Distinct gene subsets are induced at different time points after human respiratory syncytial virus infection of A549 cells

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cDNA microarray technology was applied to time course analysis of differentially expressed genes in A549 cells following human respiratory syncytial virus (HRSV) infection. Both up- and down-regulation of cellular genes were observed in a time-dependent manner. However, gene up-regulation prevailed over gene down-regulation. Virus infectivity was required as UV-inactivated virus failed to up-regulate/down-regulate those genes. At early times post-infection (0–6 h p.i.) 85 genes were up-regulated. Some of those genes were involved in cell growth/proliferation, cellular protein metabolism and cytoskeleton organization. Among the most strongly up-regulated genes at that time were the urokinase plasminogen activator (PLAU) and its receptor (PLAUR), a pleiotropic system involved in many biological processes, including chemotaxis and inflammation. Functionally related genes encoding the α- and β-chains of several integrins were also up-regulated within the first 12 h of infection. Genes up-regulated between 6 and 12 h p.i. included interferon-stimulated genes (ISGs), genes related to oxidative stress and genes of the non-canonical NF-κB pathway. At later times, genes involved in the immune response became predominant among the up-regulated genes, most of them being ISGs. Different up-regulation kinetics of cytokine and cytokine-signalling-related genes were also observed. These results highlight the dynamic interplay between the virus and the host cell and provide a general picture of changes in cellular gene expression along the HRSV replicative cycle.

INTRODUCTION

Human respiratory syncytial virus (HRSV), the prototype of the genus Pneumovirus, is an important pathogen that infects people of all ages but, whereas usually mild infections are reported in healthy adults, serious illnesses, such as bronchiolitis and pneumonia, are frequent in infants and in the elderly. Adults with heart or pulmonary disease and the immunocompromised are also at risk of suffering life-threatening infections (Falsy et al., 2005; Hall, 2001; Whimbey & Ghosh, 2000). Despite intense investigation for more than 40 years, there is currently no approved vaccine or effective therapeutic treatment for HRSV. Synagis (palivizumab), a humanized antibody specific for the HRSV fusion glycoprotein, is licensed only for immunoprophylactic treatment of high risk infants.

There is increasing evidence that much of the pathology caused by HRSV is due to the immune response to the infection. Secretion of proinflammatory cytokines and chemokines by the infected epithelial cells and immune cells (Krishnan et al., 2004), a bias toward the Th2 cellular immune response (Graham, 1995; Openshaw, 1995) and deposition of antigen–antibody immune complexes triggering complement activation (Polack et al., 2002) are cited among the possible causes behind HRSV-induced immunopathology. Therefore, in searching for better treatment and prevention of severe symptoms following HRSV infection, both inhibition of virus replication and regulation of the immune response should be considered. This implies that a profound knowledge of virus–host interactions is needed. As mentioned before, several studies have focused on different aspects of the host response against HRSV, including cytokine and chemokine release by infected cells, cellular and humoral immune responses, etc. However, other aspects of the virus–host cell interaction, such as modulation of membrane receptors or changes in intracellular molecular pathways after virus infection have not been sufficiently addressed and are poorly understood.

Tables of induced genes and functional categories of induced genes are available as supplementary material in JGV Online.
Although usually difficult to integrate and comprehend, results from microarray expression experiments can provide an overview of the events happening inside the cell following virus infection. We have used this technology to monitor expression levels of 9300 human genes at different time points after HRSV infection of the type II alveolar A549 human epithelial cells.

METHODS

Virus and cells. The human epithelial cell lines A549 and HEp-2 were grown at 37 °C in 5 % CO₂ in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum (DMEM10). The Long and A2 strains of HRSV were propagated in HEp-2 cells in DMEM with 2 % fetal calf serum (DMEM2). Viruses were purified from clarified culture supernatants by polyethylene glycol precipitation and centrifugation in a 30–45–60 % discontinuous sucrose gradient in TNE buffer (Mbiguino & Menezes, 1991). Virus titres were determined by plaque assay in HEp-2 cells layered with 0.5 % low-melting-point agarose (Conda). After 5 days, cells were fixed with 4 % formaldehyde in PBS followed by methanol, incubated with a mixture of monoclonal antibodies against the two major glycoproteins of the virus (2F, 47F, 56F, 0211G, 0211G2) (Garcia-Barreno et al., 1989; Martinez et al., 1997) and plaques were visualized using an anti-mouse IgG horseradish peroxidase-linked whole antibody (Amersham Biosciences) and 3-amino-9-ethylcarbazole (AEC) (Sigma). Virus inactivation was achieved by irradiation with UV light for 90 min and confirmed by plaque assay.

cDNA microarray analysis. A549 cells were mock-infected or infected with purified virus at an m.o.i. of 3 plaque-forming units (p.f.u.) per cell in DMEM2. After 90 min of adsorption, fresh medium was added and cells were incubated at 37 °C until RNA extraction. Immediately after the adsorption period, and at 6, 12 and 24 h post-adsorption, total RNA was extracted with TRIzol reagent (Invitrogen) and further purified with the RNeasy kit (Qiagen). A total of 40 μg of each RNA was reverse-transcribed using Oligo-(dT)15 (Promega) and labelled with Cy3-dUTP (mock samples) or Cy5-dUTP (infected samples) (Amersham Biosciences). The labelled probes were purified by using the CyScribe GFX purification kit (Amersham Biosciences) and hybridized at 55 °C for 15–18 h in SlideHyb #1 buffer (Ambion) to the 12K human CNIO cDNA microarray (Spanish National Cancer Centre, Madrid, Spain). This microarray contains about 11 500 cDNA clones in duplicate spots representing 9300 human unique genes or loci (content available at http://www.cnio.es/UserFiles/File/Biotechnologia/Genomica/ochip_v2.xls). Arrays were scanned with the G2565BA Microarray Scanner System (Agilent Technologies) (Martinez-Delgado et al., 2004; Tracey et al., 2002, 2003, 2004). The resultant images were then quantified using GenePix Pro 4.0 software (Axon Instruments). To discard label-biased results, additional dye-swap experiments were performed in which mock samples were labelled with Cy5-dUTP and infected samples with Cy3-dUTP.

Microarray data analysis. Raw data were normalized using the DNMAD (http://dnmad.bioinfo.cnio.es), an algorithm implemented from others described by Smyth & Speed (2003) and Yang et al. (2002). In a second step, the pre-processor tool included in the GEPAS package (http://gepas.bioinfo.cipf.es) was used to remove inconsistent data between replica-printed probes in the array and, in a second step, to average them (Herrero et al., 2003a, b).

Multiple testing, to identify differentially expressed genes, was run on this processed data by the use of the Significance Analysis of Microarray (SAM 2.0) routine (Tusher et al., 2001). One-class time course response analysis was carried out with the slope summary method included in SAM and a false discovery rate (FDR), reported as the q-value, was calculated. The q-value determines the fraction of potential false positives present in the final list of significant differentially expressed genes (Storey, 2002).

To discriminate between early, medium and late up-regulated genes, those previously selected with SAM were compared with each profile clustered by the Short Time-series Expression Miner (STEM) (Ernst et al., 2005). Three pre-processed datasets, corresponding to three independent experiments, were imported into STEM. Each data time series were median-averaged and then normalized so that each profile began with a gene expression ratio of 0. Experimental profiles with a minimal correlation of 0.7 with predetermined model profiles were then clustered together.

Classification of genes into consensus functional categories (biological processes) was performed with the Fatigoo tool (Al-Shahrour et al., 2004).

Quantitative RT-PCR. Results obtained by the microarray analysis were validated for some genes by TaqMan quantitative gene expression assays (Applied Biosystems) on the same sets of RNA samples. For each post-infection (p.i.) time point (0, 6, 12 and 24 h p.i.), equal amounts of total RNA from the same three independent experiments from which microarray data were obtained were mixed and 2.5 μg was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems). PCR primers and TaqMan MGB probes (FAM dye-labelled) for the following genes were products from Assays-on-demand (Applied Biosystems): PLAUR (Hs00182181 m1), PLAU (Hs00170182 m1), ACTB (Hs99999903 m1), SERPINE2 (Hs00299953m1) and LDLR (Hs00181192m1). PCRs were performed in triplicate following the manufacturer’s protocols on an ABI PRISM 7000 sequence detection system. Results were calculated and normalized by β-actin mRNA and uninfected cells at each time point using the comparative threshold method (Applied Biosystems User Bulletin no. 2).

RESULTS

Gene up-regulation predominates over gene down-regulation in A549 cells infected with HRSV

Epithelial A549 cells were infected with the Long strain of HRSV at an m.o.i. of 3 p.f.u. per cell. After virus adsorption, total RNA was extracted at 0, 6, 12 and 24 h p.i., mRNA was reverse-transcribed to Cy5-dUTP-cDNA, mixed with control Cy3-dUTP-cDNA from mock-infected cells and competitively hybridized to the 12K human CNIO microarray. Three independent experiments were performed and differentially expressed genes were identified using SAM 2.0 software (Tusher et al., 2001). Mean values of the three experiments are represented in Fig. 1(a, b). Differences in mRNA levels may reflect changes in transcription, processing and/or stability. For a q-value ≤5 %, 382 differentially expressed genes were identified, from which 303 were up-regulated and 79 were down-regulated (Fig. 1a). When only the most significant changes were considered (q-value ≤1%), 102 genes were up-regulated and 6 were down-regulated (Fig. 1b). Moreover, the largest quantitative differences in individual gene expression were seen in the category of up-regulated genes. Thus, overall, gene up-regulation predominates over gene down-regulation during HRSV infection. These changes required virus infectivity
since they did not occur in cells exposed to UV-inactivated virus (Fig. 1b) and they were not label-dependent as demonstrated by a dye-swap experiment (Fig. 1c). However, they were confirmed by infection with a different strain of HRSV (A2), which differentially altered the expression of a nearly identical set of genes at 24 h p.i. (Table 1 and supplementary Tables S1).

Genes belonging to diverse functional categories were up-regulated, indicating a profound impact of HRSV infection on cellular metabolism (supplementary Tables S2). However, a cellular response characteristic of viral infections was observed, as 21.3% of the differentially expressed genes ($q$-value $\leq 1\%$) could be classified, according to the gene ontology (GO) term, within the ‘immune response’ category, including several interferon-stimulated genes (see below).

Only six genes were found to be down-regulated ($q$-value $\leq 1\%$). Among them, it is inhibin, beta B (activin AB beta polypeptide; INHBB), which may be relevant to HRSV immunopathology as the activin/inhibin system; both members of the transforming growth factor-$\beta$ superfamily have been implicated in inflammatory processes (Okuma et al., 2005).

We further investigated up-regulation profiles looking for early, medium and late up-regulated genes. The list of genes associated with a SAM $q$-value $\leq 5\%$ were compared with different clusters of time course profiles generated with the STEM application (Ernst et al., 2005). This algorithm associates experimental profiles, normalized to 0 h, to model profiles of gene up-/down-regulation.

Eighty-five genes, classified within five different profiles, were found to be up-regulated soon after infection (0–6 h p.i.) (Fig. 2, profiles 17, 21, 22, 24 and 25). For most of them, the amount of mRNA in infected cells continued to increase up to 12 (profile 24) or 24 h p.i. (the last time point tested) (profiles 22 and 25). For 13 genes, mRNA levels remained steady from 6 to 24 h p.i. (profile 21) and in two cases mRNA levels decreased continuously after 6 h (profile 17) (supplementary Tables S1).

Sixty-two genes were significantly up-regulated between 6 and 12 h p.i. For 52 of them, mRNA levels in infected cells further increased to reach the maximum level at 24 h (profile 16). For the remaining 10 genes, mRNA levels did not vary significantly after 12 h p.i. (profile 15).

Finally, 59 genes were induced after 12 h of infection (profile 13), most of them being antiviral-response-related genes (see below).

In total, 206 genes were classified in different up-regulation profiles. Nearly half of them (98 genes) were induced $\geq 2$-fold at 24 h p.i. (supplementary Tables S1).

The PLAU/PLAUR system and cytoskeleton organization and biogenesis genes are up-regulated early after HRSV infection

Early events taking place in host cells shortly after virus infection may be crucial for the subsequent development of the infection and the concomitant host response, before extensive cytopathology and secondary cellular responses occur. We found that a relatively large number of genes ($n = 85$, $q \leq 5\%$, change $\geq 1.1$-fold) were already up-regulated within the first 6 h p.i. (supplementary Tables S1). Changes in mRNA levels compared to uninfected cells ranged from 1.1-fold (arbitrary lower threshold) to 2.8-fold at 6 h p.i.,
although, as mentioned before, higher values were reached at later time points in most cases (Fig. 2, profiles 22, 24 and 25). Up-regulated genes were classified in many GO functional categories, including transcription, apoptosis, immune response, and small-GTPase-mediated signal transduction, among others (supplementary Tables S2).

The gene most strongly up-regulated soon after infection (2.8 times by 6 h p.i.) was the urokinase plasminogen activator (PLAU) (supplementary Tables S1). The PLAU receptor (PLAUR) gene was also up-regulated at that time (2.2-fold change). Although both PLAU and PLAUR showed similar up-regulation kinetics from 0 to 6 h p.i., PLAUR mRNA levels continued to increase in infected cells to reach a mean of 7.8-fold over uninfected cell levels at 24 h p.i., while PLAU mRNA levels remained approximately the same after 6 h p.i. (Fig. 3a). As a control, β-actin (ACTB) mRNA levels did not change after HRSV infection during the time period tested (Fig. 3a).

It is worth mentioning that the PLAU/PLAUR system also includes inhibitors and interacting receptors, such as members of the serine proteinase inhibitor (Serpin) and LDL-receptor families. A modest but significant up-regulation of SERPINE2, LDLR and VLDLR genes was detected in HRSV-infected cells (Fig. 3b), indicating that the whole system is activated after virus infection. To confirm this and to validate microarray results, we selected four genes of the PLAU/PLAUR system for quantitative RT-PCR. Relative quantification to ACTB rendered results that closely mirrored those from the microarray experiments (Fig. 3c). It is worth stressing that this experiment confirmed not only the up-regulation of those genes but, most importantly, the

Table 1. Immune response-related genes up-regulated between 12 and 24 h p.i.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>GenBank accession no.</th>
<th>q-value (%)</th>
<th>Change (-fold) at 24 h p.i.*</th>
</tr>
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<tbody>
<tr>
<td>Interferon-α-inducible protein (clone IFI-15K)</td>
<td>GIP2†</td>
<td>AA406020</td>
<td>0</td>
<td>23.5 (21.7)</td>
</tr>
<tr>
<td>Interferon-β-inducible protein (clone IFI-6-16)</td>
<td>GIP3†</td>
<td>AA432030</td>
<td>0</td>
<td>8.3 (9.7)</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 5</td>
<td>CCL5</td>
<td>AA873792</td>
<td>1</td>
<td>4.9 (6.1)</td>
</tr>
<tr>
<td>Interferon-γ-inducible protein 27</td>
<td>IFI27†</td>
<td>AA149289</td>
<td>0</td>
<td>4.6 (3.8)</td>
</tr>
<tr>
<td>Guanylate-binding protein 1, interferon-inducible, 67 kDa</td>
<td>GBP1†</td>
<td>BE391804</td>
<td>0</td>
<td>3.8 (3.5)</td>
</tr>
<tr>
<td>Apolipoprotein L 3</td>
<td>APOL3</td>
<td>AA971543</td>
<td>0</td>
<td>3.7 (3.8)</td>
</tr>
<tr>
<td>Serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary)</td>
<td>SERPING1</td>
<td>AA481438</td>
<td>0</td>
<td>3.4 (1.6)</td>
</tr>
<tr>
<td>Interferon-induced protein 35</td>
<td>IFI35†</td>
<td>BE276643</td>
<td>1</td>
<td>3.3 (4.2)</td>
</tr>
<tr>
<td>2’-5’-oligoadenylate synthetase 2, 69/71 kDa</td>
<td>OAS2†</td>
<td>R34568</td>
<td>0</td>
<td>3.3 (4.5)</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I B</td>
<td>HLA-B</td>
<td>AA225198</td>
<td>1</td>
<td>3.2 (4.7)</td>
</tr>
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<td>Proteosome (prosome, macropain) subunit, β type 9 (large multifunctional protease 2)</td>
<td>PSMB9†</td>
<td>AA862434</td>
<td>0</td>
<td>3.1 (3.5)</td>
</tr>
<tr>
<td>Interferon-induced transmembrane protein 1 (9–27)</td>
<td>IFITM1†</td>
<td>AA419251</td>
<td>0</td>
<td>3.1 (2.9)</td>
</tr>
<tr>
<td>Major histocompatibility complex, class I E</td>
<td>HLA-E</td>
<td>BE272142</td>
<td>3</td>
<td>2.7 (3.3)</td>
</tr>
<tr>
<td>N-my c (and STAT) interactor</td>
<td>NMI†</td>
<td>AA279601</td>
<td>0</td>
<td>2.6 (2.3)</td>
</tr>
<tr>
<td>Interferon-induced protein with tetraicopeptide repeats 3</td>
<td>IFIT3†</td>
<td>BE207545</td>
<td>0</td>
<td>2.5 (2.8)</td>
</tr>
<tr>
<td>Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)</td>
<td>CEACAM1</td>
<td>AA411757</td>
<td>3</td>
<td>2.4 (2.1)</td>
</tr>
<tr>
<td>Tripartite motif-containing 22</td>
<td>TRIM22</td>
<td>AA083407</td>
<td>1</td>
<td>2.4 (1.4)</td>
</tr>
<tr>
<td>Interferon-γ-inducible protein 16</td>
<td>IFI16†</td>
<td>AA491191</td>
<td>0</td>
<td>2.1 (0.9)</td>
</tr>
<tr>
<td>Myeloid differentiation primary response gene 88</td>
<td>MYD88†</td>
<td>H38383</td>
<td>1</td>
<td>1.8 (1.7)</td>
</tr>
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<td>Chemokine (C-X-C motif) ligand 10</td>
<td>CXCL10</td>
<td>AA402031</td>
<td>4</td>
<td>1.7 (2.0)</td>
</tr>
<tr>
<td>Adenosine A1 receptor</td>
<td>ADORA1</td>
<td>H21045</td>
<td>1</td>
<td>1.6 (1.5)</td>
</tr>
<tr>
<td>Tumour necrosis factor (ligand) superfamily, member 13b</td>
<td>TNFSF13B</td>
<td>AA166695</td>
<td>5</td>
<td>1.5 (1.8)</td>
</tr>
<tr>
<td>Myxovirus (influenza virus) resistance 2 (mouse)</td>
<td>MX2†</td>
<td>AA035024</td>
<td>3</td>
<td>1.5 (1.4)</td>
</tr>
<tr>
<td>Diacylglycerol kinase δ 130 kDa</td>
<td>DGKD</td>
<td>AA280691</td>
<td>4</td>
<td>1.5 (1.1)</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 11</td>
<td>CXCL11</td>
<td>BX112006</td>
<td>5</td>
<td>1.4 (2.0)</td>
</tr>
<tr>
<td>GTP-binding protein 1</td>
<td>GTPBP1</td>
<td>AA026413</td>
<td>5</td>
<td>1.3 (1.4)</td>
</tr>
</tbody>
</table>

*Results from the A2 strain are shown in parentheses and correspond to a single experiment at 24 h p.i.
†ISGs.
Fig. 2. Early, medium and late gene up-regulation profiles. Genes associated with a q-value \( \leq 5\% \) were further classified in different up-regulation profiles using STEM software. Values from each time point were standardized against \( t=0 \) h p.i. The ID number of each model profile and the amount of genes in each profile (\( n \)) are represented. \( R \) and \( G \) are defined in the legend to Fig. 1.

Fig. 3. Early up-regulation of the PLAU/PLAUR system and cytoskeleton-related genes. Up-regulation profiles of genes related to the PLAU/PLAUR system and to the organization and biogenesis of cytoskeleton are represented. Each point was calculated as the mean and SD of three independent infections and microarray experiments, except for (c), which represents triplicate quantitative RT-PCR values standardized against the mRNA of the respective gene from non-infected cells and the mRNA of \( \beta \)-actin (ACTB) at each time point. Genes are represented by abbreviations (see text and supplementary Tables S1). \( R \) and \( G \) are defined in the legend to Fig. 1. (a–c) ■, PLAU; ▲, PLAUR; ●, ACTB; ◊, SERPINE2; △, LDLR; ▽, VLDLR. (d, e) ■, ITGA2; ▲, ITGA6; △, ITGA3; ▽, ITGA5; ●, ITGB4. (f) ■, PLEK2; ●, ARHGEF2; ▲, FLNA; ▽, ARPC1A; ◊, CORO1C.
kinetics of up-regulation, even for genes with modest changes in RNA expression (Fig. 3c).

Although PLAUR lacks a cytosolic domain, it can transmit intracellular signals by association with the α-chain of integrins (Ossowski & Aguirre-Ghiso, 2000). Four different integrin α-chains (ITGA2, ITGA3, ITGA5 and ITGA6) and one β-chain (ITGB4) were up-regulated in the first 12 h p.i. (Fig. 3d, e), suggesting that both systems, PLAU/PLAUR and integrins, may interact during HRSV infection. ITGA2 and ITGA6 were up-regulated earlier (0–6 h p.i.) than ITGA3, ITGA5 and ITGB4 (6–12 h p.i.). However, mRNA levels from these latter three chains continued to increase up to a maximum at 24 h p.i. (Fig. 3e).

Integrins are involved in cytoskeletal organization and signal transduction (Giancotti & Ruoslahti, 1999). Interestingly, among the early up-regulated genes, five are involved in cytoskeleton organization and biogenesis (Fig. 3f), indicating that HRSV infection might influence the structure and/or dynamic of that intracellular compartment shortly after infection.

**Up-regulation of the non-canonical NF-κB activation pathway, oxidative-stress-related genes and ISGs became apparent after 6 h p.i.**

Sixty-two genes were significantly up-regulated ($q \leq 5\%$, change $\geq 1.1$-fold) between 6 and 12 h p.i. (supplementary Tables S1). Changes in mRNA levels from infected cells relative to non-infected cells ranged from 1.1-fold (arbitrary lower threshold) to 3.3-fold at 12 h p.i. Cellular protein metabolism, the immune response, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism, apoptosis and transcription were the functional categories containing the largest number of genes (supplementary Tables S2). The homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1) was the gene that showed the highest up-regulation (mean change at 12 h p.i. = 3.3-fold). Other highly up-regulated genes were those encoding the tumour necrosis factor receptor superfamily member 12A (TNFRSF12A), the methionine-tRNA synthetase (MARS), the α- and β-chains of some integrins (ITGA3, ITGA5, ITGB4) (Fig. 3e), two ISGs (the interferon-induced protein with tetracopeptid repeat 1 – IFIT1 – and the 2′-5′-oligoadenylate synthetase-like – OASL) and the metallothioneins 2A (MT2A) and 1X (MT1X) (supplementary Tables S1).

In addition to IFIT1 and OASL, four additional immune-response-related ISGs were up-regulated at this time point, the interferon-stimulated transcription factor 3, gamma 48 kDa (ISGF3G), interleukin 15 (IL15), the interferon-induced protein with tetracopeptide repeats 5 (IFIT5) and the myxovirus resistance 1 protein (MX1) (Fig. 4a).

Metallothioneins comprise a superfamily of low-molecular-mass proteins, rich in cysteine residues, with a high capacity to bind metallic ions. These proteins have been implicated in an increasing array of physiological processes from metal homeostasis and protection against oxidative damage to immunoregulation (Borghesi & Lynes, 1996). Fig. 4(b) shows the up-regulation of two isoforms of metallothioneins, MT2A and MT1X, starting 6 h p.i. and continuing until 24 h p.i.

It has been reported recently that HRSV infection rapidly activates the non-canonical NF-κB activation pathway characterized by the association and subsequent translocation to the nucleus of NF-κB2 and RelB (Choudhary et al., 2005). In that study, the mitogen-activated protein kinase kinase 14 (MAP3K14)/NF-κB-inducing kinase (NIK) also associated with NF-κB2 (p52) and was translocated to the nucleus. In agreement with these results, we found that NFKB2, RELB and MAP3K14 genes were up-regulated to

![Fig. 4. ISGs, metallothioneins and non-canonical NF-κB pathway genes up-regulated at medium times p.i. Each point was calculated as the mean and SD of three independent infections and microarray experiments. Genes are represented by abbreviations (see text and supplementary Tables S1). R and G are defined in the legend to Fig. 1. (a) □, IFIT1; ▲, OASL; ▼, ISGF3G; ○, IL15; ●, IFIT5; □, MX1. (b) ■, MT2A; ▲, MT1X. (c) ■, NFKB2; ▲, MAP3K14; ▼, RELB.](http://vir.sgmjournals.org)
the same level at 6 h p.i. with nearly identical kinetics (Fig. 4c).

**Different cytokines and cytokine receptors have different up-regulation kinetics**

Some of the genes up-regulated at 6 h p.i. included the chemokine CCL2 (MCP-1) and the interleukin 4 receptor alpha (IL4R). In addition to CCL2, the inflammatory-response-related genes of the chemokine CCL7 (MCP-3), the suppressor of cytokine signalling 3 (SOCS3) and the tumour necrosis factor alpha-induced protein 6 (TNFAIP6) were also up-regulated at this time point (Fig. 5 and supplementary Tables S1).

In contrast, the chemokines CCL5 (RANTES), CXCL10 (IP-10) and CXCL11 (I-TAC) were up-regulated at later times (Fig. 5a).

The interleukin-15 (IL15) and the alpha chain of its receptor (IL15RA) were also up-regulated after 6 or 12 h p.i. (Fig. 5b). It is interesting that IL15 and IL15RA were up-regulated with similar kinetics, in agreement with reports in which co-ordinate expression of both genes was needed for some biological functions mediated by IL15 (Dubois et al., 2002).

**Up-regulation of genes involved in antigen processing and presentation and genes related to the immune response, mostly ISGs, predominates at late times post-infection (12–24 h)**

Fifty-nine genes were up-regulated between 12 and 24 h p.i. \( (q \leq 5\%, \text{ change } \geq 1.1\text{-fold}) \) in a range from 1.1-fold (arbitrary lower threshold) to 23.5-fold (supplementary Tables S1). When all the genes up-regulated at any time after infection were classified by GO, three main functional categories were observed, according to the number of genes included: (i) cellular protein metabolism, (ii) regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism, and (iii) transcription (supplementary Tables S2). However, genes belonging to the ‘immune response’ category became increasingly more numerous as infection progressed \((11.29\% \text{ of all up-regulated genes between 6 and 12 h p.i.}, \text{ reaching } 44.1\% \ (n = 26) \text{ of all up-regulated genes in the 12–24 h period}. \) At this time, the ‘immune response’ category became predominant (Fig. 6). This indicates that a strong cellular response of immunity-related genes was induced at late post-infection times. Further analysis of those genes revealed that 50% \( (n = 13) \) of them were ISGs (de Veer et al., 2001) (Table 1).

Antigen processing and presentation to the immune system is a classical response of infected cells to viral infections. In this study, several genes related to this process were up-regulated late in infection (Fig. 7). Five were ubiquitination-related genes, two form part of the proteosome and two were major histocompatibility complex (MHC) class I genes.
DISCUSSION

The time course of differential gene expression in an epithelial cell line from the respiratory tract infected with HRSV was analysed using cDNA microarrays to gain insight into the complexity of virus–cell interactions and to add information that may help to counteract virus infection and its pathological manifestations. A time course approach was carried out as distinct subsets of genes might be differentially expressed as infection and host-cell responses progress. The design of the experiments and controls described in this study minimize the detection of ‘false-positive’ results that may be associated with microarray technology: (1) the experiments were done in triplicate and the results were analysed using microarray-specific software to discover false-positive results, (2) each gene was analysed at four different times p.i., (3) up-regulation was not observed with inactivated virus, (4) a dye-swap experiment was performed to discard dye-biased results, (5) results at 24 h p.i. were confirmed by infection with a different strain of HRSV (A2), and finally (6) the kinetics of up-regulation of four selected genes, two of which were up-regulated around or less than twofold, were closely reproduced by qRT-PCR.

The PLAU/PLAUR system

An intriguing finding of this report is the potent and rapid up-regulation of the PLAU/PLAUR genes. PLAU is a serine protease that catalyses the conversion of plasminogen to plasmin upon activation by binding to PLAUR. Plasmin, in turn, degrades fibrin and prevents its extracellular deposition. Besides fibrinolysis, the PLAU/PLAUR system is involved in multiple physiological processes, including inflammatory reactions and modulation of innate and adaptive immune responses (Mondino & Blasi, 2004). PLAU/PLAUR-induced proteolysis of the extracellular matrix can mobilize and activate signalling molecules involved in inflammatory reactions, but the system also mediates proteolysis-independent effects through a variety of mechanisms, including signalling by interaction with integrins and G-protein-coupled receptors (Blasi & Carmeliet, 2002; Kucharewicz et al., 2003). An interesting finding is that PLAUR can be cleaved to yield a soluble fragment (D2D3) that has chemotactic activity for monocytes and other cell types through activation of the G-protein-coupled receptor FPRL1 (Resnati et al., 2002). In addition, this fragment prevents cell migration in response to the chemokines CCL2/MCP-1 and CCL5/RANTES by inhibiting integrin-dependent cell adhesion (Furlan et al., 2004). This finding suggests that D2D3 may play an important role in cell migration and chemokine cross-regulation during inflammatory and pathological manifestation of many diseases (Mondino & Blasi, 2004). The diverse processes mediated by the PLAU/PLAUR system, including airway remodelling and inflammation, makes it a likely candidate to be involved in the lung pathology caused by HRSV infection, although further analyses are required to address this question.

Cytoskeleton organization and biogenesis

Viruses use different elements of the cytoskeleton for entry, replication, intracellular transport and budding (Sodeik, 2000). Several studies have implicated intermediate filaments, actin and microtubules, as well as proteins that regulate cytoskeleton functions, in the infectious cycle of HRSV (Burke et al., 1998, 2000; Garcia-Barreno et al., 1988; Gower et al., 2005; Huang et al., 2005; Kaliewaard et al., 2005; Ulloa et al., 1998). Five cytoskeleton-related genes were up-regulated soon after infection in this study, indicating that reorganization of this intracellular compartment may start to take place at this time point. ARHGGEF2 (GEF-H1), one of the two most highly up-regulated
cytoskeleton-related genes, has been reported to have a central role in cytoskeleton reorganization by linking cellular actin polymerization and contractility to changes in microtubule dynamics (Krendel et al., 2002).

As integrins are involved in cytoskeleton organization and signalling, it is possible that the changes in the PLAU/PLAUR system and in the cytoskeleton-related genes observed in this study are both related.

**Chemokines and cytokine signalling**

Some cytokine and cytokine-related genes were also up-regulated during the first 6 h p.i. The genes encoding chemokines CCL2 (MCP-1) and CCL7 (MCP-3) were among them. By contrast, CCL5 (RANTES), CXCL10 (IP-10) and CXCL11 (I-TAC) were up-regulated later. These results resemble those of Zhang et al. (2001), although CCL7 was not described in that study. CCL7 has been associated to histopathology induced by HRSV challenge of mice primed with formalin-inactivated virus, since it was rapidly and strongly up-regulated in primed compared to control mice (Power et al., 2001).

IL4 and IL13 cytokines produced by Th2 type cells are potent inducers of inflammatory molecules in epithelial cells and have been related to pathological manifestations of HRSV infection (Bukreyev et al., 2005; Connors et al., 1994; Johnson et al., 2003; Tang & Graham, 1994). Both IL4 and IL13 receptors share the IL4 receptor χ-chain (IL4Rχ). IL4Rχ polymorphism has been associated with severe HRSV bronchiolitis in infants (Hoebel et al., 2003), and HRSV G glycoprotein-induced eosinophilia in mice appears to require signalling through this receptor by IL4 or IL13 (Johnson et al., 2003). IL4Rχ was up-regulated shortly after HRSV infection in A549 cells (Fig. 5b), suggesting that up-regulation of this receptor may contribute to enhanced immunopathology mediated by IL4 and/or IL13 following HRSV infection.

An interesting observation was the co-ordinate up-regulation of the IL15 and IL15RA genes. Although IL15Rβ was originally thought to be a component of a heterotrimetric receptor containing the IL2/IL15Rβ and γ chains, more recent evidence showed that IL15Rβ functions through a novel mechanism called ‘transpresentation’. By this mechanism IL15 and IL15Rα are co-ordinately expressed by antigen-presenting cells and IL15 bound to IL15Rα is presented in trans to NK or CD8+T cells expressing only the IL15Rβγ signalling component (Dubois et al., 2002). In this way, IL15 promotes growth and survival of several cell types, including NK and CD8+T lymphocytes (Van Belle & Grooten, 2005). In agreement with this, HRSV has been shown to up-regulate NK cytotoxicity in PMBC via IL15 induction (Fawaz et al., 1999). IL15 has also been linked to inflammatory processes, as it induces mast-cell migration and neutrophil adhesion to A549 cells (Jackson et al., 2005; Pelletier & Girard, 2005).

Cytokine signalling is under negative feedback regulation by a family of proteins called suppressors of cytokine signalling (SOCS) (Alexander & Hilton, 2004). SOCS3 is up-regulated in several inflammatory diseases (Alexander & Hilton, 2004). Paradoxically, overexpression of SOCS3 by gene therapy led to reduction of inflammation in murine models (Shouda et al., 2001). It has been proposed that SOCS3 is induced in an attempt to control the cytokine-signalling cascade, but that physiological levels of SOCS3 are insufficient to control proinflammatory signalling under pathological circumstances. Inefficient induction of SOCS3 by HRSV (only 1.8 times, Fig. 5b) might contribute to the lack of control of the inflammatory processes.

**Antiviral response**

The time course analysis of this study showed the induction of a potent antiviral response late after HRSV infection (12 h p.i.), characterized by up-regulation of ISGs and genes related to antigen processing and presentation. Increased expression of class I MHC and antigen-processing genes has been described in A549 cells infected by HRSV and it has been shown to be mediated by IFN-β (Garofalo et al., 1996; Jamaluddin et al., 2001). These observations were confirmed in the present study and extended to genes related to the ubiquitination pathway.

Approximately 27% of the genes up-regulated between 12 and 24 h p.i. (q<5%) were identified as ISGs. The relationship between HRSV and the interferon system is controversial. Compared with other viruses, HRSV appears to induce low amounts of IFNs and it has been reported to be less responsive to their antiviral effects, both in vitro and in vivo, although not in all reports (Collins et al., 2001). A recent study showed that IFN induction depends on the HRSV strain tested. The Long strain was a potent inducer of type I (α/β) IFN in plasmacytoid dendritic cells, while the A2 strain and four primary HRSV isolates were poor inducers (Hornung et al., 2004; Schlender et al., 2005). Both bovine RSV and HRSV (A2 strain) inhibit the induction of type I IFN by suppressing activation and nuclear translocation of interferon regulatory factor 3 (IRF-3), an effect mediated by the RSV non-structural proteins NS1 and NS2 (Bosser et al., 2003; Spann et al., 2005). In addition, HRSV A2, but not Long, inhibits Toll-like receptor 7 (TLR7)- and TLR9-mediated IFN-α/β production (Schlender et al., 2005). Suppression of IFN induction is not the only mechanism used by HRSV to avoid the IFN-mediated antiviral response. Type I IFN signal transduction is also blocked by HRSV NS1 and NS2 proteins of both Long and A2 strains by inhibiting Stat2 expression (Lo et al., 2005; Ramaswamy et al., 2004, 2006). Despite all of this, as mentioned above, HRSV A2 infection is able to induce antigen-processing and class I MHC genes through the induction of IFN-β in A549 cells (Garofalo et al., 1996; Jamaluddin et al., 2001). In our study, although the IFN-β gene was not present in the microarray test, the same subset of ISGs were up-regulated by both the Long and A2 strains (Table 1). A possible explanation for these findings is that the initial infection triggers the
interferon response before inhibitory levels of the virus products counteracting that response are reached. Biologically active IFN-β has been detected as early as 3 h p.i. with HRSV (Jamaluddin et al., 2001), while it takes 10–14 h to reach sufficient levels of NS1 and/or NS2 proteins to block activation and translocation of IRF-3 (Spann et al., 2005). Another possible explanation is that many ISGs can be induced by dsRNA, a product generated during RNA virus infections, in an IFN-independent way (Sen, 2001). The best studied dsRNA signalling pathway is that mediated by TLR3 (Sen & Sarkar, 2005). In this regard, it is interesting that the expression of CCL5 has been reported to be induced by HRSV directly through TLR3 signalling and did not require IFN signalling through the IFN-α/β receptor (Rudd et al., 2005). A third pathway for the induction of ISGs has been reported in Sendai-virus-infected cells that cannot respond to either IFN-α/β or dsRNA (Guo et al., 2000). Further studies are necessary to elucidate the mechanism(s) by which HRSV infection induces such a potent ISG response.

In summary, we have reported that different subsets of genes are up-regulated at different time points following HRSV infection, reflecting the impact of the virus in the cell metabolism and the subsequent defence response of the host cell. Since microarrays are only semi-quantitative assays, the differences in mRNA levels between infected and non-infected cells reported here should not be taken as absolute figures. Nevertheless, the large amount of data generated by the microarrays provides interesting clues to many processes taking place in HRSV-infected cells, opening new lines of study and experimentation. In that respect, to assess the relevance of those changes for cell and/or virus biology, it would be interesting to test the possibility that some of the genes up-regulated after HRSV infection are targets for potential inhibition of virus replication and/or modulation of the host response, avoiding pathological consequences.

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