

SHORT COMMUNICATION

Replication of *Bovine respiratory syncytial virus* in murine cells depends on type I interferon-receptor functionality

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***Bovine respiratory syncytial virus (BRSV)* is able to counteract the alpha/beta interferon (IFN- α/β)-mediated antiviral response for efficient replication in a host-specific manner. Mice models have been developed for experimental infection with human, but not bovine, respiratory syncytial virus strains. Here, it is shown that BRSV can replicate efficiently on primary cell cultures derived from type I IFN receptor-deficient, but not from wild-type, IFN-competent, mice. However, BRSV infection was not enhanced in mice devoid of the type I IFN receptor. These results show that type I IFN is a major host-range determinant for infection at the cellular level, but that other factors control virus replication and pathology *in vivo*.**

Human respiratory syncytial virus (HRSV) and *Bovine respiratory syncytial virus (BRSV)* are two closely related viruses that infect the respiratory tract of young children and calves, respectively (Larsen, 2000; Collins *et al.*, 2001). HRSV and BRSV are the major cause of bronchiolitis and pneumonia in infants and calves, resulting in substantial economic losses. They belong to the genus *Pneumovirus*, family *Paramyxoviridae*, and are enveloped viruses with a single-, negative-strand RNA genome of about 15 kb that encodes 11 proteins.

Alpha/beta interferons (IFN- α/β) represent the first line of host defence against viruses, as both IFN- α and IFN- β trigger immediate innate antiviral activities (Conzelmann, 2005). All members of the IFN- α/β family have a single common receptor, IFN- α/β R, composed of two chains (IFNAR1 and IFNAR2). Binding of IFN- α/β to its receptor results in a signalling cascade leading to the formation of a protein complex (STAT1, STAT2 and IRF9) named IFN-stimulated factor 3 (ISGF3) that translocates to the nucleus. ISGF3 binds to DNA sequences (IFN-stimulated response elements or ISRE) present in the promoters of hundreds of genes and promotes their transcription. The sensors triggering IFN production include members of the Toll-like receptor (TLR) family present at the cell surface or in endosomal compartments, namely TLR3, TLR4, TLR7, TLR8 and TLR9 (Jefferies & Fitzgerald, 2005). Signalling through TLRs leads to IRF3, IRF7 and NF- κ B activation, factors controlling IFN- α/β gene transcription (Moynagh, 2005).

Respiratory syncytial virus (RSV) has been reported to be a poor inducer of IFN- α/β and partially resistant to its antiviral activity, indicating that this virus has evolved strategies to overcome the IFN system in order to establish a productive infection (Conzelmann, 2005). However, the capacity of HRSV to inhibit production of IFN- α in epithelial cells varies among virus strains (Schlender *et al.*, 2005). Recombinant deletion mutants of RSV demonstrated that the two non-structural proteins NS1 and NS2 cooperatively suppress the induction of IFN- α/β and the IFN-mediated antiviral response (Schlender *et al.*, 2000; Bossert *et al.*, 2003; Spann *et al.*, 2004). It has been determined that NS1 and NS2 block activation of IRF3 in infected cells (Bossert *et al.*, 2003) and that NS2 downregulates STAT2 expression (Ramaswamy *et al.*, 2004, 2006; Lo *et al.*, 2005). Furthermore, the host range of RSV infection depends upon the

ability of NS1 and NS2 to neutralize the IFN- α/β response of the host cell (Bossert & Conzelmann, 2002; Valarcher *et al.*, 2003). NS proteins of BRSV might have evolved with the bovine host to optimally counteract bovine-cell antiviral responses.

HRSV replicates at low levels in the respiratory tract of rodents and these animals do not exhibit overt respiratory-tract disease, even if weight loss is observed when high titres of virus are used for inoculation. However the absence of a functional type I IFN pathway does not result in increased virus replication *in vivo*, indicating that IFN is not the sole host range-limiting factor (Johnson *et al.*, 2005). Previous trials to infect mice with BRSV have been poorly reported, probably because of the inefficiency of this infection. However, recently, Almeida *et al.* (2004) have detected BRSV replication at low levels after experimental inoculation of wild-type BALB/c mice. We therefore wondered whether BRSV replication in mice could depend on its ability to escape from the host IFN response.

To address this issue, we took advantage of mice deficient in a functional type I IFN receptor (Müller *et al.*, 1994). We derived primary cells from type 1 IFN receptor-competent (IFN- α/β R^{+/+}) or -deficient (IFN- α/β R^{-/-}) mice. IFN- α/β R^{-/-} and IFN- α/β R^{+/+} cells were isolated from either lung or muscle tissue of 1-day-old pups and passaged once in Eagle's minimal essential medium (Gibco) containing 10 % gamma-irradiated fetal calf serum before BRSV infection. Primary cell cultures were plated on six-well plates and infected with BRSV at an m.o.i. of 10⁻² for 2 h at 37 °C with the BRSV Maryland strain grown on bovine turbinate cells. We monitored the propagation of BRSV on IFN- α/β R^{-/-} or IFN- α/β R^{+/+} cells by immunostaining with anti-F mAbs (Serotec) and a secondary fluorescein isothiocyanate-conjugated anti-mouse Ig (P.A.R.I.S.). Mock- or BRSV-infected cells were fixed with paraformaldehyde (4 %) at 0, 4, 8 and 12 days post-infection. As shown in Fig. 1(a), BRSV propagated on IFN- α/β R^{-/-} mouse-derived lung cells as efficiently as on bovine turbinate cells, but no virus was detected on IFN- α/β R^{+/+} cells. At 8 days post-infection, about 50 % of the cell monolayer was infected, and all of the cells were infected at 12 days post-infection. The formation of syncytia was revealed by staining cell nuclei with the fluorescent dye Hoechst 33342 (Fig. 1a). Viral titres were determined to be 1.5±0.5×10³ p.f.u. ml⁻¹ at 8 days post-infection with IFN- α/β R^{-/-} cells, which are equivalent to viral titres obtained with bovine-cell infections (data not shown). Only traces of virus were detected in IFN- α/β R^{+/+} cells infected with BRSV at 8 days post-infection or later. Similar results were obtained when using cells derived from muscles (data not shown) and when using two other BRSV strains, A2Gelfi and W2, that were isolated from calves (Valarcher *et al.*, 2000) [results obtained with A2 strain are shown in Fig. 1(b)]. All of the virus strains used in this study were able to replicate on Madin–Darby bovine kidney (MDBK) cells (Fig. 1b), which are IFN-competent (Schlender *et al.*, 2000; Bossert & Conzelmann, 2002).

As BRSV did not replicate on IFN- α/β R^{+/+} cells, it is expected that BRSV NS proteins are unable to block either IFN- α/β synthesis in murine cells or IFN-induced antiviral pathways. Cells derived from IFN- α/β R^{-/-} mice have lost the capacity to respond to IFN-induced stimuli, but might be still able to synthesize IFN- α/β . We thus analysed the secretion of type I IFN in IFN- α/β R^{-/-} cells infected with BRSV. Type I IFN was monitored by a biological assay (Riffault *et al.*, 1996). As shown in Table 1, IFN was detected in the supernatants of IFN- α/β R^{-/-} cells infected

with BRSV at 8 and 12 days post-infection, but not in IFN- α/β R^{+/+} infected cells. These results showed clearly that BRSV was unable to inhibit IFN synthesis in murine cells after infection. Besides, BRSV induced a higher level of IFN- α/β in lung primary-cell cultures, where it also replicated more rapidly than in muscle primary-cell culture. These findings point towards a positive correlation between virus replication and IFN- α/β induction.

The data presented here show clearly that BRSV can replicate on IFN-incompetent murine primary cells as efficiently as on bovine cells, the virus escaping from the innate antiviral response. Furthermore, IFN was synthesized in IFN- α/β R^{-/-} cells infected with BRSV, showing that BRSV was unable to block IFN synthesis in these cells. The NS1 and NS2 proteins were shown to block the synthesis of IFN (Schlender *et al.*, 2000; Spann *et al.*, 2004). Our results suggest strongly that bovine NS1 and NS2 proteins are unable to interact with murine cellular factors involved in the IFN-activation pathway. The resulting establishment of antiviral mechanisms could explain, at least in part, the poor ability of BRSV to infect mice.

To investigate the factors limiting virus infection in mice, we first aimed to select an appropriate BRSV strain. For that purpose, we compared in BALB/c mice the Maryland BRSV strain with a field strain, 3761, isolated from infected calves and never passaged in cell culture (Gilles Meyer, ENVT). Mice were infected intranasally with 10⁴ p.f.u. Maryland or 500 p.f.u. isolate 3761 at day 0 and sacrificed at various time points up to 23 days post-infection. Weight and clinical symptoms (ruffled fur) were recorded daily. Virus replication in lung was determined by using semi-quantitative RT-PCR to amplify the nucleoprotein gene [primer sequences as described by Boxus *et al.* (2005)] on RNA samples normalized for their β -actin mRNA content. We did not observe symptoms of disease or weight loss, and recorded little (if any) inflammatory infiltrate in bronchoalveolar lavage (data not shown). However, semi-quantitative PCR showed some level of virus replication in lung, peaking at day 5 after infection and disappearing after 8 days. Interestingly, isolate 3761 replicated more strongly and for a longer period of time (up to day 12) than the Maryland strain (data not shown). We therefore decided to infect IFN- α/β R^{+/+} and IFN- α/β R^{-/-} mice with the BRSV 3761 field isolate. We performed two independent experiments with four mice per genetic status and time point. Neither the level of virus replication nor the kinetics of replication were altered significantly by the absence of a functional IFN-signalling pathway (Fig. 2). Besides, both IFN- α/β R^{+/+} and IFN- α/β R^{-/-} mice failed to show clinical symptoms (data not shown). By using the same mouse strains, similar findings were obtained previously with HRSV (Johnson *et al.*, 2005). These and our data point to more complex mechanisms than type I IFN controlling virus replication and pathology *in vivo*.

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References

- Almeida, R. S., Domingues, H. G., Coswig, L. T., D'Arce, R. C. F., de Carvalho, R. F. & Arns, C. W. (2004).** Detection of bovine respiratory syncytial virus in experimentally infected balb/c mice. *Vet Res* **35**, 189–197.
- Bossert, B. & Conzelmann, K.-K. (2002).** Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells. *J Virol* **76**, 4287–4293.
- Bossert, B., Marozin, S. & Conzelmann, K.-K. (2003).** Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. *J Virol* **77**, 8661–8668.
- Boxus, M., Letellier, C. & Kerkhofs, P. (2005).** Real time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. *J Virol Methods* **125**, 125–130.
- Collins, P. L., Chanock, R. M. & Murphy, B. R. (2001).** Respiratory syncytial virus. In *Fields Virology*, 4th edn, pp. 1443–1485. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.
- Conzelmann, K.-K. (2005).** Transcriptional activation of alpha/beta interferon genes: interference by nonsegmented negative-strand RNA viruses. *J Virol* **79**, 5241–5248.
- Jefferies, C. A. & Fitzgerald, K. A. (2005).** Interferon gene regulation: not all roads lead to Tolls. *Trends Mol Med* **11**, 403–411.
- Johnson, T. R., Mertz, S. E., Gitiban, N., Hammond, S., Legallo, R., Durbin, R. K. & Durbin, J. E. (2005).** Role for innate IFNs in determining respiratory syncytial virus immunopathology. *J Immunol* **174**, 7234–7241.
- Larsen, L. E. (2000).** Bovine respiratory syncytial virus (BRSV): a review. *Acta Vet Scand* **41**, 1–24.
- Lo, M. S., Brazas, R. M. & Holtzman, M. J. (2005).** Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. *J Virol* **79**, 9315–9319.
- Moynagh, P. N. (2005).** TLR signalling and activation of IRFs: revisiting old friends from the NF- κ B pathway. *Trends Immunol* **26**, 469–476.
- Müller, U., Steinhoff, U., Reis, L. F. L., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. & Aguet, M. (1994).** Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918–1921.
- Ramaswamy, M., Shi, L., Monick, M. M., Hunninghake, G. W. & Look, D. C. (2004).** Specific inhibition of type I interferon signal transduction by respiratory syncytial virus. *Am J Respir Cell Mol Biol* **30**, 893–900.
- Ramaswamy, M., Shi, L., Varga, S. M., Barik, S., Behlke, M. A. & Look, D. C. (2006).** Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. *Virology* **344**, 328–339.
- Riffault, S., Eloranta, M. L., Carrat, C., Sandberg, K., Charley, B. & Alm, G. (1996).** Herpes simplex virus induces appearance of interferon-alpha/beta-producing cells and partially interferon-alpha/beta-dependent accumulation of leukocytes in murine regional lymph nodes. *J Interferon Cytokine Res* **16**, 1007–1014.

Schlender, J., Bossert, B., Buchholz, U. & Conzelmann, K.-K. (2000). Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize alpha/beta interferon-induced antiviral response. *J Virol* **74**, 8234–8242.

Schlender, J., Hornung, V., Finke, S. & 7 other authors (2005). Inhibition of Toll-like receptor 7- and 9-mediated alpha/beta interferon production in human plasmacytoid dendritic cells by respiratory syncytial virus and measles virus. *J Virol* **79**, 5507–5515.

Spann, K. M., Tran, K. C., Chi, B., Rabin, R. L. & Collins, P. L. (2004). Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected]. *J Virol* **78**, 4363–4369.

Valarcher, J.-F., Schelcher, F. & Bourhy, H. (2000). Evolution of bovine respiratory syncytial virus. *J Virol* **74**, 10714–10728.

Valarcher, J.-F., Furze, J., Wyld, S., Cook, R., Conzelmann, K.-K. & Taylor, G. (2003). Role of alpha/beta interferons in the attenuation and immunogenicity of recombinant bovine respiratory syncytial viruses lacking NS proteins. *J Virol* **77**, 8426–8439.

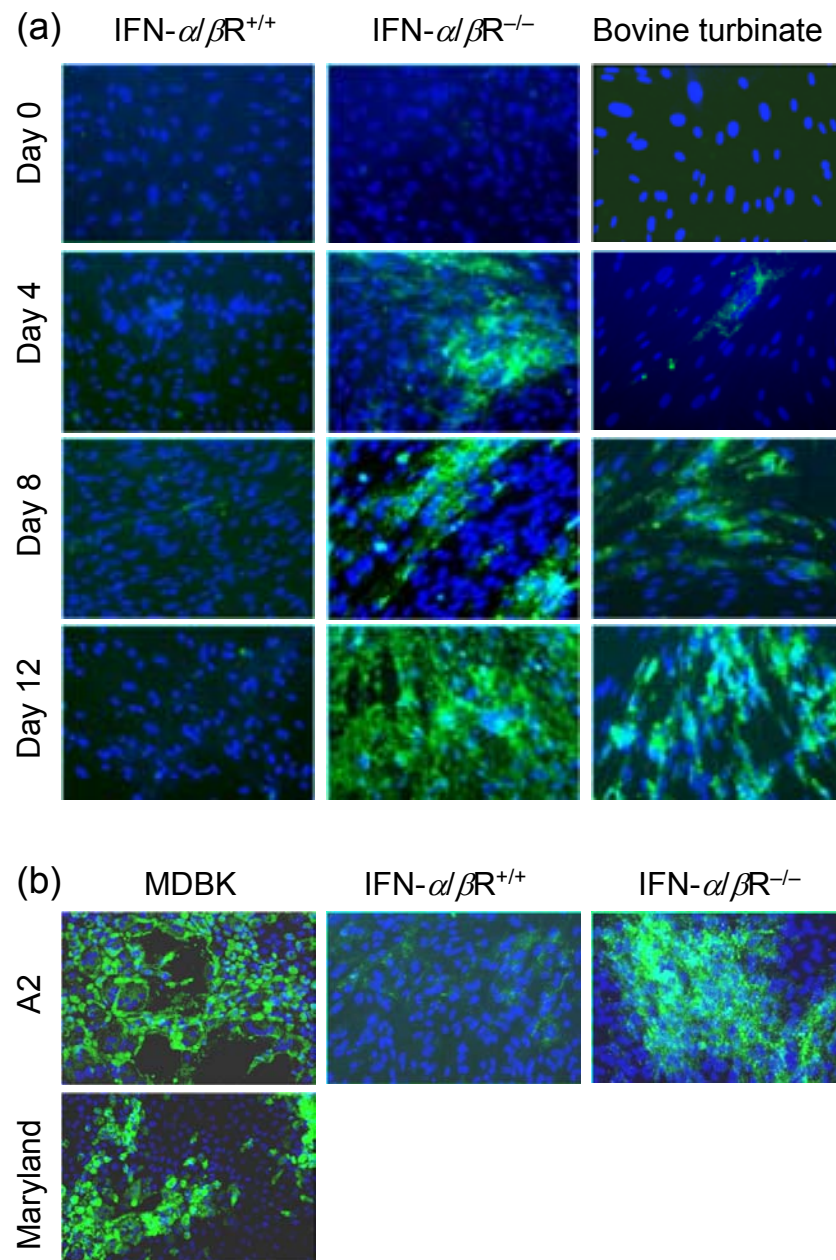


Fig. 1. (a) Propagation of BRSV strain Maryland on bovine turbinate, IFN- α/β R^{+/+}- and IFN- α/β R^{-/-}-derived primary-cell cultures. BRSV replication was visualized by immunostaining with anti-F mAbs at 0, 4 and 8 days post-infection, as indicated (left panels), and cell nuclei were stained with Hoechst 33342 dye. (b) Infection of IFN-competent bovine MDBK cells, mouse IFN- α/β R^{+/+}- and IFN- α/β R^{-/-}-derived primary-cell cultures by the BRSV A2 and Maryland strains. The presence of virus was revealed as in (a). Original magnification $\times 400$.

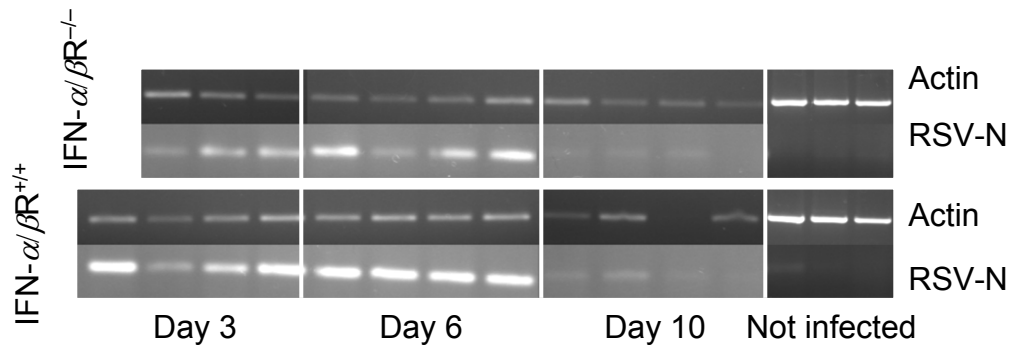


Fig. 2. Infection of IFN- α/β R^{+/+} and IFN- α/β R^{-/-} mice with BRSV field isolate 3761. Mice were infected intranasally with 500 p.f.u. virus at day 0 and sacrificed at day 3, 6 or 10 post-infection. Virus replication in lung was determined by using semi-quantitative RT-PCR to amplify the nucleoprotein gene from RNA samples normalized for their β -actin mRNA content. Individual mouse PCR results are shown (four mice per group and time point).

Table 1. Induction of type I IFN by BRSV in IFN- α/β R^{+/+}- and IFN- α/β R^{-/-}-derived mouse cells

IFN- α/β titres were monitored by a biological assay using the L929 murine cell line with vesicular stomatitis virus as challenge virus. An IFN- α/β reference calibrated against an international standard was included in each assay to calculate sample IFN- α/β titres (IU ml⁻¹).

Infection	IFN- α/β R ^{+/+}		IFN- α/β R ^{-/-}	
	Lung	Muscle	Lung	Muscle
Mock	0	0	0	0
BRSV Maryland				
4 days	0	0	0	0
8 days	0	0	160	6.5
12 days	0	0	480	91