus. Genes with products that contribute to glycosphingolipid biosynthesis (B4GALT1, B3GNT5, FUT4), and arginine and proline metabolism (MAOA, SAT1, GLUL, CKMT1B) were also expressed late in RV-infected cells.

By 48 h post-infection (Supplementary File 14) a group of genes was identified that was up-regulated by rhinovirus but was down regulated by influenza virus, demonstrating a clear difference between the two viruses. Genes in this group included CLDN1, FGF2, IL1A, IL1B, CCL20, and ID1. Caludin 1 (CLDN1) is a component of tight junctions that are required for maintaining or restoring epithelial integrity. CCL20 is strongly chemoattractant for lymphocytes but only weakly for neutrophils. An early neutrophil response is triggered by influenza virus but the intensity of this response is paramount since complete depletion of neutrophils is also lethal. Essentially a balanced neutrophil response is needed for protection from influenza virus, since an over-exuberant response was recently implicated as being key to lethal influenza infection (Brandes et al., 2013). The fact that rhinovirus uniquely triggers CCL20 might be an indicator that it is able to evade massive neutrophil-mediated inflammation while harnessing its protective effects. Concurrently, CCL20 is able to recruit lymphocytes and dendritic cells to the site of the infection. Dendritic cells undergo maturation and migrate to the lymph nodes where an effective adaptive response to rhinovirus may develop.

Host genes up-regulated by influenza virus

At late times post-infection, over 200 genes were specifically up-regulated during influenza virus infection (Fig. 2 and Supplementary Files 14–16). By 48 to 60 h post-infection, genes that contribute to the biosynthesis of steroids or other lipids (SCD, FADS2, ACSS2, C14orf1, MVK, FDFT1, DHCR7, DHCR24), genes that contribute to oxidation and reduction chemistry (PCSK9, IFI30, AKR1C3, ALDOC), and genes that are part of adenosine metabolism (ADA) are up-regulated. Genes up-regulated by influenza, but down-regulated by rhinovirus were also identified. These genes included PPARGC1A (that encodes a transcriptional coactivator of PPAR-γ that regulates genes with roles in energy metabolism), PLAT, and ABCA1.

Host genes up-regulated by rhinovirus and influenza virus co-infection

Our study revealed that 132 genes were up-regulated and 310 genes were down- regulated after co-infection with both viruses, based on our criteria of 1%FDR (FDR < 0.01) and fold-change of log 2 ± 1.5 (Fig. 1D). The up-regulated genes returned GO terms for regulation of cell proliferation, protein amino acid phosphorylation, cell motility, phosphate metabolic processes, JAK-STAT phosphorylation and signaling, cytokine signaling, and lymphocyte proliferation ($p < 0.005$). The 310 down-regulated genes returned GO terms for other broad cellular processes such as transcription, histone acetylation, RNA metabolism, and the cell cycle ($p < 0.007$). The complete GO term lists along with representative genes are presented in Supplementary File 17. Many of the 132 and 310 genes were also expressed in the single-virus infections (data not shown), but at levels that did not meet the criteria for differential expression, suggesting that many of the biological processes also pertained to the single virus infections.

A milder clinical course after rhinovirus infection?

Close comparison of the timing as well as the differentially regulated sets of genes that comprise the host response to infection by different viruses may point to differences in viral pathogenesis that could help explain why clinical rhinovirus infections are typically milder than influenza infections. Genes associated with cell cycle control, apoptosis regulation, cell migration and tissue repair all emerge from this view of the data. We speculate that the net effect of these processes may contribute to an accelerated resolution of simple rhinovirus infections compared to influenza infections.

For example, the p53 pathway was detected after rhinovirus infection but not after influenza infection as shown in Fig. 3 simply because rhinovirus-infected cells rather than influenza cells showed more pathway genes differentially expressed starting early and continuing throughout the study. Consistent with the influenza literature, apoptosis signaling via the p53 pathway does become active (Turpin et al., 2005; Terrier et al., 2012; Nailwal et al., 2015), but more slowly than infection by rhinovirus at least in the case of the BEAS-2B cells we studied as shown in Supplementary File 18. This pathway is commonly active after viruses infect mammalian cells, probably triggered by a stress response, leading to cell cycle arrest at the G_1 or G_2 check points as well as a commitment to apoptosis. Early cell cycle arrest would inhibit early viral replication which may be reflected in the different time course of rhinovirus and influenza replication we observed. Rhinovirus replication was slower than influenza infection. Rhinovirus replication increased slowly, peaking at 72 h post-infection, while influenza replication peaked early and declined by 72 h (Fig. 1A). In rhinovirus infected cells, p53 activation may also have initiated a non-inflammatory form of apoptosis 12 h before influenza infected cells, which would aid in clearance of the infected cell, without causing overt inflammation. Supplementary File 18 visualizes the broad p53 pathway and directly compares mRNA levels for key regulators from each virus as a function of time. Increased transcription of PMAIP1 (or NOXA) was detected 8–12 h after infection with rhinovirus but not by influenza virus. PMAIP1 (NOXA) encodes a pro-apoptotic Bcl-2 homolog (Oda et al, 2000) which may commit the cell to the intrinsic apoptosis pathway very early post-infection. Other differentially expressed genes include those leading to p53 phosphorylation (CHK2), those that contribute to the outcomes of cell cycle arrest at G_1 (14-3-3-a; cyclinD), and others that like NOXA, may reinforce commitment to the intrinsic apoptosis pathway: PUMA, SCOTIN, and PERP. A commitment to apoptosis is also shown by differential expression of the FAS receptor (FASR) and TNFRSF10A (Fig. 5; Supplementary File 18). FASR equips the infected cell to respond to Fas ligand that could be presented by an activated T cell, while TNFRSF10A, a member of the TNF receptor family can transduce a cell death signal after activation by TNFSF10/TRAIL, representing yet another path to apoptosis (Walczak et al., 1997; Pan et al., 1997). This suggests that rhinovirus-infected BEAS-2B cells are also primed for extrinsic initiation of programmed cell death since FAS-FASL was differentially expressed by 48 h even while RV infection was continuing to rise (Fig. 1A and Supplementary File 14). We speculate that if apoptosis occurs before the release of virus particles, it could help limit the spread of the infection. In contrast, influenza infection likely triggers pyroptotic form of cell death that is more inflammatory and less contained than simple apoptosis (Bergsbaken et al., 2009). Pyroptotic cell death is mediated by activation of caspase 1 that was detected by 12 h post-infection (Supplementary File 11) resulting in production of IL-1a and IL-1b, that while protective may also result in tissue damage if unrestrained. Caspase 1 activation is largely mediated through the NOD-like receptor signaling pathway that turns on after IV infection rather than RV infection (Fig. 3). Supplementary File 18 shows that influenza infection also activates genes in the intrinsic apoptosis pathway, but with qualitative and temporal differences compared with rhinovirus. Influenza virus may actively interfere with apoptosis. Munoz-Fontela et al. (2011) infected p53-deficient mice with influenza virus and noted delayed cytokine and anti-viral gene responses in lung and bone marrow as well as decreased dendritic cell activation and virus-specific CD8 T cell immunity. But proteins induced by influenza virus or respiratory syncytial virus stabilize p53 protein either by interfering with MDM2-mediated ubiquitination (Wang et al., 2012) or by inducing the G_1 check point (Bian et al., 2012).

The suggestion that these two pathogens regulate apoptosis differently is readily testable and could point to new therapeutic targets.

A second distinction of rhinovirus infected cells is suggested by the up-regulation of the integrins ITGA5 and ITGB8 (Fig. 5). ITGA5 is a receptor for fibronectin and fibrinogen, and it has also been linked to the promotion of cell migration (Qin et al., 2011; Turner et al., 2008). ITGB8 has a role in human airway epithelial proliferation while in the brain it has been reported to alter the activation of Rho GTPases to promote glioblastoma cell invasiveness (Reyes et al., 2013). These integrins may enhance cell–cell and cell-extracellular matrix interactions and play a role in epithelial proliferation (Kenny and Connelly, 2014) and contribute to efficient presentation of viral antigens to macrophages or dendritic cells which would then carry processed antigens to the lymph nodes directing the adaptive immune response. Cell–cell interactions are essential for rhinovirus infected cells to present FasR at the cell membrane to Fas-L expressing cells, leading to the removal of the FasR-expressing infected cells. A pro-migratory role has also been proposed for MAP4K4 signaling (Fig. 5) which may also contribute to the TNFα signaling cascade (Collins et al., 2006).

Rhinovirus-infected cells also launch tissue repair processes, especially directed towards the basement membrane. This is shown by the differential expression of the non-fibrillar collagens COL4A6, COL4A2, and COL4A1 that are the principal collagens of the basement membrane (Fig. 5). We speculate that during infection with rhinovirus, some epithelial cells may repair or prevent damage to the basement membrane, even if others are removed after apoptosis. The laminin subunit gamma 2 is encoded by LAMC2 gene while the laminin alpha 3 chain is encoded by LAMA3 both of which were up-regulated in rhinovirus infected cells (Fig. 5). Together they form laminin 5, which is an integral part of the anchoring filaments that connect epithelial cells to the underlying basement membrane and their up-regulation is consistent with tissue repair or remodeling and cell migration (Mizushima et al., 1998). Id1 expression was detected early, 2 and 4 h after rhinovirus infection (Supplementary Files 8 and 9). This basic helix-loop-helix transcription factor appears to have a role in promoting cell migration and proliferation (Nishiyama et al., 2005; Li et al., 2004) which is consistent with a protective response triggered by rhinovirus but not influenza virus. Very early expression of Id1 may

prime the rhinovirus-infected cell for a potential survival response early, before a potentially host-damaging innate response can start. This might take the form of proliferation or migration to secondary lymphoid organs where adaptive immunity could be triggered. Id1 expression becomes even stronger at late times for example 60 h (Supplementary File 15) which may complement the other late wound healing responses observed with rhinovirus.

The soluble factors produced in common after viral infection were typically chemotactic, but rhinovirus-infected cells produced a broader range of factors that illustrated the breadth of tissue repair and remodeling that can be orchestrated by an epithelial cell. CCL5, CXCL10, and CXCL11 mRNAs were differentially expressed by both viruses and these are all chemotactic for T cells, with some activity to attract eosinophils, basophils, monocytes, macrophages, and NK cells. IL-15 and IL15RA were both differentially expressed in common as well (Fig. 5). IL-15RA is the receptor that binds and presents IL-15 via cell-to-cell contact or juxtacrine signaling to target cells (Jakobisiak et al., 2011; Olsen et al., 2007) and it leads to proliferation and activation of natural killer cells. IL8 was also produced after infection by both viruses, and it is chemotactic for neutrophils, monocytes, and it can induce phagocytosis activity. Substantially more diverse soluble factors were documented after rhinovirus infection that could contribute to tissue repair or remodeling. Rhinovirus infection up-regulated IL-24 which can activate STAT1 and STAT3 in target cells and contribute to wound healing (Wang and Liang, 2005). FGF2 (basic fibroblast growth factor) is a normal component of basement membranes and thus may contribute to coordinating its repair (Ornitz and Itoh, 2001). The broadly proinflammatory cytokines IL1A and IL1B were up-regulated by rhinovirus, but not by influenza virus infection (Bankers-Fulbright et al., 1996). Caspase 1 (CASP1) which activates the pro-protein of IL1B, was up-regulated after rhinovirus infection or by rhinovirus plus influenza virus. Rhinovirus infected cells also over-expressed CCL20, which is chemotactic for lymphocytes and neutrophils (Hieshima et al., 1997). The roles for several other factors produced after rhinovirus infection are unclear (LIF, BMP2, EREG, INHBA), but may contribute to repair or remodeling.

Limitations of the present analysis

The gene expression networks that are perturbed after an epithelial cell line is infected by rhinovirus or influenza virus clearly represents an oversimplification of the complex biology that takes place in vivo. First, we only investigated single types of rhinovirus and influenza virus, H1N1. At a minimum, our results and conclusions only pertain to these viral serotypes. Peripheral blood monocytes, macrophages, dendritic cells, and lymphocytes all respond to infecting viruses directly, or to the cytokines and chemokines produced by other infected cells, and those interactions were not part of this study. Genes that were down-regulated during the time-course were identified in Fig. 1 and listed in Supplementary File 3, but they were not fully integrated into Fig. 5. Other genes that were defined as discrete subsets (Supplementary Files 6 and 7) but were only expressed for 12–24 h were not integrated into the pathway time course shown in Fig. 5. These gene subsets included some that were specific for influenza (patterns 5, 7, 10, 11, 16, 19) and one that was specific for rhinovirus (pattern 18). These short-time constant genes were listed in Supplementary File 7. Integration of the down-regulated and short time-constant genes will be the subject of a future report. While we focused our study on influenza and rhinovirus, additional viruses that infect respiratory epithelial cells are known including respiratory syncytial virus, adenovirus, picornavirus, or coronavirus (Duerkop and Hooper, 2013). Investigation of single or co-infection of respiratory epithelial cells with these viruses in addition to rhinovirus and influenza virus will provide us with a greater understanding of the complexity of host response to respiratory infections. Influenza virus replicates early and rapidly triggers inflammatory responses that would be likely to lead to substantial tissue damage that may be slow to repair, while rhinovirus infection results in a slower innate response, coupled with induction of wound healing processes that may help achieve a level of inflammation that is optimal to resolve infection while preventing wide-spread tissue destruction. These possibilities await biological validation in suitable gene knock-down and knock-out models in vitro and in vivo.

Materials and methods

Cell culture and viral infection

Human bronchial epithelial cells (BEAS-2B; ATCC® CRL-9609™) were grown in Lonza (Cat# CC-3170) bronchial epithelial cell growth medium in a 37 °C incubator with 5% CO_2 and 90% humidity. The day prior to RV16 infection, one 75 cm^2 flask of BEAS-2B cells was treated with 2 ml of 0.25 mg/ml Trypsin /EDTA (Lonza, cat# CC-5012) at 37 °C until the cells completely detached from the flask bottom. Then 6 ml of TNS (Trypsin Neutralizing Solution, Lonza cat# CC-5002) were added to inactive the trypsin. The BEAS-2B cells were transferred to a 15 ml tube and centrifuged for 5 min at 800 rpm; the supernatant medium was removed by aspiration, and cells were re-suspended with 10 ml of the growth medium. The BEAS-2B cells were counted using a Beckman Coulter Counter, and diluted with growth medium to a density of 2.8×10^5cells/ml. BEAS-2B cells were seeded into 12-well plates at a density of 2.8×10^5 cells/well and placed overnight in the 37 °C incubator with 5% CO_2 and 90% humidity. On the day of infection, the rhinovirus strain RV16 (RV) and the influenza virus (IV) (H1N1, strain A/WS/33, ATCC IV-1520) stocks were removed from a −80 °C freezer, and diluted in serum-free medium. RV16 was chosen for this work since it is approved by the FDA for human induced cold studies and because it was used in a previous induced cold transcriptomic study (Proud et al., 2008). The influenza A virus strain H1N1 was chosen because it is a common cause of human flu and because the BEAS-2B cell line was shown to be permissive for infection by both RV16 as well as influenza A virus strain H1N1 in vitro (Ueki et al., 2013). The results and conclusions we present are thus limited to RV16 and influenza virus A, H1N1.

After removing the cell culture medium from the BEAS-2B cells, 300 µl of the diluted virus (either RV, IV or RV+IV) was added to each well. A similar volume of medium alone was added to the control wells. A multiplicity of infection of 2 was used for each virus. All plates were incubated at 33 °C with 5% CO_2 and 90% humidity. After a two hour incubation, 1.2 mls of the cell culture medium were added to each well while 3 h later, the same amount of medium was added to all other samples. All infected and uninfected (control) samples were collected at the scheduled time points (2, 4, 8, 12, 24, 36, 48, 60 and 78 h post-infection) and processed according to a randomized schedule to minimize the possibility of experimentally induced batch effects. Selected cytokines were assayed in culture media at the indicated times with the Milliplex Human Cytokine Magnetic Bead Panel Kits (Millipore, Billerica MA USA).

RNA isolation and microarray data reduction

Total RNA (including small RNAs) was isolated from BEAS-2B cells with QIAgen (Helden, Germany) miRNeasy Mini columns using the manufacturer's protocol. In brief, monolayer BEAS-2B cells were rinsed once with ice-cold phosphate buffered saline (pH 7.4) followed by extraction in QIAzol lysis reagent and brief vortexing. Lysates were frozen at −80 °C until RNA isolation. For RNA isolation, lysates were thawed on ice and then extracted once in chloroform. Ethanol was added to the aqueous phase of the extract and this mixture was added

to RNeasy spin columns. Columns were washed to remove contaminants and purified total RNA was eluted with nuclease-free water. RNA quantity was determined using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA quality and integrity was confirmed using the Agilent 2100 BioAnalyzer (Santa Clara, CA). Purified RNA was stored at −80 °C until use in the Genechip experiments. For the Genechip experiments, 250 ng of total RNA was converted to Genechip targets using the Affymetrix HT 3′ IVT Express (Santa Clara, CA) protocol optimized for use on the Beckman Coulter Biomek FXp automation workstation (Indianapolis, IN). Labeled target was hybridized overnight to Affymetrix U219-96 plates followed by washing, staining, and scanning on the Affymetrix GeneTitan instrument following the manufacturer's protocol. To verify the microarray results, selected mRNAs were quantitated by RT-PCR. RT-PCR was performed with 1 μg of total RNA and the iScript cDNA synthesis kit (Bio-Rad). A 1:20 dilution of cDNA was used in the RT-PCR reaction. Quantitative RT-PCR was carried out in a 10 μl reaction volume with gene-specific primers and β-actin using RT2 SYBR Green ROX qPCR Mastermix (Qiagen). The qPCR conditions were 50 °C for 2 min, and 45 cycles of 95 °C for 15 s, 59 °C for 45 s, 72 °C for 30 s on the ABI HT 7600 PCR instrument. All samples were assayed in quadruplicate. The differences in expression of specific gene products were evaluated using a relative quantification method where the expression of a specific cellular gene was normalized to the reference gene, β-actin. For viral genes, the expression was compared to its time-matched control sample. The primers used for verification of selected mRNAs from the microarray study and the viral RNAs are listed in Supplementary File 1.

Bioinformatics analysis: The overall plan for the microarray data analysis, calculation of differentially expressed genes and construction of networks is outlined in Supplementary File 2, and more details have been published (Cho et al., 2011; Brown et al., 2014). The nine point time-course combined with four experimental series (rhinovirus-infected; influenza-infected; infection with both viruses; mock-infected) with five biological replicates per time point generated 180 ⁎.cel files for analysis. Pearson correlations were calculated which revealed large changes in the overall pattern of gene expression by 24 h, noticeable differences between rhinovirus (RV) and influenza virus (IV) gene expression starting by 12 h, and no outliers (data not shown). Differentially expressed genes were calculated between the control and the virus-infected samples and false-discovery rates were also calculated by our published methods. A false discovery rate of <0.01 and differential expression of $\log 2 \pm 1.5$ were adopted for this analysis. Differentially expressed genes were derived by comparing infected cell profiles to mock infected profiles. We displayed the overall time-course of expression by using hierarchical cluster analysis (not shown). Instead of using heat maps for defining subsets of differentially expressed genes, we used a more objective and comprehensive approach and derived subsets of differentially expressed genes by a non-negative matrix factorization-based clustering method (Kim et al., 2011; see also Hofree et al., 2013). This clustering method not only allows us to automatically capture dynamic gene expression patterns over multiple conditions, but it also generates the protein sub-networks that correspond to the captured dynamic patterns. In addition, since statistical values such as p-values were computed and applied as a single statistical test, we avoided applying multiple statistical tests which can influence the false positive rate.

Functional enrichment analysis

To identify cellular processes for genes of interest, enrichment analysis of gene ontology biological processes and KEGG pathways was performed by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang et al., 2009). The biological processes significantly ($p<0.01$) enriched by the differentially expressed genes identified at each time point were summarized (see *Results and discussion*). The KEGG pathways that were enriched by genes having specific temporal patterns of expression were also derived (see *Results and discussion*). For biological processes linked to multiple redundant terms, only the most representative terms assigned to the largest numbers of genes were chosen. Predicted interactions between transcription factors and their potential target genes were identified from GeneGO Metacore ver 6.7. In Metacore, the options 'direct interaction' and 'transcriptional regulation' were selected to retrieve transcription factors and their targets.

Gene expression omnibus accession

The data from this study was placed in the GEO database under accession GSE71766.

Acknowledgments

We acknowledge support from the Procter & Gamble – Institute for Systems Biology Research Collaboration. We thank Naeha Subramanian, Peter Askovich, Krupa Navalkar and Kathie Walters for critical reading of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2015.08.014.

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