

# Induction of type I interferons and interferon-inducible Mx genes during respiratory syncytial virus infection and reinfection in cotton rats

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Respiratory syncytial virus (RSV) is the primary cause of bronchiolitis in young children. In general, RSV is considered to be a poor inducer of type I (alpha/beta) interferons (IFNs). Measurement of active type I IFN production during infection *in vivo* is demanding, as multiple IFN subtypes with overlapping activities are produced. In contrast, Mx gene expression, which is tightly regulated by type I IFN expression, is easily determined. This study therefore measured Mx expression as a reliable surrogate marker of type I IFN activity during RSV infection *in vivo* in a cotton rat model. It was shown that expression of Mx genes was dramatically augmented in the lungs of infected animals in a dose- and virus strain-dependent manner. The expression of Mx genes in the lungs was paralleled by their induction in the nose and spleen, although in spleen no simultaneous virus gene expression was detected. Reinfection of RSV-immune animals leads to abortive virus replication in the lungs. Thus, type I IFN and Mx gene expression was triggered in reinfected animals, even though virus could not be isolated from their lungs. Furthermore, it was demonstrated that immunity to RSV wanes with time. Virus replication and Mx gene expression became more prominent with increasing intervals between primary infection and reinfection. These results highlight the role of type I IFN in modulation of the immune response to RSV.

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## INTRODUCTION

Respiratory syncytial virus (RSV) is a negative-strand RNA virus of the family *Paramyxoviridae*. It causes acute respiratory tract infections, resulting in substantial morbidity and some mortality in humans, particularly at the extremes of age (Thompson *et al.*, 2003; Welliver, 2003; Falsey *et al.*, 2005). RSV is generally considered to be a poor inducer of type I (alpha/beta) interferon (IFN- $\alpha/\beta$ ) in comparison with other RNA viruses (Hall *et al.*, 1978; Roberts *et al.*, 1992). Two non-structural proteins of RSV, NS1 and NS2, are known to suppress IFN production (Bossert *et al.*, 2003; Spann *et al.*, 2004). Surprisingly, however, RSV is found to induce high-level expression of IFN- $\beta$  in cultures of various human respiratory epithelial cells and fibroblasts (Garofalo *et al.*, 1996; Jamaluddin *et al.*, 2001). RSV also induces high levels of IFN- $\alpha$  in different subsets of dendritic cells (Hornung *et al.*, 2004; Schlender *et al.*, 2005; Guerrero-Plata *et al.*, 2006; Wang *et al.*, 2006).

Type I IFN production plays an important role in limiting RSV-induced pathology during infection (Durbin *et al.*, 2002). Studies of type I IFN production *in vivo* are complicated by the large number of type I IFN genes

involved. IFN- $\alpha$  genes are represented by a multigenic family of intronless genes clustered in a 400 kb region, also containing the only IFN- $\beta$  gene, on human chromosome 9 and on a syntenic region of chromosome 4 in the mouse (Diaz *et al.*, 1994). They have been shown to be coordinately induced by viruses in human and mouse cells, but to differ in the level of expression of their individual mRNAs (Hiscott *et al.*, 1984). Secreted type I IFNs act through a common receptor, which consists of two subunits (IFNAR-1 and IFNAR-2) present in virtually all cells (Stark *et al.*, 1998; Smith *et al.*, 2005). Receptor binding leads to a signalling cascade, which results in activation of interferon-stimulated genes. The best-studied interferon-stimulated genes with antiviral properties are 2',5'-oligoadenylate synthetase (2',5'-OAS)/RNaseL, protein kinase R (PKR) and the Mx genes. In contrast to 2',5'-OAS and PKR, Mx expression is stimulated exclusively by IFN- $\alpha/\beta$  or IFN- $\delta$  (Holzinger *et al.*, 2007) and does not respond to other cytokines such as interleukin (IL)-1 or tumour necrosis factor (TNF)- $\alpha$  (Simon *et al.*, 1991). Accordingly, Mx expression has been shown to be an excellent marker for type I IFN activation in clinical settings (Roers *et al.*, 1994; Forster *et al.*, 1996; Halminen *et al.*, 1997).

Here, we established the value of measuring mRNA induction of the cotton rat Mx genes to monitor the production of type I IFNs during RSV infection *in vivo*. We showed that expression of Mx1 and Mx2 mRNAs was strongly induced in the lungs of infected animals. In most cases, a parallel induction of cotton rat IFN- $\alpha$ 1 and IFN- $\beta$  was detected. The ability to trigger type I IFN and Mx gene expression depended on the virus strain used. In RSV-immune animals, expression of these genes occurred at early time points after a new virus challenge and was influenced by the time interval between primary infection and rechallenge.

## METHODS

**Virus preparations and cell cultures.** HEp-2 and Vero cells (ATCC CCL-23 and CCL-81, respectively) were used to prepare virus stocks. Virus titres for all RSV stocks were determined by plaque assay on HEp-2 cells. Uninfected cell culture supernatants (mock preparations) were used as a control. RSV strain A/Long (ATCC VR-26) virus stock ( $3.3 \times 10^7$  p.f.u. ml<sup>-1</sup>) was prepared in HEp-2 cells and stabilized with sucrose and fetal calf serum. UV inactivation was carried out by exposure of the virus stock to UV light until no infectious virus was detected. Primary human RSV isolates A074 (group A), B006 and B007 (group B) were recovered from hospitalized children and provided from the laboratory of Dr Val Hemming of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA). All RSV clinical isolates as well as human RSV strains A2 (ATCC VR-1540) and Long were first amplified by two passages in HEp-2 cells and then titrated before preparing IFN-free virus stocks in Vero cells (m.o.i.=0.1) for the experiment in which different virus isolates were used to infect animals.

**Animals.** An inbred cotton rat (*Sigmodon hispidus*) colony was maintained at Virion Systems, Inc., Rockville, MD, USA. The cotton rats were housed in sterile conditions and fed a diet of rodent food and water. The cotton rat colony was maintained free of paramyxoviruses, including RSV, and rodent viruses. The animals were 4–6 weeks old and weighed approximately 100 g at the onset of experiment. All animal experimentation procedures were carried out following NIH and USDA guidelines and were approved by the Virion Systems, Inc. Institutional Animals Care and Use Committee.

**Infection.** Cotton rats were inoculated intranasally under isoflurane anaesthesia with 100  $\mu$ l RSV suspension containing the indicated number of p.f.u. Uninfected rats and rats inoculated with either uninfected cell culture supernatant (mock) or with UV-inactivated virus suspensions were used as controls. Rats were sacrificed by carbon dioxide inhalation at the indicated intervals thereafter. Tissues (lung, spleen and turbinates) were dissected and snap frozen in liquid nitrogen.

**Analysis of tissue mRNA by RT-PCR.** Total RNA was isolated using a Qiagen RNA isolation kit and treated with DNase I (Qiagen) to remove traces of contaminant DNA. Total RNA (1  $\mu$ g) from each tissue sample was utilized for the preparation of cDNA using oligo(dT) primers and Super Script II reverse transcriptase (Invitrogen). The relative quantities of mRNA for each gene of interest were determined by semi-quantitative PCR (Blanco *et al.*, 2002) or by real-time quantitative PCR (qPCR) using iQ SYBR Green Supermix (Bio-Rad) and a MyiQ Single-Colour Real-Time PCR Detection System (Bio-Rad). The sense (S) and antisense (AS) primers and probes (P) for each analysed gene were as follows: IFN- $\beta$ ,

5'-GCAGCATTTCAGAATGTCTAGAGC-3' (S), 5'-ATCTTAATAAGTCTTCCCATGATGG-3' (AS) and 5'-TCCATCTCGTTACGGAGTTTATCC-3' (P). Due to differences in the sequences of the F gene for RSV types A and B, we generated two sets of primers for amplification of the F gene: type A: 5'-CAGACTACTAGAGATTACCAGGGAAT-3 (S) and 5'-TGCTCCTAGAGATGTGATAACGGAGC-3' (AS); type B: 5'-CAGATTGTTGGAAATCACCAGAGAAT-3' (S) and 5'-TAGCTCCAAGAGAAGTAATTACTGAGC-3' (AS). Both sets amplified the same region of the gene with equal efficiency. The probe for detection of the F gene amplicon of both RSV types was the same: 5'-GGTGTAACCTACACCTGTAAGCAC-3'. The primers and probes for the NS1 gene were: RSV/NS1 type A: 5'-GCAGCAATTCGTTGAGTATG-3' (S), 5'-GATCAAATCCAAGTAATTCAG-3' (AS) and 5'-CATTGTGTTTGTGCATGTTATTAC-3' (P); type B: 5' GTCAATTCAGTACTGAGCATGA-3' (S) and 5' GAGATCAAGCCCAAGTAAATC-3' (AS); and for the NS2 gene: RSV/NS2 type A: 5'-GACACAACCCACAATGATA-3' (S), 5'-CATGGATTGAGATCATACTTG-3' (AS) and 5'-CAACTATGAAATGAACTATTGCAC-3' (P). The primers and probes for the other genes assessed in this study have been published previously (Blanco *et al.*, 2002; Pletneva *et al.*, 2006). PCR conditions were as follows: IFN- $\alpha$ , 62 °C for annealing and 30 s for extension; IFN- $\beta$ , 55 °C and 30 s; F gene, 60 °C and 30 s; NS1 and NS2 genes, 60 °C and 30 s;  $\beta$ -actin, 65 °C and 30 s. The optimum number of cycles for each particular gene in lung tissue was determined empirically. Briefly, pilot PCRs were performed using positive lung cDNA control samples for each gene, and the number of cycles needed to achieve the strongest unsaturated signal was used in subsequent experiments. The number of cycles used for each gene was as follows: IFN- $\alpha$ , 28 cycles; IFN- $\beta$ , 26 cycles; Mx1 and Mx2, 19 cycles; F, 22 cycles in lungs and turbinates and 26 cycles in the spleen; NS1 and NS2, 19 cycles in lungs and turbinates and 26 cycles in the spleen. In cases where the cycle numbers were different from those mentioned above, correct numbers are indicated in the legend of the figure.  $\beta$ -Actin was included as a housekeeping gene to control for differences in cDNA amount for each treatment during the amplification reaction. Standard Southern blotting was utilized to analyse mRNA expression for each gene.

**Preparation of protein extracts and Western blot analysis.** Whole-cell extracts were prepared from lung tissue using a lysis buffer containing 20 mM Tris/HCl (pH 7.9), 100 mM NaCl, 1% NP-40, 4 mM dithiothreitol, 7.5 mM NaF, 2 mM EDTA, 0.5 mM PMSF and protease inhibitors. Cell extracts were subjected to 8% SDS-PAGE, transferred to Immobilon-P membranes (Millipore) and probed with the indicated antibodies. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies and reagents for enhanced chemiluminescence detection were obtained from Amersham.

**Statistical analysis.** Results are expressed as means  $\pm$  SEM. Statistical significance was calculated using Student's *t*-test as indicated in the legends.

## RESULTS

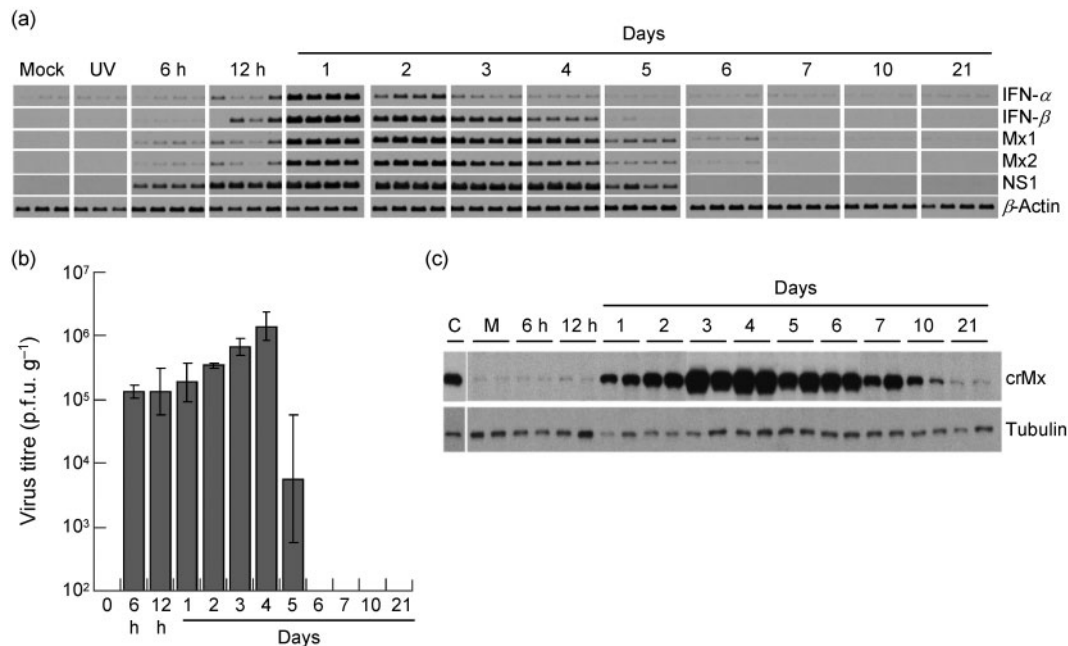
### RSV infection induces Mx proteins in cotton rat lungs

We previously cloned the cotton rat Mx1 and Mx2 genes and found that they exhibited considerable similarity to the human and mouse genes (Pletneva *et al.*, 2006). Using cotton rat cell lines, we further verified that these genes were responsive to type I IFN but not to type II IFN (IFN- $\gamma$ ) or other inflammatory cytokines such as IL-1 and TNF- $\alpha$  (data not shown). As the expression of these genes

accurately reflected the presence of active type I IFN, we analysed their expression *in vivo* during infection with RSV. Groups of cotton rats were infected intranasally with  $3 \times 10^6$  p.f.u. per 100 g body weight; on the indicated days post-infection (p.i.), four animals from each group were sacrificed and lung samples were trisected for determination of viral titre and gene and protein expression. Steady-state levels of host mRNA for IFN- $\alpha$ , IFN- $\beta$ , Mx1 and Mx2 and of viral NS1 mRNA were analysed by RT-PCR (Fig. 1a). RSV infection induced a rapid increase in the steady-state levels of all tested genes. In particular, expression of both Mx genes was detected as early as 6 h p.i., whereas IFN- $\alpha$ / $\beta$  mRNAs were detected at 12 h p.i. Expression of the two IFN genes analysed peaked on days 1 and 2 p.i. and decreased to almost undetectable levels by day 7. Both Mx mRNAs showed overlapping patterns of induction peaking on days 2 and 3 p.i. and decreasing to almost undetectable levels by day 7. Replicating virus was required for activation of mRNA expression of type I IFN and Mx genes, as induction of these genes was not detected when animals were inoculated with UV-inactivated virus. In addition, mRNA expression for most of these genes was undetectable or very low (in the case of IFN- $\alpha$ ) in

mock-infected rats sacrificed at different times post-treatment. mRNA expression for the host genes paralleled the presence of NS1 mRNA (Fig. 1a) and virus in the lungs (Fig. 1b), and preceded the peak of lung pathology, which occurs on day 6 after infection (Prince *et al.*, 1999).

In parallel, lung samples from RSV-infected animals were analysed for the expression of Mx proteins by Western blotting using monoclonal antibody M143 prepared against human MxA, which recognizes the N-terminal region of Mx proteins from most species, including mouse, rat and cotton rat (Flohr *et al.*, 1999; Pletneva *et al.*, 2006) (Fig. 1c). The presence of a band that co-migrated with human MxA protein used as a control was detected as early as 12 h p.i. and was not detected in mock-treated animals. As cotton rat Mx1 and Mx2 proteins have similar estimated molecular masses (74.7 and 74.9 kDa, respectively), it was not possible to discriminate between their individual levels of expression during infection in lung samples by this methodology. The peak expression of cotton rat Mx proteins occurred on day 4 and had declined by day 7 when no virus could be isolated from the lungs (Fig. 1b). After day 7 p.i., the amount of Mx protein gradually decreased but was still detectable at 21 days p.i.



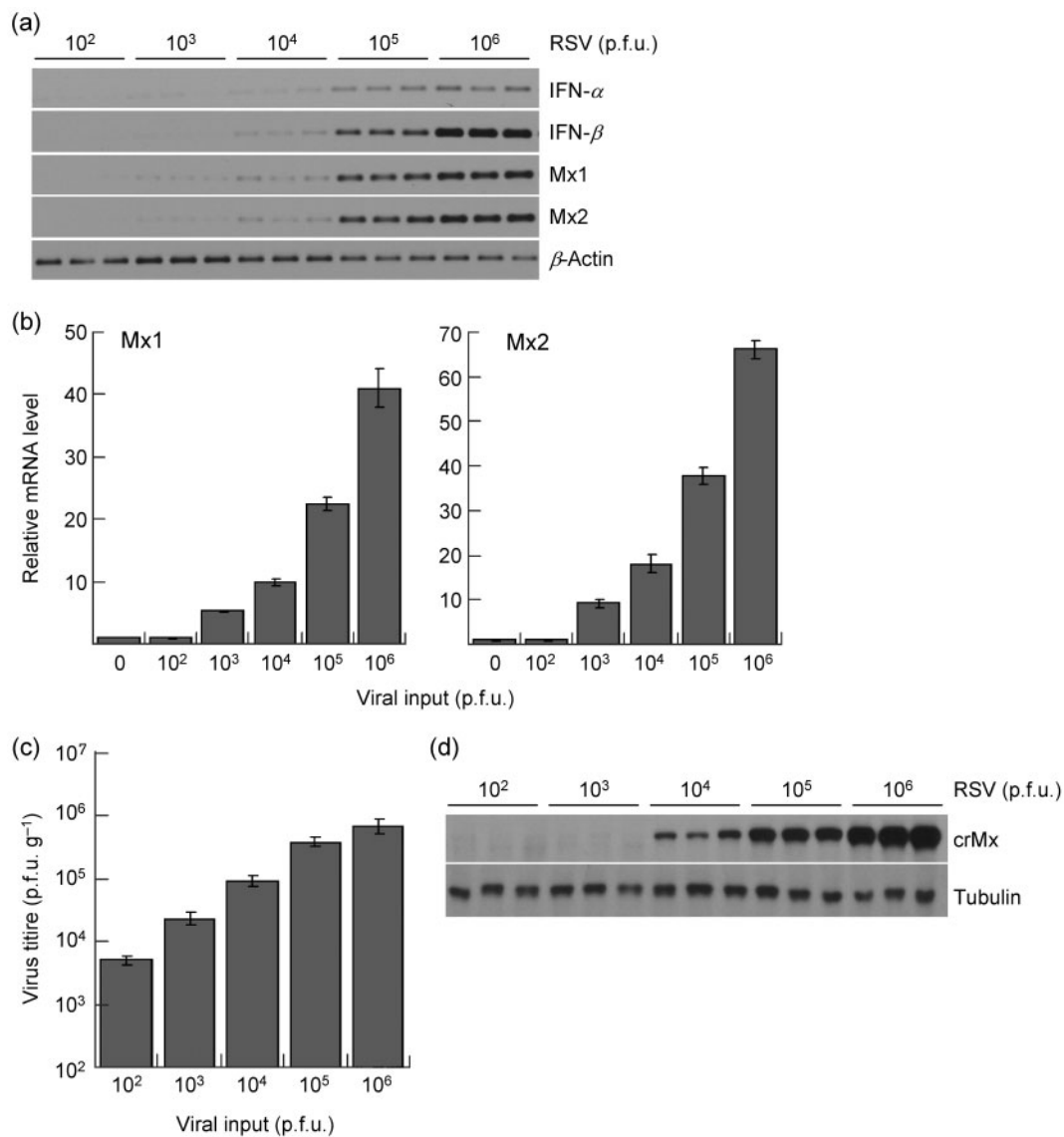
**Fig. 1.** (a) Time course of IFN- $\alpha$ , IFN- $\beta$ , Mx1, Mx2 and NS1 mRNA expression in lungs of cotton rats infected with the A/Long strain of RSV, as assessed by RT-PCR analysis. Mock or UV-inactivated (UV) preparations of the virus were used as controls. Three control animals and four infected animals per time point were sacrificed. Each lane represents one animal. One result for each mock and UV-inactivated group is included as the representative result for the entire time course. (b) RSV replication in the lungs of cotton rats sacrificed at the indicated times p.i. Viral titres were determined by plaque assay and mean titres  $\pm$  SEM are shown. For day 5 p.i., two out of four rats did not have detectable virus in the lung. (c) Western blot analysis of the expression of cotton rat Mx proteins (crMx) at the indicated times p.i. in the lungs of infected cotton rats. Each lane represents one animal. Western blotting of tubulin was included as a loading control. C, MxA protein loaded as a control; M, mock infection. Results are representative of two independent experiments.

The expression of type I IFN, Mx1 and Mx2 mRNAs was evaluated by semi-quantitative RT-PCR (Fig. 2a) and qRT-PCR for Mx1 and Mx2 (Fig. 2b) on day 3 (peak Mx mRNA expression) after inoculation of cotton rats with different doses of RSV. IFN- $\alpha$ , IFN- $\beta$ , Mx1 and Mx2 mRNAs were induced in the lungs of infected cotton rats in a dose-dependent manner, correlating with the replication of RSV in the lung (Fig. 2c); the smallest inoculum that generated detectable production was  $10^3$  p.f.u. per 100 g body weight (Fig. 2b). Expression of Mx proteins was also tightly correlated with levels of mRNA (Fig. 2d). Taken together, these data demonstrated that RSV induced strong expression

of Mx protein and, therefore, of active type I IFN in the lungs of cotton rats, and that their expression was tightly associated with viral infection in a dose-dependent manner.

### Induction of Mx genes *in vivo* is dependent on RSV strain

The differential ability of RSV isolates to induce expression of IFN- $\alpha$  has recently been demonstrated *in vitro* using human plasmacytoid dendritic cells (Hornung *et al.*, 2004; Schlender *et al.*, 2005). As we hypothesized that the expression of Mx genes was directly associated with the



**Fig. 2.** (a) Dose-response analysis of Mx1 and Mx2 mRNA levels by semi-quantitative RT-PCR during RSV infection (total p.f.u./Long per 100 g body weight as indicated) in lungs of cotton rats, performed on day 3 p.i. (b) qRT-PCR of Mx1 and Mx2 gene expression. (c) RSV replication in the lungs of cotton rats after infection with the indicated dose of virus and sacrificed on day 3 p.i. (d) Dose-response analysis of the expression of cotton rat Mx proteins in the lungs of RSV-infected animals on day 3 p.i. Western blotting of tubulin was included as a loading control.

total amount of active type I IFN produced *in situ*, we next determined the extent of type I IFN and Mx gene induction and viral gene expression (F and NS1 genes) in the lungs of animals infected with different RSV isolates of subtype A, including the prototype RSV strains A/Long and A/A2 and the clinical isolate A/074-91, and two clinical isolates of the RSV subtype B, B006-92 and B007-92. Animals were infected with equal amounts of each isolate ( $10^6$  p.f.u. per 100 g body weight) and sacrificed on days 1 and 4 p.i. All isolates replicated with similar kinetics, as shown by the levels of expression of the viral F gene (Fig. 3a) or NS1 gene (Fig. 3b, bottom graph), which were higher on day 4 than on day 1, and by the equivalent viral titres in the lungs on day 4 (Fig. 3c).

Importantly, all isolates of subtype A induced stronger IFN- $\alpha/\beta$  mRNA expression than isolates of subtype B on day 1, whereas the mRNA levels of type I IFN genes induced by each strain were comparable on day 4 (Fig. 3a). In addition, comparison of Mx1 and Mx2 mRNA levels revealed that the A/A2 strain was the strongest activator of these genes on day 1 and also the fastest suppressor of their steady-state mRNA levels on day 4 p.i. (Fig. 3a and Fig. 3b, top graph), as all other strains induced peak levels of Mx1 and Mx2 mRNA on day 4. Importantly, the observations described at the level of Mx mRNA were paralleled precisely by the Mx protein expression analysed in the same samples by Western blotting (Fig. 3d). Samples obtained from animals infected and sacrificed on day 1 showed robust Mx expression when infected with the A/A2 strain of RSV but not with the other strains. In addition, on day 4 p.i., all strains but A/A2 showed high levels of Mx protein expression, confirming the inhibitory effect on type I IFN of this strain.

These data (i) demonstrated the differential ability of different strains of RSV to induce type I IFN *in vivo*, (ii) confirmed previously reported results that showed the ability of strain A/A2, in contrast to A/Long, to suppress the production of type I IFN, and (iii) underscore the utility of measuring Mx mRNA levels as the most accurate indication of total active type I IFN production *in vivo*, as these differences are not appreciated by measuring only the expression of selected type I IFN forms.

### Expression of Mx genes in different tissues following RSV infection

We evaluated the expression of type I IFN and Mx mRNAs in turbinates of infected cotton rats, as this is another tissue where sustained RSV replication takes place. In addition, we chose to analyse expression of the same genes in the spleen. In the spleen, amongst other lymphoid tissues, RSV-specific lymphocytes are generated by interacting with antigen-presenting cells including plasmacytoid dendritic cells, which migrate from peripheral tissues into the T-cell areas and exert their function by producing type I IFN.

Steady-state levels of mRNA for all of the genes analysed in the turbinates were robust after infection with either the A/

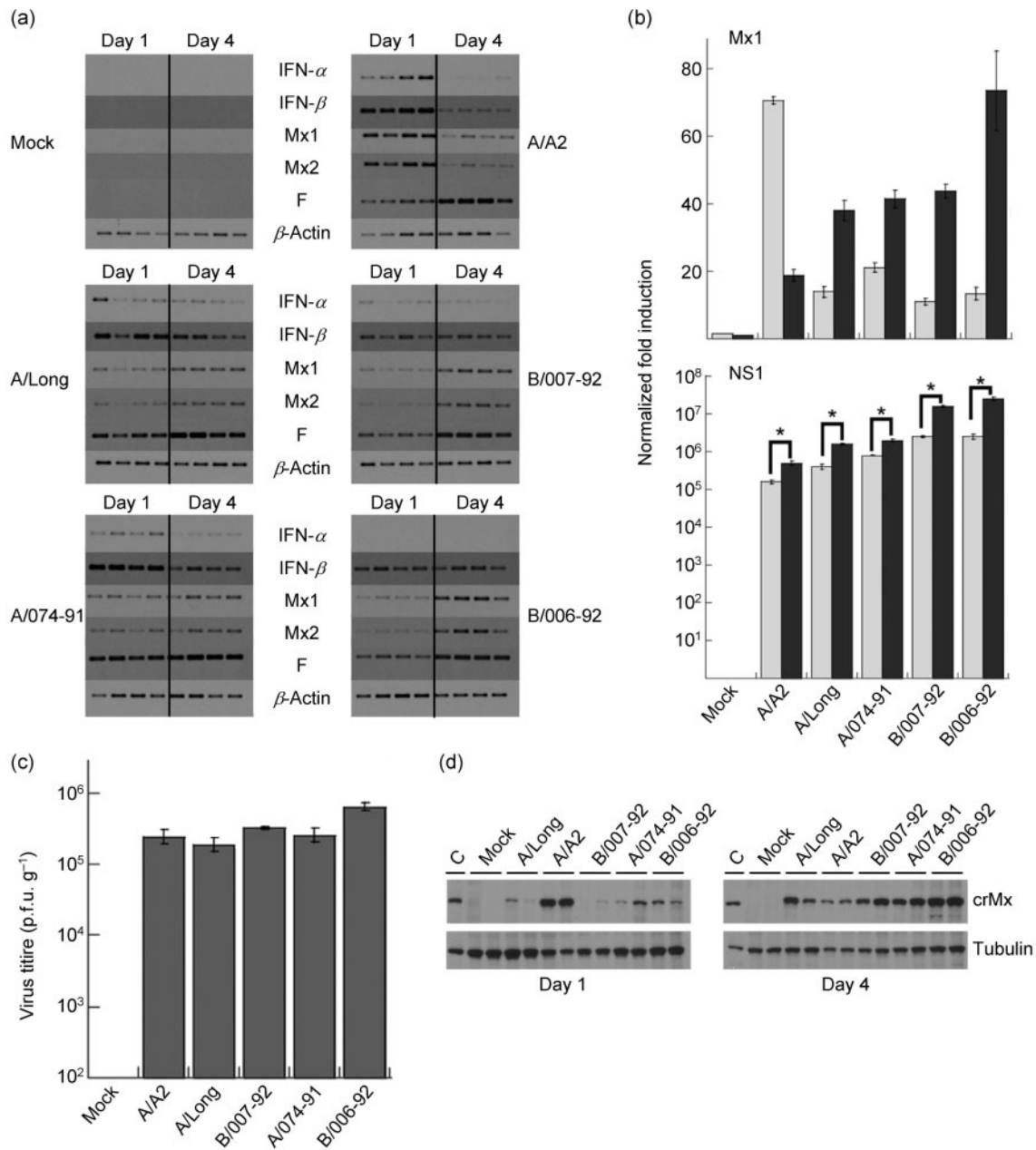
A2 or A/Long strain of RSV. However, for both viruses, greater expression levels of mRNA for all genes were seen on day 4 compared with day 1 p.i. (Fig. 4, top panel). This contrasted with our results for expression of the same genes in the lungs, where RSV A/A2 but not RSV A/Long induced stronger Mx expression on day 1.

In the spleen (Fig. 4, bottom panel), analysis of the expression of mRNA for the same cluster of genes revealed variable expression of type I IFN but a clear pattern of mRNA expression for both Mx genes, with peak mRNA levels on day 1 p.i. for A/A2 and on day 4 p.i. for A/Long – a pattern correlating with that in the lungs. In addition, no viral gene expression was detected, consistent with previous observations that RSV does not replicate outside the respiratory tract (Prince *et al.*, 1978). Our results clearly indicated that, following viral infection, different target tissues respond to RSV infection producing different levels of type I IFN, most likely due to differential utilization of an innate virus detection mechanism. In addition, we were able to detect virus-specific production of type I IFN in the spleen by analysing the induction of Mx mRNA. To our knowledge, this is the first time that type I IFN production following RSV infection has been measured in the spleen *in vivo*.

### Expression of type I IFN and Mx genes during RSV reinfection

Reinfections with RSV are common. The effect of the length of time between infection and reinfection on type I IFN induction remains unclear. An analysis of the expression of type I IFN and Mx genes during primary infection and reinfection in the cotton rat model was conducted following the indicated time schedule (Fig. 5a). Briefly, animals of the same age were separated into three groups: a primary infection group was mock infected on day -60 and then infected once with  $10^6$  p.f.u. RSV A/Long per 100 g body weight on day 0; a secondary infection group [Sec (21)] was infected twice, on day -21 and day 0; and another secondary infection group [Sec (60)] was infected twice, on day -60 and day 0. Three to four animals per group were sacrificed on days 1, 4 and 7, and lung samples were collected for determination of viral titres and mRNA analysis. As expected, virus was isolated only from lungs of those animals with the primary infection (data not shown). In addition, these animals showed the strongest mRNA induction for all genes analysed, and had detectable viral and Mx genes on day 7.

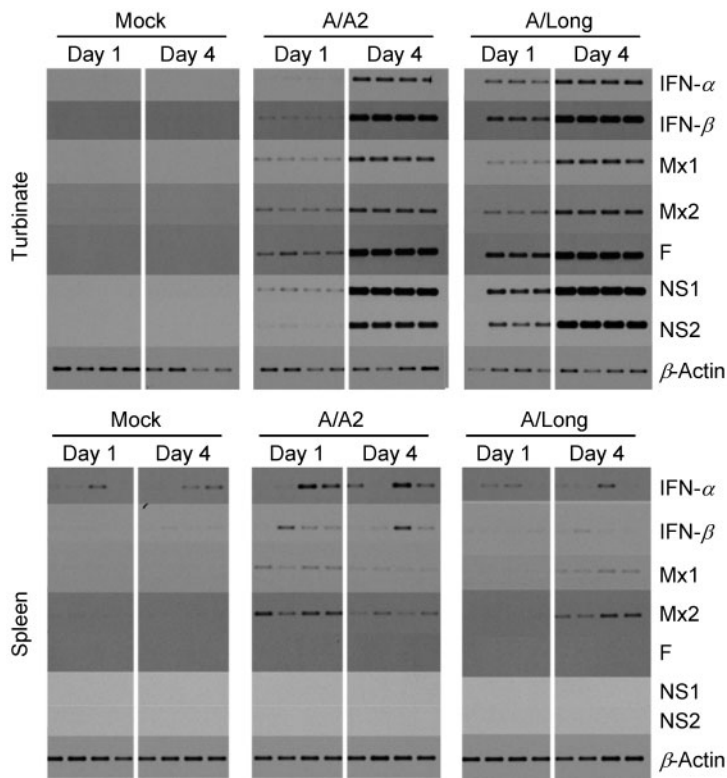
As previously reported (Prince *et al.*, 1999), secondary infection did not yield any virus amplification, although abortive replication was detected (as indicated by the measurable expression of all viral genes on day 1). Most importantly, the abortive viral gene expression was sufficient for the induction of type I IFN and Mx gene expression (Fig. 5b). Furthermore, after comparison of the two secondary infection groups, it was evident that expression of type I IFN, Mx and viral genes was stronger



**Fig. 3.** Induction of type I IFN and Mx genes by different RSV isolates. Groups of cotton rats were inoculated with  $10^6$  p.f.u. of each isolate (RSV A/A2, A/Long and A/074-91, B/007-92 and B/006-92) per 100 g body weight or mock infected and sacrificed on days 1 and 4 p.i. (a) RT-PCR analysis of IFN- $\alpha$ , IFN- $\beta$ , Mx1, Mx2 and RSV F mRNA levels in the lungs.  $\beta$ -Actin is shown as a control for the total amount of RNA used in each reaction. (b) qRT-PCR expression for Mx1 (top) and NS1 (bottom) gene expression on days 1 (light-grey bars) and 4 (dark-grey bars) p.i. for each strain of RSV. Significant differences were seen in the expression of NS1 when comparing expression of NS1 on days 1 and 4 (\*,  $P < 0.001$ ). An identical profile to that of Mx1 was obtained for Mx2 (data not shown). (c) RSV replication in the lungs of cotton rats on day 4 after infection with the indicated RSV isolate. (d) Western blot analysis showing expression of cotton rat Mx proteins (crMx) in the lungs of animals infected with the indicated RSV isolates or mock infected and sacrificed on days 1 and 4 p.i. Western blotting of tubulin was included as a loading control. Each lane in (a) and (c) represents one animal. The results are representative of two independent experiments.

in animals reinfected after 60 days of primary challenge than in those reinfected after 21 days. The difference was clear for transcription of the viral NS1 and NS2 genes, as

their mRNA was detected in all rats on day 4 in the Sec (60) infection group, whereas it was almost undetectable on the same day in animals of the Sec (21) infection group. Less



**Fig. 4.** Induction of type I IFN and Mx genes during RSV A/A2 and A/Long infection in nasal turbinates and spleen. Cotton rats were infected intranasally with the indicated isolate of RSV or were mock infected. Animals were sacrificed on days 1 and 4 p.i. and total RNA was extracted from the turbinate and spleen. RT-PCR analysis of turbinate (upper panel) and spleen (lower panel) was performed for host (IFN- $\alpha$ , IFN- $\beta$ , Mx1 and Mx2) and viral (NS1, NS2 and F) genes as indicated. A total of four animals per group is shown.

evident but also significant (as seen by qRT-PCR; Fig. 5c) was the difference in expression of Mx genes on day 1 post-challenge. Taken together, these data demonstrated that abortive RSV replication, seen during secondary infection in the lung of cotton rats, is sufficient to activate type I IFN production. In addition, we demonstrated that both abortive viral gene expression and type I IFN can be influenced by the length of the time period between infections with RSV, suggesting that time between infections is an important factor in determining the extent of abortive viral gene expression, IFN production and most likely the outcome of the disease.

## DISCUSSION

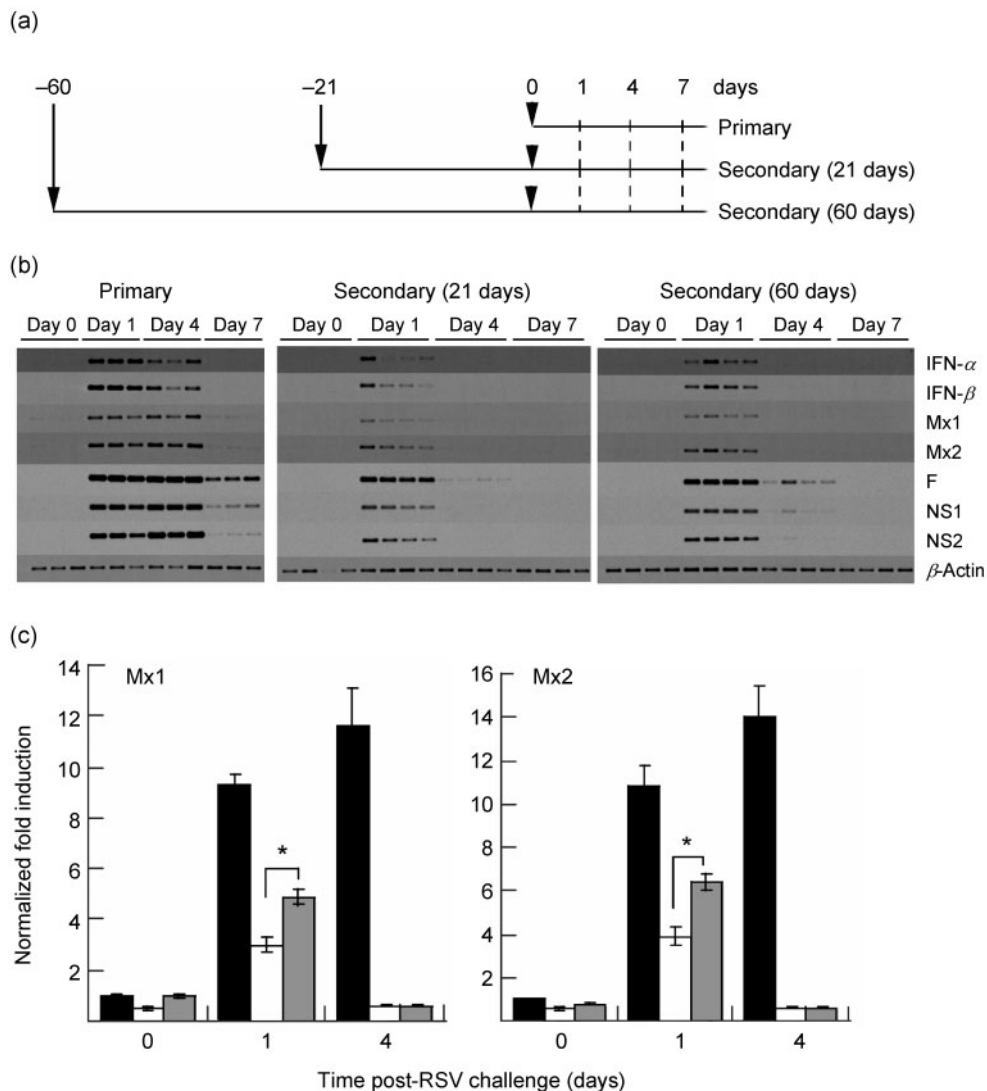
In humans infected with RSV, the detection of type I IFN is sporadic in nasopharyngeal secretions (Hall *et al.*, 1978, 1981; McIntosh, 1978; Isaacs, 1989; Taylor *et al.*, 1989; Nakayama *et al.*, 1993). However, these studies are difficult to interpret considering the nature of the samples analysed (nasal washes, nasal aspirates and sera), variations in subject age, timing of sample isolation and IFN measurement techniques. The fact that all of these studies demonstrated the presence of type I IFN in a portion of the nasopharyngeal samples tested suggests that type I IFN plays an important but transient role during RSV infection.

Production of type I IFN in response to RSV infection has been studied previously in other systems. Many different factors seem to determine the production of type I IFN by

RSV, including cell type and viral strain (Garofalo *et al.*, 1996; Jamaluddin *et al.*, 2001; Hornung *et al.*, 2004; Schlender *et al.*, 2005; Guerrero-Plata *et al.*, 2006; Wang *et al.*, 2006). However, it is clear from the literature that RSV stimulates type I IFN to a lesser extent than other human respiratory viruses, such as influenza virus, human parainfluenza virus 1 and human metapneumovirus (Hall *et al.*, 1978; Chonmaitree *et al.*, 1981; Roberts *et al.*, 1992; Guerrero-Plata *et al.*, 2006).

*In vivo* expression of type I IFN and Mx genes in response to influenza infection in the cotton rat has been studied previously (Pletneva *et al.*, 2006) and their antiviral potential tested (Stertz *et al.*, 2007). We have now shown that, as with influenza virus, RSV infection activates type I IFN and Mx gene expression in the lung relatively early during infection (6–12 h p.i.).

Importantly, in infections with RSV A/A2, we showed that the peak of type I IFN and Mx mRNA and Mx protein preceded the peak of viral replication (Fig. 3a and b). Recently, induction of type I IFN by RSV was shown to be virus strain-dependent (Schlender *et al.*, 2005). In various types of human cell, including epithelial cell lines (A549, HEp-2 and 293) and freshly isolated human B cells, T cells, monocytes and plasmacytoid dendritic cells, only RSV A/Long induced high levels of IFN- $\alpha$ , whereas the other isolates tested (including RSV A/A2) were at least tenfold less efficient at IFN- $\alpha$  induction. Our results extend those of Schlender *et al.* (2005) by further demonstrating that RSV A/Long has a lesser ability to counteract cellular



**Fig. 5.** Induction of type I IFN and Mx genes in the lungs of cotton rats during infection and reinfection with RSV A/Long. (a) Experimental details of primary infection and secondary reinfections at 21 or 60 days after the first challenge. Groups of cotton rats were inoculated intranasally with  $10^6$  p.f.u. virus suspension per 100 g body weight for the secondary (21 days) and secondary (60 days) groups on days -21 and -60, respectively, or with an identical volume (100  $\mu$ l) of a mock preparation for the primary group on day -60. At 21 or 60 days after primary inoculation, three to four animals per group were sacrificed at the day 0 time point and the rest of the animals in all groups were inoculated with the same dose of virus and sacrificed at the indicated times after the second inoculation. (b) Semi-quantitative RT-PCR analysis of induction of the host genes IFN- $\alpha$ , IFN- $\beta$ , Mx1 and Mx2 and the viral genes NS1 (24 cycles), NS2 (24 cycles) and F.  $\beta$ -Actin is shown as a control for the total amount of RNA used in each reaction. (c) qRT-PCR of the levels of Mx1 (left) and Mx2 (right) mRNA in the lungs of animals infected (primary; black bars) and reinfected at 21 days (white bars) or 60 days (grey bars) after the primary infection.  $\beta$ -Actin was used as a reference gene. The mean  $\pm$  SEM for four animals per group is shown. Significant differences were seen in the expression of Mx when comparing the indicated groups (\*,  $P < 0.001$ ).

mechanisms of type I IFN production (thus producing type I IFN and Mx for a longer period of time) than the RSV A/A2 strain *in vivo*.

We demonstrated that RSV infection also induced type I IFN and Mx mRNA expression in tissues other than the

lung. First, we performed an analysis of nasal turbinates, as RSV replicates robustly in the nose of cotton rats with a peak viral titre on days 3–5 p.i. (Prince *et al.*, 1978). In contrast to our results for the expression of type I IFN in the lungs, both RSV A/A2 and A/Long followed a similar profile of expression in nasal turbinates, with the strongest

expression on day 4 p.i. This implies that, during RSV infection, there is a differential type I IFN response depending on the target tissue.

In addition to their antiviral effect, type I IFNs play pleiotropic roles in immunomodulation, at both innate and adaptive immune levels (Theofilopoulos *et al.*, 2005). Adjuvants currently under development exert part of their effect by inducing local expression of type I IFN through Toll-like receptors (Seya *et al.*, 2006), some of them previously tested in cotton rats (Prince *et al.*, 2003; Boukhvalova *et al.*, 2006). Moreover, IFN- $\alpha$  has been tested as an adjuvant for influenza vaccines and was demonstrated to potentiate immunity to the virus (Bracci *et al.*, 2006; Tovey *et al.*, 2006). Understanding the mechanisms that determine the tissue-specific induction of type I IFNs by RSV could become decisive at the time of determining the route of immunization for RSV vaccines.

We analysed type I IFN production through Mx mRNA expression in the spleen, as this organ mounts a specific immunological response to RSV and no viral replication was detected in this organ (Richter *et al.*, 2005). Interestingly, the pattern of Mx1 and Mx2 mRNA expression in the spleen after infection with RSV A/Long and A/A2 reflected exactly the pattern of mRNA expression seen in the lung, with A/A2 having the strongest induction on day 1 and A/Long showing stronger expression on day 4 p.i. These results suggest that antigen-presenting cells draining from the lung, but not from the turbinate, are most likely those secreting type I IFN and activating Mx expression in the spleen.

RSV reinfections in humans are frequent, and their severity in general decreases with each subsequent infection but in some cases is comparable to the first encounter (Henderson *et al.*, 1979). The implications of multiple reinfections on RSV-induced type I IFN production have not been evaluated previously. In the cotton rat model of RSV reinfection, it has been demonstrated that abortive replication takes place in immune animals (Boukhvalova *et al.*, 2007). In our study, viral gene expression (NS1 is the most sensitive marker of viral persistence) was detected even 4 days after secondary challenge. Importantly, abortive viral replication was followed by transient induction of type I IFN and Mx gene expression in the lung and this coincided with the pathology described previously during secondary infection in the cotton rat (Prince *et al.*, 1999). More importantly, we showed for the first time that both abortive viral replication and type I IFN production were enhanced when the time between consecutive RSV infections was prolonged, clearly indicating a decay in the immunity to RSV.

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