



# Myxoma virus selectively disrupts type I interferon signaling in primary human fibroblasts by blocking the activation of the Janus kinase Tyk2

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## ABSTRACT

Poxviruses currently are known to disrupt Jak-STAT signal transduction induced by interferon (IFN) through two distinct mechanisms: (1) secreted poxviral IFN decoy receptors that prevent the initiation of IFN signaling from type I or II receptors at the cell surface; and (2) poxviral phosphatase that dephosphorylates STAT1 intracellularly. Here, we report a novel mechanism by which poxviruses can inhibit Jak-STAT signaling in response to type I IFN. Myxoma virus (MV) is a highly species-restricted member of the poxvirus family that infects only rabbits under the natural setting. Interestingly, primary human fibroblasts support a permissive MV infection that is only partially sensitive to the antiviral state induced by type I IFN. In this study we show that when type I IFN is added to primary human fibroblasts following MV infection, the tyrosine phosphorylation of the Janus kinase Tyk2 is specifically blocked, thereby preventing the subsequent activation of downstream STAT1 and STAT2. In stark contrast, type II IFN-induced activation of Jak1, Jak2 and STAT1 remains largely unaffected in MV-infected human fibroblasts. Unlike the de-activation of STAT1 by the poxvirus phosphatase, which is delivered into the cell by the input virions, the Tyk2 inhibition by MV infection requires new viral gene expression. Thus, our study documents a previously unrecognized immune evasion mechanism exploited by a poxvirus to selectively disrupt the type I IFN-Jak-STAT signaling cascade.

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## Introduction

Interferons (IFN), mainly type I (IFN- $\alpha/\beta$ ) and type II IFN (IFN- $\gamma$ ), are part of a powerful ancient defense system that enables multicellular organisms to combat invading viruses (Hengel et al., 2005; Sen, 2001; Takaoka and Yanai, 2006). Binding of type I IFN to its cell surface IFN- $\alpha$  receptor (IFNAR) triggers tyrosine phosphorylation of the Janus kinases Tyk2 and Jak1 followed by tyrosine phosphorylation of the transcription factors STAT1 and STAT2. The activated STAT1 and STAT2 heterodimerize and together with IFN-regulatory factor 9 (IRF-9) migrate to the nucleus to activate the transcription of IFN-stimulated genes (ISGs) through the IFN-stimulated responsive element (ISRE) (Stark et al., 1998; Takaoka and Yanai, 2006). On the other hand, binding of type II IFN to its cell surface IFN- $\gamma$  receptor (IFNGR) activates Jak1 and Jak2 resulting in the tyrosine phosphorylation of only STAT1, but not STAT2. As a result, the tyrosine phosphorylated STAT1 homodimerizes and migrates to the nucleus to

trigger ISG transcription through the  $\gamma$ -activated sequence (GAS) (Samuel, 2001; Stark et al., 1998). Thus, the signal transduction of type I versus type II IFN is mediated distinctly through IFNAR-Tyk2/Jak1-STAT1/STAT2/IRF9-ISRE and IFNGR-Jak1/Jak2-STAT1-GAS, respectively. Consequently, the expression of multiple ISG products such as ISG15, myxovirus resistance (Mx) and 2'-5'-oligoadenylate synthetase (OAS) leads to the establishment of an antiviral state (Katze et al., 2002; Randall and Goodbourn, 2008; Samuel, 2001; Stetson and Medzhitov, 2006).

Given that host immune defenses have co-evolved with viruses (Hengel et al., 2005; Levy and Garcia-Sastre, 2001), all viruses are likely to have evolved at least some countermeasures to block the IFN-Jak-STAT defense pathway as part of their survival strategies (Takaoka and Yanai, 2006; Weber et al., 2004). For example, herpes simplex virus 1 downregulates the expression of the cell surface receptors IFNAR and IFNGR (Chee and Roizman, 2004; Eisemann et al., 2008). Downstream of the IFN receptors, Japanese encephalitis virus and West Nile virus inhibit Tyk2 and Jak1 tyrosine phosphorylation (Guo et al., 2005; Lin et al., 2004) while human cytomegalovirus and adenovirus decrease Jak1 expression (Miller et al., 1999; Shi et al., 2007). Further downstream of the cascade, simian virus 5 and human parainfluenza virus type 2 have been shown to target STAT1 and STAT2 for proteasomal degradation, respectively (Andrejeva et al., 2002). In addition, while human

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metapneumovirus and measles virus suppress the tyrosine phosphorylation of STAT1 and STAT2 (Dinwiddie and Harrod, 2008; Takeuchi et al., 2003), rabies virus phosphoprotein P and Ebola virus block the nuclear translocation of tyrosine phosphorylated STAT1 and STAT2 (Brzozka et al., 2006; Reid et al., 2006). In view of the power of such evolutionary pressures, these instances appear to further emphasize the notion that the IFN-Jak-STAT signaling cascade is a critical interface that hosts and viruses are constantly competing to dominate.

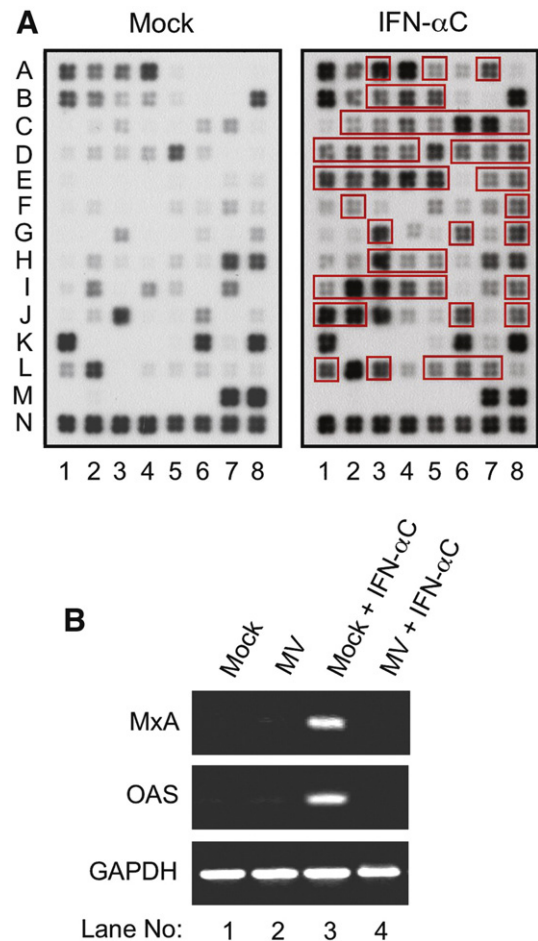
Myxoma virus (MV) is the causative agent of a deadly veterinary disease known as myxomatosis, specifically in the European rabbit (Fenner and Ratcliffe, 1965; Kerr and McFadden, 2002). Interestingly, recent work shows that MV not only productively infects primary human fibroblasts, but also is partially resistant to the antiviral state induced by type I IFN, while it is fully inhibited by type I IFN plus TNF (Wang et al., 2008). However, little is known about how the IFN-Jak-STAT signaling cascade that induces the antiviral state might be perturbed by MV infection. MV is a poxvirus and member of the leporipoxvirus genus (Moss, 2001). Containing large DNA genomes, the poxvirus family members all encode a unique repertoire of immune evasion molecules that systematically subvert key host immune defenses, including chemokine, interleukin, complement, apoptosis and Toll-like receptor signaling (Seet et al., 2003). In particular, poxviruses have been shown to antagonize a wide range of IFN-induced antiviral ISGs. For example, vaccinia virus E3L inhibits the antiviral functions of protein kinase R (PKR), OAS (Katze et al., 2002; Seet et al., 2003) and ISG15 (Guerra et al., 2008), thus showing a multi-pronged immune evasion property for the poxvirus family. However, in terms of subversion of the IFN-Jak-STAT signaling cascade itself, only two distinctly different mechanisms have been described thus far for the poxvirus family. In the first, poxviruses including MV, vaccinia virus, ectromelia virus and camelpox virus express soluble IFN receptor homologs, which act to scavenge IFN ligands and thereby prevent the initiation of IFN signal transduction at the cell surface (Randall and Goodbourn, 2008). In the second, a dual specificity phosphatase, such as the vaccinia VH1 protein, dephosphorylates STAT1 following type II IFN stimulation (Najarro et al., 2001) or both STAT1 and STAT2 following type I IFN treatment (Mann et al., 2008), thereby blocking the IFN-transduced signal at the intracellular STAT level. However, it remains to be demonstrated how other evasion strategies would be employed by the poxvirus family members to subvert the IFN-Jak-STAT pathway following IFN ligation with its receptors at the cell surface.

Here we show that in primary human fibroblasts, MV infection blocks type I IFN-induced tyrosine phosphorylation of the upstream Janus kinase Tyk2, thereby preventing the activation of its downstream transcription factors STAT1 and STAT2. In contrast, type II IFN-induced tyrosine phosphorylation of Jak1, Jak2 and STAT1 is not significantly affected. Thus, we report a novel poxvirus immune subversion mechanism to selectively disrupt the type I IFN-Jak-STAT signaling cascade in virus-infected cells.

## Results

### *MV infection inhibits antiviral ISG expression induced by exogenous type I IFN in primary human fibroblasts*

MV infection has been shown to be partially resistant to the antiviral state induced by type I IFN in CCD-922Sk primary human skin fibroblasts (Wang et al., 2008). Therefore, we began by defining the type I IFN responsiveness of CCD-922Sk fibroblasts. To this end, we stimulated the cells for 4 h with IFN- $\alpha$ C, a member of the type I IFN family, and isolated total cellular RNA in order to analyze ISG expression by use of the IFN- $\alpha$ / $\beta$  response cDNA SuperArray. We observed that, of the total of 96 genes included in the SuperArray format, 50 genes were upregulated by IFN- $\alpha$ C (Fig. 1A and Table 1). In



**Fig. 1.** MV infection inhibits type I IFN-induced antiviral ISG expression in primary human fibroblasts. (A) CCD-922Sk fibroblasts were stimulated with IFN- $\alpha$ C for 4 h and the total cellular RNA was then isolated for ISG induction profiling using the IFN- $\alpha$ / $\beta$  response cDNA SuperArray. The ISGs upregulated by IFN- $\alpha$ C are circumscribed in red lines. (B) CCD-922Sk fibroblasts were infected with MV for 6 h; subsequently, the MV-infected cells were stimulated with IFN- $\alpha$ C for 4 h. The total cellular RNA was then isolated for RT-PCR analysis of the mRNAs of MxA and OAS (isoform 1). GAPDH was used as control.

particular, we noted that the typical antiviral ISGs, such as ISG15, MxA and OAS, were clearly induced following IFN- $\alpha$ C stimulation (Table 1). These results indicate that CCD-922Sk fibroblasts *per se* are capable of mounting the expected type I IFN responses. Importantly, these data imply that MV's partial resistance to type I IFN might be related to the MV inhibition of type I IFN-induced antiviral signaling in CCD-922Sk cells.

To ascertain whether ongoing MV infection might inhibit the antiviral ISG response induced by subsequent exposure to type I IFN, we infected CCD-922Sk fibroblasts with MV for 6 h and then stimulated the infected cells with IFN- $\alpha$ C for 4 h. We then isolated total cellular RNA and employed RT-PCR to analyze the induction of genes MxA and OAS, which were chosen here for the purpose of representing the classical IFN-induced antiviral signaling response (Samuel, 2001). As shown in Fig. 1B, IFN- $\alpha$ C strongly upregulated mRNA expression of MxA (top panel, lane 3) and OAS (middle panel, lane 3) in mock-infected cells. In contrast, the same IFN- $\alpha$ C treatment was unable to induce MxA (Fig. 1B, top panel, lane 4) and OAS (Fig. 1B, middle panel, lane 4) mRNA expression following MV infection. Similar results were obtained from other adult and fetal primary human fibroblasts (data not shown). Collectively, these results show that MV infection inhibits the type I IFN-induced antiviral ISG expression in primary human fibroblasts.

**Table 1**  
Genes upregulated by IFN- $\alpha$ C in primary human fibroblasts

Array position	GenBank access No.	Gene symbol	Expression fold increase
A3	NM_012068	AFT5	1.60
A5	NM_004281	BAG3	7.40
A7	NM_006820	IFI44L	333.84
B3	NM_001755	CBFB	2.67
B4	NM_004064	CDKN1B	2.77
B5	NM_033133	CNP	11.11
C2	NM_006736	DNAJB2	5.02
C3	NM_006304	SHFM1	2.32
C4	NM_005101	ISG15	32.12
C5	NM_002038	G1P3	33.54
C6	NM_002053	GBP1	4.96
C7	NM_004120	GBP2	3.07
C8	NM_000161	GCH1	8.78
D1	NM_002196	H19	8.30
D2	NM_002116	HLA-A	3.99
D3	NM_005514	HLA-B	4.22
D4	NM_002145	HOXB2	2.29
D6	NM_005531	IFI16	4.47
D7	NM_005532	ISG12	77.84
D8	NM_006332	IFI30	34.60
E1	NM_005533	IFI35	21.76
E2	NM_006417	IFI44	21.98
E3	NM_001548	IFIT1	106.15
E4	NM_001547	IFIT2	367.94
E5	NM_001549	IFIT3	55.32
E7	NM_003641	IFITM1	6.35
E8	NM_006435	IFITM2	8.70
F2	NM_000629	IFNAR1	4.12
F8	NM_006764	IRFD2	5.44
G3	NM_002199	IRF2	2.75
G6	NM_001572	IRF7	6.20
G8	NM_006084	IRF9	3.01
H3	NM_017554	PARP14	14.55
H4	NM_001648	KLK3	11.58
H5	NM_005561	LAMP1	2.79
I1	NM_020310	MNT	5.13
I2	NM_002462	MXA	2.18
I3	NM_002463	MX2	49.36
I4	NM_002467	MYC	1.88
I5	NM_002468	MYD88	3.17
I8	NM_002534	OAS1	21.58
J1	NM_002535	OAS2	32.26
J2	NM_003733	OASL	6.67
J6	NM_003690	PRKRA	1.68
J8	NM_002818	PSME2	4.84
L1	NM_005419	STAT2	2.11
L3	NM_003810	TNFSF10	44.08
L5	NM_006074	TRIM22	4.36
L6	NM_021616	TRIM34	3.87
L7	NM_003331	TYK2	5.48

*MV infection inhibits type I IFN-induced nuclear translocation of STAT1 in primary human fibroblasts*

The inhibition of type I IFN-induced expression of antiviral ISGs in MV-infected CCD-922Sk fibroblasts led us to first focus on STAT1, a primary mediator involved in mounting the IFN antiviral signaling response (Randall and Goodbourn, 2008; Takaoka and Yanai, 2006). To ascertain whether or not MV infection inhibited STAT1 nuclear translocation, a hallmark feature of STAT1 activation (Randall and Goodbourn, 2008; Samuel, 2001), we infected CCD-922Sk fibroblasts with MV for 6 h and then treated the MV-infected cells with IFN- $\alpha$ C for 30 min. We employed immunofluorescence microscopy to assess the nuclear accumulation of STAT1 at the single cell level. As expected, STAT1 was distributed predominantly in the cytoplasm under the basal state (Fig. 2, top row), but was overwhelmingly mobilized to the nucleus after the IFN- $\alpha$ C stimulation in mock-infected cells (Fig. 2, second row). In contrast, neither MV infection alone nor IFN- $\alpha$ C treatment of MV-infected cells was able to induce the translocation of STAT1 into the nucleus in the infected cells

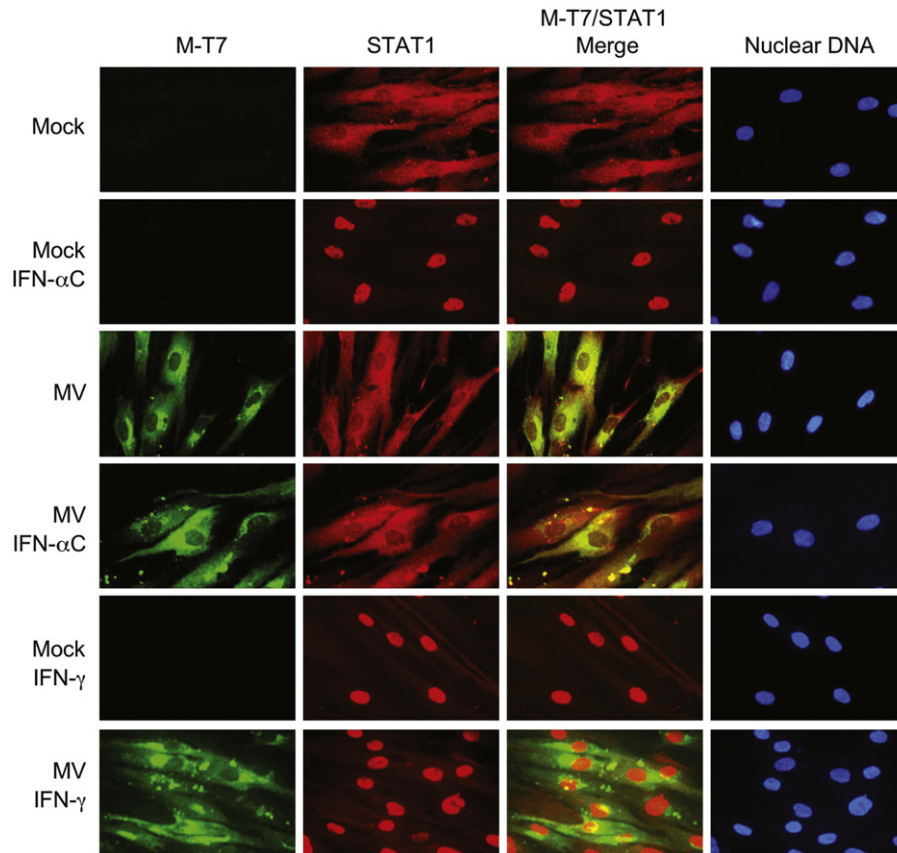
(Fig. 2, third and fourth row, respectively). We confirmed MV infection by immunostaining of the infected cell monolayers for the expression of M-T7, an early MV gene product, as the viral infection marker (Mossman et al., 1996). We observed that the blockade of IFN- $\alpha$ C-induced STAT1 nuclear translocation specifically occurred in M-T7-positive cells (Fig. 2, fourth row). Thus, this result demonstrates an unequivocal correlation between MV infection and impairment in the nuclear mobilization of STAT1 following type I IFN stimulation.

Next, to determine whether or not the MV-mediated inhibition of STAT1 nuclear translocation was limited to type I IFN, we infected CCD-922Sk fibroblasts with MV for 6 h and then stimulated the MV-infected cells with type II IFN for 30 min. As shown in Fig. 2 (fifth row), IFN- $\gamma$  treatment of mock-infected cells mobilized STAT1 into the nucleus. However, unlike IFN- $\alpha$ C, this IFN- $\gamma$ -induced nuclear mobilization of STAT1 was not blocked by MV infection (Fig. 2, bottom row). Taken in *toto*, the above results indicate that MV infection selectively inhibits the STAT1 nuclear translocation induced by type I but not type II IFN in primary human fibroblasts.

*MV infection inhibits type I IFN-induced tyrosine phosphorylation of STAT1 in primary human fibroblasts*

Tyrosine phosphorylation is a key step in STAT1-mediated IFN signal transduction (Randall and Goodbourn, 2008; Stark et al., 1998). Therefore, to determine whether or not MV infection suppressed tyrosine phosphorylation of STAT1, we infected CCD-922Sk fibroblasts with MV for 6 h and then stimulated the MV-infected cells with IFN- $\alpha$ C for 30 min. Subsequently, we conducted immunoprecipitation of STAT1 $\alpha$ , a key functional isoform of STAT1 (Stark et al., 1998), out of the whole cell extracts using an anti-STAT1 $\alpha$  antibody. As revealed by Western blotting using an anti-phosphotyrosine antibody, IFN- $\alpha$ C treatment resulted in a strong band of STAT1 tyrosine phosphorylation in mock-infected cells (Fig. 3A, top left panel, lane 3). In contrast, the same IFN- $\alpha$ C treatment failed to induce STAT1 tyrosine phosphorylation in MV-infected cells (Fig. 3A, top left panel, lane 4). We then examined the impact of MV infection on STAT1 tyrosine phosphorylation following IFN- $\gamma$  stimulation. As expected, IFN- $\gamma$  caused a strong tyrosine phosphorylation signal in mock-infected cells (Fig. 3A, top right panel, lane 3). Significantly, IFN- $\gamma$ -induced STAT1 tyrosine phosphorylation was not inhibited by MV infection (Fig. 3A, top right panel, lane 4).

Of note, it has been shown that certain viruses, such as measles virus and Ebola virus, do not suppress IFN-induced STAT1 tyrosine phosphorylation *per se* but instead prohibit nuclear translocation of the tyrosine phosphorylated STAT1 (Palosaari et al., 2003; Reid et al., 2006). Therefore, we determined the subcellular localization features of the tyrosine phosphorylated STAT1 induced by IFN- $\gamma$  in MV-infected cells. For this purpose, we took advantage of an activation status-specific antibody which detects STAT1 only when Tyr-701 is phosphorylated. As shown by immunofluorescent antibody staining, tyrosine phosphorylated STAT1 was barely discernable in mock-infected CCD-922Sk fibroblasts prior to IFN- $\gamma$  stimulation (Fig. 3B, top row) whereas a strong immunofluorescence signal for tyrosine phosphorylated STAT1 was detected in the nucleus following IFN- $\gamma$  treatment (Fig. 3B, second row). Of note, MV infection itself did not cause any tyrosine phosphorylation of STAT1 (Fig. 3B, third row). Remarkably, the IFN- $\gamma$ -induced nuclear localization of tyrosine phosphorylated STAT1 was unaffected by MV infection (Fig. 3B, bottom row). In contrast, we were unable to detect any immunofluorescence signal for tyrosine phosphorylated STAT1 in either the nucleus or the cytoplasm of MV-infected cells following IFN- $\alpha$ C stimulation (data not shown). This is consistent with the Western blotting data (Fig. 3A, top left panel). Collectively, these results further demonstrate that MV infection only inhibits STAT1 tyrosine phosphorylation initiated by type I IFN but does not interfere with the



**Fig. 2.** MV infection blocks the STAT1 nuclear translocation induced by type I but not type II IFN in primary human fibroblasts. Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN- $\alpha$ C or IFN- $\gamma$  for 30 min as indicated. Double immunofluorescence staining was conducted to visualize the expression of MV early gene M-T7 (green) and the subcellular localization of STAT1 protein (red).

nuclear translocation of tyrosine phosphorylated STAT1 induced by type II IFN.

*MV gene expression is required for inhibition of type I IFN-induced tyrosine phosphorylation of STAT1 in primary human fibroblasts*

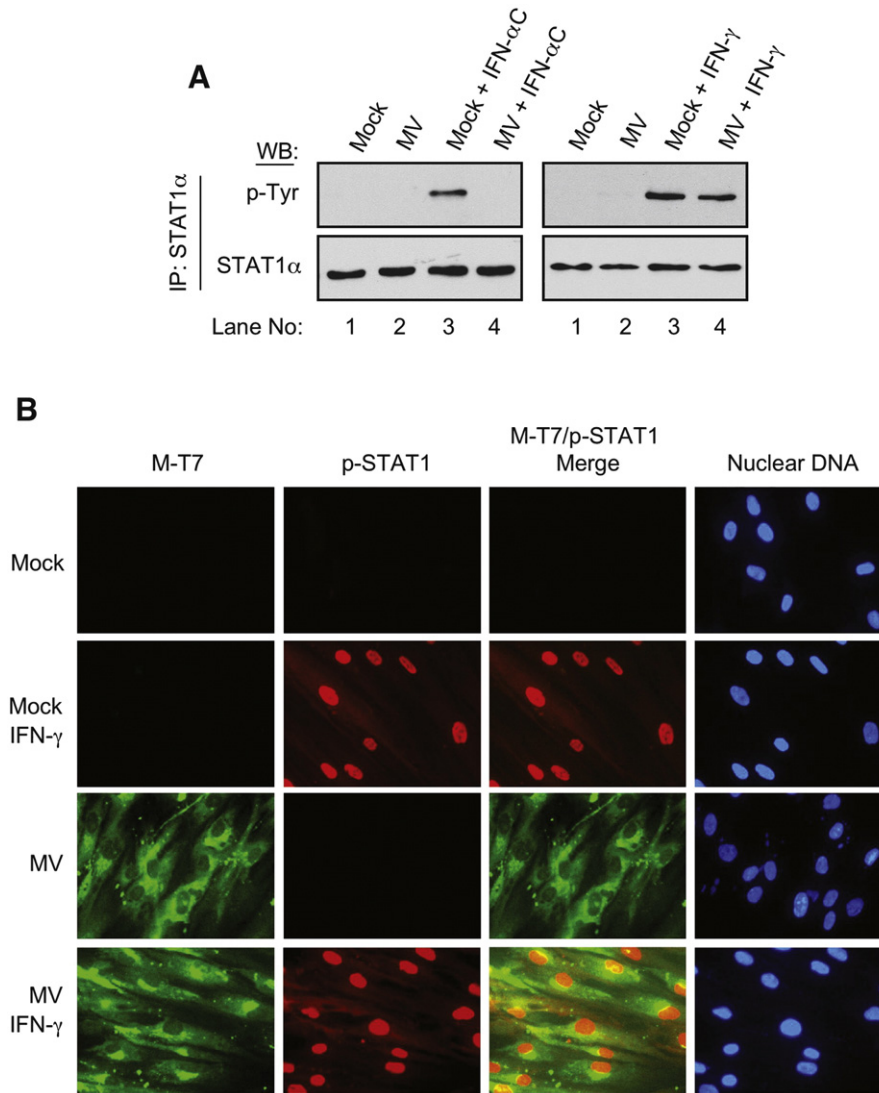
The inhibition of IFN-induced STAT1 tyrosine phosphorylation by vaccinia virus through VH1 phosphatase is a rapid, viral dose-dependent, but viral gene expression-independent process, because the phosphatase is delivered by the input virions. Thus, infection of HeLa or 3T3 cells with vaccinia virus at an MOI of 10 for 1 h to allow for the release of VH1 phosphatase from the virion into the cytoplasm was sufficient for the inhibitory effect on either type I or type II IFN signal transduction (Mann et al., 2008; Najarro et al., 2001). Given that MV I1L (also referred to as M069L) encodes a phosphatase homologous to vaccinia VH1 phosphatase (Mossman et al., 1995a), we infected CCD-922Sk fibroblasts with MV at an MOI of 0.5, 5 and 10 for 1 and 6 h and then challenged the MV-infected cells with IFN- $\alpha$ C for 30 min. We observed that IFN- $\alpha$ C-induced tyrosine phosphorylation of STAT1 was not inhibited by MV at 1 h either at an MOI of 0.5 (Fig. 4A, top panel, lane 4), 5 (Fig. 4A, third panel, lane 4) or 10 (Fig. 4A, fifth panel, lane 4). However, IFN- $\alpha$ C-induced tyrosine phosphorylation of STAT1 was fully suppressed in MV-infected cells at 6 h at either an MOI of 5 (Fig. 4A, third panel, lane 5) or 10 (Fig. 4A, fifth panel, lane 5). Thus, these late appearing inhibitory effects imply that the incoming MV virion components such as I1L phosphatase are unlikely to be responsible for suppressing STAT1 phosphorylation.

Of particular note, Western blot only revealed a minor reduction in tyrosine phosphorylation of STAT1 in the cells infected with MV at an

MOI of 0.5 for 6 h (Fig. 4A, top panel, lane 5). We reasoned that the observed inefficient suppression of STAT1 tyrosine phosphorylation under the low MOI infection condition was due to a reduced number of cells being productively infected. To confirm this, we conducted immunofluorescent antibody staining for both MV infection marker M-T7 and tyrosine phosphorylated STAT1. We found that at an MOI of 0.5, roughly one fourth of the cells were infected, as identified by M-T7 expression (Fig. 4B, second row). As expected, IFN- $\alpha$ C treatment caused a strong nuclear accumulation of tyrosine phosphorylated STAT1 (Fig. 4B, third row). Significantly, we observed that among the IFN- $\alpha$ C-treated cell population, only those that expressed M-T7 showed inhibition of immunofluorescent staining for tyrosine phosphorylated STAT1 (Fig. 4B, bottom row). These data clearly demonstrate that MV expresses or induces a strong suppressor of STAT1 tyrosine phosphorylation following type I IFN stimulation in a manner dependent on MV gene expression.

To examine this further, we prepared UV-inactivated MV that was no longer able to synthesize significant levels of *de novo* viral proteins as indicated by the suppression of synthesis of an early MV protein M-T7 (Fig. 4C, top panel) and was unable to produce infectious MV progeny (data not shown). We then infected CCD-922Sk fibroblasts with the UV-inactivated MV for 6 h and stimulated the infected cells with IFN- $\alpha$ C for 30 min. We observed that unlike replication-competent MV, UV-inactivated MV failed to inhibit IFN- $\alpha$ C-induced tyrosine phosphorylation of STAT1 (Fig. 4D, top panel). These data show that MV gene expression is required for the inhibition of tyrosine phosphorylation of STAT1 in response to type I IFN.

Next, to further dissect whether early or late MV proteins mediate the inhibition of type I IFN signaling, we then performed MV



**Fig. 3.** MV infection inhibits the STAT1 tyrosine phosphorylation induced by type I but not type II IFN in primary human fibroblasts. (A) Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN-αC or IFN-γ for 30 min as indicated. The cell lysates were then immunoprecipitated (IP) with anti-STAT1α antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1α antibodies. (B) Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN-γ for 30 min. Double immunofluorescence staining was conducted to visualize the expression of MV early gene M-T7 (green) and the subcellular localization of tyrosine phosphorylated STAT1 (red).

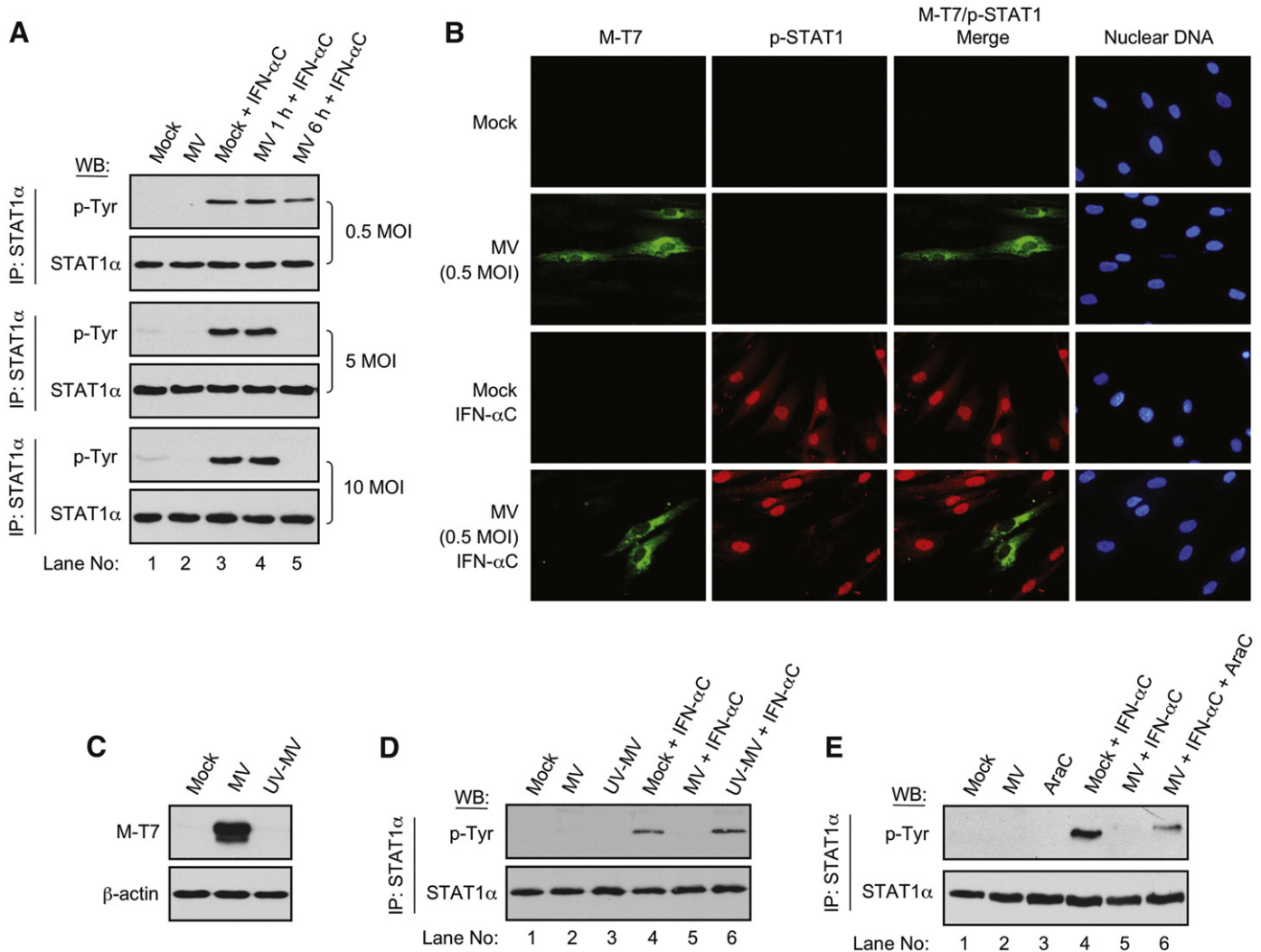
infection in the presence of cytosine arabinoside (AraC), which blocks viral late gene expression but has no inhibitory impact on viral early gene expression (Boshkov et al., 1992). We observed that when MV late gene expression was blocked by AraC, MV infection failed to completely inhibit IFN-αC-induced tyrosine phosphorylation of STAT1 (Fig. 4E, top panel, lane 6). Taken in aggregate, these data suggest that a full-fledged suppression of type I IFN signaling by MV infection in primary human fibroblasts requires a combinatorial action of both MV-encoded early and late gene products.

#### *MV infection does not inhibit type I IFN binding to cell surface IFN-α receptor in primary human fibroblasts*

The first step in IFN signal transduction is the binding of IFN to its cell surface cognate receptors (Randall and Goodbourn, 2008; Stark et al., 1998). Although type I and type II IFN transduce through distinct cell surface receptors, i.e. IFNAR and IFNGR, respectively, both receptor cascades converge downstream on STAT1 (Samuel, 2001; Stark et al., 1998). Thus, the inhibition of IFN-αC-induced, but not IFN-γ-induced, tyrosine phosphorylation of STAT1 in MV-infected

cells suggests that MV infection might mediate a selective block at the type I IFN–IFNAR binding stage. Functional IFNAR is comprised of two subunits, IFNAR1 and IFNAR2 (Stark et al., 1998). As a number of viruses downregulate IFN receptors as a way to subdue IFN antiviral effects (Chee and Roizman, 2004; Joseph and Look, 2001), we therefore sought to first examine the levels of IFNAR expression. To this end, we infected CCD-922Sk fibroblasts with MV for 6 h. The biotin labeling of cell surface proteins was then carried out with Sulfo-NHS-Biotin. Subsequently, the cell extracts from the biotin-labeled cells were prepared for the separate evaluation of IFNAR1 and IFNAR2 by immunoprecipitation utilizing anti-IFNAR1 or anti-IFNAR2 antibodies followed by Western blotting with an HRP-streptavidin conjugate. As revealed in Fig. 5A, there were no apparent differences in either IFNAR1 (top panel) or IFNAR2 (middle panel) expression between mock and MV-infected cells. The specificity of this cell surface biotin labeling was confirmed using an intracellular protein USF2 as a control (Fig. 5A, bottom panel). These data clearly indicate that MV infection does not modulate cell surface IFN-α receptor levels in CCD-922Sk fibroblasts.

To further ascertain whether or not MV infection affected the capacity of the host IFNAR to bind exogenous type I IFN, we then

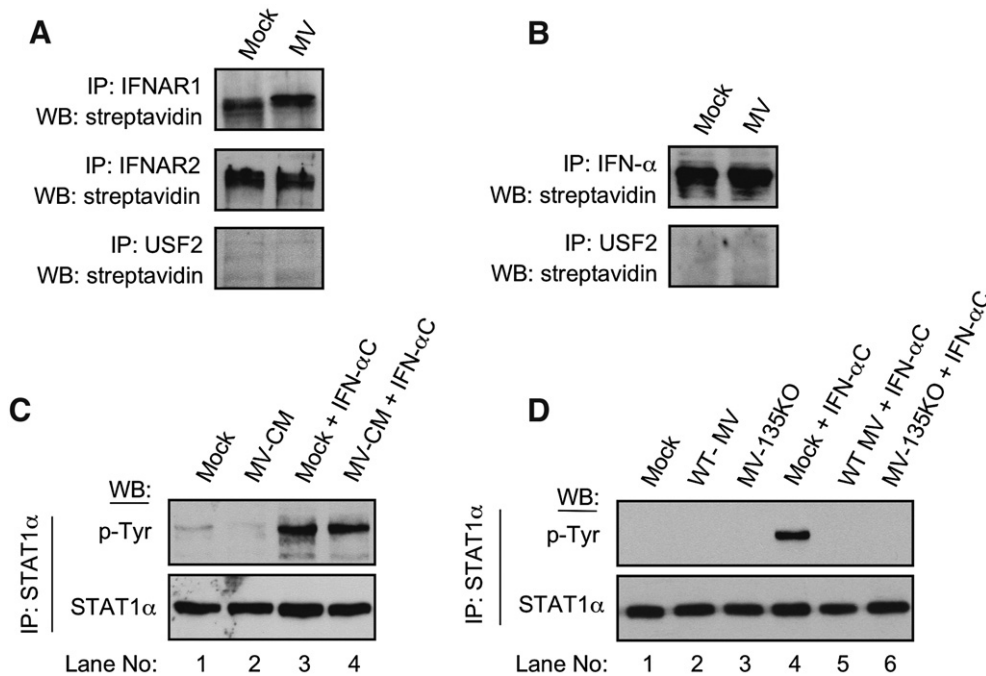


**Fig. 4.** MV gene expression is necessary for the inhibition of the STAT1 tyrosine phosphorylation induced by type I IFN in primary human fibroblasts. (A) CCD-922Sk fibroblasts of mock and MV infections at different MOIs as indicated were left alone or stimulated with IFN- $\alpha$ C for 30 min. The cell lysates were immunoprecipitated (IP) with anti-STAT1 $\alpha$  antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1 $\alpha$  antibodies. (B) CCD-922Sk fibroblasts of mock and MV infection at an MOI of 0.5 were left alone or stimulated with IFN- $\alpha$ C for 30 min. Double immunofluorescence staining was conducted to visualize the expression of MV early gene M-T7 (green) and the subcellular localization of tyrosine phosphorylated STAT1 (red). (C) CCD-922Sk fibroblasts were mock-infected or infected with MV or UV-inactivated MV (UV-MV) as indicated and the cell lysates were immunoblotted for the expression of MV early gene M-T7 using anti-M-T7 antibody. (D) CCD-922Sk fibroblasts of mock and MV or UV-MV infections as indicated were left alone or stimulated with IFN- $\alpha$ C for 30 min. The cell lysates were then immunoprecipitated (IP) with anti-STAT1 $\alpha$  antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1 $\alpha$  antibodies. (E) CCD-922Sk fibroblasts of mock and MV infections carried out in the absence or presence of AraC as indicated were left alone or stimulated with IFN- $\alpha$ C for 30 min. The cell lysates were then immunoprecipitated (IP) with anti-STAT1 $\alpha$  antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1 $\alpha$  antibodies.

incubated MV-infected CCD-922Sk fibroblasts with biotinylated IFN- $\alpha$ C. Following the preparation of the cell extracts, immunoprecipitation was performed using anti-IFN- $\alpha$  antibody followed by Western blotting with an HRP-streptavidin conjugate. As shown in Fig. 5B (top panel), comparable levels of IFN- $\alpha$ C were bound to the cell surface of both mock and MV-infected cells. The detection specificity for the biotinylated IFN- $\alpha$ C bound to the cell surface was again tested by using the intracellular protein USF2 (Fig. 5B, bottom panel). Thus, these data confirm that the host IFNAR retains the ability to bind exogenous type I IFN following MV infection. Significantly, these results further suggest that there are no MV-encoded molecules that would capture additional exogenous type I IFN on the infected cell surface.

To explore this further, we then sought to determine whether the impaired tyrosine phosphorylation of STAT1 was caused by the interference of secreted IFN-binding molecules encoded by infecting MV in a manner demonstrated for other poxviruses. For instance, vaccinia virus secretes soluble IFN- $\alpha$  viroreceptors which bind to both

infected and uninfected cell surface, thereby hindering type I IFN from binding to the host IFN- $\alpha$  receptors (Alcami et al., 2000). To examine whether or not this is the case for MV, we infected CCD-922Sk fibroblasts with MV for 6 h. The MV-conditioned media (MV-CM) was subsequently transferred to uninfected cells. After the cells were incubated with MV-CM for 1 h to ensure surface binding by any potential viral IFN decoy receptors in MV-CM, IFN- $\alpha$ C was added subsequently for 30 min. We observed that MV-CM treatments had no inhibitory effect on tyrosine phosphorylation of STAT1 following IFN- $\alpha$ C treatment (Fig. 5C, top panel). To further determine whether MV encodes any secreted IFN-binding protein(s) that would act mainly in a free form in the culture medium, we pre-incubated IFN- $\alpha$ C with the above MV-CM for 1 h. We then transferred the IFN-containing medium to fresh CCD-922Sk cells for 30 min. We observed that the IFN- $\alpha$ C pre-incubated with MV-CM invoked tyrosine phosphorylation of STAT1 as efficiently as did with the mock-conditioned medium (data not shown). Taken together, the above results indicate that MV is most probably not in a position to encode any type I IFN binding



**Fig. 5.** MV infection does not affect type I IFN binding to the cell surface IFN- $\alpha$  receptors in primary human fibroblasts. (A) CCD-922Sk fibroblasts were mock or MV-infected for 6 h and cell surface molecules were biotinylated with Sulpho-NHS-Biotin. The cell lysates were then immunoprecipitated (IP) with anti-IFNAR1 or anti-IFNAR2 antibodies. Western blots (WB) were probed with a horseradish peroxidase (HRP)-conjugated streptavidin. Immunoprecipitation with the antibody against an intracellular protein USF2 was used as the negative control. (B) CCD-922Sk fibroblasts were mock or MV-infected for 6 h. Then, biotinylated-IFN- $\alpha$ C was incubated with the cells at 4 °C for 1 h to examine the binding capacity of the cell surface IFNAR. Subsequently, the cell lysates were immunoprecipitated (IP) with an anti-IFN- $\alpha$  antibody. Western blots (WB) were probed with a horseradish peroxidase (HRP)-conjugated streptavidin. Immunoprecipitation with the antibody against an intracellular protein USF2 was used as the negative control. (C) CCD-922Sk fibroblasts treated by mock and MV-conditioned medium (MV-CM) were left alone or stimulated with IFN- $\alpha$ C for 30 min as indicated. The cell lysates were immunoprecipitated (IP) with anti-STAT1 $\alpha$  antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1 $\alpha$  antibodies. (D) CCD-922Sk fibroblasts of mock, WT MV and MV-135KO infections as indicated were left alone or stimulated with IFN- $\alpha$ C for 30 min. The cell lysates were then immunoprecipitated (IP) with anti-STAT1 $\alpha$  antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT1 $\alpha$  antibodies.

proteins that would act in an extracellular manner to neutralize type I IFN in the human cell culture environment.

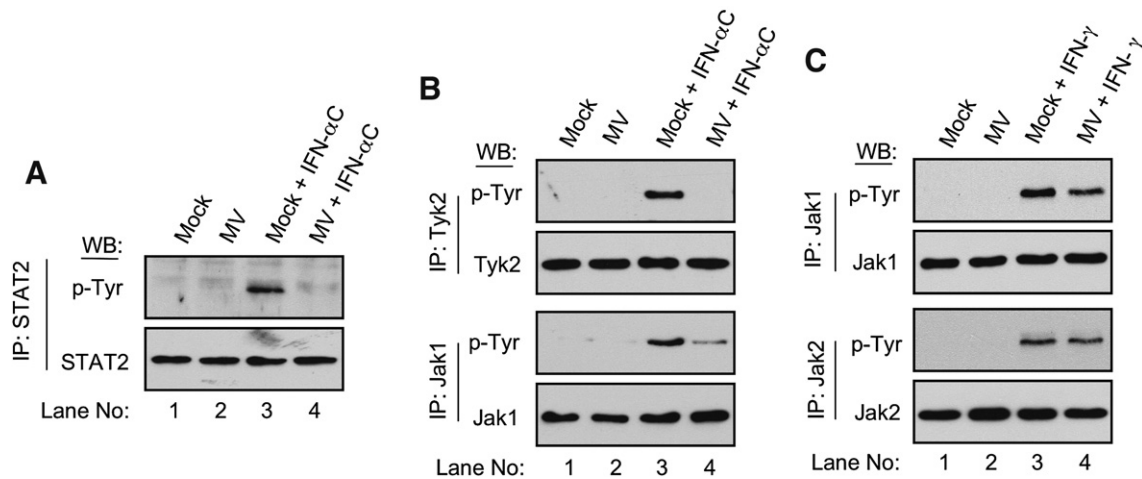
Notably, systematic sequence analysis of MV genome has revealed that there is only one MV gene designated M135R homologous to the vaccinia virus gene B18R that is known to encode a potent type I IFN decoy receptor (Alcami et al., 2000; Barrett et al., 2007). Thus, to formally demonstrate that no MV-encoded type I IFN decoy receptor is involved in MV-mediated inhibition of type I IFN signaling, we infected CCD-922Sk fibroblasts for 6 h with both wild type MV and a recombinant MV in which the M135R gene has been knocked out (MV-135KO) and then stimulated the infected cells with IFN- $\alpha$ C for 30 min. Significantly, we observed that tyrosine phosphorylation of STAT1 was equally inhibited in both wild type MV and MV-135KO-infected cells following IFN- $\alpha$ C stimulation (Fig. 5D, lanes 5 and 6). Taken collectively, our results thus clearly show that in primary human fibroblasts, MV-mediated blockade of the type I IFN signaling is not due to the interference by MV-encoded IFN decoy receptor at the cell surface IFN- $\alpha$  receptor binding stage.

#### *MV infection inhibits type I IFN-induced tyrosine phosphorylation of Tyk2 in primary human fibroblasts*

Type I IFN-induced activation of STAT1 is triggered by tyrosine phosphorylation of the upstream Janus kinases Tyk2 and Jak1, which are pre-associated with the cytoplasmic tails of IFNAR1 and IFNAR2, respectively (Randall and Goodbourn, 2008; Stark et al., 1998). Nonetheless, in terms of the requirements for STAT1 activation *per se*, the transcription factor STAT2, an essential component of IFN-stimulated gene factor 3 (ISGF3) complex, must be first tyrosine phosphorylated by the Janus kinases in order to create a docking site so that subsequent STAT1 tyrosine phosphorylation can occur (Randall and

Goodbourn, 2008; Stark et al., 1998). Thus, as a necessary step in further pinpointing how MV inhibits the type I IFN signaling cascade, we examined whether or not MV infection caused the suppression of STAT2 tyrosine phosphorylation. For this purpose, we conducted a STAT2 immunoprecipitation from the MV-infected CCD-922Sk fibroblasts following IFN- $\alpha$ C treatment for 30 min. As expected, IFN- $\alpha$ C stimulation resulted in strong tyrosine phosphorylation of STAT2 in mock-infected cells (Fig. 6A, top panel, lane 3). In contrast, IFN- $\alpha$ C-induced tyrosine phosphorylation of STAT2 was suppressed by MV infection (Fig. 6A, top panel, lane 4). However, the total protein levels of STAT2 remained unchanged in both mock and MV-infected cells (Fig. 6A, bottom panel). Together, these results suggest that the upstream Janus kinases are the likely molecular target(s) for MV-mediated inhibition of the type I IFN signaling.

To further investigate this inference, we stimulated mock and MV-infected CCD-922Sk fibroblasts with IFN- $\alpha$ C for 15 min and then examined the tyrosine phosphorylation status of Tyk2 and Jak1. We observed that IFN- $\alpha$ C treatment induced strong tyrosine phosphorylation of Tyk2 (Fig. 6B, top panel, lane 3) and Jak1 (Fig. 6B, third panel, lane 3) in mock-infected cells. Significantly, MV infection not only abrogated IFN- $\alpha$ C-induced tyrosine phosphorylation of Tyk2 (Fig. 6B, top panel, lane 4), but also considerably decreased IFN- $\alpha$ C-induced tyrosine phosphorylation of Jak1 (Fig. 6B, third panel, lane 4). Nevertheless, the total protein levels of Tyk2 (Fig. 6B, second panel) and Jak1 (Fig. 6B, fourth panel) were not affected by MV infection. Furthermore, UV-inactivated MV was unable to inhibit IFN- $\alpha$ C-induced tyrosine phosphorylation of Tyk2 or Jak1 (data not shown). Taken collectively, these data complement the MV-mediated suppression of IFN- $\alpha$ C-induced tyrosine phosphorylation of STAT1 (Fig. 3A, top left panel), but would also predict that IFN- $\gamma$ -induced tyrosine phosphorylation of STAT1 might be concurrently reduced because



**Fig. 6.** MV infection abrogates Tyk2 tyrosine phosphorylation induced by type I IFN in primary human fibroblasts. (A) Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN- $\alpha$ C for 30 min. The cell lysates were immunoprecipitated (IP) with anti-STAT2 antibody. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-STAT2 antibodies. (B) Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN- $\alpha$ C for 15 min. The cell lysates were immunoprecipitated (IP) with anti-Tyk2 or anti-Jak1 antibodies. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-Tyk2 or anti-Jak1 antibodies. (C) Mock and MV-infected CCD-922Sk fibroblasts were left alone or stimulated with IFN- $\gamma$  for 15 min. The cell lysates were immunoprecipitated (IP) with anti-Jak1 or anti-Jak2 antibodies. Western blots (WB) were probed with anti-phosphotyrosine (p-Tyr) or anti-Jak1 or anti-Jak2 antibodies.

Jak1 is also critically involved in the IFN- $\gamma$ -induced tyrosine phosphorylation of STAT1 (Samuel, 2001; Stark et al., 1998). This prediction contradicts the unhindered tyrosine phosphorylation of STAT1 in MV-infected cells following IFN- $\gamma$  stimulation as shown in both Fig. 3A (top right panel) and Fig. 3B (bottom panel).

In an effort to reconcile this apparent contradiction, we sought to examine the tyrosine phosphorylation profiles of Jak1 and Jak2 following IFN- $\gamma$  stimulation of MV-infected CCD-922Sk fibroblasts. Jak1 is also pre-associated with IFNGR1 while Jak2 is pre-associated with IFNGR2 (Randall and Goodbourn, 2008; Stark et al., 1998). We observed that IFN- $\gamma$  stimulation resulted in potent tyrosine phosphorylation of Jak1 (Fig. 6C, top panel, lane 3) and Jak2 (Fig. 6C, third panel, lane 3) in mock-infected cells. In contrast, IFN- $\gamma$ -induced tyrosine phosphorylation of Jak1 (Fig. 6C, top panel, lane 4) or Jak2 (Fig. 6C, third panel, lane 4) was not significantly suppressed by MV infection. Thus, these differential tyrosine phosphorylation patterns of Jak1 induced by IFN- $\alpha$ C versus IFN- $\gamma$  in MV-infected cells suggest that the selective inhibition of tyrosine phosphorylation of Tyk2 is the pivotal evasion strategy employed by MV to disrupt the type I IFN-Jak-STAT signaling in primary human fibroblasts.

## Discussion

Poxviruses are a family of large cytoplasmic DNA viruses that have considerable medical and veterinary importance (Esposito and Fenner, 2001). Although a number of antiviral measures are involved in the host defense against poxvirus infection, type I IFN remains to be one of the key innate factors during poxviral pathogenesis, species barriers and cellular tropism (McFadden, 2005; Seet et al., 2003; Takaoka and Yanai, 2006; Wang et al., 2004). Hence, uncovering the countermeasures employed by poxviruses against the infected host IFN response is essential to better understand poxvirus pathobiology. In this study, we have shown that MV infection selectively disrupts the type I IFN-Jak-STAT signaling cascade in primary human fibroblasts. This occurs mainly through the functional blockade of Tyk2, a key Janus kinase upstream of STAT1 and STAT2, thereby preventing a type I IFN-transduced signal from inducing the expression of antiviral ISGs. This evasion strategy is not only different from the poxviral IFN decoy receptors aimed at antagonizing host IFN ligands extracellularly (Randall and Goodbourn, 2008), but is also distinct from the previously reported poxviral phosphatase that has been shown to

dephosphorylate STAT1 and STAT2 intracellularly (Mann et al., 2008; Najarro et al., 2001).

Tyk2 is the first member of the Janus kinase family to be characterized as a prototypic non-receptor tyrosine kinase (Firmbach-Kraft et al., 1990). The criticality of Tyk2 in type I IFN signal transduction was initially demonstrated in human fibroblast cell lines by genetic complementation (Muller et al., 1993; Velazquez et al., 1992). More recently, it has been further demonstrated that type I IFN fails completely to activate STAT1 and STAT2 in the human cells derived from a patient genetically deficient in Tyk2 (Minegishi et al., 2006). In contrast, two independent groups have shown that the murine cells derived from Tyk2 knockout mice nevertheless retain the capability of initiating tyrosine phosphorylation of STAT1 and STAT2 in responding to type I IFN, particularly at a relatively high concentration (Karaghiosoff et al., 2000; Shimoda et al., 2000). Taken together, these studies clearly indicate that the requirement for Tyk2 in the type I IFN signaling cascade is more stringent in human than in mouse cells. In this report, we have observed that in primary human fibroblasts, MV infection abrogated the tyrosine phosphorylation of not only the upstream kinase Tyk2, but also downstream STAT1 and STAT2 following IFN- $\alpha$ C stimulation. Taken collectively, our data reveals that Tyk2 is the functionally essential signaling molecule targeted by MV to antagonize type I IFN-Jak-STAT signaling in virus-infected primary human fibroblasts.

Notably, although only a moderate inhibition of IFN- $\gamma$ -induced tyrosine phosphorylation of Jak1 was observed following MV infection, IFN- $\alpha$ C-induced tyrosine phosphorylation of Jak1 was substantially suppressed. This is an interesting observation given that Jak1 is a common kinase used by both type I and type II IFN pathways (Rodriguez et al., 1998; Stark et al., 1998). In the type II IFN pathway, binding of IFN- $\gamma$  to its cell surface receptor brings IFNGR1-associated Jak1 and IFNGR2-associated Jak2 into close proximity, resulting in Jak2 activation. The activated Jak2 then triggers tyrosine phosphorylation of Jak1 (Stark et al., 1998; Takaoka and Yanai, 2006). According to this canonical model, it is not immediately clear why MV-invoked inhibition of Tyk2 should affect IFN- $\gamma$ -induced activation of Jak1. Of relevance in this regard, a similar reduction in IFN- $\gamma$ -induced tyrosine phosphorylation of Jak1 was found in the cells genetically deficient in Tyk2 gene (Karaghiosoff et al., 2000). Currently, the exact mechanism(s), as to why in some cell systems Tyk2 affects IFN- $\gamma$ -induced activation of Jak1, still remain elusive. Nevertheless, since broad cross-talk between type I and type II IFN signaling components have been

recognized, such as the involvement of Tyk2 in the IFNAR–IFNGR superstructure (Karaghiosoff et al., 2000; Schroder et al., 2004; Takaoka et al., 2000), the partial reduction in IFN- $\gamma$ -induced tyrosine phosphorylation of Jak1 in MV-infected primary human fibroblasts might imply that MV-blocked Tyk2 would present some hindrance for Jak2 to maximally activate Jak1. Further experimental investigation will be needed to test this hypothesis.

On the other hand, type I IFN binding to its cell surface receptor juxtaposes IFNAR1-associated Tyk2 and IFNAR2-associated Jak1, causing Tyk2 tyrosine phosphorylation through activated Jak1 (Gauzzi et al., 1996). The activated Tyk2 then cross-phosphorylates Jak1, further activating each other in turn for signal amplification (Stark et al., 1998). Based on these considerations, it seems highly plausible that the vigorous blockade we observed of IFN- $\alpha$ C-induced Jak1 tyrosine phosphorylation is due to the lack of the Tyk2-driven cross-phosphorylation of Jak1 resulting from MV-caused blockade to Tyk2 activation.

Our discovery that MV specifically abolishes IFN- $\alpha$ C-induced Tyk2 activation suggests the following scenarios that might explain MV's inhibitory effects. Firstly, we have observed that the expression of IFN- $\alpha$  receptor *per se* or IFN binding to the cell surface is not affected by MV infection in primary human fibroblasts. Secondly, a higher concentration of type I IFN did not show any saturating effect on the reversal of Tyk2 activation in MV-infected cells (data not shown). Thirdly, Tyk2 is known to phosphorylate the IFNAR1 subunit first before it can activate STAT1 and STAT2 (Stark et al., 1998). In this regard, we have not detected any IFNAR1 tyrosine phosphorylation following IFN- $\alpha$ C stimulation in MV-infected cells (data not shown). Fourthly, we have found that UV-inactivated MV fails to block IFN- $\alpha$ C-induced tyrosine phosphorylation of Tyk2 (data not shown). Fifthly, the deletion of the gene for the sole predicted type I IFN decoy receptor M135R has no effect on MV-mediated inhibition of Jak-STAT1 activation in response to type I IFN stimulation. On the basis of these results, we speculate that Tyk2 is the prime target by MV infection and that viral protein(s) expressed during MV infection interacts directly or indirectly with Tyk2, consequently hindering Tyk2 from being activated upon type I IFN binding to its cell surface IFN- $\alpha$  receptor. Alternatively, MV infection in primary human fibroblasts may trigger the expression or activation of a cellular inhibitor of Tyk2, thereby preventing Tyk2 from being accessible to the type I IFN-transduced stimuli. Such robust inhibitors as suppressor of cytokine signaling (SOCS) have been increasingly recognized as important negative regulators for the Jak-STAT signaling system under various physiological conditions (Yoshimura et al., 2007). In particular, it has been recently shown that tumor-induced SOCS3 physically binds to Tyk2, thereby suppressing type I IFN signal transduction in mouse models (Zeng et al., 2008). Further studies will be required to dissect the above potential scenarios.

Vaccinia virus VH1 phosphatase inhibits the IFN-Jak-STAT signal transduction rapidly by specific targeting of STAT1 tyrosine phosphorylation induced by either type I or type II IFN in a viral gene expression-independent manner. In particular, VH1 phosphatase does not inhibit the tyrosine phosphorylation of the upstream Janus kinases (Mann et al., 2008; Najjarro et al., 2001). In contrast, MV-mediated disruption of the IFN-Jak-STAT signaling pathway requires viral gene expression. Significantly, MV infection mainly blocks the Janus kinase Tyk2 in the context of the type I IFN signaling cascade, but has no significant inhibitory effect on type II IFN-induced tyrosine phosphorylation of Jak1 and Jak2. On the other hand, MV I1L and vaccinia virus VH1 phosphatases do share a common essential feature in that I1L and VH1 are both critically required for a normal productive infection of MV and vaccinia virus, respectively (Liu et al., 1995; Mossman et al., 1995a). Thus, our results suggest that, except for being central to the poxviral life, the superimposed anti-host functions of poxviral phosphatases may vary markedly among individual poxviruses. This is of interest considering the fact that VH1 homologous

genes are highly conserved among the poxvirus family members (Mossman et al., 1995a; Najjarro et al., 2001).

Poxviruses are adept at secreting various viral IFN decoy receptors to inactivate host IFN (Randall and Goodbourn, 2008; Seet et al., 2003). Interestingly, vaccinia virus-encoded type I IFN decoy receptors can bind IFN- $\alpha/\beta$  from broad species including cow and human (Alcami et al., 2000). In contrast, MV-encoded type II IFN binding proteins are rabbit-specific. As such, the IFN- $\gamma$  receptor mimetic encoded by an early MV gene M-T7 binds only to rabbit IFN- $\gamma$ , but not to mouse or human IFN- $\gamma$  (Mossman et al., 1995b). In this study, we have confirmed that no viral IFN decoy receptors encoded by MV interfere with the action or the binding of either human IFN- $\alpha$ C or IFN- $\gamma$  (data not shown) to the cell surface IFN receptors of CCD-922Sk fibroblasts. Of further functional relevance in this regard, we have noted that MV infection in primary rabbit embryo fibroblasts (pREFs) are resistant to rabbit type I IFN (Supplementary Fig. 1A, top panel) whereas VSV infection in pREFs are highly sensitive to the antiviral state induced by rabbit type I IFN (Supplementary Fig. 1A, bottom panel). By analogy, human type I IFN fails to effectively contain MV infection (Supplementary Fig. 1B, top panel) but is able to fully control VSV infection in primary human fibroblasts (Supplementary Fig. 1B, bottom panel). Thus, from an evolutionary point of view, these analogies suggest that MV's ability to antagonize type I IFN signaling may play an important role in the modulation of a host cell's permissiveness to MV infection outside of the rabbit species.

The natural host range of MV is restricted to the rabbit species, such that even live MV injection cannot cause clinical disease in humans (Fenner and Ratcliffe, 1965). Therefore, deconstructing why MV can disrupt the IFN-Jak-STAT signaling in human cells will provide essential information for the development of MV as a novel tumor-killing agent in oncolytic virotherapy (Lun et al., 2005; Lun et al., 2007; Woo et al., 2008; Wu et al., 2008). Furthermore, these studies will also shed light on the dynamics of viral tropism at the cellular, tissue and organismal levels in general when a virus encounters a non-evolutionary host (McFadden, 2005; Virgin, 2007).

## Materials and methods

### Cell culture, IFN, primary antibodies and viral infections

Primary human skin fibroblasts CCD-922Sk (kindly provided by Dr. Scot Roberts) were maintained in MEM with 10% FBS, penicillin (100 u/ml) and streptomycin (100  $\mu$ g/ml). For the preparation of primary rabbit embryo fibroblasts (pREFs), 21-day-old New Zealand White rabbit embryos were employed according to the standard 3T3 protocol (Todaro and Green, 1963). Human IFN- $\alpha$ C and IFN- $\gamma$  were obtained from PBL Biomedical Laboratories. Rabbit type I IFN was prepared in primary rabbit kidney cells as previously described (Mozes and Vilcek, 1981). Immunodetecting primary antibodies were from the following sources: phospho-STAT1 (Tyr-701) antibody: Upstate Biotechnology; Jak1, Jak2, Tyk2, STAT1 $\alpha$ , STAT2, IFN- $\alpha$ , IFNAR1, IFNAR2, USF2 and phosphotyrosine antibodies: Santa Cruz Biotechnology;  $\beta$ -actin antibody: Sigma; anti-MV M-T7 antibody was previously described (Mossman et al., 1996). MV (Lausanne strain) with or without *lacZ* gene inserted at an innocuous intergenic site under the control of a late viral promoter was previously described (Oggenorth et al., 1992). UV-inactivated MV was prepared utilizing a Stratagene Stratelinker. The preparation of GFP-tagged wild type MV (MV-gfp) or the MV with knockout of M135R gene (MV-135KO-gfp) was previously described (Barrett et al., 2007). When indicated for the MV infection in the presence of AraC (Sigma), the drug was used at 40  $\mu$ g/ml. MV infections were conducted at a multiplicity of infection (MOI) of 5 unless specified otherwise. Vesicular stomatitis virus expressing GFP (VSV-gfp) was described previously (Stojdl et al., 2003).

### SuperArray analysis

For SuperArray analysis, CCD-922Sk fibroblasts grown in 25 cm<sup>2</sup> flasks were stimulated with IFN- $\alpha$ C at 200 U/ml for 4 h. Total cellular RNA was isolated using the RNA purification kit from SABiosciences. Five micrograms of total RNAs from each sample were reverse transcribed and cDNA probes were generated by biotin-16-dUTP (Roche) incorporation employing the AmpoLabeling-LPR kit (SABiosciences) according to the product instructions. The labeled cDNA probes were hybridized to the nylon membranes of GEArray Q Series SuperArray (HS-054, SABiosciences) according to the manufacturer's protocol. The SuperArray format contained a total of 96 genes representing various signaling components and target molecules related to human IFN- $\alpha$ / $\beta$  response pathways. The membrane-bound cDNA probes were detected using an alkaline phosphatase-conjugated streptavidin and subsequently visualized by CDP-Star chemiluminescence substrate (SABiosciences). The hybridization signals were recorded on X-ray films and digitalized using an Epson densitometer scanner. Image analysis and data acquisition were carried out through the web-based integrated GEArray Expression Analysis Suite provided by SABiosciences. The IFN- $\alpha$ C-induced genes in the experimental group were considered to be of biological importance when the induction change was greater than 1.5-fold cut-off criteria compared to the control group.

### RT-PCR

CCD-922Sk fibroblasts grown in 25 cm<sup>2</sup> flasks were infected with MV for 6 h. The MV-infected cells were then stimulated with IFN- $\alpha$ C at 200 U/ml for 4 h. Subsequently, the cells were lysed following various treatments as specified and total cellular RNA was isolated using the RNeasy kit (Qiagen). Reverse transcription was performed using Superscript reverse transcriptase (Gibco BRL). Primer sequences for human MxA, OAS and GAPDH as well as RT-PCR conditions were described (Fujii et al., 1999; Stewart et al., 2006).

### Immunofluorescence microscopy

For immunofluorescent staining, CCD-922Sk fibroblasts were grown on coverslips. After infection with MV for 6 h, the infected fibroblasts were stimulated for 30 min with either IFN- $\alpha$ C or IFN- $\gamma$  at 200 U/ml as specified. Subsequently, the cells were fixed in cold methanol for 10 min, washed in PBS and blocked in 5% normal goat serum for 50 min. For the double immunofluorescent staining of STAT1 and MV early gene product M-T7, mouse monoclonal anti-STAT1 and rabbit anti-MV M-T7 antibodies were applied at room temperature for 1 h followed by a 50-min secondary detection with Texas Red-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG, respectively. For the double immunofluorescent staining of phospho-STAT1 (Tyr-701) and M-T7, rabbit anti-phospho-STAT1 and mouse monoclonal anti-MV M-T7 antibodies were applied at room temperature for 1 h followed by a 50-min secondary detection with Texas Red-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG, respectively. Subsequently, the nuclear DNA of immunostained cells were counterstained with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Molecular Probes) according to the product specification. The secondary antibody conjugates were from Jackson ImmunoResearch Laboratories. The fluorescence images were taken with a Leica DMIRE2 microscope.

### Immunoprecipitation and Western blotting

For tyrosine phosphorylated forms of STAT1, STAT2, Tyk2, Jak1 and Jak2, CCD-922Sk fibroblasts were maintained in 25 cm<sup>2</sup> flasks. After infection with MV for 6 h, the infected fibroblasts were stimulated for 15 or 30 min with either IFN- $\alpha$ C or IFN- $\gamma$  at 200 U/ml as specified.

Subsequently, whole cell extracts were prepared using the commercial formula of RIPA lysis buffer as recommended in the immunoprecipitation protocol by Santa Cruz Biotechnology. The whole cell extracts were cleared for 1 h at 4 °C with control IgG and protein-A/G-Sepharose (Santa Cruz Biotechnology). Subsequent immunoprecipitations were performed with the indicated antibodies at 4 °C overnight. The immune complexes were precipitated with protein-A/G-Sepharose and then rendered for electrophoresis on 7.5% SDS-polyacrylamide gels. After the electrophoretically separated immunoprecipitates were transferred to Hybond-C nitrocellulose (Amersham Pharmacia Biotech), Western blotting analysis was then performed with respective primary antibodies. Thereafter, bands were visualized utilizing secondary HRP-conjugated antibodies and ECL system (NEN Life Science Products).

### Detection of cell surface IFN- $\alpha$ receptor IFNAR1 and IFNAR2 and IFN- $\alpha$ C binding

CCD-922Sk fibroblasts were first infected with MV for 6 h and then washed thoroughly with cold PBS (pH 8.0). For biotinylation of the cell surface protein molecules, the washed cells were incubated with Sulfo-NHS-Biotin (Pierce) in PBS for 30 min according to the product specifications. Thereafter, the whole cell extracts were prepared and the immunoprecipitates achieved with anti-IFNAR1 or anti-IFNAR2 antibodies were subjected to electrophoresis and subsequent Western blotting with HRP-streptavidin conjugate. For the IFN- $\alpha$ C binding experiment, IFN- $\alpha$ C was first biotinylated as described (Plett et al., 2000). Then, the biotinylated IFN- $\alpha$ C was incubated with both mock and MV-infected CCD-922Sk cells at 4 °C for 1 h. After washing in PBS, the cell extracts were prepared and the immunoprecipitates obtained with an anti-IFN- $\alpha$  antibody were rendered to Western blotting with HRP-streptavidin conjugate following SDS-PAGE.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.02.013.

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