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Orf virus inhibits interferon stimulated gene expression and modulates the JAK/STAT signalling pathway

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ABSTRACT

Interferons (IFNs) play a critical role as a first line of defence against viral infection. Activation of the Janus kinase/signal transducer and activation of transcription (JAK/STAT) pathway by IFNs leads to the production of IFN stimulated genes (ISGs) that block viral replication. The *Parapoxvirus, Orf virus* (ORFV) induces acute pustular skin lesions of sheep and goats and is transmissible to man. The virus replicates in keratinocytes that are the immune sentinels of skin. We investigated whether or not ORFV could block the expression of ISGs. The human gene *GBP1* is stimulated exclusively by type II IFN while *MxA* is stimulated exclusively in response to type I IFNs. We found that *GBP1* and *MxA* were strongly inhibited in ORFV infected HeLa cells stimulated with IFN-γ or IFN-α respectively. Furthermore we showed that ORFV inhibition of ISG expression was not affected by cells pretreated with adenosine N1-oxide (ANO), a molecule that inhibits poxvirus mRNA translation. This suggested that new viral gene synthesis was not required and that a virion structural protein was involved. We next investigated whether ORFV infection affected STAT1 phosphorylation in IFN-γ or IFN-α treated HeLa cells. We found that ORFV reduced the levels of phosphorylated STAT1 in a dose-dependent manner and was specific for Tyr701 but not Ser727. Treatment of cells with sodium vanadate suggested that a tyrosine phosphatase was responsible for dephosphorylating STAT1-p. ORFV encodes a factor, ORFV057, with homology to the vaccinia virus structural protein VH1 that impairs the JAK/STAT pathway by dephosphorylating STAT1. Our findings show that ORFV has the capability to block ISG expression and modulate the JAK/STAT signalling pathway.

1. Introduction

Interferons (IFNs) are a multifunctional family of cytokines that play a critical role as a first line of defence against viral infection. Type I IFNs α/β are induced in virus-infected cells by engagement of viral molecules, in particular nucleic acids, with pattern recognition receptors (Takeda and Akira, 2005; Kawai and Akira, 2007). Most human cell types can produce type I IFNs including keratinocytes that have evolved as immune sentinels within skin (Debenedictis et al., 2001; Liu et al., 2012; Nestle et al., 2009). These cytokines induce an antiviral state in virus infected and neighbouring cells through autocrine or paracrine mechanisms. IFN-γ is produced by T lymphocytes, natural killer cells and plasmacytoid DC that are recruited to skin by inflammatory mediators released from infected cells (Schroder et al., 2004; Debenedictis et al., 2001). IFN-γ has immunostimulatory as well as immunomodulatory roles in innate and adaptive immunity and like type 1 IFNs is able to inhibit viral replication directly.

The anti-viral state involves the upregulation of numerous interferon-stimulated genes (ISGs) that inhibit various stages of viral replication (MacMicking, 2012; Schoggins et al., 2011; Sadler and Williams, 2008; Liu et al., 2012). Type I IFNs induce ISGs through the interferon stimulated response element (ISRE) while IFN-γ induces ISGs through the interferon-gamma activated sequence (GAS) (Takaoka and Yanai, 2006). Both responses lead to the upregulation of hundreds of effector molecules, many of which inhibit and kill viruses and most of these effectors are stimulated by both responses (Schoggins et al., 2011; Liu et al., 2012). The receptors for IFN-α/β and IFN-γ signal through Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways.

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Type I IFN induced signalling initially results in the phosphorylation of the receptor-associated kinases JAK1 and tyrosine kinase 2 (Tyk2) that phosphorylate STAT1 and STAT2, respectively. Phosphorylated STAT1 and STAT2 form a heterodimer that translocates to the nucleus forming the heterotrimeric transcription factor complex IFN-stimulated genes factor 3 (ISGF3) with IFN regulatory factor (IRF)-9 that induces ISGs via the ISRE (Takaoka and Yanai, 2006; Audsley and Moseley, 2013). In comparison the JAK/STAT signalling pathway mediated by IFN-γ does not involve Tyk2 nor STAT2 but involves the phosphorylation of the transcriptional factor STAT1 that homodimerises and translocates to the nucleus where it binds to GAS, inducing the upregulation of ISGs (Takaoka and Yanai, 2006). In addition, Type I IFN signalling is also mediated through the p38 mitogen activated protein (MAP) kinase pathway (Halfmann et al., 2011).

The importance of the induction of ISGs as part of the host's anti-viral defences is highlighted by the fact that many viruses have evolved strategies to overcome the JAK/STAT signalling pathway so as to inhibit the production of the IFN effector responses. A number of viruses inhibit the JAK/STAT pathway induced by IFN-α/β however, many inhibit the JAK/STAT pathway induced by both type I and type II IFNs (Audsley and Moseley, 2013). Many viruses are known to target STAT1. The poxvirus *Vaccinia virus*, targets the activated form of STAT1 using a phosphatase that is part of the virion structure (Hakes et al., 1993; Najarro et al., 2001). *Hepatitis C virus* upregulates protein phosphatase 2A that impairs IFN-α induced anti-viral activity through inhibition of STAT1 tyrosine phosphorylation (Shanker et al., 2013). Measles virus V protein blocks JAK1 mediated phosphorylation of STAT1 (Caignard et al., 2007) and tick-borne flavivirus blocks phosphorylation of Tyk2 and JAK1 (Best et al., 2005). *Mumps virus* and *Newcastle disease virus* also target STAT1 by proteasomal degradation (Kubota et al., 2001; Huang et al., 2003). A number of other viruses target both STAT1 and STAT2 (Audsley and Moseley, 2013). HCV core protein blocks the JAK/STAT signalling pathway by upregulating cellular SOCS3 (Bode et al., 2003).

Orf virus (ORFV) is the prototype species of the *Parapoxvirus* genus of the *Poxviridae* family that induces acute pustular skin lesions in sheep and goats and is transmissible to man (Haig and McInnes, 2002; Fleming et al., 2015). The virus replicates in keratinocytes and epithelial cells of the oral mucosa. ORFV lesions normally persist for about 6–8 weeks. There is currently nothing known about the effects of ORFV on the induction of ISGs. We show here that ORFV has the capability to inhibit the transcriptional activation of guanylate binding protein (*GBP1*) by IFN-γ and the transcriptional activation of *MxA* by IFN-α. Furthermore we show that this could be as a consequence of ORFV reducing the levels of phosphorylated STAT1 in virus infected cells and our data suggests that this is related to a viral structural protein.

2. Materials and methods

2.1. Cells

HeLa cells were maintained in DMEM supplemented with fetal bovine serum (FBS) and penicillin/streptomycin/kanamycin (PSK). Primary lamb testis (LT) cells were maintained in minimum essential medium (MEM) (GIBCO, Invitrogen) and supplemented with FBS at 10% for growth and 5% for culture maintenance. LT cells were supplemented with PSK solution. HeLa cells and LT cells were incubated at 37 °C in a humidified 7% CO_2 atmosphere.

2.2. Virus

ORFV strain NZ2 (Robinson et al., 1982) was propagated in LT cells.

2.3. Inhibition of the IFN response: quantitative RT-PCR

HeLa cells, in 6-well plates were infected with ORFV at an MOI of 10 for 30 min on ice followed by 1 h at 37 °C. Cells were then treated with IFN-γ or IFN-α. Each assay was performed in duplicate and repeated 3 times. Cells were lysed for total mRNA extraction (RNeasy, Qiagen) and cDNA synthesis (SuperScript III, Invitrogen). Transcript analysis was carried out using Power-Mix SYBR Green (Applied Biosystems). Primers used: MxA (forward) 5′-TTC AGC ACC TGA TGG CCT ATC-3′, MxA (reverse) 5′-TGG ATG ATC AAA GGG ATG TGG-3′, hGBP1 (forward) 5′-CAC AGG CAA ATC CAT CCT GA-3′, hGBP1 (reverse) 5′-GCA CAC ACC ACA TCC AGA TT-3′, GAPDH (forward) 5′-CTC TGC TGA TGC CCC CAT GTT C-3′, GAPDH (reverse) 5′-GGT GGT GCA GGA GGC ATT GCT G-3′. MxA primer sequences are described in Jorns et al. (2006), GBP1 primer sequences were obtained from qPrimerDepot and GAPDH primer sequences are described in Wise et al. (2007). All primers were synthesised by Life Technologies.

2.4. Antibodies and cytokines

Donkey anti-goat IRDye 800CW, donkey anti-rabbit IRDye 800CW and donkey anti-mouse IRDye 680RD were obtained from Li-Cor, Biosciences. Mouse anti-STAT1 endogenous, mouse anti-STAT1 phosphorylated (tyrosine 701), mouse anti-STAT1 phosphorylated (serine 727) were obtained from BD Biosciences. Rabbit-anti-mouse-HRP was obtained from Dako Cytomation. Goat anti-actin was obtained from Santa Cruz. Rabbit-anti-goat-HRP and mouse-anti-FLAG-M2-HRP were obtained from Sigma–Aldrich. Rabbit anti-ORFV119 was manufactured by Mimotopes. Goat-anti-rabbit-HRP was obtained from Life Technologies. Monoclonal purified mouse antibody and rabbit-anti-GFP were obtained from Abcam. Polyclonal purified rabbit antibody and human IFN-α and human IFN-γ were obtained from GIBCO, Invitrogen.

2.5. Modulation of the IFN signalling pathway

HeLa cells were infected with ORFV in 6-well plates as described above. Each assay was performed in duplicate and repeated at least 3 times. After 30 min incubation at 4 °C, 1 ml of medium was added and cells incubated at 37 °C for 30 min. In all assays IFN (in 400 μl PBS) was added 60 min after virus was first added to cells. Cells were washed with PBS after 30 min of IFN treatment. Following this wash step, 1 ml of medium containing 10% FBS was added to the cells for the remainder of the assay. In all assays the addition of IFN to cells was taken as time-point 0. At the completion of the assay, medium was removed and cells washed twice with PBS. Cells were then removed from the wells using sterile rubber scrapers, washed with 1 ml of PBS before transfer to 2 ml microfuge tubes. Cells were centrifuged at 3000 × *g* for 3 min at 4 °C. Cell pellets were re-suspended in ice-cold PhosphoSafe (Novagen) extraction reagent by aspiration and incubated for 15 min on ice with vortexing 4 times for 30 s. Lysates were then centrifuged at 10,000 × *g* for 3 min at 4 °C and supernatants stored at −80 °C for Western blot analysis.

Where HeLa cells were pretreated with sodium orthovanadate (Na_3VO_4) (Sigma–Aldrich), cells were incubated with 100 mM (final concentration) Na_3VO_4 for 4 h prior to infection with ORFV. Where HeLa cells were treated with adenosine N1-oxide (ANO) (Sigma–Aldrich) cells were incubated with ANO 10 μg/ml (final concentration) for 6 h and then infected with ORFV.

2.6. Cloning

The ORFV gene *ORFV057* that encodes the polypeptide ORFV057 was amplified from plasmid pOV56 of ORFV strain NZ2 (Mercer et al., 1987) using the polymerase chain reaction

(PCR) and custom made DNA primers 057 N-term 5′-CCGCGCTAGCGCCACCATGGGCGATAAGAGCGAGTGGTAC and 057 C-term 5′-CGCTCTAGATTACTTGTCATCGTCGCTCTTGTAGTCGGAC-GGCGAGTCGCAGACGAACAT.

PCR reactions were carried out as follows: 200–500 ng template DNA, 1 unit Pwo (Roche), 1 unit Pwo buffer, 200 mM dNTPs, 200 mM of both N and C term primers, 5 μl DMSO, 5 mM $MgSO_4$ (final vol 50 μl). A BioRad iCycler was used for amplification of DNA using the following settings: 95 °C for 5 min, then 30 cycles of 95 °C for 1 min, 62 °C for 1 min, 72 °C for 45 sec. The PCR product was purified from agarose using the QIA quick Gel extraction kit (Qiagen), digested with *Sma*I and *Xba*I, dephosphorylated and ligated into the vector pEGFP-CI (Clontech) to produce an EGFP-057 fusion. The plasmid was transformed in competent *E. coli* JM110.

2.7. Transfection

Cells were seeded at 1×10^6 (6-well plates) and incubated O/N. FuGene-HD (Promega) and 3 μg of DNA were then added to medium containing 10% FCS and incubated for 15 min at R/T. The ratio of FuGene-HD (μl):DNA (μg) was 3:1. After washing the cells in PBS the transfection reagent:DNA:medium mix in 300 μl/well was added to cells. Transfected cells were incubated for 24 h at 37 °C in 7% CO_2. The transfection efficiency was determined by enhanced green fluorescent protein (EGFP) detection.

2.8. Western blot analysis

Cell lysates were added to SDS-PAGE loading buffer at a 1:1 ratio (total vol 20 μl) and analysed on an SDS-PAGE (8–12%). Molecular weight markers used were Precision Protein Plus All Blue (BioRad) for fluorescent detection. Proteins were separated using the Bio Rad Mini Protean 3-cell system and transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Bioscience). Following washing in PBS, membranes were dried, re-hydrated in PBS and transferred into Odyssey blocking buffer for 60 min at R/T. Primary antibodies were added to the membrane and incubated at R/T for 1–1.5 h. Membranes were washed in Odyssey wash buffer. Membranes were incubated with secondary antibodies at R/T for 1 h. Protein bands were visualised by fluorescence on the Odyssey Fc imaging system. Protein bands were quantitated using Image Studio software (Li-Cor) and normalised to actin.

2.9. Statistical analyses

All statistical analyses were performed using a Student's *t*-test with *p*-values less than 0.05 considered significant.

3. Results

3.1. ORFV infection results in inhibition of IFN stimulated gene expression

The production of ISGs by host cells is a critical mechanism to inhibit viral replication early in infection. We examined the ability of ORFV to inhibit ISG expression. The gene *GBP1* was used as a marker for type II ISGs, as it is upregulated by IFN-γ but not induced by type I IFNs (Ostler et al., 2014). The gene *MxA* is a sensitive marker of type I IFN activity and is stimulated exclusively through the ISRE1 (Holzinger et al., 2007).

HeLa cells are permissive for ORFV replication (Diel et al., 2010) and are responsive to IFN with the induction of ISGs (Najarro et al., 2001; Chang et al., 2004). HeLa cells were infected with ORFV and stimulated with either IFN-γ or IFN-α. RNA was extracted at 6.5 h post-IFN stimulation. The levels of specific mRNAs were determined by quantitative RT-PCR. The results showed that ORFV infection caused a significant reduction in the levels of GBP1 mRNA induced by IFN-γ of 70% (Fig. 1a) and MxA mRNA induced by IFN-α of 95% (Fig. 1b).

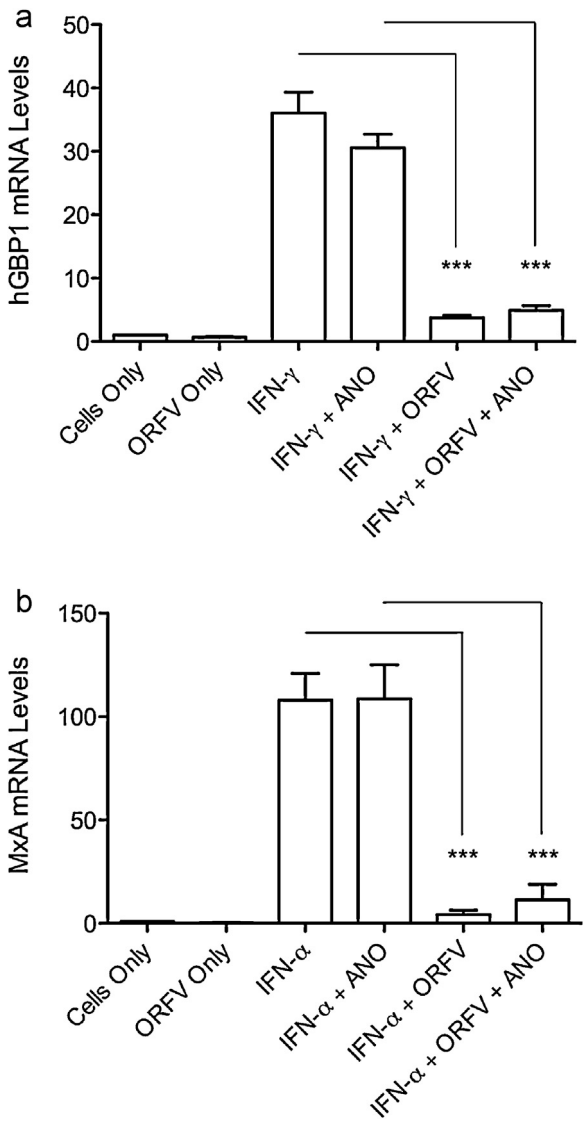

Fig. 1. ORFV inhibits the induction of IFN stimulated genes. (A) ORFV inhibits induction of the IFN-γ induced gene hGBP1. HeLa cells, at 10^6 cells/well, were pretreated with or without adenosine N_1-oxide (ANO) for 6 h prior to infection. Cells were then infected at an MOI of 10. Cells were then stimulated with IFN-γ at a final concentration 0.0025 μg/ml. Cells were incubated for 6.5 h post-IFN treatment at which time RNA was extracted and analysed by qRT-PCR using primers to detect GAPDH and hGBP1. (B) ORFV inhibits IFN-α induction of MxA. HeLa cells, at 2×10^6 cells/well, were pretreated with or without ANO as described above and with virus infected at an MOI of 10. Cells were stimulated with IFN-α at a final concentration 0.01 μg/ml. Cells were incubated for 6.5 h post-IFN treatment at which time RNA was extracted and analysed by qRT-PCR using primers to detect GAPDH and MxA. Asterisks indicate results that are significantly different (***$p < 0.001$; Student's *t*-test). The data are shown as mean ± SE where $n = 3$ (combined data from 3 repeated assays) and are relative to GAPDH.

Several viruses inhibit ISGs by structural proteins incorporated into the virion particle and others by new viral gene expression. We examined both of these possibilities by inhibiting ORFV mRNA translation with the inhibitor adenosine N1 oxide (ANO). ANO has been shown to inhibit poxvirus mRNA translation due to selective incorporation of the adenosine analogue into viral mRNA and is a potent inhibitor of poxvirus replication whilst having no reported effects on eukaryotic cells (Kane and Shuman, 1995). The quantity

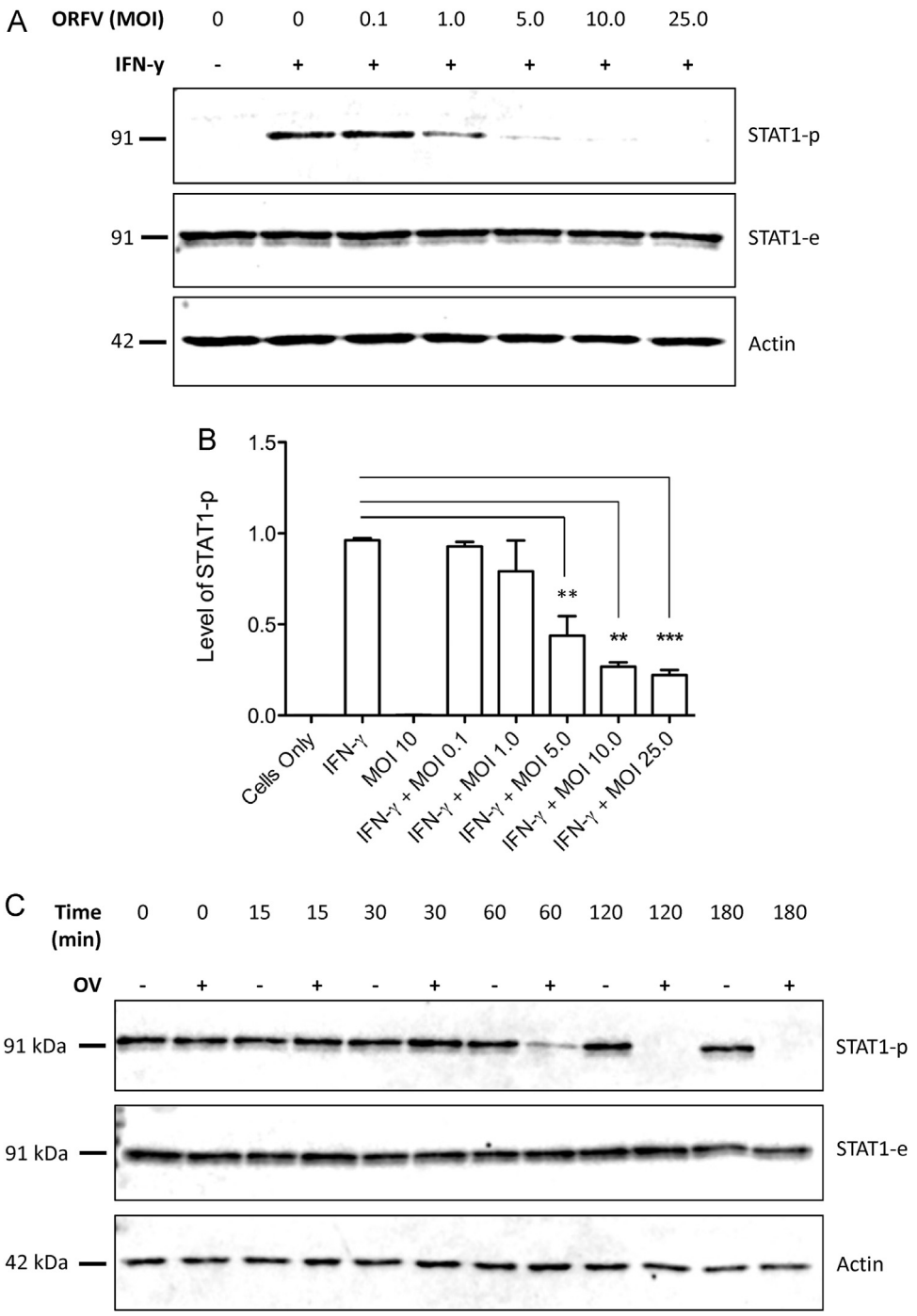

Fig. 2. ORFV infection results in a dose-dependent dephosphorylation of STAT1 in IFN-γ treated cells. (A) HeLa cells, at 10^6 cells/well, were infected with ORFV over a range of MOIs. IFN-γ was added at a final concentration of 0.0025 μg/ml at 60 min post-infection. After 90 min IFN-γ treatment, cell lysates were analysed by Western blotting. Proteins were detected with antibodies against phosphorylated STAT1 (Tyr701) (STAT1-p), endogenous STAT1 (STAT1-e) and actin. Proteins were visualised by fluorescence on the Odyssey Fc imaging system (Li-Cor). (B) Protein bands were quantitated from 5 repeated assays using Image Studio software (Li-Cor) and normalised to actin. The data are shown as mean ± SE where $n = 5$. Asterisks indicate results that are significantly different (**$p < 0.01$, ***$p < 0.001$; Student's t-test). (C) ORFV-induced dephosphorylation of STAT1 occurs rapidly. HeLa cells, at 10^6 cells/well, were infected with ORFV at an MOI of 10 (OV). IFN-γ was added at a final concentration of 0.0025 μg/ml. Cells were lysed at the time-points shown post-IFN-γ treatment and lysates analysed by Western blotting. Proteins were detected with monoclonal mouse antibodies against STAT1-p, STAT1-e and actin and visualised as described above.

of ANO was optimised by inhibition of expression of an ORFV early gene ORFV119 (shown in Fig. 6). We showed that pre-treatment of cells with ANO did not affect the ability of ORFV to inhibit ISGs induced with IFN-γ or IFN-α (Fig. 1a and b) suggesting that a protein that is not produced by new viral gene synthesis was inhibiting the induction of ISGs.

3.2. *ORFV infection results in a dose-dependent reduction in phosphorylation of IFN-γ stimulated STAT1*

We hypothesised that ORFV was inhibiting the induction of ISGs by impairing the JAK/STAT signalling pathway. Many viruses either dephosphorylate STATs, degrade STATS, sequester STATs or block

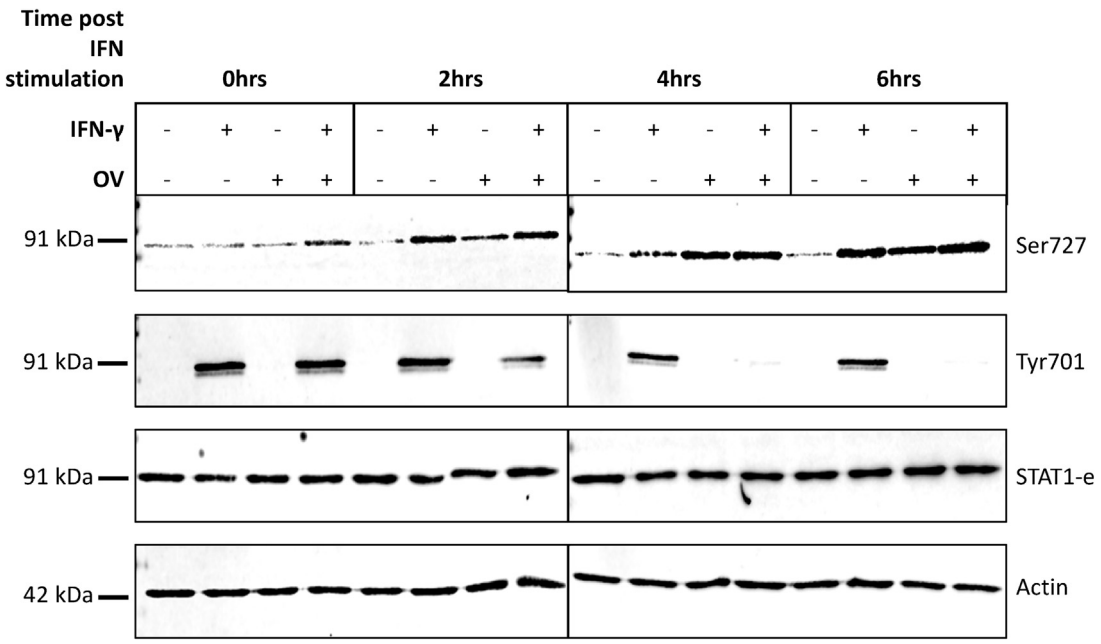

Fig. 3. ORFV does not dephosphorylate STAT1 at Ser727 in IFN-γ treated cells. HeLa cells, at 10^6 cells/well, were infected with ORFV at an MOI of 25 (OV). IFN-γ was added at a final concentration of 0.0025 μg/ml at 60 min post-infection. Cells were lysed at the time points shown post-IFN stimulation and lysates analysed by Western blotting. Proteins were detected with antibodies against STAT1-p (Tyr701) and STAT1-p (Ser727), STAT1-e and actin. Protein bands were visualised by fluorescence.

STAT phosphorylation (Audsley and Moseley, 2013; Najarro et al., 2001. Activation of the JAK/STAT pathway by IFN-γ stimulates a phosphorylation cascade, resulting in the phosphorylation of STAT1 residue Tyr701. Phosphorylation of STAT1 at Tyr701 by JAK is critical for the activation of this factor and regulates the dimerisation of STATs as an essential prerequisite for subcellular localisation to the nucleus and DNA binding (Haspel and Darnell, 1999; Ito et al., 1999; Decker and Kovarik, 2000). IFN-γ also stimulates the phosphorylation of STAT1 at Ser727 within the C-terminus by a mechanism that does not involve JAK. Preliminary experiments were conducted to examine the dose responsiveness of HeLa cells to IFN-γ. The results showed that levels of phosphorylated STAT1 at Tyr701 increased in a dose-dependent manner with increasing amounts of IFN-γ (data not shown) and that STAT1 was not phosphorylated in the absence of IFN-γ. Very low amounts of IFN-γ (0.00125 μg/ml) were shown to stimulate the JAK/STAT pathway and this increased in proportion to the amount of IFN-γ present.

We then investigated whether ORFV affected the phosphorylation of STAT1 at Tyr701 in cells activated with IFN-γ. HeLa cells were infected with ORFV over a range of MOIs (0.1–25 pfu/ml) and then stimulated with IFN-γ. The results showed that ORFV reduced the levels of phosphorylated STAT1 at Tyr701 in a dose-dependent manner, whilst the levels of endogenous STAT1 remained unchanged (Fig. 2a). At an MOI of 5, the levels of phosphorylated STAT1 were reduced significantly ($p < 0.01$) compared with uninfected IFN-γ stimulated cells (Fig. 2b). At an MOI of 10 and 25 the levels of activated STAT1 were reduced by around 4 and 5-fold respectively, compared with uninfected IFN-γ stimulated cells ($p < 0.01$).

Next we investigated the time post-IFN-γ stimulation at which ORFV exerted its effects on STAT1 phosphorylation. HeLa cells were infected with ORFV at an MOI of 10 and stimulated with IFN-γ. The results showed that the reduction in phosphorylation of STAT1 by ORFV occurred rapidly following IFN stimulation of infected cells. Reduced levels of phosphorylated STAT1 were observed at 60 min post-IFN stimulation when compared with uninfected IFN-γ treated cells (Fig. 2c). As above, the endogenous levels of STAT1 remained unchanged. The results show that reduced phosphorylation of STAT1 begins at between 30 and 60 min post-IFN stimulation and continues beyond 3 h post-IFN treatment.

3.3. ORFV does not reduce phosphorylation of STAT1 at Ser727 in IFN-γ stimulated cells

The phosphorylation of STAT1 at the C-terminal Ser727 is known to increase the transcriptional activity of STAT1 and differentially affects specific target gene expression where cells are stimulated with IFN-γ (Kovarik et al., 2001). In addition, phosphorylation of STAT1 at Ser727 is induced by mitogen-activated protein kinases (MAPKs) (Kovarik et al., 1999; Decker and Kovarik, 2000). MAPKs are involved in stress responses and prime STATs prior to induction of the JAK/STAT signalling pathway by IFN-γ. We investigated whether ORFV infection affected STAT1 phosphorylation at Ser727. HeLa cells were infected with ORFV at an MOI of 10 and stimulated with IFN-γ before lysates were analysed with antibodies specific for phosphorylation of either Tyr701 or Ser727 of STAT1. Cells were incubated for 0, 2, 4 and 6 h post-IFN treatment. Surprisingly the results showed increasing levels of STAT1 phosphorylated at Ser727 in all infected cells from 2 h post-IFN-γ stimulation. There was no detectable reduction in Ser727 phosphorylation levels over the duration of the assay (Fig. 3). It was also evident that ORFV alone was increasing STAT1 phosphorylated at Ser727 but not Tyr701. Consistent with previous data, ORFV affected STAT1 phosphorylation at Tyr701 at 2 h post-IFN stimulation and STAT1 phosphorylation at Tyr701 was undetectable at 4 and 6 h post-IFN treatment (Fig. 3). The levels of endogenous STAT1 remained unchanged over the 6 h infection period.

3.4. ORFV infection results in reduced phosphorylation of STAT1 in IFN-α stimulated cells

Stimulation of the JAK/STAT pathway by IFN-α leads to the phosphorylation of STAT1 and STAT2 and the formation of a heterodimer. The heterodimer interacts with IRF9 in the nucleus to produce the activation complex ISGF3. As for IFN-γ induced signalling, STAT1 phosphorylation at Tyr701 is a critical event in

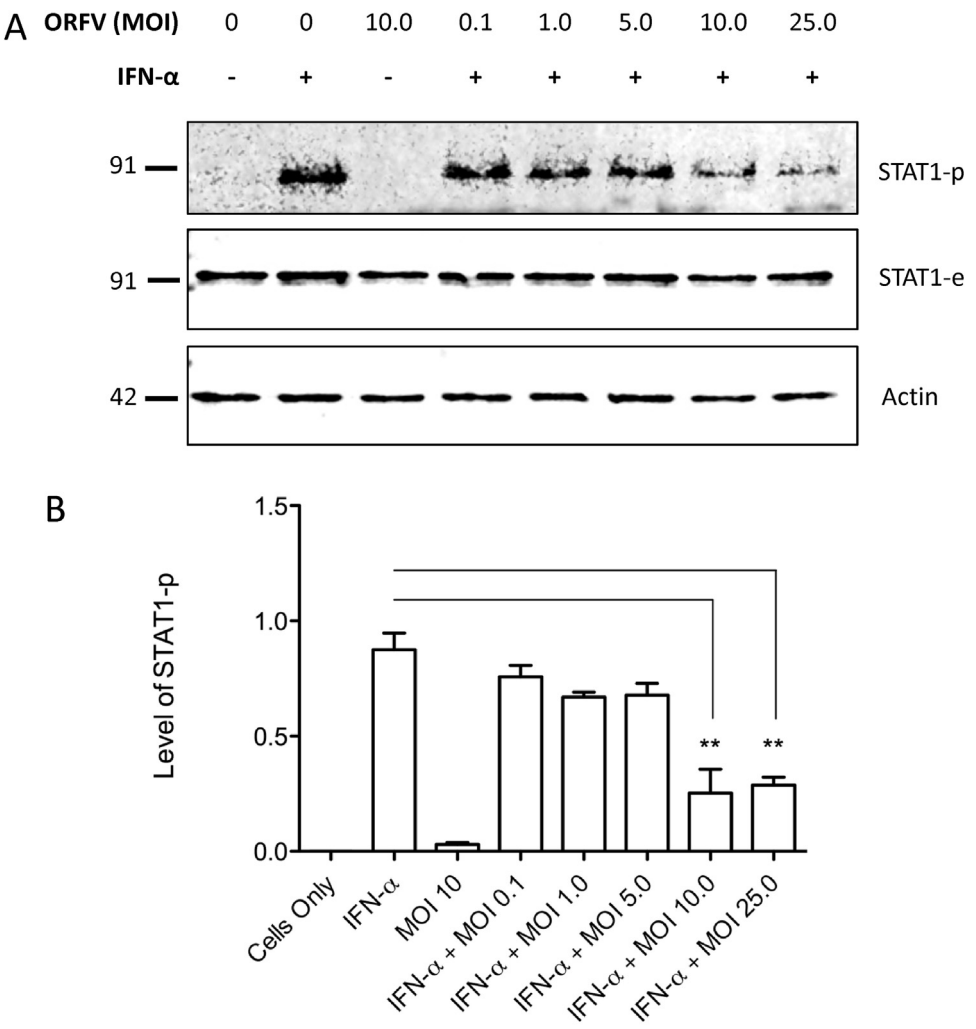

Fig. 4. ORFV infection results in dephosphorylation of STAT1 in IFN-α treated cells. (A) HeLa cells, at 10^6 cells/well, were infected with ORFV over a range of MOIs. IFN-α was added at a final concentration of 0.01 μg/ml at 60 min post-infection. After 90 min IFN treatment cell lysates were analysed by Western blotting. Proteins were detected with antibodies against STAT1-p (Tyr701), STAT1-e and actin. Protein bands were visualised by fluorescence (Odyssey Fc). (B) Protein bands were quantitated for 3 repeated assays using Image Studio software and normalised to actin. The data are shown as mean ± SE where $n = 3$. Asterisks indicate results that are significantly different (**$p < 0.01$; Student's *t*-test).

the activation of the JAK/STAT pathway by IFN-α. We investigated whether ORFV affected STAT1 phosphorylation at Tyr701 in IFN-α stimulated cells. HeLa cells were infected with ORFV over a range of MOIs (0.1–25). Cells were then stimulated with IFN-α. The results showed that ORFV was again able to modulate the phosphorylation levels of STAT1 when cells were stimulated with IFN-α (Fig. 4). This effect was most pronounced at an MOI of 10 and 25 ($p < 0.01$) (Fig. 4b). The ability of ORFV to reduce phosphorylation of STAT1 (Tyr701) was not significant at lower MOIs of 1.0 and 5.0. As above the levels of endogenous STAT1 remained unchanged regardless of the MOI, demonstrating that STAT1 phosphorylation is reduced in response to viral infection.

3.5. Sodium vanadate blocks the effects of ORFV on STAT1 phosphorylation

Previous assays had shown that levels of phosphorylated STAT1 (Tyr701) reduced during viral infection while endogenous (total) levels of STAT1 remained unchanged. This suggested that the virus was not degrading STAT1-p, but affecting the phosphorylation levels of this molecule by a mechanism such as dephosphorylation. In order to investigate this possibility HeLa cells were pretreated with Na_3VO_4, a specific inhibitor of tyrosine phosphatases (Huyer et al., 1997). The results showed that in the absence of Na_3VO_4, ORFV was able to reduce phosphorylated STAT1 to undetectable levels, whereas in the presence of Na_3VO_4 phosphorylated STAT1-p (Tyr701) levels remained unchanged (Fig. 5).

3.6. Adenosine N_1-Oxide does not inhibit the ability of ORFV to reduce phosphorylation of STAT1

Pretreating HeLa cells with ANO suggested that an ORFV structural protein was involved in inhibiting ISG expression. To test whether inhibition of ORFV protein translation also affected its ability to reduce STAT1-p levels at Tyr701, cells were treated with ANO. Prior to infection, HeLa cells were pretreated with ANO for 6 h and then infected with ORFV at an MOI of 10 and stimulated with IFN-γ. The results showed that ANO did not inhibit the ability of ORFV to reduce the levels of phosphorylated STAT1 in IFN-γ treated cells, whilst it clearly blocked the synthesis of the ORFV early gene product ORFV119 (Fig. 6). Furthermore, cells pretreated with ANO showed minimal changes in the levels of endogenous STAT1 when compared with untreated cells. The results suggested that an ORFV structural protein is able to modulate STAT1 phosphorylation.

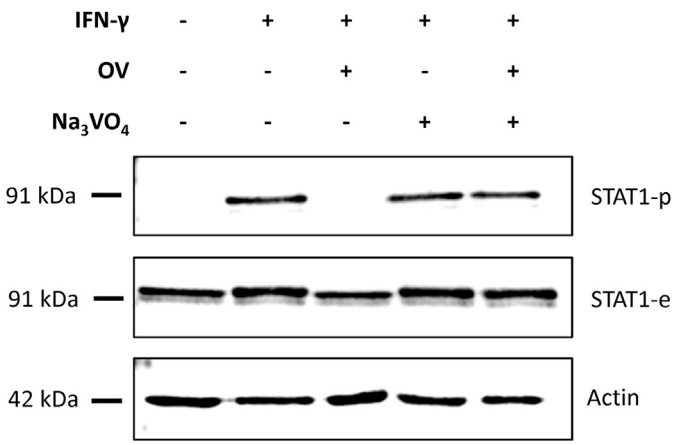

Fig. 5. Sodium vanadate blocks dephosphorylation of STAT1 by ORFV. HeLa cells, at 10^6 cells/well, were treated with Na_3VO_4 (final concentration 100 mM) for 4 h at 37 °C and then infected with ORFV at an MOI of 10 (OV). Cells were treated with IFN-γ at a final concentration of 0.0025 μg/ml at 60 min post-infection. Cells were incubated for 90 min post-IFN stimulation and cell lysates analysed by Western blot. Proteins were detected with antibodies against phosphorylated STAT1-p (Tyr701), STAT1-e and actin. Protein bands were visualised by fluorescence. The result shown is representative of 3 repeated assays.

3.7. ORFV057 reduces STAT1 phosphorylation

The ORFV gene *ORFV057* encodes a putative polypeptide (ORFV057) that shares 41% amino acid identity with the vaccinia virus encoded factor VH1 and contains a conserved phosphatase motif (Delhon et al., 2004; Mercer et al., 2006). VH1 is known to block ISG expression by dephosphorylating STAT1 (Najarro et al., 2001). We investigated whether or not the putative tyrosine phosphatase ORFV057 could dephosphorylate activated STAT1. HeLa cells were transfected with pEGFP-057 or pEGFP-C1 (empty vector control) and incubated for 24 h. Transfection efficiencies were determined by cells expressing GFP from three independent repeats. For EGFP-C1, transfection efficiencies were 94%, 86% and 93% with an average of 91% and for pEGFP-057 transfection efficiencies were 85%, 81% and 87%, with an average of 84.3%. Where cells were transfected with pEGFP-057 and treated with IFN-γ, STAT1-p levels were reduced on average by 26.9% ($p = 0.047$) compared with pEGFP-C1 transfected cells treated with IFN- γ (Fig. 7a). It was noted that although the STAT1-p levels of pEGFP-057 (Fig. 7a, lane 6) were reduced compared with the levels of STAT1-p in the empty vector control (Fig. 7a, lane 5), STAT1-p levels in both of these samples were considerably less than the IFN-γ treated non-transfected control cells (Fig. 7a, lane 4). It would appear that the transfection process itself was reducing the levels of total STAT1 in transfected cells compared with untransfected cells and having an effect on STAT1-p expression.

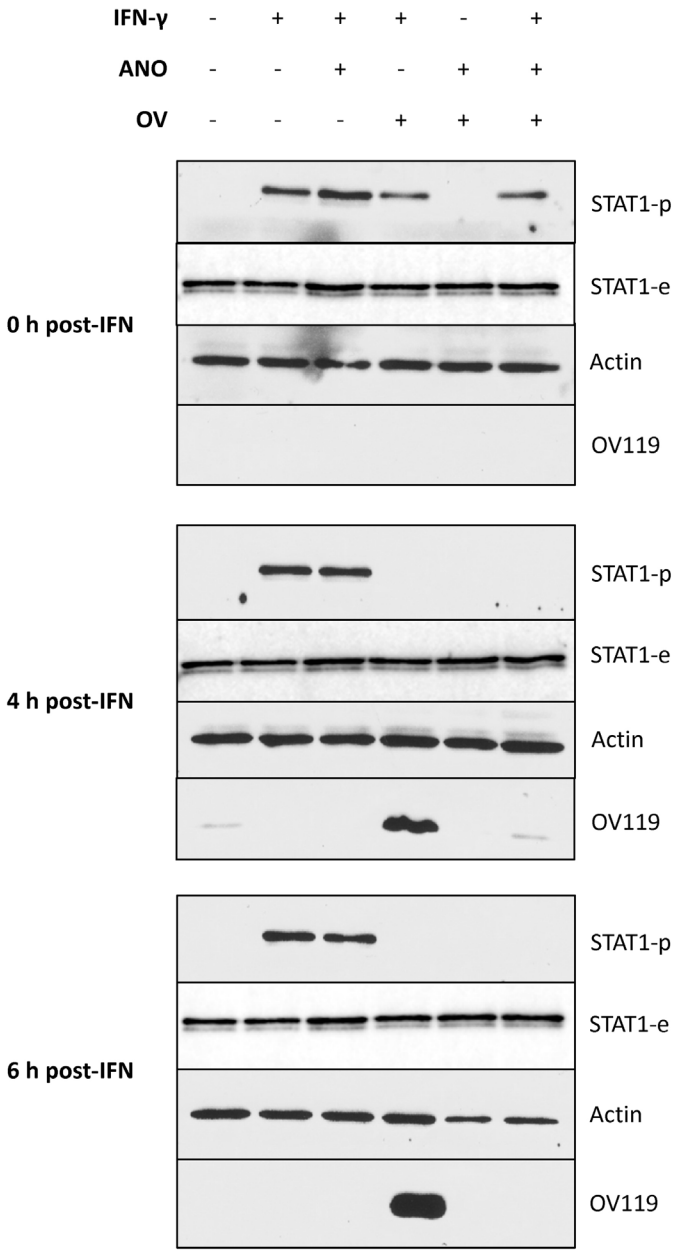

Fig. 6. Inhibition of ORFV induced dephosphorylation of STAT1 with adenosine N_1-Oxide. HeLa cells, at 10^6 cells/well were treated with ANO (final concentration of 10 μg/ml) for 6 h prior to infection with ORFV at an MOI of 10 (OV) and stimulated with IFN-γ at a final concentration of 0.0025 μg/ml at 60 min post-infection. Cells were lysed at 0, 4 and 6 h post-IFN stimulation and lysates analysed by Western blotting. Proteins were detected with antibodies against STAT1-p (tyr701)), STAT1-e, OV119 and actin. Protein bands were visualised by fluorescence. The result shown is representative of 4 repeated assays.

4. Discussion

The induction of IFN effectors through the JAK/STAT signalling pathway is a critical innate response that allows the host to limit or eliminate viral spread before the adaptive responses are mobilised. In the last 15 years many viruses have been reported to inhibit the JAK/STAT pathway through a wide range of mechanisms further highlighting the importance of this pathway to both host defence and virus survival.

ISGs are induced through the JAK/STAT pathway by type I and type II IFNs and through the p38 mitogen-activated protein kinase (MAPK) by type I IFNs (Halfmann et al., 2011). In this study we found that ORFV was able to inhibit the expression of ISGs induced by, both type I and type II IFNs in HeLa cells. We hypothesised that this effect could most likely be explained by modulation of the JAK/STAT1 signalling pathway, since inhibition of ISGs were observed for both type I and type II, IFNs. STAT1 is critical in IFN signalling by both type I and type II IFNs and is targeted by many viruses using diverse mechanisms. Initially we examined the levels of endogenous STAT1 and phosphorylated STAT1 in ORFV infected cells stimulated with IFN-γ or IFN-α. Phosphorylation at Tyr701 is critical for the translocation of this factor to the nucleus and its function. We showed that ORFV was not affecting the endogenous levels of STAT1 but it significantly reduced the levels of STAT1 phosphorylated at Tyr701. Our initial findings which suggested that an ORFV structural protein was involved in inhibiting the induction of

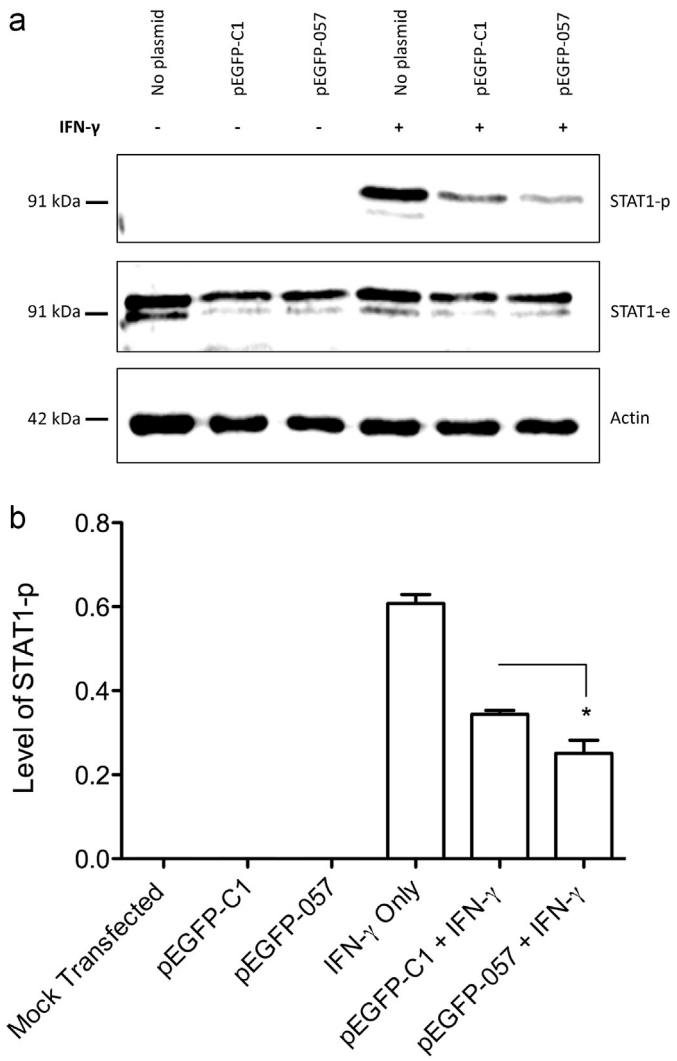

Fig. 7. ORFV057 reduces phosphorylated STAT1 in IFN-treated cells. (A) HeLa cells, at 10^6 cells/well were transfected with FuGene-HD and 3 μg of either pEGFP-C1 control plasmid DNA or pEGFP-057 plasmid DNA. Transfected cells were incubated at 37 °C for 24 h and then stimulated with IFN-γ (final concentration 0.0025 μg/ml) for 2.5 h at which time cell lysates were analysed by Western blotting using antibodies against STAT1-p (Tyr701), STAT1-e and actin. Protein bands were visualised by fluorescence. (B) Protein bands were quantitated from 3 repeated assays using Image Studio software. The data are shown as mean ± SE where $n = 3$. Asterisks indicate results that are significantly different ($^*p < 0.05$; Student's *t*-test).

ISGs was supported by our observations in which the treatment of cells with ANO had no effect on the ability of ORFV to dephosphorylate STAT1. Our observations pointed towards a protein that was either produced by the virus or a cellular protein that was induced by the virus that was either specifically blocking phosphorylation of Tyr701 or specifically dephosphorylating STAT1 at Tyr701. Specific inhibition of tyrosine phosphatases by sodium vanadate was compelling evidence that a tyrosine phosphatase was dephosphorylating STAT1.

Our results show that ORFV infection does not reduce phosphorylation at Ser727 and in fact enhances phosphorylation of this residue and this occurs in IFN-γ treated and untreated cells. In infected cells not treated with IFN-γ this is most likely due to a stress response. It is widely reported that a number of factors including inflammation and virus infection of cells can lead to phosphorylation of STAT1 at residue Ser727 but not Tyr701 through the p38 MAPK pathway (Kovarik et al., 2001). This priming event enhances the transcriptional response once IFN-γ provides the stimulus for tyrosine phosphorylation of STAT1 (Kovarik et al., 1998, 1999; Stoiber et al., 1999). In addition, STAT1 is also phosphorylated at Ser727 by IFN-γ and only recently has the serine kinase chromatin-associated cyclin-dependent kinase 8 been implicated (Bancerek et al., 2013). In this case serine phosphorylation is linked to gene specific transcription. In view of the phosphorylation of STAT1 at Ser727 by various factors it is intriguing that the vaccinia virus VH1 phosphatase has evolved the capability to dephosphorylate this molecule at Ser727 as well as Tyr701 (Najarro et al., 2001). We have found no evidence that this is also the case in ORFV infected cells. Interestingly although IFN-α stimulation of cells also leads to phosphorylation of Ser727 on STAT1 this does not enhance the transcriptional activity of ISGF3 (Kovarik et al., 2001).

In addition to poxviruses, a number of members of other virus families are known to inhibit phosphorylation of STAT1 at Tyr701 that include *Hepatitis C virus* (Shanker et al., 2013), *Measles virus* (Caignard et al., 2007), *Rinderpest virus*, *Hendra virus* and *Nipah virus* (Audsley and Moseley, 2013). As described above this will prevent dimerisation of STAT1 where the signalling pathway is induced by IFN-γ or the formation of the heterodimer (STAT1-STAT2) where the signalling pathway is induced by type 1 IFNs. Many viruses also target STAT1 by other mechanisms such as proteasomal degradation and sequestering STAT1 in cytoplasmic aggregates. In addition, viruses also target STAT2 using similar mechanisms to that used against STAT1 and many viruses target both STAT1 and STAT2 (Audsley and Moseley, 2013). VH1 has also been shown to inhibit the phosphorylation of STAT2 induced by type 1 IFNs (Mann et al., 2008). We have not investigated whether ORFV inhibits the phosphorylation of STAT2.

Our data clearly showed that ISG expression and phosphorylation of STAT1 (Tyr701) induced by both IFN-γ and IFN-α was almost completely inhibited by the ORFV particle and did not involve new viral gene expression in the infected cell. As described above a likely candidate from genetic and bioinformatics analysis of the ORFV genome is *ORFV057*, a gene encoding a putative protein with homology to VH1 that contains a tyrosine phosphatase motif. In addition, this protein is expressed from a late promoter which suggests that it may be packaged in the virion. We showed that ORFV057 has phosphatase activity against STAT1 by transient expression but more rigorous experiments are required to establish if it alone was responsible for the inhibition of ISG expression. It is also possible that ORFV may be inducing a cellular protein that dephosphorylates STAT1.

Apart from VH1, poxviruses have evolved other mechanisms to inhibit the JAK/STAT signalling pathway. *Vaccinia virus* produces a secreted IFN decoy receptor that acts exogenously preventing IFN receptor binding and signalling (Seet et al., 2003). *Myxoma virus* selectively disrupts type I IFN signalling in primary human fibroblasts by blocking the tyrosine phosphorylation of the Janus kinase Tyk2 thus preventing the subsequent activation of downstream STAT1 and STAT2 but not type II IFN induced activation of Jak1, Jak2 and STAT1 (Wang et al., 2009). This mechanism requires new viral gene expression. Intriguingly ORFV may have evolved a second mechanism to suppress the JAK/STAT pathway by secreting a viral version of interleukin-10 (IL-10) from infected cells (Fleming et al., 1997; Imlach et al., 2002; Haig et al., 2002). Cellular IL-10 inhibits the expression of both IFN-α and IFN-γ induced genes by suppressing tyrosine phosphorylation of STAT1 (Ito et al., 1999). As described above this results in suppression of IFN-induced assembly of STAT factors to specific promoter motifs on IFN-α and IFN-γ inducible genes and is thought to occur by the ability of IL-10 to induce expression of the gene, suppressor of cytokine signalling 3 (SOCS3). ORFV IL-10 could potentially inhibit the JAK/STAT1 pathway, not only in cells in which the virus replicates, but also in immune cells that mediate the Th1-type adaptive responses.

This is the first report that describes inhibition of ISG expression by a parapoxvirus. ORFV has evolved a mechanism to impair the JAK/STAT pathway by dephosphorylating STAT1 in virus-infected cells that does not require new viral gene expression. A candidate gene could be *ORFV057* however further work is required to characterise its functional activities and effects on the JAK/STAT signalling pathway.

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