

# Gamma Interferon-Induced Interferon Regulatory Factor 1-Dependent Antiviral Response Inhibits Vaccinia Virus Replication in Mouse but Not Human Fibroblasts<sup>∇</sup>

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**Vaccinia virus (VACV) replicates in mouse and human fibroblasts with comparable kinetics and efficiency, yielding similar titers of infectious progeny. Here we demonstrate that gamma interferon (IFN- $\gamma$ ) but not IFN- $\alpha$  or IFN- $\beta$  pretreatment of mouse fibroblasts prior to VACV infection induces a long-lasting antiviral state blocking VACV replication. In contrast, high doses of IFN- $\gamma$  failed to establish an antiviral state in human fibroblasts. In mouse fibroblasts, IFN- $\gamma$  impeded the viral replication cycle at the level of late gene transcription and blocked the multiplication of VACV genomes. The IFN- $\gamma$ -induced antiviral state invariably prevented the growth of different VACV strains but was not effective against the replication of ectromelia virus. The IFN- $\gamma$  effect required intact IFN- $\gamma$  receptor signaling prior to VACV infection through Janus kinase 2 (Jak2) and signal transducer and activator of transcription 1 (STAT1). The permissive state of IFN- $\gamma$ -treated human cells was unrelated to the VACV-encoded IFN decoy receptors B8 and B18 and associated with a complete disruption of STAT1 homodimer formation and DNA binding. Unlike human fibroblasts, mouse cells responded with long-lasting STAT1 activation which was preserved after VACV infection. The deletion of the IFN regulatory factor 1 (IRF-1) gene from mouse cells rescued efficient VACV replication, demonstrating that IRF-1 target genes have a critical role in VACV control. These data have implications for the understanding of VACV pathogenesis and identify an incongruent IFN- $\gamma$  response between the human host and the mouse model.**

Vaccinia virus (VACV) is a prototypical member of the genus *Orthopoxvirus* of the *Poxviridae* family (47) and shares more than 90% nucleotide identity with variola virus, the causative virus of smallpox. VACV is the live vaccine used for vaccination against variola virus. Although VACV bears the name resembling the alleged origin species (vacca [Latin for cow]), the natural host of VACV is still elusive (23). In cell culture, VACV can replicate in many cell types (45, 47). Pathogenesis and immune responses have been studied in various animal species and compared with the situation in humans (63). For myxoma virus, a natural poxviral pathogen of South American rabbits, the interferon (IFN) pathway is a critical determinant of host range. Myxoma virus is unable to replicate in mouse cells but gains this ability in the absence of the IFN- $\alpha/\beta$  receptor or STAT1 (74). Experiments in mice have demonstrated that the expression of mouse IFN- $\gamma$  by VACV strongly enhances viral clearance and promotes immune responses, thus producing VACV vaccines that are apparently safe, even in immunodeficient animals (21, 35, 39). Conversely, the expression of a soluble mouse IFN- $\gamma$  receptor by VACV increased virus virulence in mice (68).

IFNs are cytokines that stimulate the induction of an anti-

viral state of cells. Upon the binding of IFNs to their receptors, the activation of the Janus kinase (Jak)-signal transducer and activator of transcription (STAT) signaling cascades leads to the transcription of IFN-inducible target genes with antiviral effector functions (reviewed in reference 59). IFNs are divided into three classes, type I (IFN- $\alpha$  and - $\beta$ ), type II (IFN- $\gamma$ ), and the recently described type III IFNs (36). Although type I and type II IFNs have partially overlapping biological functions, they engage different signaling cascades, activating distinct transcription factors recognizing specific promoter elements. Upon the binding of type I IFN, IFN- $\alpha$  receptor chain 1 and 2 (IFNAR1, IFNAR2) dimerize, leading to the activation of the preassociated kinases tyrosine kinase 2 (Tyk2) and Jak1, which subsequently induce the tyrosine phosphorylation of STAT2 and STAT1. The phosphorylation induces the formation of a STAT2:STAT1 heterodimer which together with IFN regulatory factor 9 (IRF-9) (also called IFN-stimulated gene factor 3 gamma [ISGF3 $\gamma$ ] or p48) forms the transcriptionally active heterotrimer ISGF3. ISGF3 binds to IFN-stimulated response elements (ISRE) to activate the corresponding promoters.

IFN- $\gamma$  induces the phosphorylation of STAT1 via the two IFN- $\gamma$  receptor chain 1 (IFNGR1)- and IFNGR2-preassociated kinases Jak1 and Jak2. The activation results in the generation of STAT1:STAT1 homodimers called gamma-activated factors, which bind to gamma-activated sequence (GAS) consensus elements to induce the transcription of IFN- $\gamma$ -inducible genes (reviewed in reference 8). Besides STAT-con-

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taining complexes, IRF proteins constitute a family of transcription factors that regulate the expression of a diverse set of IFN-stimulated target genes (69). Among the IFN-induced genes, the indoleamine-2,3-deoxygenase (IDO), inducible nitric oxide synthetase (iNOS), RNA-dependent protein kinase (PKR), and RNase L genes exhibit direct antiviral properties against VACV (12, 26, 42, 70).

Viral IFN antagonists highlight antiviral potency and secure viral replication (29). VACV devotes at least eight genes to the inhibition of the IFN network (25). B8 and B18 are soluble IFN decoy receptors for type II and type I IFNs, respectively (2, 7, 67). Despite the fact that both VACV IFN decoy receptors can bind IFNs from an unusually broad range of species, both interact poorly with mouse IFNs (2, 67). The VACV protein E3 is a double-stranded RNA binding protein that inhibits the double-stranded RNA- and B-DNA-dependent induction of type I IFN and in conjunction with K3 prevents the activity of PKR (6, 9, 75). N1, A46, and A52 have been reported to inhibit the Toll-like receptor (TLR)-dependent induction of type I IFN (3, 13, 81). Finally, the phosphatase VH1 encoded by the gene *HIL* inhibits the IFN-signaling cascade via the tyrosine dephosphorylation of STAT1 (50).

Herein we characterize a novel IFN- $\gamma$ -inducible pathway establishing a tight and long-lasting antiviral state preventing VACV replication. This host cell response is functional in mouse fibroblasts but is inefficient in human fibroblasts. The anti-VACV defense mechanism critically depends on IRF-1. Our findings imply that the mouse model overestimates the antiviral efficacy of IFN- $\gamma$  responses when data are extrapolated to the situation in the human host.

## MATERIALS AND METHODS

**Cells and cytokines.** Human MRC-5 fibroblasts (ATCC CCL-171), primary human foreskin fibroblasts (28), HeLa cells (ATCC CCL-2), African green monkey CV-1 cells (ATCC CCL-70), mouse RAW-264.7 macrophages (ATCC TIB-71), M2-10B4 mouse cells (ATCC CRL-1972), immortal STAT2- (53) and STAT1-deficient mouse fibroblasts (16), crisis-immortalized IFNAR1-deficient mouse embryonic fibroblasts (MEF) (generated from primary IFNAR1-deficient MEF) (83), Jak2-deficient MEF (51), and primary MEF (prepared according to a standard protocol) (4) from IFN- $\beta^{-/-}$  (17), IRF-1 $^{-/-}$  (44), C57BL/6, and BALB/c embryos were grown in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum, streptomycin, penicillin, and 2 mM glutamine. Mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, streptomycin, penicillin, and 2 mM glutamine.

IFNs (mouse IFN- $\alpha$  [12100-1], mouse IFN- $\beta$  [12400-1], mouse IFN- $\gamma$  [12500-1], human IFN- $\alpha$  [11100-1], and human IFN- $\gamma$  [11500-2]) were purchased from PBL Biomedical Laboratories, New Jersey. Cells were treated with 500 U/ml of IFN starting 48 h before infection or as otherwise indicated. According to the manufacturer's product information, human IFN- $\gamma$  contains  $1.59 \times 10^7$  U antiviral activity per mg, whereas mouse IFN- $\gamma$  contains  $1.02 \times 10^7$  U per mg.

**Viruses, infection conditions, and virus titration.** The VACV strains Western Reserve (WR), Elstree-BN (kindly provided by Bavarian Nordic, Munich, Germany), ectromelia virus (ECTV) strain MP-Nü (kindly provided by Heinz Ellerbrock, Robert Koch Institut, Berlin, Germany), early *lacZ* expressing vaccinia viral early promoter P-KIL (MVA-P<sub>KIL</sub>LZ) (14), late *lacZ* expressing modified VACV Ankara (MVA) viral late promoter P11 (MVA-LZ) (66), VACV  $\Delta$ B8R, and the corresponding revertant virus (68) were used. VACV titers were determined by a standard plaque assay using CV-1 cells. VACV was used at an initial infection of 0.05 PFU/cell. At the indicated time points postinfection (p.i.), the plates were frozen at  $-80^{\circ}\text{C}$ . Virus was released from cells by ultrasonication and titrated. Plaques were counted 30 to 36 h p.i. by microscopic inspection. Each sample was titrated at least in duplicate, and all replication analysis was performed twice ( $n = 2 \cdot 2$ ). Shown in Fig. 1, 2, 4, 6, and 7 are the arithmetic means with the standard deviations.

**RT-PCR.** Expression analysis of viral transcripts by semiquantitative reverse transcriptase PCR (RT-PCR) was done as described previously (38). Briefly, total RNA was extracted using an RNeasy minikit (Qiagen). The RNA was digested with DNase I before semiquantitative RT-PCR was performed using the OneStep RT-PCR kit (Qiagen) with serial 10-fold dilutions (as indicated) of total RNA as a template. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific primer set has been described previously (38). The following primers were used to amplify the known vaccinia virus early gene C11R coding for the vaccinia virus growth factor (72) and the known late gene F17R (77): VACVearly (C11R)<sub>for</sub> (5'-CATTGCGCCGATAGTGGAACGC-3'), VACVearly (C11R)<sub>rev</sub> (5'-ATCTCCCTCTGGACCGCAT-3'), VACVlate (F17R)<sub>for</sub> (5'-AGATAAACCCCTCATCGCCGC-3'), and VACVlate (F17R)<sub>rev</sub> (5'-CACGTTGTCGCGATTAGCCG-3').

**Northern blot analysis.** Northern blotting was performed as described previously (37). Briefly, total RNA was extracted at the time points indicated in the legend to Fig. 3 and conditions using an RNeasy minikit (Qiagen). Total RNA was separated by standard agarose gel electrophoresis and subsequently transferred to nylon membranes. The probe was prepared by PCR using the VACVlate (F17R) primers and digoxigenin-labeled dUTP (Roche).

**DNA hybridization by slot blot analysis.** Cells were lysed overnight at  $56^{\circ}\text{C}$  in proteinase K buffer (final concentration, 12.5 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], and 100  $\mu\text{g}$  proteinase K [Roche]). DNA was prepared by phenol-chloroform extraction and isopropanol precipitation. DNA (350 ng and indicated dilutions thereof) was transferred onto nylon filters using the SRC 072/0 manifold for slot blotting (Schleicher & Schuell). The probe was prepared by PCR using the VACVlate (F17R) primer set and digoxigenin-labeled dUTP (Roche).

**EMSA.** For the electrophoretic mobility shift assay (EMSA), extractions were performed according to established protocols (46). Cells were infected (5 PFU/cell) and lysed in cytosolic extraction buffer (20 mM HEPES [pH 7.4], 10 mM KCl, 0.2% NP-40, 0.1 mM EDTA, 10% glycerol, 0.1 mM Na-vanadate, 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 1 mM dithiothreitol [DTT], Complete protease inhibitors [Roche, Mannheim, Germany]). The extracts were centrifuged at  $16,000 \times g$  for 16 s at  $4^{\circ}\text{C}$ , and the supernatants were collected, centrifuged for 10 min, and used as cytosolic extracts for the EMSA. The pellets were washed in phosphate-buffered saline (PBS) and suspended in nuclear extraction buffer (20 mM HEPES [pH 7.6], 420 mM KCl, 0.1 mM vanadate, 20% glycerol, 1 mM EDTA, 0.1 mM PMSF, 1 mM DTT, Complete protease inhibitors [Roche, Mannheim, Germany]). After incubation on ice for 25 min the extracts were centrifuged at  $16,000 \times g$  for 25 min at  $4^{\circ}\text{C}$ , and the supernatants were used as nuclear extracts. Both extracts were frozen immediately in liquid nitrogen until final use. Nuclear or cytosolic lysates were incubated with 1 ng ( $\sim 50,000$  cpm) of  $^{32}\text{P}$ -labeled M67 GAS probe (27) for 20 min at room temperature. The DNA-protein complexes were separated on 4.7% polyacrylamide, 22.5 mM Tris-HCl, 22.5 mM borate, and 50  $\mu\text{M}$  EDTA gels, fixed and finally visualized by autoradiography. Supershifts were performed with a STAT1 antibody (Santa Cruz).

**Immunoblotting.** Immunoblotting was performed according to standard procedures. Briefly, cells were infected (5 PFU/cell), and equal amounts of cells were washed in PBS and lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% [vol/vol] IGEPAL [Sigma], 1% [vol/vol] Na-deoxycholate, 0.1% [vol/vol] SDS, 1 mM DTT, 0.2 mM PMSF, 1  $\mu\text{g}/\text{ml}$  leupeptin and pepstatin, 50 mM NaF, 0.1 mM Na-vanadate, and Complete protease inhibitor [Roche, Mannheim, Germany]). The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. The filters were probed with anti-phospho-STAT1 (Cell Signaling, Danvers, MA), STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA), and  $\beta$ -actin (Sigma-Aldrich, Taufkirchen, Germany). The proteins were visualized using peroxidase-coupled secondary antibodies and the ECL-Plus chemiluminescence system (Amersham, Buckinghamshire, United Kingdom).

**$\beta$ -Galactosidase staining.** The cells were washed with PBS and fixed for 20 min in 3% (wt/vol) paraformaldehyde. The fixed cells were washed with PBS and subsequently stained with X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) solution (66 mM  $\text{Na}_2\text{HPO}_4$ , 33 mM  $\text{NaH}_2\text{PO}_4$ , 1.3 mM  $\text{MgCl}_2$ , 3 mM ferricyanide, 3 mM ferrocyanide, and 1 mg/ml X-Gal [pH 7.3]).

## RESULTS

**IFN- $\gamma$  induces an efficient antiviral state against VACV replication in mouse but not in human fibroblasts.** VACV strain WR multiplication levels in MRC-5 human fibroblasts and mouse fibroblasts were found to be comparable, yielding virus

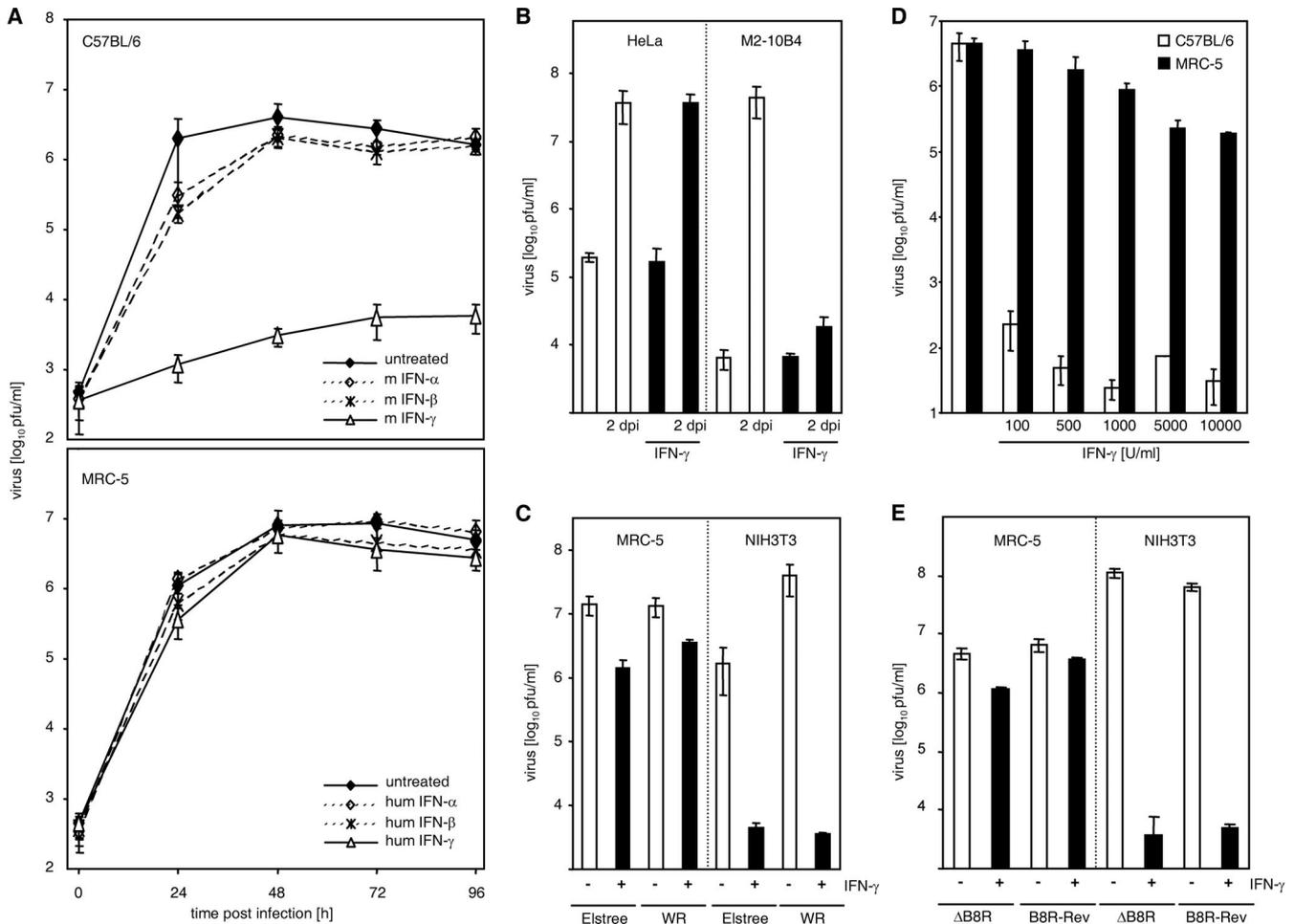


FIG. 1. IFN- $\gamma$  blocks VACV replication in mouse but not human cells. (A) C57BL/6 MEF (top panel) and human MRC-5 cells (bottom panel) were either mock treated (closed diamonds) or incubated with 500 IU/ml IFN- $\alpha$  (open diamonds, dashed line), IFN- $\beta$  (asterisks, dashed line), or IFN- $\gamma$  (open triangles) for 48 h before infection with VACV WR at 0.05 PFU/cell. Human cells were treated with human IFNs, and mouse cells were treated with murine IFNs. VACV progeny titers were determined at the indicated time points p.i. by plaque assay on CV-1 cells. Shown is one representative experiment of at least 3. Each data point represents the arithmetic mean of two duplicate samples titrated at least in duplicate ( $n = 2 \cdot 2$ ). The standard deviation is indicated for each data point. (B) Mouse M2-10B4 cells (right panel) and human HeLa cells (left panel) were incubated with IFN- $\gamma$  (500 U/ml IFN) or mock treated for 48 h before infection with VACV WR at 0.05 PFU/cell. The VACV progeny was determined 48 h p.i. (C) Human MRC-5 and mouse NIH 3T3 cells were infected with VACV strain WR or strain Elstree (0.05 PFU/cell) after preincubation with IFN- $\gamma$  (500 U/ml IFN) or mock treatment for 48 h. The VACV progeny was determined after 72 h. (D) The antiviral effect of IFN- $\gamma$  is dose dependent. Human MRC-5 (black bars) and C57BL/6 MEF (white bars) were pretreated with the indicated concentrations of IFN- $\gamma$  (indicated as U/ml) 48 h before cells were infected at 0.05 PFU/cell with VACV WR. The virus yield was determined after 3 days. (E) Human MRC-5 and mouse NIH 3T3 fibroblasts were infected with  $\Delta$ B8R-VACV or the corresponding revertant virus at 0.05 PFU/cell after incubation with IFN- $\gamma$  (500 U/ml IFN) or mock treatment. The VACV progeny was determined after 72 h.

titers of approximately  $10^7$  PFU/ml at 48 h p.i. (Fig. 1A) and allowing a comparison of the species-specific constraints of the antiviral potency of IFNs against IFN-conditioned mouse and human cells. Exposure of the cells to IFN- $\alpha$  or IFN- $\beta$  had only marginal antiviral effects against VACV, irrespective of the host species (Fig. 1A). Surprisingly, the effect of mouse IFN- $\gamma$  in mouse fibroblasts was several orders of magnitude stronger than the antiviral potency of human IFN- $\gamma$  in human MRC-5 fibroblasts when used at saturating IFN concentrations (Fig. 1A). The IFN- $\gamma$ -mediated blockade of VACV replication was also observed in mouse M2-10B4 (Fig. 1B) and NIH 3T3 cells (Fig. 1C), and the virtual inability of human IFN- $\gamma$  to block VACV in human cells was also reproduced in HeLa cells (Fig. 1B) and primary human foreskin fibroblasts (data not shown).

The lack of VACV replication in IFN- $\gamma$ -treated mouse cells was not a consequence of cell death, as IFN- $\gamma$ -treated cells could be stained with crystal violet even 48 h after VACV infection, indicating viability (data not shown).

The IFN- $\gamma$ -dependent inhibition of VACV replication in mouse cells was effective against the two independent VACV strains WR and Elstree (Fig. 1C) and was achieved in a dose-dependent manner (data not shown). Even very high concentrations of human IFN- $\gamma$  on human MRC-5 cells failed to reach an antiviral efficacy comparable to that observed with 100-fold-lower concentrations of mouse IFN- $\gamma$  on mouse cells (Fig. 1D). To rule out that the human IFN- $\gamma$  preparation in use does not elicit the anticipated antiviral activity, we controlled its antiviral potency against vesicular stomatitis virus (VSV).

The IFN- $\gamma$  preparation used was confirmed to be fully biologically active as indicated by the inhibition of VSV replication between 5 and 8 log<sub>10</sub> orders of magnitude in MRC-5 cells treated with 500 U/ml IFN- $\gamma$  (data not shown).

The VACV gene *B8R* encodes the viral IFN- $\gamma$  decoy receptor which has a rather limited proviral activity in mice (73), raising the possibility of antagonizing the IFN- $\gamma$  treatment of human cells more potently than mouse cells. We addressed this explanation by use of a VACV mutant lacking the gene *B8R*. Interestingly, IFN- $\gamma$  was not able to block the replication of the  $\Delta$ B8R-VACV mutant in MRC-5 cells, suggesting that IFN- $\gamma$  fails to establish an antiviral effect even when having undiminished access to IFNGR (Fig. 1E).

Interestingly, the pronounced antiviral potency of IFN- $\gamma$  in mouse fibroblasts appeared to be selective for VACV and was not observed for herpes simplex virus type 1 F (data not shown), murine cytomegalovirus (MCMV) (83), or the orthopoxvirus ECTV, the replication of which was reduced less than 1 log<sub>10</sub> (Fig. 2).

Taken together, IFN- $\gamma$ , but not type I IFN, strongly impairs VACV replication in mouse but not human fibroblasts, suggesting that host-dependent constraints downstream of IFNGR triggering exist that are able to terminate VACV replication.

**IFN- $\gamma$  blocks VACV replication at the level of viral late gene expression.** To define at which step the VACV replication cycle becomes arrested in the IFN- $\gamma$ -conditioned MEF, transcription levels of VACV, the early C11R gene and the late F17R gene were compared by semiquantitative RT-PCR and Northern blotting. While the C11R transcripts were only modestly reduced in IFN- $\gamma$ -conditioned cells, late F17R gene expression was found to be drastically reduced by RT-PCR (Fig. 3A) and Northern blot analysis (Fig. 3B). Next, we tested the impact of IFN- $\gamma$  on the replication of VACV genomes by slot blot hybridization (Fig. 3C), which revealed an approximately 25-fold reduction of viral genomes in IFN- $\gamma$ -pretreated cells, leaving the possibility that replicating VACV genomes had contributed to the observed differences in the C11R transcription levels determined by RT-PCR. To exclude the influence of replicating viral genomes, we used recombinant MVA viruses carrying the *Escherichia coli lacZ* reporter gene under the control of MVA-P<sub>KILL</sub>LZ or MVA-LZ to determine the step in the viral life cycle targeted by IFN- $\gamma$  in mouse fibroblasts. We observed a striking inhibition of viral-late-promoter-driven reporter gene expression upon IFN- $\gamma$  treatment but not of early-promoter-driven gene expression. Taken together, the data are consistent with the notion that IFN- $\gamma$  blocks preferentially late gene transcription and VACV genome replication but only marginally early gene expression or earlier steps of VACV growth (Fig. 3).

**STAT1 and JAK2 are essential for the IFN- $\gamma$ -dependent inhibition of VACV replication.** The IFN- $\gamma$ -inducible antiviral enzymes iNOS and IDO have been reported to execute antiviral activities against VACV in macrophages and human cells, respectively (26, 33). The inability of mouse NIH 3T3 cells to respond to IFN- $\gamma$  stimulation by producing significant amounts of nitrite or L-kynurenine, the metabolite produced by iNOS or IDO, respectively (data not shown), led us to conclude that an additional effector mechanism(s) must exist in mouse fibroblasts that effectively blocks VACV replication.

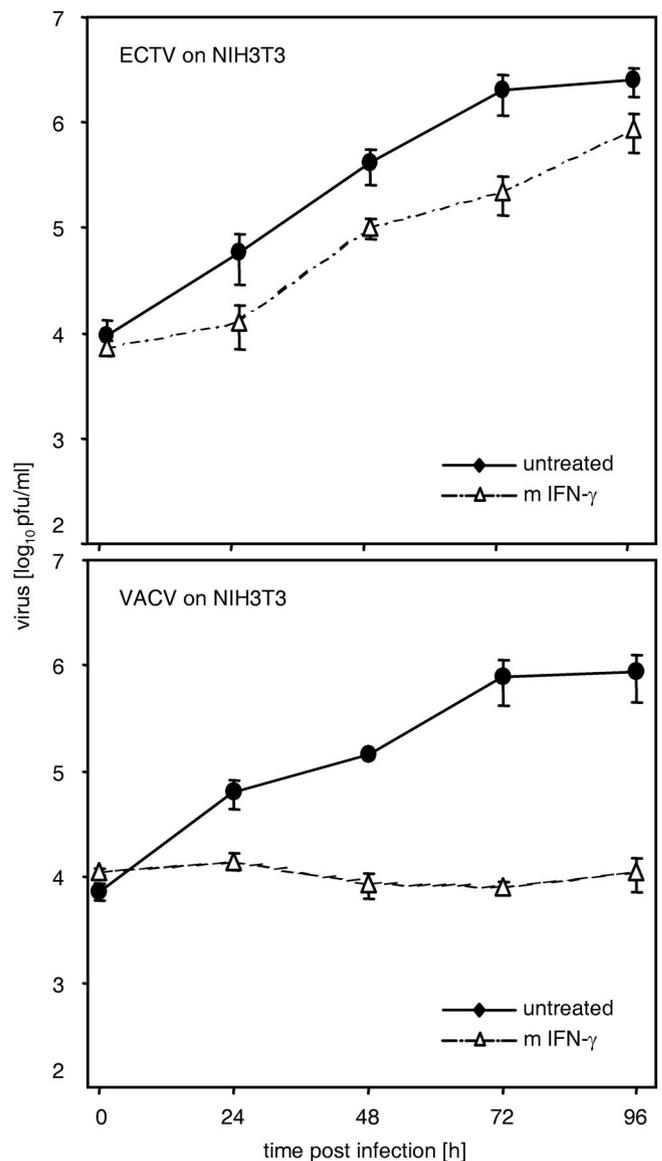


FIG. 2. ECTV can replicate in IFN- $\gamma$ -conditioned mouse fibroblasts. Comparison of ECTV (top panel) with VACV strain WR (bottom panel) replication in mouse NIH 3T3 cells that had or had not been treated with IFN- $\gamma$  (500 U/ml; 48 h preincubation). Virus titers at the indicated times p.i. were determined by plaque assay on CV-1 cells. Each data point represents the arithmetic mean of two experiments titrated in duplicate ( $n = 2 \cdot 2$ ). The standard deviation is indicated for each data point.

The fact that B8R did not affect the phenotypic differences between mouse and human fibroblasts (Fig. 1E) prompted us to define critical host components of IFN-signaling cascades with regard to their relative significance for VACV inhibition. IFN- $\gamma$  did not exert any antiviral activity against VACV in *stat1*<sup>-/-</sup> and *jak2*<sup>-/-</sup> fibroblasts (Fig. 4), suggesting an indispensable role for the canonical IFNGR-Jak-STAT1 pathways for the induction of the antiviral status preventing VACV replication (Fig. 4, top panel).

VACV replication in IFN- $\gamma$ -pretreated immortalized IFNAR-deficient MEF and immortalized STAT2-deficient MEF was

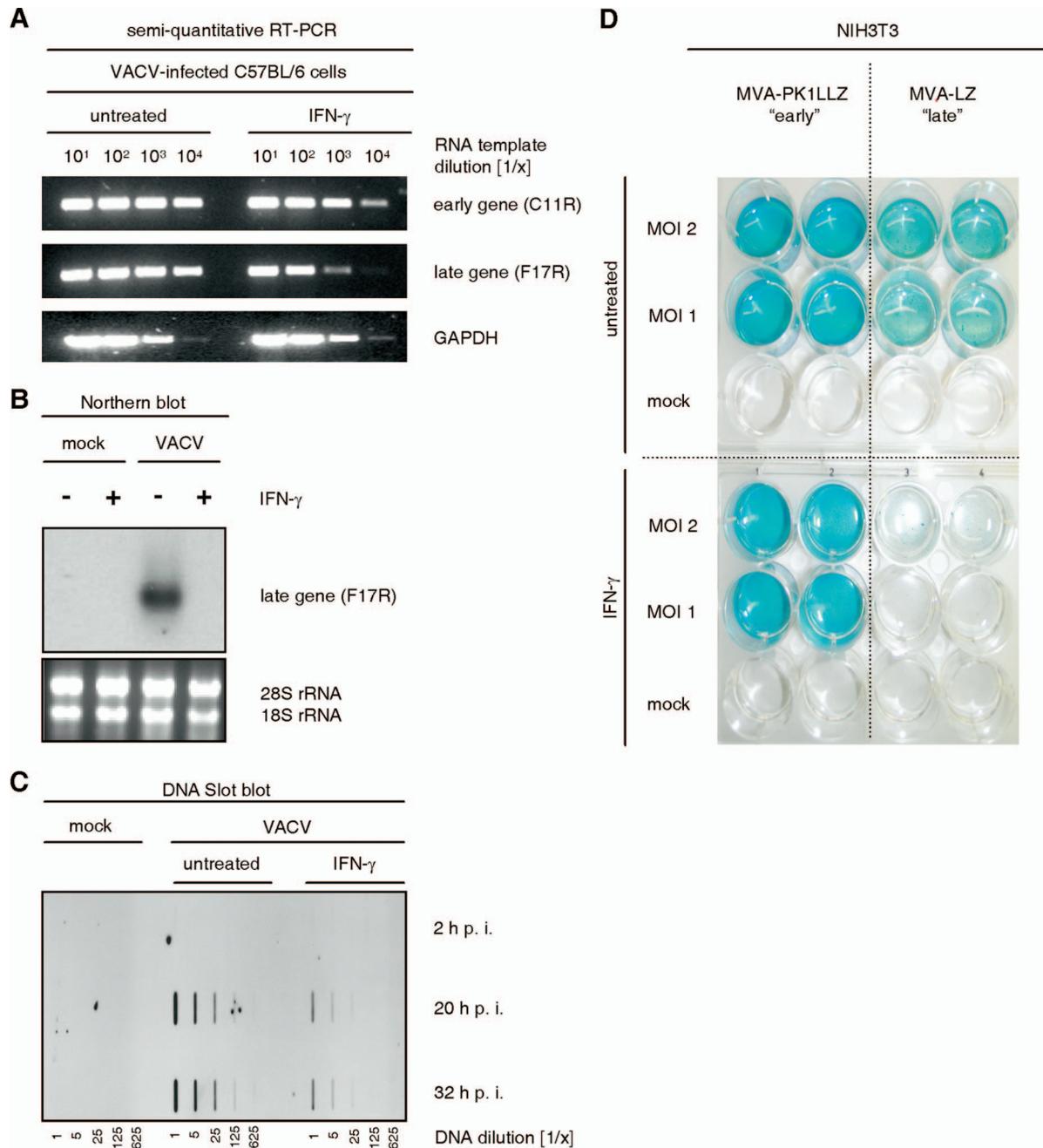


FIG. 3. IFN- $\gamma$  blocks VACV replication on the level of late gene expression. (A) Semiquantitative RT-PCR analysis of VACV early and late genes. C57BL/6 MEF were preincubated for 48 h with 500 U/ml IFN- $\gamma$  and subsequently infected with 1 PFU/cell VACV WR. Eighteen hours later, total RNA was extracted. The indicated serial (10-fold) RNA template dilutions were subjected to RT-PCR with gene-specific primers for C11R (early) and F17R (late) transcripts. Comparable RNA concentrations were confirmed by GAPDH-specific semiquantitative RT-PCR. (B) The treatment and infection conditions were as described for panel A, but VACV late gene expression was analyzed by Northern blotting with an F17R-specific DNA probe as described in Materials and Methods. (C) C57BL/6 MEF were treated with 500 U/ml IFN- $\gamma$  for 48 h. Cells were infected with 1 PFU/cell VACV WR. At 2, 20, and 32 h p.i., the cells were lysed and DNA was prepared. The indicated serial (fivefold) DNA dilutions were transferred by slot blotting on nylon membranes. VACV genomic DNA was detected by hybridization with a digoxigenin-labeled F17R-specific DNA probe. (D) NIH 3T3 cells were preincubated with 500 U/ml IFN- $\gamma$  for 48 h or left untreated. The cells were then either mock infected or infected with 1 or 2 PFU/cell with early *lacZ* expressing MVA-P<sub>K11</sub>LLZ (14) or late *lacZ* expressing MVA MVA-LZ (66) recombinant viruses. After 8 h, cells were stained with X-Gal solution as described in Materials and Methods. MOI, multiplicity of infection.

clearly rescued compared with that of wild-type (wt) fibroblasts (Fig. 4), albeit at 100- to 1,000-fold-lower levels compared with mock-treated cells. This suggested that IFNAR-dependent signals contribute to IFN- $\gamma$  response, although type I IFN on its

own had only a minimal impact on VACV growth (Fig. 1A). Specifically, impaired IFN- $\gamma$  responses in type I IFN-deficient cells could be due to considerably decreased levels of STAT1 protein resulting from these gene deficiencies (53, 65). The

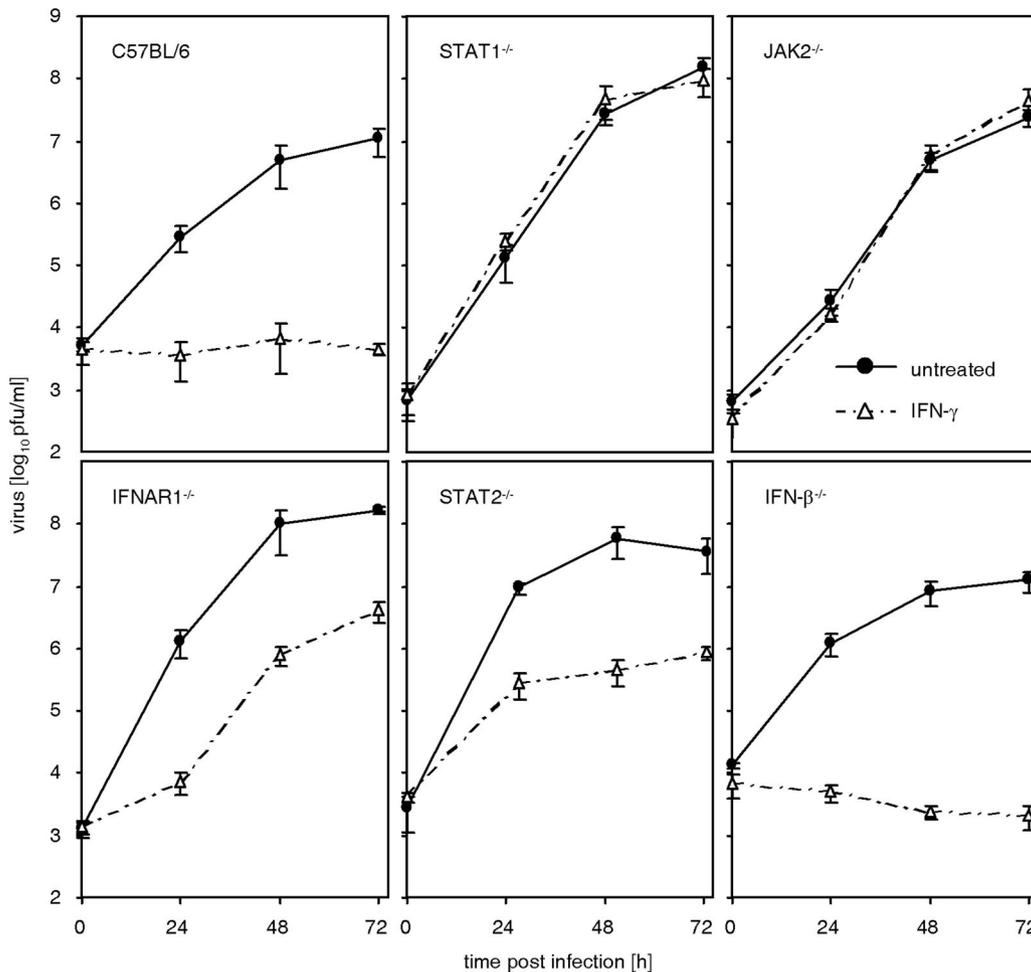


FIG. 4. The IFN- $\gamma$ -induced antiviral state requires the expression of STAT1 and JAK2. The level of replication of VACV WR was determined after infection at 0.05 PFU/cell, comparing untreated (solid line, filled circles) and IFN- $\gamma$ -incubated (500 U/ml; 48 h preincubation) (dashed line, open triangles) fibroblast cell lines lacking STAT1, IFNAR1, or STAT2. VACV replication was also tested under identical conditions using primary MEF from C57BL/6 mice and MEF deficient for Jak2 and IFN- $\beta$ .

finding that the anti-VACV activity of IFN- $\gamma$  was unaltered in IFN- $\beta$ -deficient MEF compared to that of wt MEF (Fig. 4) supports the notion that STAT1 levels are critical in preventing VACV replication rather than synergistic signals transmitted by IFNAR.

**IFNAR signaling fails to induce an antiviral state blocking VACV replication.** Due to the unclear role of type I IFN signaling, which is unable to mount a significant anti-VACV response alone but may contribute to the efficiency of IFN- $\gamma$  (see Fig. 4), we investigated whether or not type I IFN can induce IFNAR1-dependent Jak-STAT signaling upon VACV infection. Although functional redundancies between type I and type II IFNs exist and a subset of induced target genes is known to overlap (11), the potency of type I- versus type II-IFN-mediated inhibition of VACV replication in mouse fibroblasts was strikingly different. VACV expression of the type I IFN decoy receptor B18 could explain the virtual absence of antiviral-state induction in IFN- $\alpha$ -treated human as well as mouse fibroblasts. To this end, we performed an ISRE-luciferase reporter gene assay using a stably transfected mouse fibroblast cell line monitoring IFN- $\alpha$ -inducible transcription (83). The supernatant of VACV strain WR-infected cells pre-

vented IFN- $\alpha$ -stimulated ISRE transcription efficiently, while ISRE responsiveness was fully intact in the presence of supernatant from cells infected with a mutant virus lacking the *B18R* gene ( $\nu\Delta B18R$ ) (67) (data not shown). The wt VACV blocked IFN- $\alpha$ -dependent STAT2 phosphorylation (data not shown) and the formation of ISRE-binding ISGF3 complexes even in IFN- $\gamma$ -pretreated mouse cells in a virus dose-dependent manner, shown by EMSA analysis (data not shown). In contrast,  $\nu\Delta B18R$  did not block IFN- $\alpha$ -dependent STAT2 phosphorylation (data not shown), indicating that B18 is essential and sufficient for blocking the IFN- $\alpha$ -dependent signal transduction. To test whether the deletion of VACV gene *B18R* or *B8R* affected the antiviral efficiency of IFN, the replication of VACV mutants lacking either gene was assessed using IFN-primed MEF (data not shown). IFN- $\alpha$  did not have any antiviral activity in MEF when lacking B18 or B8, suggesting that host genes effective against VACV replication become induced by IFN- $\gamma$  but not by type I IFN.

**Differential activation and VACV-mediated inhibition of the IFN- $\gamma$  signaling cascade in human versus mouse fibroblasts.** Given that Jak2 and STAT1 are indispensable to establish the

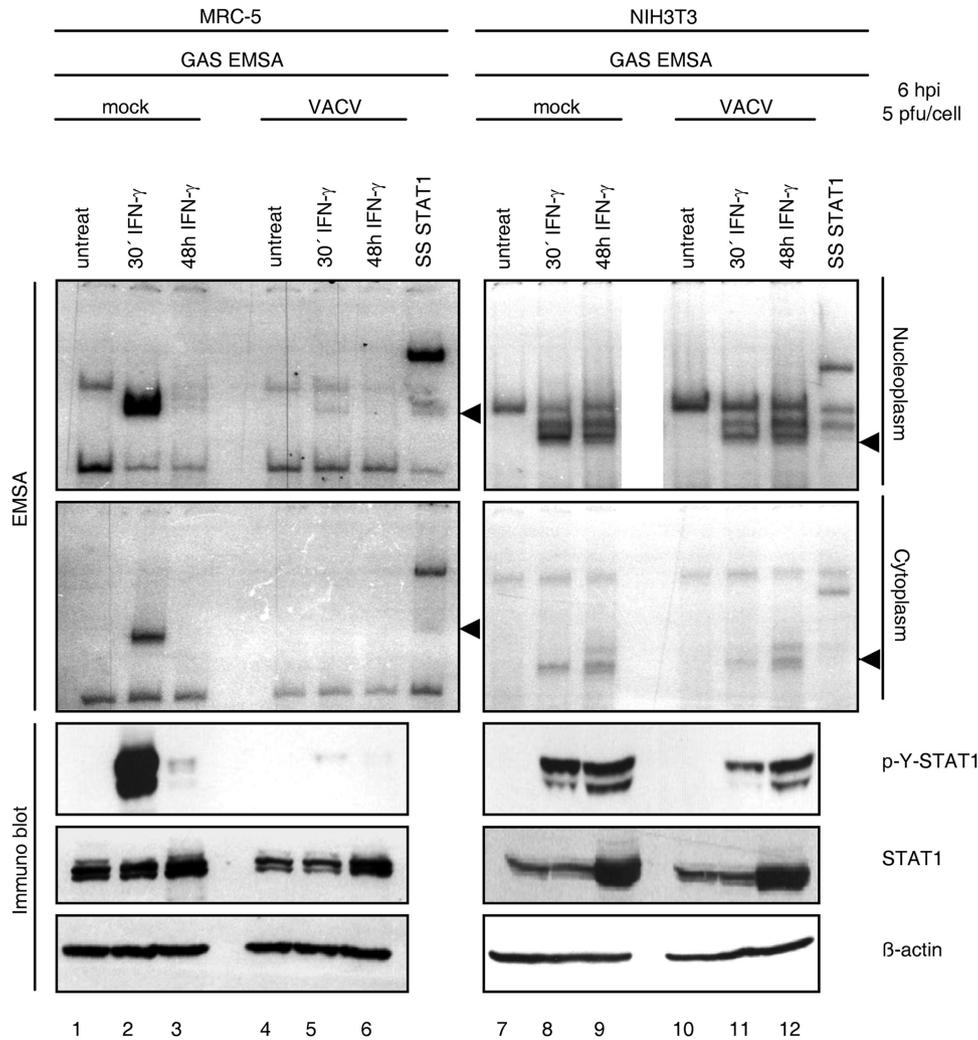


FIG. 5. IFN- $\gamma$  induces a sustained STAT1 activation only in mouse but not human fibroblasts, and VACV incompletely interferes with STAT1 activation in mouse fibroblasts. EMSA analysis using a  $^{32}\text{P}$ -labeled GAS probe reveals a long-lasting formation of STAT1 homodimers in IFN- $\gamma$ -pretreated mouse NIH 3T3 (right panels) but not human MRC-5 (left panels) fibroblasts. Cells were mock infected or infected at 5 PFU/cell with VACV for 6 h. Cells were incubated with 500 U/ml IFN- $\gamma$  for 30 min or preincubated with 500 U/ml IFN- $\gamma$  for 48 h. Lysates were fractionated and equivalent amounts of nucleoplasmic or cytoplasmic extracts were subjected to EMSA analysis. STAT1 protein complexes were identified by shifting when adding a STAT1-specific antibody. STAT1 complexes are indicated by arrowheads. The detection of proteins by immunoblotting was performed with the same lysates. Antibodies specific for human and P-Tyr STAT1 were used. Immunoblots were reprobed for STAT1 and  $\beta$ -actin.

IFN- $\gamma$ -mediated inhibition of VACV replication in MEF (Fig. 4), we surmised that VACV could fail to block IFN- $\gamma$ -mediated Jak-STAT signal transduction in mouse cells. The capacity of IFN- $\gamma$  to induce tyrosine phosphorylation and DNA binding of STAT1 was analyzed under two different experimental conditions. A short exposure with IFN- $\gamma$  for 30 min was compared with an extended pretreatment of cells over 48 h, the latter reproducing the conditions used before in the replication analysis. In both human MRC-5 and mouse NIH 3T3 fibroblasts, a short treatment with IFN- $\gamma$  induced tyrosine phosphorylation and the DNA binding of STAT1 (Fig. 5, lane 2 and 8). Extended IFN- $\gamma$  exposure induced IDO expression in MRC-5 cells (data not shown), while under these conditions levels of activated and GAS-binding STAT1 were detected only in NIH 3T3 cells but not in MRC-5 cells (Fig. 5, compare lane 3 with lane 9). This finding indicates the incongruent kinetics of the

feedback-inhibition of Jak-STAT signaling after IFN- $\gamma$  stimulation. The difference in STAT1 deactivation after sustained IFN- $\gamma$  incubation was not restricted to NIH 3T3 and MRC-5 fibroblasts but was also observed when comparing M2-10B4 cells with HeLa cells (data not shown).

In VACV-infected MRC-5 cells, the IFN- $\gamma$ -stimulated tyrosine phosphorylation of STAT1 and DNA binding was almost completely blocked (Fig. 5, lanes 5 and 6). In VACV-infected NIH 3T3 fibroblasts, the levels of tyrosine phosphorylation of STAT1 were lowered moderately compared to those of the mock-infected cells. In striking contrast to that of the VACV-infected MRC-5 cells, however, the formation of STAT1 dimers and binding to DNA was detected readily in NIH 3T3 cells infected with VACV exposed to IFN- $\gamma$  for 30 min or 48 h (Fig. 5, lanes 11 and 12), indicating a largely intact ability to transduce STAT1-dependent signals. Collectively,

these data demonstrate the different capacities of mouse versus human fibroblasts to transmit IFN- $\gamma$ -induced signals upon VACV infection. The molecular basis for the antiviral effect of IFN- $\gamma$  in mouse NIH 3T3 fibroblasts includes a sustained activation of STAT1 compared to human MRC-5 fibroblasts. In addition to this intrinsic difference of the host response to IFN- $\gamma$ , VACV abrogates the tyrosine phosphorylation of STAT1 more efficiently in human than in mouse cells.

**Pretreatment with IFN- $\gamma$  is required and sufficient to establish a stable antiviral state.** In the replication analyses shown above cells were routinely pretreated with IFN for 48 h followed by VACV infection with a low infectious dose (0.05 to 0.1 PFU/cell) before virus replication was monitored. Our finding that the IFN- $\gamma$  priming of cells for 48 h induces a sustained activation of STAT1 in mouse but not in human fibroblasts prompted us to assess the relative impact of the preincubation for the observed antiviral effect. The effect of adding IFN before the infection of cells and then maintaining IFN levels during VACV replication was compared with (i) IFN treatment starting only at the time of VACV infection and (ii) adding IFN before infection and removing it when the cells were infected (Fig. 6). IFN- $\alpha$  treatment only affected VACV replication marginally, irrespective of the time of addition (Fig. 6, top left panel). In contrast, IFN- $\gamma$  elicited an optimal antiviral effect when the cells were preincubated before infection and the presence of IFN- $\gamma$  was maintained after infection (Fig. 6, top right panel). The complete removal of IFN- $\gamma$  by the repetitive washing of cells yielded a similar antiviral effect that was as efficient as when IFN- $\gamma$  was maintained (Fig. 6, bottom right panel). The addition of IFN- $\gamma$  at the time point of infection inhibited VACV replication, albeit with a 50-fold-lower efficiency (Fig. 6, bottom left panel) compared with the protocol that included the IFN- $\gamma$  priming of cells before infection. Given the low infection dose used, allowing the IFN- $\gamma$  exposure of uninfected bystander cells during the first round of VACV replication, this result suggested that IFN- $\gamma$  has little if any antiviral potency without the preincubation of cells before infection. To test this, IFN- $\gamma$  was added simultaneously to NIH 3T3 cells infected at 2 PFU/cell to allow only one round of VACV replication. No inhibition of VACV replication was observed up to 48 h p.i., followed by a marginal inhibition seen at 72 h p.i. without IFN- $\gamma$  pretreatment (data not shown).

In conclusion, these data indicated that the pretreatment of mouse fibroblasts is necessary and sufficient to gain the full IFN- $\gamma$ -mediated antiviral effect. The inhibition of VACV replication by IFN- $\gamma$  in mouse fibroblasts is induced prior to VACV infection and the expression of VACV antagonists counteracting Jak-STAT signaling, revealing an intrinsic difference in the regulation of this signaling pathway between mouse and human fibroblasts.

**IRF-1 is essential for the IFN- $\gamma$ -dependent inhibition of VACV replication.** Many genes stimulated by STAT1 in the context of IFNGR activation require cooperative effects with further transcription factors such as IRF-1. Moreover, the promoter of the *irf-1* gene contains a binding site for STAT1 dimers, leading to an accumulation of IRF-1 in IFN- $\gamma$ -treated cells (54). IRF-1 is essential for a fraction of ISGs (15) and contributes substantially to the expression of further ISGs. To test whether IRF-1 is required to establish the antiviral effect

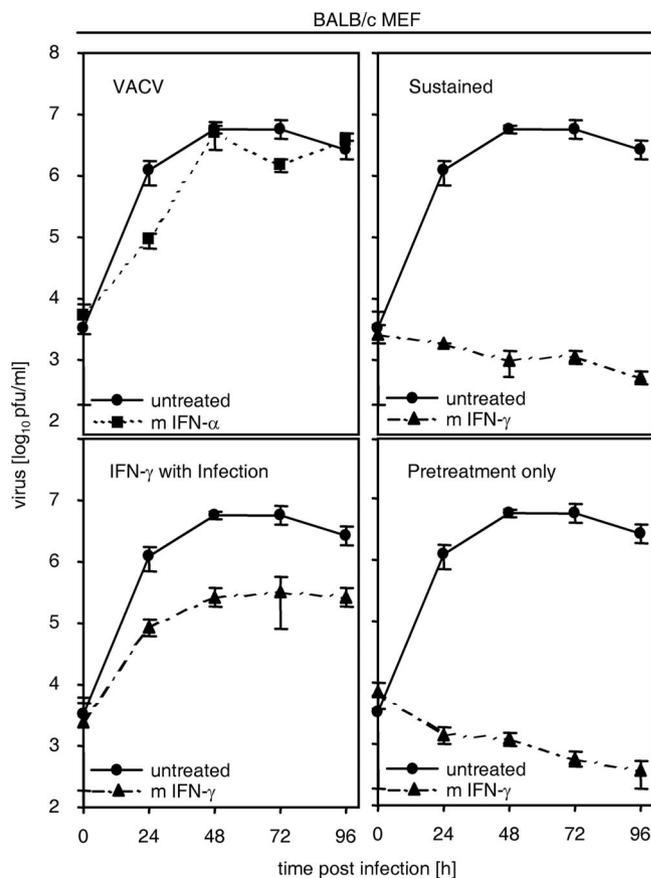


FIG. 6. IFN- $\gamma$  preincubation is essential and sufficient to block VACV growth in mouse fibroblasts. Comparison of VACV WR replication in BALB/c MEF (infected at 0.05 PFU/cell) under various conditions of IFN treatment. Cells were incubated with 500 IU/ml IFN- $\alpha$  (top left panel) or 500 U/ml IFN- $\gamma$  (top right panel) for 48 h prior to VACV infection, and IFN exposure was maintained thereafter (top panels). Bottom left panel, IFN- $\gamma$  treatment was started at the time point of VACV infection and maintained. Bottom right panel, cells were incubated with 500 U/ml IFN- $\gamma$  for 48 h prior to VACV infection before IFN-containing medium was removed and cells were washed and incubated in the absence of IFN.

of mouse fibroblasts against VACV, we tested VACV replication in *irf-1*<sup>-/-</sup> cells. VACV replication after the preincubation of MEF with IFN- $\gamma$  was restored by two orders of magnitude in the absence of IRF-1 (Fig. 7) compared to that of wt MEF, demonstrating that IRF-1 is necessary but not fully sufficient for the generation of the IFN- $\gamma$ -inducible antiviral state.

## DISCUSSION

VACV is relatively resistant to type I IFN in most cells (52). Here, we document a fundamental difference in the ability of mouse and human fibroblasts to prevent VACV replication upon IFN- $\gamma$  but not IFN- $\alpha/\beta$  pretreatment. The data presented show that IFN- $\gamma$  treatment of mouse but not human cells blocks VACV production and that there is a sustained activation of STAT1 leading to the formation of gamma-activated factor only in mouse cells. In mouse cells, the establishment of an antiviral state upon IFN- $\gamma$  stimulation required intact IFN- $\gamma$ R signaling via Jak2 and STAT1. Moreover, IRF-1

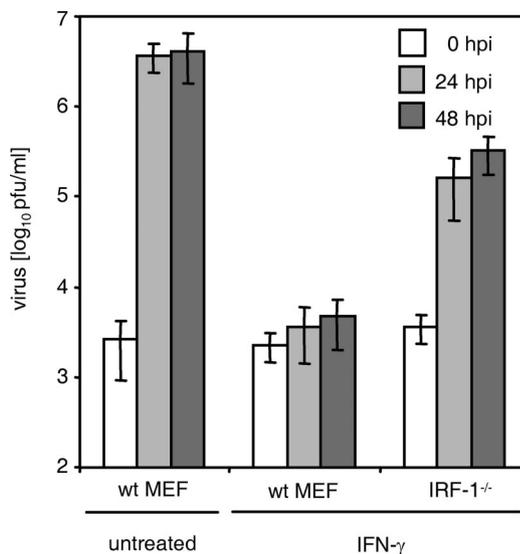


FIG. 7. IRF-1 is essential for the IFN- $\gamma$ -dependent inhibition of VACV replication. Direct comparison of the antiviral efficiency of IFN- $\gamma$  (500 U/ml; 48 h preincubation) in IRF-1-deficient MEF and wt MEF 24 (light gray bars) and 48 h p.i. (dark gray bars).

was crucial to inhibit VACV genome replication, and the inhibition was mediated before the transition of VACV early to late gene expression.

**Induction of ISGs with anti-VACV efficacy.** The induction of ISGs via the canonical Jak/STAT1-signaling cascade plays an important role in mediating the biological effects of IFN- $\gamma$  (64). In addition, STAT1-independent pathways contribute to a complete IFN- $\gamma$  response (22, 55, 61). The analysis of STAT1- and Jak2-deficient cells revealed that the anti-VACV effect in IFN- $\gamma$ -treated mouse fibroblasts relies solely on the former pathway. Previously, two classical effector enzymes that are activated downstream of IFN- $\gamma$ R, iNOS and IDO, were reported to mediate the direct inhibition of VACV replication (33, 70). Remarkably, both activities could not be detected in IFN- $\gamma$ -treated mouse fibroblasts, excluding any essential role for them inhibiting VACV replication in this setting. Many more of the very large set of ISGs can be ruled out by the fact that the factor responsible for inhibiting VACV replication is induced only by IFN- $\gamma$  and independent of type I IFNs. This is noteworthy given that the latter were shown to control myxoma virus replication in mouse fibroblasts and thus to restrict the host range of this leporipoxvirus (74). Multiple IFN-induced genes contain both ISRE and GAS consensus elements in their promoters. ISRE sequences binding STAT complexes like ISGF3 overlap with IRF-E consensus sites that bind preferentially IRFs (34). The fact that the antiviral potency of IFN- $\gamma$  is reduced drastically but incompletely in IRF-1-deficient cells (Fig. 7) points toward a target gene(s) that contains IRF-E consensus sites as well as STAT1 dimer-binding GAS motifs. In this context, it is interesting that an IRF-1 dependency of CpG-DNA induced the inhibition of VACV-MVA late gene expression and has been documented in myeloid dendritic cells (60). Moreover, a clinical study found a significant association between single nucleotide polymorphisms in the human *irf-1* gene region and VACV-induced adverse events upon VACV

immunization (58), pointing toward an important role for IRF-1 in the immune response toward VACV.

A subgroup of genes induced by IFN- $\gamma$  requires both STAT1 and IRF-1 for transcriptional activation. Among such coregulated genes are the IFN- $\gamma$ -inducible cytoplasmic guanylate-binding proteins (GBP) that are members of the p65 GTPase gene family with putative roles in the resistance to intracellular pathogens (10, 43). The IFN- $\gamma$ -induced initiation of the transcription of both *gbp1* and *gbp2* in mouse fibroblasts was shown to be absolutely dependent on STAT1 and active at low levels in the absence of IRF-1 (56), thus resembling the genetic requirements of the IFN- $\gamma$ -mediated inhibition of VACV replication in MEF. The IFN- $\gamma$ -induced target genes also include type I IFNs (82) and downstream enzymes with a prominent antiviral function like PKR and 2-5A synthetase (20, 57, 62). Only at first glance PKR and 2-5A synthetase may appear to be candidates for effectors against VACV, but the fact that type I IFNs representing potent inducers of both factors completely failed to prevent VACV replication suggests that these enzymes are unlikely to be the IFN- $\gamma$ -induced factors that block VACV replication. In fact, VACV replication was found to be indistinguishable in wt MEF and MEF lacking PKR and RNase L, and the susceptibility of mice to fatal infection with VACV was unaffected by the absence of these factors (78). The cloning and overexpression of cDNAs from IFN- $\gamma$ -stimulated MEF in human cells might allow the identification of the ISG factors that inhibit VACV replication.

**IFN- $\gamma$ -induced STAT1 signaling in orthopoxvirus-infected cells.** IFN- $\gamma$  represents a key cytokine mediating protection against infection with orthopoxviruses (32). Consequently, the viruses have evolved several strategies to counteract IFN- $\gamma$  (29, 57). Specifically, the VACV IFN- $\gamma$ -binding protein B8 competes with and blocks the binding of IFN- $\gamma$  to its receptor (2, 48), and the VACV VH1 virion phosphatase blocks IFN- $\gamma$ R signaling by binding and dephosphorylating human STAT1 (50). The analysis of  $\nu\Delta B8R$ , a VACV mutant lacking the *B8R* gene (Fig. 1), proved that the expression of this inhibitor was not sufficient to mediate proviral effects in our test system for VACV replication, irrespective of the host species investigated. In contrast, the strong downregulation of STAT1 tyrosine phosphorylation and subsequent dimer formation upon IFN- $\gamma$  stimulation observed in VACV-infected MRC-5 cells is fully compatible with the activity of a viral component reversing STAT1 activation. While the inhibition of STAT1 activation was clear in VACV-infected MRC-5 cells, it was only visible weakly in mouse fibroblasts (Fig. 5), raising the possibility that VH1 has species-dependent preferences for STAT substrates. An even more striking difference between human and mouse fibroblasts was observed after a prolonged exposure of cells to IFN- $\gamma$ . While mouse cells contained high levels of tyrosine-phosphorylated STAT1 and GAS complexes, in human MRC-5 fibroblasts, the abundant STAT1 protein existed only in a deactivated state (Fig. 5). This finding points to a basic, species-specific difference in the regulation of STAT1-signaling pathways leading to a sustained induction of STAT1/GAS-mediated antiviral gene expression in mouse but not human fibroblasts. Apparently, VACV is not equipped to disrupt this STAT1-mediated pathway, while ECTV is able to replicate well under these conditions. Likewise, another mouse-adapted large DNA virus, MCMV, efficiently blocks the IFN- $\gamma$  sensi-

tivity of VACV following coinfection (M. Trilling and H. Hengel, unpublished observation), demonstrating that the antiviral block against VACV is amenable to viral counterregulation. In contrast to MCMV and ECTV, VACV is subjected to this actively maintained antiviral state in murine cells due to its insufficient equipment with counteracting factors. Conversely, it is tempting to speculate that the IFN- $\gamma$  responsiveness of the so-far-unknown original host of VACV must differ from the inherent IFN- $\gamma$  response pattern of mouse fibroblasts.

Several host factors regulate STAT signaling cascades, such as the protein inhibitor of activated STAT proteins (40), several phosphotyrosine phosphatases, and members of the suppressors of cytokine signaling (80). It is tempting to speculate that the presence of such a dominant host-derived attenuator of human STAT1 has a broader impact on the antiviral potency of IFN- $\gamma$  to inhibit the replication of further human pathogenic viruses. Moreover, this factor could represent an important distinguishing feature between the human and the mouse IFN- $\gamma$  response.

**Potential implications for VACV studies in the mouse model.** VACV and ectromelia virus have each been studied extensively in mice (5, 18). The host range of VACV is much broader than that for ECTV, and although both viruses can be lethal in mice, mice are particularly sensitive to ECTV and less than 1 PFU can be fatal in some strains. Nonetheless, VACV differs from ECTV in that the VACV spread from the primary site of infection is more efficiently restricted in immunocompetent mice (31, 76, 79). The administration of exogenous IFN- $\gamma$  prevents lethal respiratory VACV infection in mice and reduces the virus titer in the lungs about 1,000-fold (41). In contrast, ECTV disseminates via the lymphatics and undergoes a primary viremia that is followed by an extensive virus multiplication in visceral organs. The release of virus progeny results in a secondary viremia and systemic rash (19). Given the different course of ECTV and VACV replication in laboratory mice, it is possible that the much stronger impact of IFN- $\gamma$  on VACV replication *in vitro* has consequences for the limited productivity of infection and altered disease pathogenesis *in vivo*. IFN- $\gamma$  is secreted in response to virus infection by NK cells as well as major subsets of T cells, implying that VACV is exposed to IFN- $\gamma$  continuously in the early and later phases of infection in mice. Specifically, IFN- $\gamma$  produced by CD4<sup>+</sup> T cells was demonstrated to be essential to control VACV replication after challenge infection (1), corroborating the earlier finding that mice deficient for IFN- $\gamma$ R exhibit an increased susceptibility to infection by VACV but not to VSV (30) or Semliki Forest virus (49). Beyond the implications for poxvirus pathogenesis, our findings could be of interest in a further context of VACV application. Several mouse models exhibit an enhanced selectivity of human tumor cells for infection by oncolytic VACV vectors (24, 71). Based on our data, it is tempting to speculate that in a murine environment that is protected from VACV multiplication due to the antiviral activity of mouse IFN- $\gamma$ , human tumor cells remain unprotected and become unavoidably targets of VACV destruction. A generally poor antiviral responsiveness of environmental cells to IFN- $\gamma$ , as observed here with human fibroblasts, could eliminate this seemingly selective VACV replication in tumor tissue when translated into such a situation in human patients.

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