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The highly virulent variola and monkeypox viruses express secreted inhibitors of type I interferon

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ABSTRACT Variola virus (VARV) caused smallpox, one of the most devastating human diseases and the first to be eradicated, but its deliberate release represents a dangerous threat. Virulent orthopoxviruses infecting humans, such as monkeypox virus (MPXV), could fill the niche left by smallpox eradication and the cessation of vaccination. However, immunomodulatory activities and virulence determinants of VARV and MPXV remain largely unexplored. We report the molecular characterization of the VARV- and MPXV-secreted type I interferon-binding proteins, which interact with the cell surface after secretion and prevent type I interferon responses. The proteins expressed in the baculovirus system have been purified, and their interferon-binding properties characterized by surface plasmon resonance. The ability of these proteins to inhibit a broad range of interferons was investigated to identify potential adaptation to the human immune system. Furthermore, we demonstrate by Western blot and activity assays the expression of the type I interferon inhibitor during VARV and MPXV infections. These findings are relevant for the design of new vaccines and therapeutics to smallpox and emergent virulent orthopoxviruses because the type I interferon-binding protein is a major virulence factor in animal models, vaccination with this protein induces protective immunity, and its neutralization prevents disease progression.—Fernández de Marco, M. M., Alejo, A., Hudson, P., Damon, I. K., Alcami, A. The highly virulent variola and monkeypox viruses express secreted inhibitors of type I interferon. *FASEB J.* 24, 1479–1488 (2010). www.fasebj.org

Key Words: immune modulation • cytokine • infection • smallpox • vaccine

THE *POXVIRIDAE* ARE a family of large-dsDNA viruses that replicate in the cytoplasm of infected cells. Most members of the genera *Orthopoxvirus* (OPV), *Parapoxvirus*, *Molluscipoxvirus*, and *Yatapoxvirus* infect humans either exclusively, for example, variola virus (VARV) and molluscum contagiosum virus, or zoonotically, such as monkeypox virus (MPXV), vaccinia virus (VACV), or

Yaba-like disease virus (YLDV). The consequences of these infections range from severe disease associated with high mortality to more benign localized infections such as seen with VACV infections of dairy cattle handlers in Brazil (1). VACV was the vaccine used to eradicate smallpox and is the prototypic member of the poxvirus family.

Two OPVs may cause severe disease in humans. VARV is the causative agent of smallpox, which was declared to be eradicated in 1980 as a result of the World Health Organization Smallpox Global Eradication Campaign, becoming the first and only viral disease eradicated by vaccination efforts (2). MPXV infects both humans and nonhuman primates, likely has a rodent reservoir, and is an emerging infectious disease, with cases observed in Africa and the United States (3). The deliberate release of VARV would have catastrophic consequences on global public health, considering that the majority of the human population has not been vaccinated or boosted in recent years, so there is a need to define the mechanisms of smallpox pathogenesis in order to develop intervention strategies (2). In addition, the reduced level of herd immunity against OPVs increases the possibility of infection with zoonotic OPVs, exemplified by VACV and cowpox virus infections in South America and Europe, respectively, and the more virulent MPXV, endemic in Central and West Africa, and the recent epidemic in the United States (3, 4).

Viral strategies to evade the immune response are likely pathogenesis determinants of smallpox and monkeypox (5, 6) and may also modulate an immunopathological reaction responsible for the toxemia reported in

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individuals suffering from severe smallpox and the adverse effects after smallpox vaccination (7).

The innate immune response is the first line of immune defense. One of its main effectors are interferons (IFNs), a family of multifunctional cytokines that are secreted from cells and inhibit virus replication *via* their direct antiviral and indirect immunoregulatory activities (8). Type I IFNs are induced by viral infection of almost any cell type and include various IFN α subtypes, IFN β and IFN ω among others. All type I IFNs bind to a common and widely expressed heterodimeric receptor and induce signaling through the Janus protein tyrosine-kinase and signal transducers and activators of transcription (STAT) pathway. Type I IFNs act by directly inducing an antiviral state in the cell (9) and have immunoregulatory activity (10). IFN γ , the only member of type II IFNs, is induced by antigen-stimulated lymphocytes and activates natural killer and cytotoxic T cells that destroy infected cells. Type III IFNs (IFN λ) are interleukin 10 (IL-10)-related cytokines with antiviral activity that are produced on cell infection by most cell types, including plasmacytoid dendritic cells (11). Although type III IFNs bind to a unique heterodimeric IFN λ receptor complex, they induce a type I IFN signaling pattern (12).

The central role of IFNs in antiviral defense is reinforced by the fact that most viruses interfere with IFN signaling pathways at different levels (8, 13). Poxviruses express intracellular proteins that target this pathway, such as the eIF-2 α homologue K3 (14) and the double-stranded RNA-binding protein E3 (15). An IFN evasion strategy particular to poxviruses is the expression of secreted IFN decoy receptors, including the IFN α / β -binding protein (IFN α / β BP) (16–19) and IFN γ receptor (20, 21), which bind IFNs with high affinity and prevent their interaction with cellular receptors. The IFN α / β BP, encoded by the VACV strain Western Reserve (WR) *B18R* gene, is an immunoglobulin (Ig) superfamily glycoprotein of sequence unrelated to type I IFN receptors (22, 23). It is secreted from infected cells and acts both in solution and when associated with the cell surface, preventing the establishment of an antiviral state in surrounding uninfected cells (17). Uniquely among IFN receptors, and indicative of their potential host range, the poxvirus-encoded IFN decoy receptors bind IFNs from a broad range of host species (19). Deletion of the IFN α / β BP gene from the VACV WR genome attenuates the virus in a mouse model of infection (19, 24), and it has been recently demonstrated that the IFN α / β BP is also essential for ectromelia virus (ECTV) virulence and that vaccination with the IFN α / β BP induces protective immunity (25).

In this study we demonstrate that VARV and MPXV express a soluble IFN α / β BP, which binds to the cell surface and protects cells from the antiviral effects of IFN. A detailed characterization of the interaction of the VARV and MPXV IFN α / β BPs with a broad range of type I IFNs from different species and with type III IFNs was carried out to identify potential differences with VACV and adaptation to the human IFN system.

The implications of these findings for the improvement of vaccines and therapeutics against smallpox and emergent virulent poxviruses is discussed.

MATERIALS AND METHODS

Cells and reagents

HeLa, A549, and BSC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 5–10% fetal calf serum (FCS). Sf21 and High FiveTM cells (Invitrogen, Carlsbad, CA, USA) were grown in TC-100 medium with 10% FCS. Vesicular stomatitis virus (VSV) strain Cocal was obtained from W. James (Oxford University, Oxford, UK). Recombinant hIFN α -G, hIFN α -J1, hIFN α -K, hIFN α -B2, hIFN α -F, hIFN ω , mIFN α -A, mIFN β , and Rhesus/cynomolgus IFN α -2 were from PBL InterferonSource (Piscataway, NJ, USA). hIFN α -A, hIL-10, mL-10, and mL28A were from PeproTech (Rocky Hill, NJ, USA). hIFN β was from Calbiochem (San Diego, CA, USA) or PBL InterferonSource. hIFN α -2b (Intron A) was from Schering-Plough (Kenilworth, NJ, USA). Recombinant hIL-28A and hIL-29 were from R&D Systems (Minneapolis, MN, USA).

Cloning and expression of viral IFN α / β BP

The VACV strain WR *B18R*, MPXV strain MSF6 *B16R*, and VARV strain BSH1975 *B17R* genes were amplified by PCR. MPXV and VARV template DNAs were provided by H. Meyer (Bundeswehr Institute of Microbiology, Munich, Germany) and the U.S. Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA), respectively. The region amplified corresponded to the coding region without the predicted signal peptide. PCR products were cloned into pAL7, a modified pFastBac plasmid bearing the honeybee mellitin signal peptide at the 5' region and a C-terminal V5-His tag. The plasmids generated were pMF1 (VARV B17), pMF2 (VACV B18), and pMF6 (MPXV B16). The sequence of the inserts was confirmed. The MPXV *B16R* gene from strain MSF6 was identical to the sequence published for MPXV strain Zaire 96 (26). Handling of VARV DNA was performed under World Health Organization (WHO) permission and in accordance with WHO guidelines. Recombinant baculoviruses were obtained using the Bac to Bac system (Invitrogen). Recombinant baculovirus expressing ECTV semaphorin homologue (SEMA) will be described elsewhere (unpublished results).

Generation of MPXV- and VARV-infected cell supernatants

BSC-40 cells were infected with MPXV West African (MPXV USA2003-WI-044) and Congo Basin (MPXV RCG2003-RCG-358) strains at 10 PFU/cell in Opti-MEM (Invitrogen), and media were harvested at 48 h postinfection. Media were centrifuged at 1000 *g* for 5 min and UV inactivated. VARV (Bangladesh 1975) infections were performed in a similar way under BSL4 laboratory conditions, and media were collected at 30 h postinfection. The supernatant was γ -irradiated (5×10^6 rad) on ice for 4 h and safety tested to demonstrate no viral growth in 2 sequential passages. VARV reagents were made in 2000, using a WHO-approved protocol. Supernatants were stored at -70°C . For all samples, 1 ml of supernatant corresponds to $\sim 1 \times 10^6$ cells.

Immunofluorescent cell staining

HeLa cells were incubated for 30 min at 37°C with supernatants from baculovirus-infected High5 cells. Unbound protein was removed with 5 washes in cold PBS, and cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Immunofluorescent detection was performed without cell membrane permeabilization using a monoclonal anti-V5 antibody (Invitrogen), followed by Alexa Fluor® 488 donkey anti-mouse IgG (Invitrogen). Cell nuclei were stained with DAPI (Calbiochem).

Inhibition of type I and type III IFN-induced STAT-1 phosphorylation

HeLa (for IFN type I) or A549 (for IFN type III) cells were incubated with supernatants from baculovirus-infected cells and thoroughly washed to remove unbound material. Subsequently, cells were incubated with 1000 U of IFN for 15 or 30 min at 37°C. Cells were lysed in Laemmli buffer and analyzed by Western blot using the anti-phospho STAT1 (Tyr701) and anti-STAT1 antibodies from Cell Signaling and anti-V5 antibody.

Inhibition of type I IFN-induced transcription

HeLa cells grown in 96-well plates were cotransfected using FuGene HD (Roche) with 100 ng/well of pSRE-Luc, pCRE-Luc, or pTAL-Luc (Mercury Pathway Profiling System; Clontech, Palo Alto, CA, USA) and 5 ng/well of pRLnull (Promega, Madison, WI, USA) plasmid bearing the *Renilla* luciferase for in-well normalization of transfection efficiency. At 24 h post-transfection, 50 U of hIFN α A was added in the presence or absence of 20 ng of the indicated purified recombinant proteins, and cells were incubated for 16 h. Luciferase activity was detected using the Dual-Glo Luciferase assay system (Promega).

Neutralization of IFN antiviral activity

Recombinant IFNs were incubated with culture supernatants or purified proteins in 5% FCS DMEM for 30–90 min at 37°C and added to HeLa cells grown in 96-well plates for 16 h. Cells were then infected with 50 PFU/cell of VSV strain cocof for 48 h (16). Cell viability was determined using the Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega).

Protein expression and purification

Supernatants from baculovirus-infected High5 cells were concentrated on a stirred ultrafiltration cell (Amicon, Danvers, MA, USA) using YM-10 membranes (Millipore, Bedford, MA, USA), and buffer was exchanged into binding buffer (50 mM phosphate, 300 mM NaCl, and 10 mM imidazole, pH 7.4) on PD-10 desalting columns (GE Healthcare, Little Chalfont, UK). The His-tagged recombinant proteins were affinity-purified using Ni-NTA (Qiagen, Valencia, CA) columns, dialyzed into PBS, and concentrated using Vivaspin 500 columns (Sartorius Stedim Biotech, Aubagne, France). Protein purity was checked on Coomassie-blue-stained SDS-PAGE and quantitated by a BCA assay (Pierce Biotechnology, Rockford, IL, USA).

Biomolecular interaction analysis by SPR

Cytokine binding specificity and affinity constants were determined using a BIAcore X biosensor (BIAcore, Uppsala,

Sweden). For ligand screening experiments, purified proteins were amine coupled to CM5 chips to ~5000 RU (5000 pg/mm²). IFNs were injected at 10 or 100 nM in HBS-EP buffer [10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4] at a flow rate of 10 μ l/min, and association and dissociation were monitored. The surface was regenerated using 10 mM glycine-HCl, pH 1.5. For kinetic analyses, proteins were immobilized at low densities (R_{\max} < 200 RU). Different concentrations of the analyte were injected at a flow rate of 30 μ l/min over 2 min and allowed to dissociate for 3–5 min. BIAcore sensorgrams were analyzed using BIAevaluation 3.2 software. Bulk refractive index changes were removed by subtracting the reference flow cell responses, and the average response of a blank injection was subtracted from all analyte sensorgrams to remove systematic artifacts. Kinetic data were globally fitted to a 1:1 Langmuir model.

RESULTS

VARV and MPXV encode secreted IFN α / β BPs that bind to the cell membrane

We expressed recombinant versions of VACV WR IFN α / β BP B18 and its orthologues VARV B17 and MPXV B16 proteins fused to V5 and 6xHis C-terminal tags (Supplemental Fig. 1). All 3 recombinant proteins were secreted from baculovirus-infected insect cells with molecular sizes of 52 kDa for VACV and MPXV proteins or 50 kDa for the VARV protein (Supplemental Fig. 2A and Fig. 2). Cell extracts obtained in the presence of tunicamycin, an inhibitor of *N*-glycosylation, showed a single band of 37 kDa in each case, corresponding to the predicted size of the unmodified proteins (Supplemental Fig. 2B).

VACV B18 binds to the plasma membrane of neighboring, uninfected cells after secretion from infected cells (17). Immunofluorescence of HeLa cell monolayers preincubated with supernatants from recombinant baculovirus-infected cells containing the mentioned proteins showed an intense staining indicative of protein binding to the cell surface for the 3 IFN α / β BPs but not with a control secreted protein, the SEMA encoded by ECTV (Fig. 1A). Confocal microscopy analysis showed a clear plasmatic membrane staining pattern for the VACV and VARV proteins (Fig. 1B) and the MPXV protein (not shown).

The VARV B17 and MPXV B16 proteins inhibit type I IFN-induced signaling

To examine whether cell surface-associated proteins prevented IFN-induced signaling, HeLa cells were incubated with recombinant proteins and washed to remove unbound protein. Addition of human IFN α (hIFN α) induced STAT-1 phosphorylation in HeLa cells preincubated with the control protein SEMA (Fig. 2A). However, cells preincubated with either VACV B18 protein or its VARV or MPXV orthologues did not show STAT-1 phosphorylation, indicating

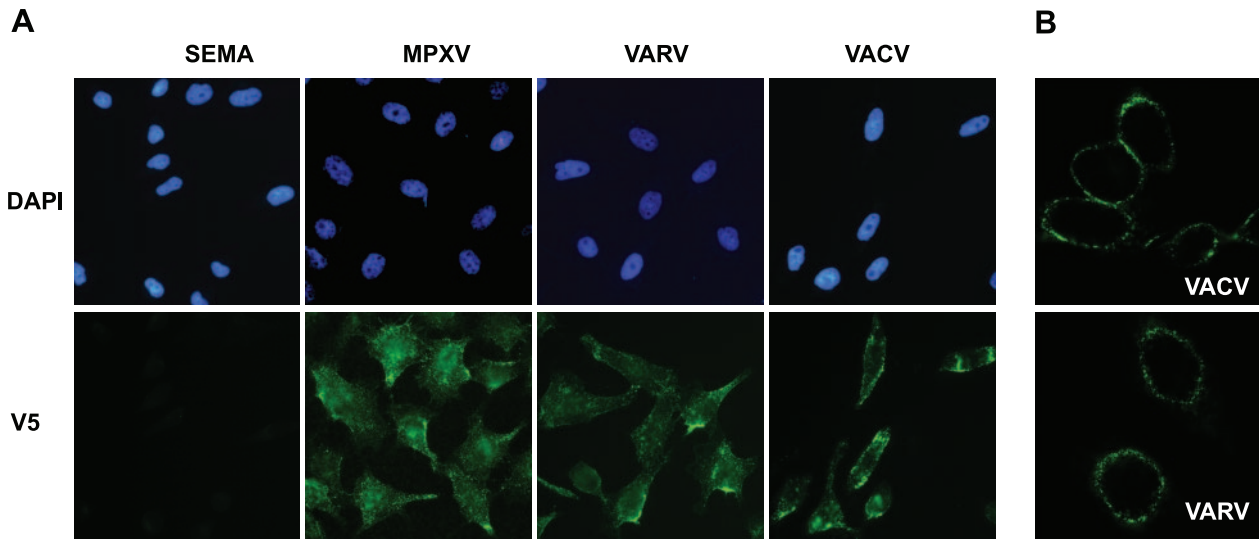


Figure 1. Cell surface binding of VACV IFN α / β BP and its VARV and MPXV orthologues. *A*) Anti-V5 immunofluorescent staining of nonpermeabilized HeLa cells incubated with supernatants containing the indicated V5-tagged recombinant proteins. Cell nuclei were visualized using DAPI. *B*) Confocal microscopy of cells incubated as before, showing plasmatic membrane localization of the indicated proteins.

that the 3 IFN α / β BPs block hIFN- α -induced signaling. All 3 proteins, but not the control protein, were detected by Western blot analysis, confirming their binding to the plasma membrane. In a similar experiment, neither the VACV nor its VARV or MPXV orthologues blocked STAT-1 phosphorylation induced by type III IFNs (IL-29 and IL-28A), which was readily detected in the absence or the presence of the viral proteins (Fig. 2*B, C*).

The VARV B17 and MPXV B16 proteins block the antiviral activity of several human type I IFNs

We next determined the ability of the IFN α / β BPs from VARV and MPXV to inhibit the antiviral activity of different human type I IFNs: hIFN α -A, hIFN α -2b, hIFN α -K, hIFN α -G, hIFN α -J1, hIFN α -F, hIFN α -B2, hIFN β , and hIFN ω (Fig. 3). IFN treatment completely abrogated VSV-induced cell killing in the presence of

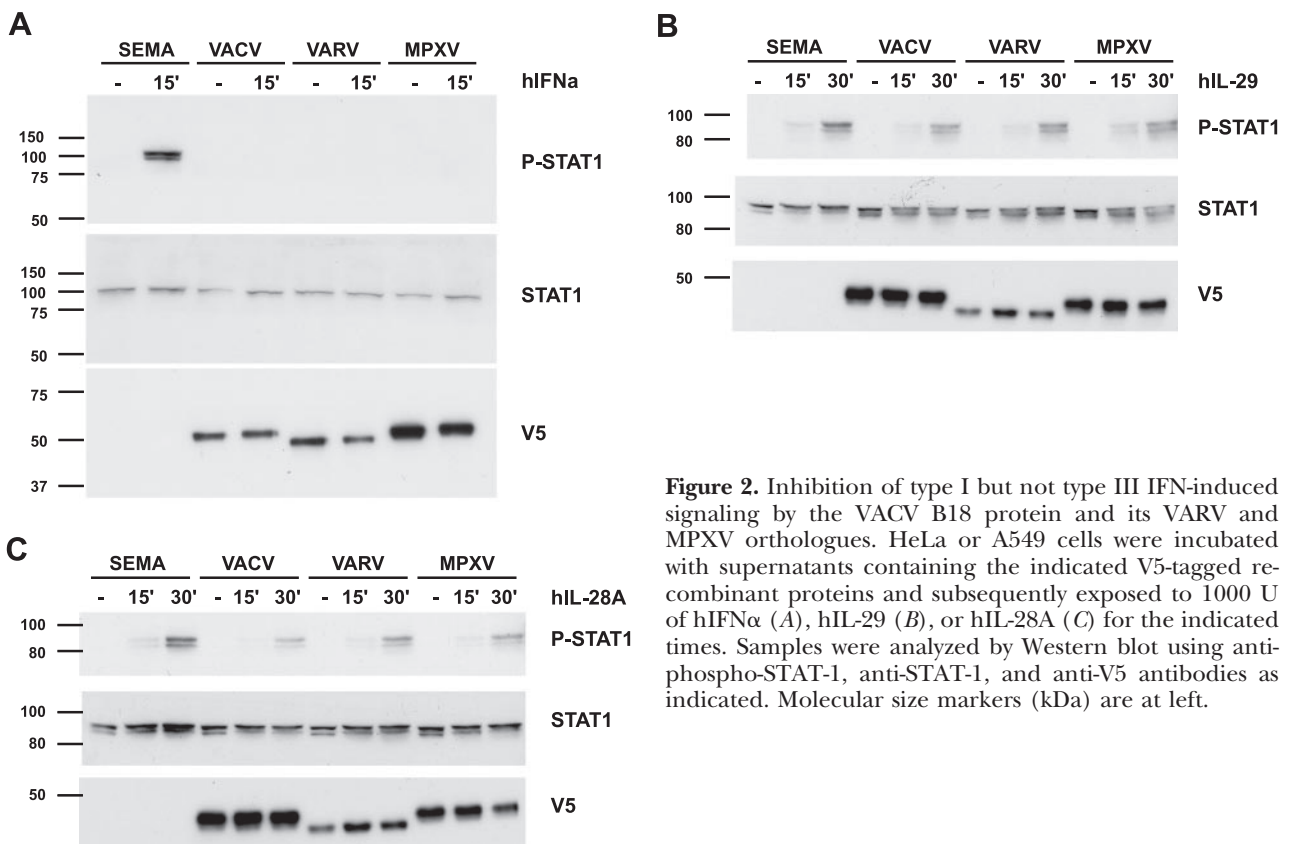


Figure 2. Inhibition of type I but not type III IFN-induced signaling by the VACV B18 protein and its VARV and MPXV orthologues. HeLa or A549 cells were incubated with supernatants containing the indicated V5-tagged recombinant proteins and subsequently exposed to 1000 U of hIFN α (*A*), hIL-29 (*B*), or hIL-28A (*C*) for the indicated times. Samples were analyzed by Western blot using anti-phospho-STAT-1, anti-STAT-1, and anti-V5 antibodies as indicated. Molecular size markers (kDa) are at left.

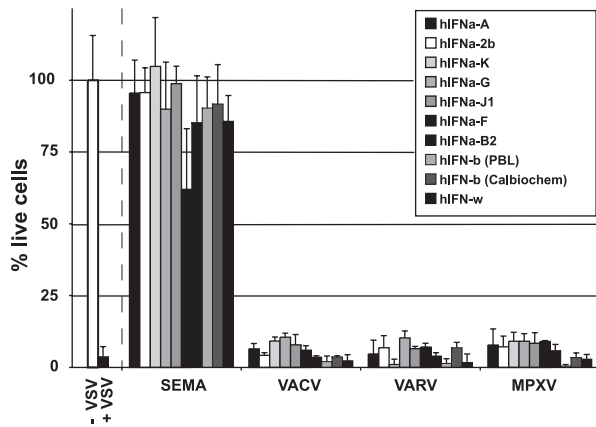


Figure 3. Inhibition of type I IFN antiviral activity by VACV IFN α / β BP and its VARV and MPXV orthologues. Supernatants containing the indicated recombinant proteins were preincubated with 50 U of each type I IFN and subsequently added to HeLa cell monolayers. After a 16 h incubation, cells were infected with VSV, and cell viability was determined after 48 h. Controls of nontreated, uninfected (-VSV), and infected (+VSV) cells are at left, separated by a dashed line. Results are means \pm SD of triplicate samples from one representative experiment.

the control protein SEMA. By contrast, preincubation of IFNs with supernatants containing the IFN α / β BP from VACV, VARV, or MPXV resulted in complete cell death, demonstrating that all 3 proteins inhibit the antiviral effect of all the human IFNs tested. This effect was dose dependent for all the IFNs tested (not shown).

Differential binding properties of the VARV B17 protein as compared to the VACV B18 protein

To further characterize the properties of VARV B17 *in vitro* in comparison to VACV B18, we purified both by affinity chromatography (Supplemental Fig. 2A). Both proteins bound similarly to all the human type I IFNs tested (hIFN α -A, hIFN α -2b, hIFN α -K, hIFN α -G, hIFN α -J1, hIFN α -F, hIFN α -B2, hIFN β , and hIFN ω) as well as to Rhesus/cynomolgus IFN α (not shown), as determined by surface plasmon resonance (SPR). Although both proteins bound the murine IFN α (mIFN α) equally well, the VARV B17 protein showed a much weaker binding to mIFN β , with a very marked difference in the dissociation time (Fig. 4, top panels). In addition, we tested human and murine type III IFNs (hIL-28A, mIL-28A, and hIL-29) as well as the more distantly related human and murine IL-10 for binding to both proteins. Although VARV B17 was not able to bind strongly to any of these molecules, VACV B18 bound to hIL-28A, mIL-28A, hIL-29, and mIL-10 (Fig. 4, bottom panels). To further evaluate these differences, the binding affinities for several of these ligands were calculated by SPR (Table 1 and Supplemental Fig. 3). The affinities of VARV B17 and VACV B18 for hIFN α -A, hIFN β , and mIFN α were found to be high (ranking from 0.1 to 5 nM), and the VARV protein bound the human ligands

with \sim 5-fold higher affinity than the VACV orthologue. However, although VACV B18 showed comparatively weaker affinities (ranking from 3 to 15 nM) for mIFN β and the type III IFNs, the VARV B17 protein showed no detectable binding to any of these ligands under the conditions used.

In a luciferase reporter assay, preincubation of hIFN α -A with either VACV B18 or VARV B17 completely abrogated the IFN-induced activation of transcription from an IFN-stimulated response enhancer element (Supplemental Fig. 4A). In addition, both purified proteins blocked the antiviral activity of all the type I hIFNs tested (hIFN α -A, hIFN α -2b, hIFN α -K, hIFN α -G, hIFN α -J1, hIFN α -F, hIFN α -B2, hIFN β , and hIFN ω) in a dose-dependent manner (Supplemental Fig. 4B), with the concentration of protein needed to inhibit the IFN activity by 50% estimated at 10–40 ng/ml for all cases (Supplemental Fig. 4C).

An IFN inhibitory activity is secreted by MPXV- and VARV-infected cells

To address whether an active IFN α / β BP is expressed and secreted during MPXV or VARV infection, BSC-1 cells were infected with strains USA or RCG of MPXV or strain BSH1975 of VARV, and supernatants were collected at 48 or 30 h postinfection. Virus present in the supernatants was inactivated to allow safe handling of the samples. Western blot analyses using a polyclonal anti-VACV B18 protein antibody showed a band of \sim 50 kDa in supernatants from cells infected with either RCG or USA MPXV strains as well as VARV, but not in mock-infected cells, and likely corresponds to the MPXV B16 protein and VARV B17 protein, respectively (Fig. 5A). Supernatants from cells infected with each MPXV strain, but not from mock-infected cells, efficiently inhibited hIFN α -2b, hIFN α -A, or hIFN β -induced antiviral activity in a dose-dependent manner (Fig. 5B). Similarly, supernatants from VARV-infected cells contained a specific type I IFN inhibitory activity (Fig. 5C).

DISCUSSION

Viral immunomodulatory mechanisms are key determinants of pathogenesis. Currently the mechanisms of immune evasion in VARV and MPXV are largely unknown and mostly inferred from data from other poxviral species. Given the relevance of both viruses as human pathogens, it is important to study the properties of the VARV and MPXV proteins with immunomodulatory potential. In this report we have characterized the secreted type I IFN inhibitors from both viral species, comparing their activity to their VACV orthologue. Moreover, we have shown the expression of both viral proteins during infection with VARV and MPXV in cell culture.

The genomes of several VARV and MPXV strains

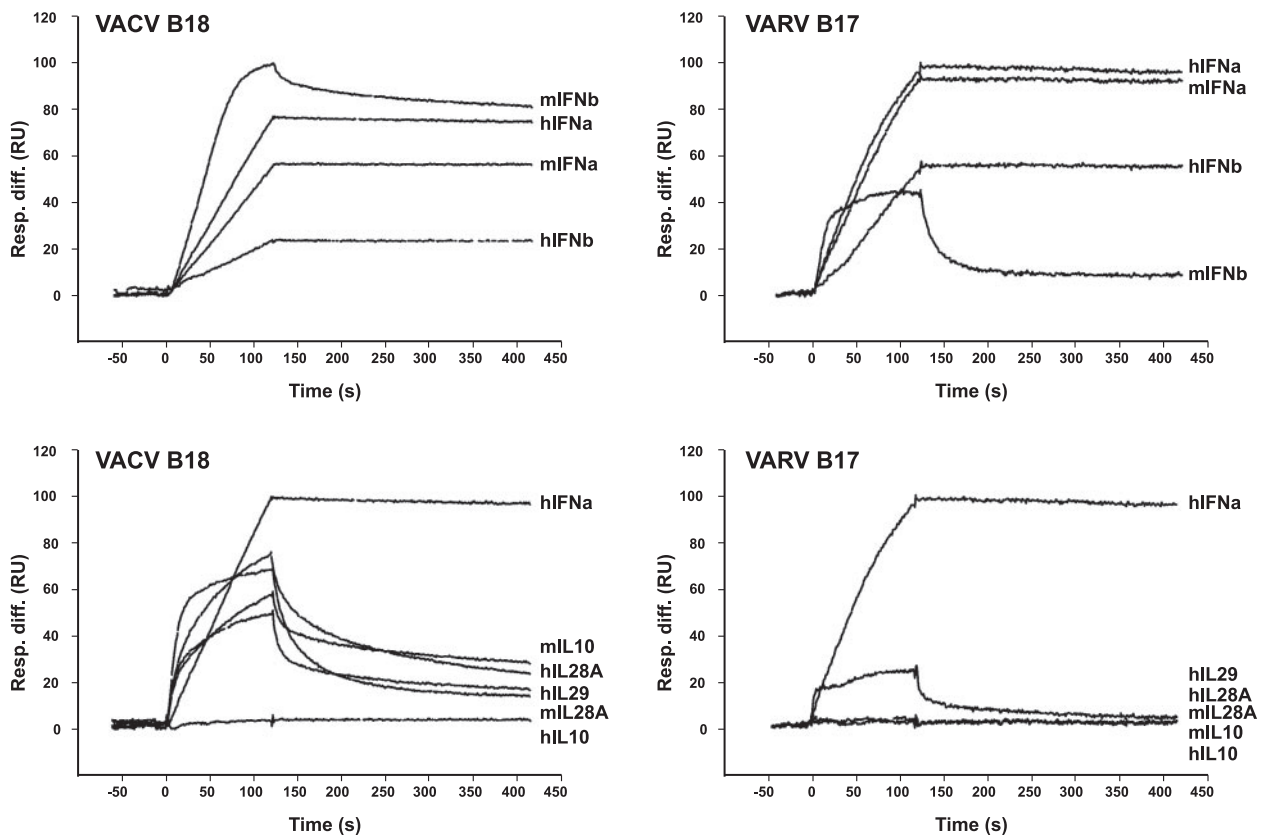


Figure 4. Differential binding of VACV B18 and VARV B17 to human and murine IFNs. Purified recombinant VACV B18 or VARV B17 were coupled to a sensor chip and screened for interactions with the indicated molecules by SPR. Top panels: binding curves of each protein to indicated human and murine type I IFNs. Bottom panels: binding curves of each protein to human and murine type III IFNs and the related human and murine IL-10. Curves correspond to injections of 10 nM hIFN α -A, 10 nM hIFN β , 10 nM mIFN α , and 100 nM of all other analytes. All curves are normalized to an arbitrary value of 100 RU for comparative purposes; hIFN α -A curve is shown as a reference. Data correspond to one representative experiment.

have been sequenced and proteins with immunomodulatory potential predicted. However, very few such proteins have been characterized so far (reviewed in refs. 27, 28). The IFN α / β BP has been shown to contribute to poxvirus pathogenesis in VACV infections (18, 19), and more recently this has been confirmed using the mousepox model, considered the best surrogate mouse model for human smallpox. In the absence of the ECTV IFN α / β BP, the LD₅₀ in susceptible mice was raised $\geq 10^7$ -fold, and this attenuation was accompanied by an increased innate and adaptive immune

response (25). Therefore, it is reasonable to speculate that the VARV and MPXV IFN α / β BPs may play a role in pathogenesis. A rapid activation of IFN-associated genes was observed in peripheral blood mononuclear cells from VARV-infected cynomolgus macaques (29). In addition, the inability to induce a strong IFN response appeared to correlate with more severe disease. This is consistent with the hypothesis that VARV regulation of IFN responses is a key determinant of smallpox pathogenesis. Here we demonstrate that VARV B17 is expressed and active as a type I IFN inhibitor during

TABLE 1. Comparison of kinetic parameters and derived affinity constants for the binding of VACV IFN type I binding protein and its VARV orthologue to IFNs

IFN	VACV B18			VARV B17		
	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_d (M)	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_d (M)
hIFN α	3.89×10^5	2.25×10^{-4}	5.8×10^{-10}	1.22×10^6	1.32×10^{-4}	1.08×10^{-10}
hIFN β	5.98×10^5	3.16×10^{-4}	5.28×10^{-9}	2.52×10^5	3.13×10^{-4}	1.24×10^{-9}
mIFN α	1.72×10^6	7.84×10^{-4}	4.55×10^{-10}	1.34×10^6	3.84×10^{-4}	2.6×10^{-10}
mIFN β	1.04×10^5	3.33×10^{-4}	3.2×10^{-9}	—	—	—
hIL28A	2.45×10^5	3.64×10^{-3}	1.49×10^{-8}	—	—	—
hIL29	1.93×10^5	2.6×10^{-3}	1.35×10^{-8}	—	—	—
mIL28A	8.32×10^5	1.12×10^{-2}	1.34×10^{-8}	—	—	—

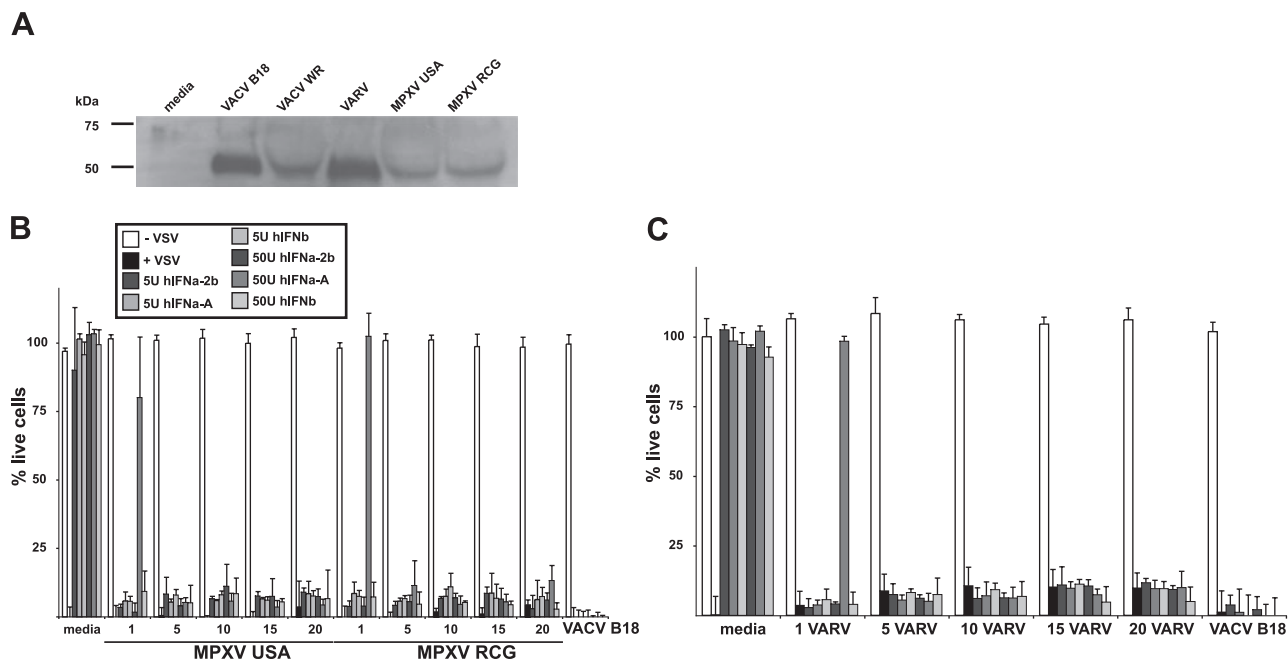


Figure 5. Expression and activity of the IFN α / β BP in supernatants from MPXV- or VARV-infected cells. *A*) Supernatants from VACV-, MPXV-, or VARV-infected cells were analyzed by Western blot using an anti-B18 protein antibody along with negative (media) and purified recombinant B18 protein (VACV B18) as indicated. *B*, *C*) Increasing amounts of supernatants from MPXV strain USA- or RCG-infected cells (*B*) or VARV strain BSH75 X or mock-infected cells (media) (*C*) were preincubated with either 5 or 50 U of type I hIFNs as indicated and added to HeLa cell monolayers for 16 h. As a control, 10 ng of purified VACV B18 protein was preincubated with IFN and added to HeLa cell monolayers as before. Cells were infected with VSV, and cell viability was determined after 48 h. Controls of nontreated, uninfected (–VSV) and infected (+VSV) cells were included in each case. Results are means \pm SD of triplicate samples from one representative experiment.

infection. To our knowledge, this is the first demonstration of expression and activity of a native-secreted immunomodulatory VARV protein. Similarly, we have shown expression of the active protein in both RCG and USA strains of MPXV during infection in cell culture. However, we cannot formally rule out the possibility that VARV or MPXV may encode additional secreted antitype I IFN activity distinct from the IFN α / β BP characterized in this report. Previously, transcription of the *B16R* gene from MPXV strain Zaire96 was described to occur early during tissue culture infection, but protein expression was not reported (30). Note that orthologues of both intracellular poxviral IFN-resistance proteins E3 and K3 are present in VARV but truncated or absent in MPXV (28). This may make MPXV far more reliant for the control of the host's immune response on the secreted type I IFN inhibitor than other poxviruses.

VACV B18 is a decoy-type I IFN receptor with broad species specificity and blocks the antiviral activity of human IFN α 1, IFN α 2, IFN α 7, IFN α 8, IFN β , IFN ω , IFN κ , and IFN ϵ (18, 19, 31). Our results show that the VARV and MPXV orthologues block the antiviral activity of 8 different human type I IFNs, with an inhibitory profile similar to that of the VACV protein. In addition, both the VARV and MPXV proteins block monkey IFN activity, indicating that its activity should be evident in the macaque model of infection.

A unique property of VACV B18 is its ability to bind

to the cell surface and prevent IFN signaling at this location (17, 18). It was suggested that secretion is the best way to transport this protein to uninfected cells to coat a surrounding uninfected tissue, which would be its most relevant site of action physiologically. We show that this property is shared by the VARV and MPXV IFN α / β BPs. Interestingly, the more distantly related secreted IFN inhibitor protein from the yatapoxvirus YLDV also binds to the cell surface (31).

The antiviral activity of type III IFN is restricted to viruses that enter through epithelia, such as those lining the lung (32). Type III IFN impairs VACV replication *in vivo* (33) and, more importantly, the YLDV orthologue of VACV B18 inhibits human type III IFN activity in cell culture (31). Because VARV and MPXV enter their host mainly through the respiratory system, it was important to determine whether the VARV or MPXV IFN α / β BP may also block type III IFN signaling. As shown before (31), we found that the VACV protein does not block type III IFN-induced signaling. Similarly, neither its MPXV nor its VARV orthologues blocked type III IFN activity. The importance of type III IFN in response against human poxviral infections is not known. YLDV is a primate virus that can be transmitted to humans causing a mild illness clearly distinct from monkeypox and smallpox, and perhaps type III IFN is more important in the local control of yatapoxvirus infections. Alternatively, other OPVs may encode different secreted immunomodula-

tors targeting type III IFNs. It is also conceivable that the intracellular blockade of type I IFN-induced responses mediated by E3L, K3L, and the VH1 phosphatase (34) is also efficient against type III IFN signaling and sufficient to prevent its antiviral activity *in vivo*. Surprisingly, we found that the VACV, but not the VARV IFN α / β BP, did bind human and murine type III IFNs. The inability of this protein to block their activity may be due to its relatively low binding affinity. This lower affinity or the nature of the recombinant proteins used may be the reason why Huang *et al.* (31) did not detect this interaction. However, this finding has important consequences for structure-function studies, especially for the determination of the motifs involved in type I *vs.* type III IFN binding in poxviral orthologues of VACV B18.

To study the potential adaptation of the VARV IFN α / β BP to its human host, we compared the binding and specific activity of the purified protein with those of VACV B18. The binding affinities of VARV B17 for hIFN α -A and hIFN β were slightly better (5-fold higher) than those of VACV B18. Although the specific activity of both proteins in IFN α and IFN β inhibition assays *in vitro* did not show major differences, a potential advantage *in vivo* in the human host cannot be excluded. Interestingly, while VARV B17 binds mIFN α with an affinity comparable to that of VACV B18, it has lost the capacity to bind murine IFN β as well as type III IFNs and IL-10. It should be noted that binding of VACV B18 to these cytokines does not block their activity (refs. 16, 31 and the present study) and is therefore probably not relevant *in vivo*. It is surprising that the OPV IFN α / β BPs have lost the ability to block mIFN β activity while retaining hIFN β -inhibitory activity since IFN β -deficient mice are more susceptible to VACV (35).

Apparent adaptation of poxvirus immunomodulatory proteins to their specific hosts has been shown for the smallpox inhibitor of complement enzyme (SPICE), a VARV orthologue of the VACV complement control protein, which contributes to pathogenesis *in vivo*. SPICE is a highly specific and potent inhibitor of human complement as compared to the VACV protein (36). This enhanced function against human complement has been ascribed to 4 amino acid changes (37), illustrating that adaptation to the human host may not require many mutations. However, the VARV immunomodulators studied to date do not necessarily have higher affinities for their human ligands. Thus, the VARV IL-18BP showed similar affinities for its human and murine ligand (38), while VARV CrmB is a better inhibitor of murine than human tumor necrosis factor (39). This suggests the relative importance of the binding affinities of various virally encoded immune response modifiers for various human immune effectors in the maintenance of VARV in a human host system and perhaps indicates that VARV has only recently (in evolutionary time) adapted to a human host. Yatapoxviruses, on the contrary, show a marked species

specificity in their secreted receptors for TNF and IFN (31, 40).

Because of the risk of emerging OPV infections and weaponization, and because the live VACV-based vaccine is contraindicated for portions of the global population, there is a considerable interest in developing novel anti-OPV vaccines (41, 42). It was previously shown that monoclonal antibodies against the secreted VACV/VARV growth factor could enhance protective immunity in a VACV intranasal mouse model of infection (43), which led the authors to propose that blockade of secreted viral immunomodulators represents an alternative to the traditional approach that prevents infection by virus neutralization. The IFN α / β BP encoded by ECTV, with properties similar to those of VARV and MPXV (16), has been shown to be a major contributor to virus virulence in mousepox (25), considered one of the best mouse models of human smallpox. The unprecedented attenuation of an ECTV mutant in the IFN α / β BP gene, with an increase of more than 10⁷-fold in the LD₅₀, clearly demonstrated that virulent OPVs require this protein to establish a smallpox-like disease and strongly suggested that neutralization of this viral IFN inhibitor is a therapeutic strategy to control disease severity. Consistent with this, vaccination with recombinant ECTV IFN α / β BP could effectively prevent fatal mousepox (25).

Our demonstration that the IFN α / β BP is actively expressed by cells infected with VARV and MPXV identifies this viral immunomodulatory protein as both a component of a protective vaccine and a target for therapeutics against virulent human poxviruses. Subunit vaccines against poxviruses are composed of structural proteins present in the virus particle and neutralize the infectivity of the virions, and the inclusion of viral-secreted virulence factors may serve as a complementary approach (25, 43). It should be noted that the smallpox vaccines Dryvax and modified virus Ankara encode truncated versions of the IFN α / β BP, which in the case of vaccinia virus Dryvax has been shown to be expressed and to bind IFN with 70-fold lower affinity than the full-length protein (17, 44). The inclusion of full-length IFN α / β BP, or, even better, a mutagenized version of the protein lacking IFN-binding activity, in the current smallpox vaccines may induce better anti-IFN α / β BP response and enhance their protective effect. In addition, monoclonal antibodies against the MPXV or VARV proteins could be helpful as a pharmacological compound in exposed individuals to prevent disease in human monkeypox and smallpox cases. This would represent an alternative to the large proportion of individuals at high risk of serious adverse effects when receiving smallpox vaccination, due to immune deficiency, atopic dermatitis, or at the extremes of age (45).

In summary, we report the expression of an active IFN α / β BP by VARV and MPXV, and the characterization of their binding properties against a broad range

of IFNs. This is the first demonstration of the expression of a soluble cytokine binding protein by VARV. The critical role of the IFN α / β BP in the pathogenesis of virulent poxviruses (25) emphasizes the relevance of these findings to understand the mechanisms of pathogenesis of smallpox and monkeypox. Furthermore, our findings identify the VARV and MPXV IFN α / β BPs as a target for vaccination and therapeutic treatment of infected individuals. **[F]**

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SUPPLEMENTAL FIGURE LEGENDS

Supportive information figure 1. Sequence alignment of B18 from VACV strain WR (GenBank accession number: YP_233082) with its VARV strain Bangladesh 1975 (B17R, GenBank accession number AAA60926) and MPXV strain Zaire_1979-005 (B16R, GenBank accession number: AAY97385) orthologues. Sequence alignments were performed with CLUSTALW at (www.ebi.ac.uk) and shaded using BOXSHADE (<http://bioweb.pasteur.fr/seqanal/interfaces/boxshade.html>). Positions presenting conservative changes are shaded in grey and non-conservative changes are shown in white letters on black background. Numbering for each protein is shown. The predicted signal peptide is indicated (SP) and Ig domains 1 to 3 for VACV B18 are delimited by bent arrows. Both VARV and MPXV encode orthologues of the VACV, encoded by genes B17R in VARV (94% amino acid identity) and B16R in MPXV (89.2% amino acid identity). The VARV B17 and MPXV B16 proteins are 85.6% identical. Most of these amino acid changes are concentrated in the N-terminal half of the protein, with 17 variable positions located in the first Ig-like domain. The VARV orthologue is predicted to have an extended N-terminus that would be included within the early mRNA mapped in VACV WR.

Supportive information figure 2. A. Different amounts of purified recombinant VACV B18 and VARV B17 proteins were separated by SDS-PAGE alongside known amounts of BSA and stained with Coomassie-blue. B. Expression and N-glycosilation of VACV IFN type I BP and its VARV and MPXV orthologues. High5 cells were not infected (-) or infected with baculoviruses bearing the genes for the indicated proteins in the absence (-) or the presence (+) of the N-glycosilation inhibitor tunicamycin. After 48

hours, the cells were harvested and samples were lysed and analysed by western blot using an anti-V5 antibody. MWM in kDa are shown on the left.

Supportive information figure 3. Fittings used for the determination of kinetic binding parameters of VACV B18 (A and C) and VARV IFN type I BP (B) for the different IFNs. In each plot, the analyte is shown on the top left. The number of curves used, and the range of concentrations in nM for each analyte are shown in brackets in the bottom right of each panel.

Supportive information figure 4. Inhibition of type I IFN by purified recombinant VACV and VARV IFN α / β BP. A. Fold luciferase activity activation of HeLa cells transfected with the indicated plasmids and stimulated with 1000U of hIFN α -A which had been preincubated or not with purified recombinant VACV B18 or VARV B17, as shown. Cells were cotransfected with phRL-null to allow for normalization of transfection efficiency. Fold activation was calculated in relationship to transfected, unstimulated cells. Means of triplicate samples from one representative experiment out of three are shown. B. Inhibition of IFN type I antiviral activity by purified recombinant VACV B18 or VARV IFN type I BP. 50U of the indicated hIFNs were preincubated with 20ng of each recombinant protein or not (- protein) and added to HeLa cell monolayers for 16 hours. After this period, the cells were infected with VSV and cell viability was determined after 48 hours. Shown are means of triplicate samples from one representative experiment out of three. C. Dose-dependent inhibition of type I IFN antiviral activity by purified recombinant VACV B18 or VARV B17. Experiments were performed using 50U of the indicated hIFN and increasing amounts of purified protein. Mean \pm SD of triplicates for each sample are shown.

SP
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VACV 0  --M--T--M--K--M--M--V--E--I--Y--F--V--S--L--L--L--L--P--H--S--Y--A--I--D--I--E--N--E--I--T--E--F--F--N--K--M--R--D--T--L--P--A--K--D--S--K--W--L--N--P--V--C--M--F--G--G
VARV 0  M--M--K--M--T--M--K--M--M--V--E--I--Y--F--V--S--L--L--L--L--P--H--S--Y--A--I--D--I--E--N--E--I--T--D--F--F--N--K--M--K--D--T--L--P--A--K--D--S--K--W--L--N--P--C--I--F--G--G
MPXV 0  --M--M--K--M--M--M--V--E--I--Y--F--V--S--L--L--L--L--P--H--S--Y--A--I--D--I--E--N--E--I--T--E--F--F--N--K--M--R--D--T--L--P--A--K--D--S--K--W--L--N--P--V--C--M--F--G--G

VACV 57 T--M--N--D--I--A--A--L--G--E--P--F--S--A--K--C--P--P--I--E--D--S--L--L--S--H--R--Y--K--D--Y--V--V--K--W--E--R--L--E--K--N--R--R--R--Q--V--S--N--K--R--V--K--E--G--D--L--W--I
VARV 60 T--M--N--M--A--A--I--G--E--P--F--S--A--K--C--P--P--I--E--D--S--L--L--S--R--R--Y--I--N--K--D--N--V--V--N--W--E--K--I--G--K--A--R--R--P--L--N--R--R--V--K--E--G--D--L--W--I
MPXV 58 T--M--N--D--I--A--A--L--G--E--P--F--S--A--K--C--P--P--I--E--D--S--L--L--S--H--R--Y--K--D--Y--V--V--K--W--E--R--L--E--K--N--R--R--R--Q--V--S--N--K--R--V--K--E--G--D--L--W--I

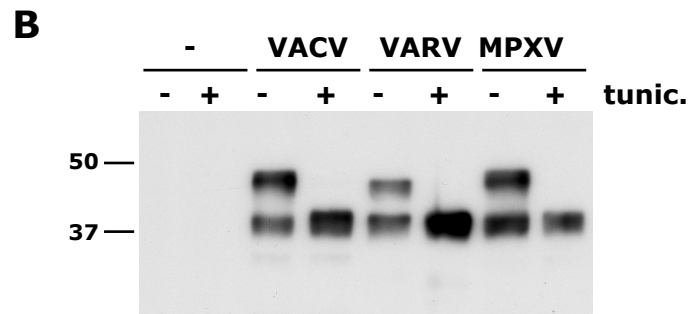
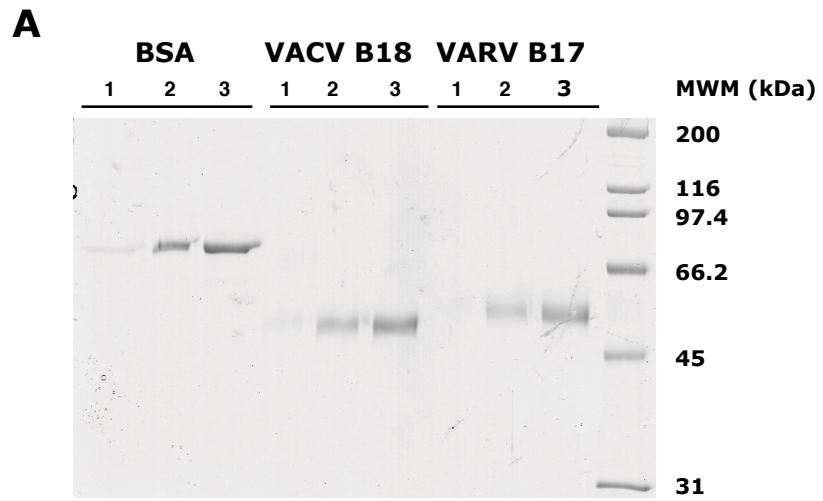
VACV 115 A--N--Y--T--S--K--F--S--N--R--R--Y--L--C--T--V--T--K--N--G--D--C--V--Q--G--I--V--R--S--H--I--R--K--P--P--S--C--I--P--K--T--Y--E--L--G--T--H--D--K--Y--G--I--D--L--Y--C--G--I--L
VARV 118 A--N--Y--T--S--N--D--S--H--R--M--T--L--C--T--V--I--T--K--N--G--D--C--I--Q--G--I--V--R--S--H--V--R--K--P--S--S--C--I--P--E--I--Y--E--L--G--T--H--D--K--Y--G--I--D--L--Y--C--G--I--I
MPXV 116 A--N--Y--T--S--K--F--S--N--R--R--Y--L--C--T--V--T--K--N--G--D--C--V--Q--G--I--V--R--S--H--V--M--K--P--S--S--C--I--P--K--T--Y--E--L--G--T--Y--D--K--Y--G--I--D--L--Y--C--G--I--L

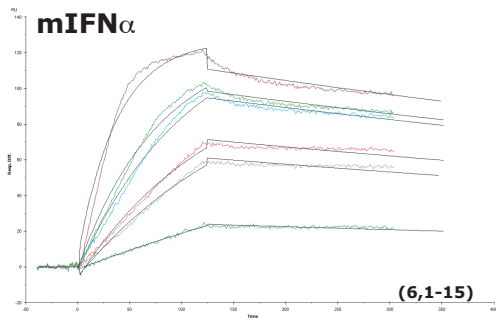
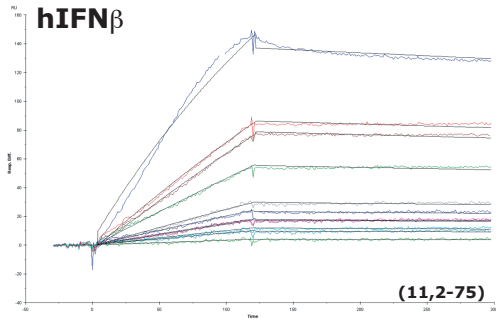
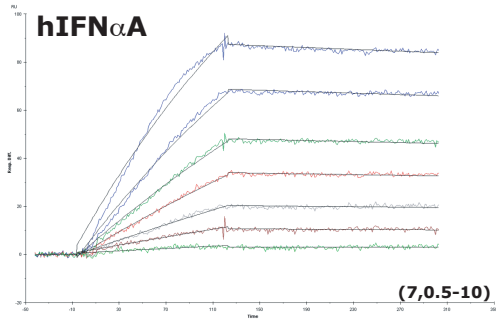
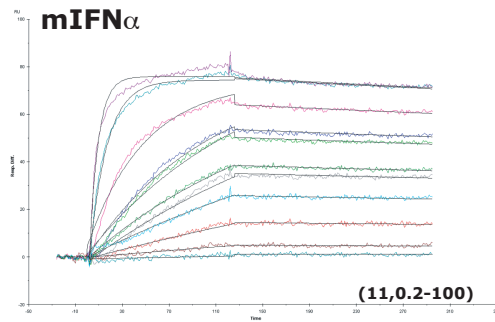
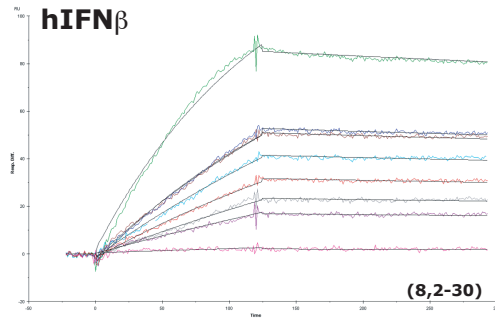
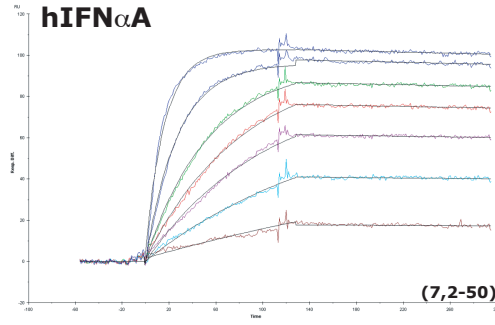
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VARV 178 Y--A--K--H--Y--N--N--I--T--W--Y--K--D--N--K--E--I--N--I--D--D--I--K--Y--S--Q--G--K--E--L--I--H--N--P--A--L--E--D--S--G--R--Y--D--C--Y--V--H--Y--D--D--V--R--I--K--N--D--I--V
MPXV 176 Y--A--K--H--Y--N--N--I--T--W--Y--K--D--N--K--E--I--N--I--D--D--F--K--Y--S--Q--G--K--E--L--I--H--N--P--E--L--E--D--S--G--R--Y--D--C--Y--V--H--Y--D--D--V--R--I--K--N--D--I--V

VACV 235 V--S--R--C--K--I--L--T--V--I--P--S--Q--D--H--R--F--K--L--I--L--D--K--I--N--V--I--G--E--P--A--N--I--T--C--T--A--V--S--T--S--L--L--I--D--D--V--L--I--E--W--E--N--P--S--G--W--L
VARV 238 V--S--R--C--K--I--L--T--V--I--P--S--Q--D--H--R--F--K--L--I--L--D--K--I--N--V--I--G--E--P--A--N--I--T--C--T--A--V--S--T--S--L--L--F--D--D--V--L--I--E--W--E--N--P--S--G--W--L
MPXV 236 V--S--R--C--K--I--L--T--V--I--P--S--Q--D--H--R--F--K--L--I--L--D--K--I--N--V--I--G--E--P--A--N--I--T--C--S--A--V--S--T--S--L--F--V--D--D--V--L--I--E--W--E--N--P--S--G--W--I

VACV 295 I--G--D--F--D--V--Y--S--V--L--T--S--R--G--G--I--E--A--T--L--Y--F--E--N--V--T--E--E--Y--I--G--N--T--Y--K--C--R--G--H--N--Y--F--E--K--T--L--T--T--V--V--L--E
VARV 298 I--G--D--F--D--V--Y--S--V--L--T--S--R--G--G--I--E--A--T--L--Y--F--K--N--V--T--E--E--Y--I--G--N--T--Y--K--C--R--G--H--N--Y--F--E--K--T--L--T--T--V--V--L--E
MPXV 296 I--G--D--F--G--V--Y--S--I--L--T--S--R--G--G--I--E--A--T--L--Y--F--E--N--V--T--E--E--Y--I--G--N--T--Y--T--C--R--G--H--N--Y--F--D--K--T--L--T--T--V--V--L--E

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A**VACV B18****B****VARV B17****C****VACV B18**