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Vaccinia Virus-Mediated Inhibition of Type I Interferon Responses Is a Multifactorial Process Involving the Soluble Type I Interferon Receptor B18 and Intracellular Components▽

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Poxviruses such as virulent vaccinia virus (VACV) strain Western Reserve encode a broad range of immune modulators that interfere with host responses to infection. Upon more than 570 in vitro passages in chicken embryo fibroblasts (CEF), chorioallantois VACV Ankara (CVA) accumulated mutations that resulted in highly attenuated modified vaccinia virus Ankara (MVA). MVA infection of mice and of dendritic cells (DC) induced significant type I interferon (IFN) responses, whereas infection with VACV alone or in combination with MVA did not. These results implied that VACV expressed an IFN inhibitor(s) that was functionally deleted in MVA. To further characterize the IFN inhibitor(s), infection experiments were carried out with CVA strains isolated after 152 (CVA152) and 386 CEF passages (CVA386). Interestingly, neither CVA152 nor CVA386 induced IFN-α, whereas the latter variant did induce IFN-β. This pattern suggested a consecutive loss of inhibitors during MVA attenuation. Similar to supernatants of VACV- and CVA152-infected DC cultures, recombinantly expressed soluble IFN decoy receptor B18, which is encoded in the VACV genome, inhibited MVA-induced IFN-α but not IFN-β. In the same direction, a B18R-deficient VACV variant triggered only IFN-α, confirming B18 as the soluble IFN-α inhibitor. Interestingly, VACV infection inhibited IFN responses induced by a multitude of different stimuli, including oligodeoxynucleotides containing CpG motifs, poly(I:C), and vesicular stomatitis virus. Collectively, the data presented show that VACV-mediated IFN inhibition is a multistep process involving secreted factors such as B18 plus intracellular components that cooperate to efficiently shut off systemic IFN-α and IFN-β responses.

Upon viral infection of a host, the pathogen is usually sensed by antigen-presenting cells such as dendritic cells (DC), which then secrete cytokines and, depending on the pathogen load, induce adaptive immunity (41). Two major DC subtypes exist: myeloid DC (mDC; also called conventional DC), which play a crucial role in antigen presentation and activation of lymphocytes and NK cells, and plasmacytoid DC (pDC), which can produce large amounts of type I interferons (IFNs) and other cytokines upon appropriate stimulation and show only moderate antigen presentation capacities (9, 12, 16, 20). Murine mDC are characterized by the expression of the surface markers CD11c and CD11b, whereas pDC express CD11c and B220 (10, 20, 45). Although most cell types can produce type I IFN upon in vitro infection, pDC are the major type I IFN producers upon in vivo infection with certain viruses (22). Type I IFNs (which in mice comprise 14 IFN-α isoforms and 1 IFN-β) are proinflammatory cytokines initially defined by their ability to induce resistance to viral infection (21, 28, 31, 39). All type I IFNs bind one common type I IFN receptor (IFNAR) (28, 43) and constitute a first line of defense against many viral infections to ensure the initial survival of the host. The impact of type I IFNs in viral infections is immense. Treatment of mousepox virus-infected mice with type I IFN neutralizing antibodies affects virus elimination (32), and pretreatment with IFN abrogates the infection (42). Infections of IFNAR-deficient mice with virulent vaccinia virus (VACV) strain Western Reserve result in about 10^3-fold higher virus titers than do infections of wild-type mice, and moreover, knockout mice succumb to the infection (56). Interestingly, upon infections with low-virulence VACV strain Lancy, wild-type mice clear the infection whereas the virus persists in IFNAR-deficient mice (30). Thus, modulation or inhibition of the host type I IFN response upon infection is crucial for the virus to replicate and spread.

Many viruses, such as VACV, influenza viruses A and B, Thogoto virus, herpes simplex virus type 1, human immunodeficiency virus, and Epstein-Barr virus, have evolved evasion strategies to prevent the induction of IFNs and/or IFNAR signaling (3, 4, 58, 59).

Poxviruses use a multitude of different strategies to evade immune surveillance. They harbor a large double-stranded DNA genome that contains more than 200 genes (27). Several VACV genes encode proteins that act as cytokine analogues, as molecular decoys to block the activity of host cytokines, and as soluble receptors, binding complement factors, cytokines, and chemokines in solution or at the cell surface. So far, VACV-encoded receptor homologues are known that bind interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α),

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IFN-α/β, and IFN-γ (1–3, 5). Furthermore, VACV expresses antagonists that interfere with major intracellular signaling pathways, e.g., factors that inhibit Toll-like receptor (TLR) signaling, which can lead to the induction of IFN responses. One such factor, A46, consists of a Toll/IL-1 receptor domain and competes with various different Toll/IL-1 receptor domain-containing TLR adaptor molecules (48). Moreover, VACV proteins K1, N1, and B14 have been reported to inhibit NF-κB signaling (17, 18, 23, 44).

Modified vaccinia virus Ankara (MVA) was attenuated by serial passaging of chorioallantois VACV Ankara (CVA) in chicken embryo fibroblasts (CEF) (35). During these in vitro passages, MVA accumulated many point mutations and deletions. The MVA genome is characterized by six major deletions that include open reading frames that encode proteins that regulate virus-host interaction and viral immune-modulatory genes. In total, MVA lost approximately 30 kb of the parental viral genome, which corresponds to approximately 15% (7, 36). As a consequence, MVA infection of most mammalian cells is abortive (14). Its severe replication deficiency in most cells of human origin, its avirulence in humans and even in immunocompromised animals, and its particular immunogenic properties make MVA a promising vaccine candidate for use in animals and humans. We recently showed that MVA, unlike VACV, does induce significant IFN responses upon immunization of mice or treatment of DC cultures in a TLR-independent manner (57). Nevertheless, although VACV-infected mDC do not secrete IFN, they produce other cytokines such as IL-6, IL-1, and IL-12, and the induction of these cytokines is presumably TLR2 dependent (61, 62).

Here we show that, upon VACV infection of mice or DC cultures, viral factors actively inhibit the induction of type I IFN responses. Analyzing MVA ancestor viruses that were isolated during attenuation in CEF passages, we show that IFN inhibition is a multistep process involving one secreted and likely several intracellular viral proteins that inhibit systemic IFN-α and IFN-β responses. Experiments involving the DNA replication inhibitor 1-β-D-arabinofuranosylcytosine (AraC) or UV-irradiated virus indicated that VACV-mediated IFN inhibition depended on newly synthesized early viral gene products. Interestingly, VACV infection inhibited type I IFN responses that were induced by a whole variety of different stimuli. Finally, infection with VACV, but not MVA, also impaired DC maturation. This latter mechanism can have an impact on the activation of adaptive immune responses.

MATERIALS AND METHODS

Mice and viruses. All of the mice used in this study were bred under specific-pathogen-free conditions at the Zentrale Tierhaltung of the Paul-Ehrlich-Institut. C57BL/6 mice were purchased from Harlan. Mouse experimental work was carried out with 8- to 12-week old mice in compliance with German animal welfare regulations.

MVA (cloned isolate F6 at CEF passage 584) and CVA at passage 152 (CVA152) and passage 386 (CVA386), which originated from passage 382 (36), were used and were originally provided by Anton Mayr (University of Munich, Munich, Germany). VACV strain Western Reserve was originally provided by Bernard Moss (NIH, Bethesda, MD). VACV strain Western Reserve deletion mutant vAA6 lacking B18R has been described previously (6). Vaccinia viruses were propagated and titrated on CEF and purified by centrifugation through sucrose by standard methodologies (49). The vesicular stomatitis virus (VSV) M2 strain used in this study has been shown before to be a particularly strong type I IFN-inducing variant of the HR strain of wild-type VSV (50) and was kindly provided by John Bell (University of Ottawa, Ottawa, Ontario, Canada). VSV strain M2 was grown on BHK-21 cells and plaque assayed on Vero cells (12).

Cell isolation and culture. Bone marrow (BM) cells were isolated by flushing the femur and tibia with RPMI medium supplemented with 10% fetal calf serum. Upon red blood cell lysis, cells were washed and seeded at a density of 2×10^6/ml in medium supplemented with Flt3-L (100 ng/ml; R&D Systems). Flt3-L-supplemented BM cell cultures were cultivated for 8 days with one medium change at day 4 by replacing half of the medium with fresh cytokine-supplemented medium (Flt3-L pDC).

Flow cytometric analysis and cell enrichment. Flt3-L pDC were stained with anti-B220–phycoerythrin (PE)–Cy5.5 monoclonal antibody (MAb), anti-CD11c–allophycocyanin MAb, anti-CD69–PE MAb, and anti-CD86–fluorescein isothiocyanate MAb (all from BD PharMingen). Cells were analyzed by flow cytometry (LSR II; BD), and data were analyzed with DIVA software.

In vitro stimulation and quantification of cytokine production. For stimulation, in vitro differentiated Flt3-L pDC were seeded at 1×10^6/well in 24-well culture plates in 1 ml medium or at 2×10^5/well in 96-well culture plates in 200 μl medium. CpG-containing oligodeoxynucleotide 2216 (5′-ggGGGA**CG**AT**CG** TCggggG-3′ [small letters, phosphorothioate linkage; bold letters, CpG dinucleotides; capital letters, phosphodiester linkage 3′ of the base]; Sigma-ARK) was used at a concentration of 10 μg/ml. For transfection of 2 μg poly(I:C) (pI:C; Sigma-Aldrich), the reagent Fugene (Roche) was used according to the manufacturer's instructions. AraC was purchased from Sigma and used at a concentration of 40 μg/ml. Recombinant IFN-α (HyCult Biotechnology) was used at the indicated concentrations. For UV irradiation of virus, a UV irradiation chamber (Herolab) was used. Irradiation with an energy of 75 mJ/cm^2 usually took approximately 10 s. Recombinant B18 (rB18) has been described previously (47), and recombinant viral TNF receptor (rCrmD) was produced in the same way. After stimulation of DC cultures, cell-free supernatant was collected and analyzed with enzyme-linked immunosorbent assay (ELISA) kits allowing the determination of mouse IFN-α or mouse IFN-β (PBL Biomedical Laboratories).

Northern blotting. NIH 3T3 cells were mock infected or infected with VACV, CVA152, CVA386, or MVA at a multiplicity of infection (MOI) of 5. Total RNA was isolated with TRIzol reagent (Invitrogen) by following the manufacturer's instructions. Total RNA was separated by electrophoresis in 1% agarose formaldehyde gels. RNA was transferred onto positively charged nylon membranes (Roche Diagnostics). Riboprobes for detection of B18R-specific RNA were synthesized by in vitro transcription with PCR products generated from VACV DNA templates with the following primers (sequences are shown in 5′-to-3′ orientation): HLPEI 143, ATG ACG ATG AAA ATG ATG GTA C; HLPEI 144, CTA ATA CGA CTC ACT ATA GGG AGA GAC ACA AAT ACC TAC GGT TAC TG. The reverse primer contained a T7 RNA polymerase promoter-recognition sequence (underlined). Digoxigenin (DIG)-labeled riboprobes were obtained by in vitro transcription with T7 RNA polymerase (Roche Diagnostics) with PCR-generated DNA fragments as templates. In vitro RNA labeling, hybridization, and signal detection were carried out according to the manufacturer's instructions (DIG RNA labeling kit and anti-DIG detection chemicals; Roche Diagnostics), applying 68°C for hybridization and a high-stringency wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate buffer.

Sequencing. For isolation of viral DNA, purified virus was treated with proteinase K (Roche) and DNA was prepared by phenol-chloroform extraction. Amplification of B18R was performed with the following primers (sequences are shown in 5′-to-3′ orientation): fw, ATG ACG ATG AAA ATG ATG GTA CA; rev, TTA CTC CAA TAC TAC TGT AGT TG. The 5′ region was amplified with the following primers (sequences are shown in 5′-to-3′ orientation): B18R 5′ for, ATG ACA CAT TTT ACA ATA GAC ACG; B18R 5′ rev, GAA TAG CAA TAA CAA TAA TGA TAC G. PCR products were cloned in pCR2.1-TOPO (Invitrogen), and three independent clones per virus variant were sequenced (MWG).

RESULTS

VACV down-modulates MVA-induced type I IFN responses. As shown previously (57), highly attenuated MVA, but not VACV, was able to induce IFN-α responses upon immunization of mice (Fig. 1A) and after in vitro stimulation of Flt3-L pDC cultures (Fig. 1B). To explore the impact of VACV infection on MVA-induced IFN responses, coinfection experiments were carried out. Mice were inoculated intravenously

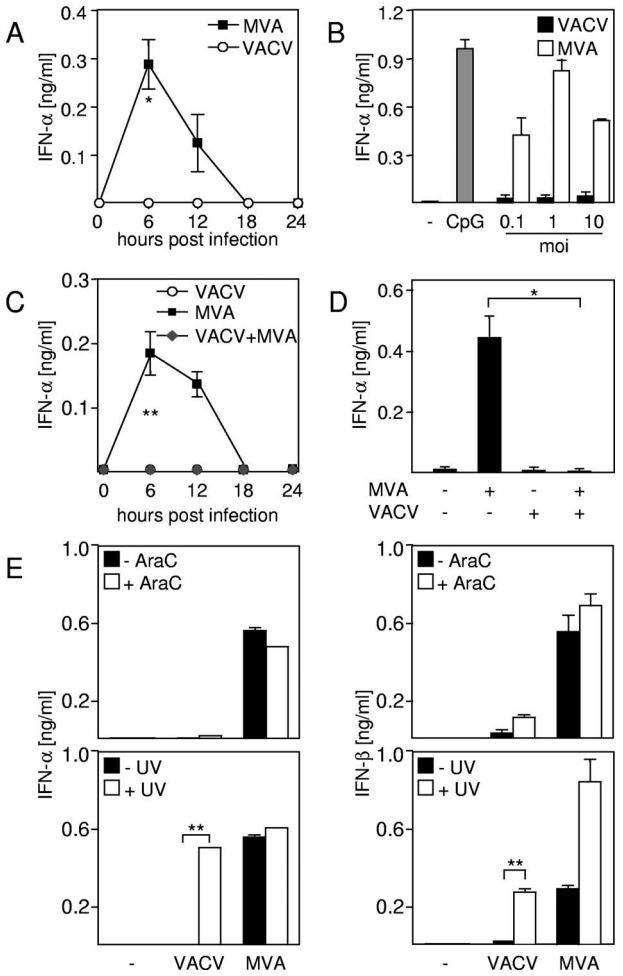

FIG. 1. VACV infection inhibits MVA-induced IFN responses. (A) C57BL/6 mice were inoculated i.v. with 1×10^7 PFU of MVA (black squares) or 1×10^5 PFU of VACV (open circles). Serum was collected at the indicated times after immunization and analyzed for IFN-α by an ELISA method ($n = 3$ or 4). (B) Flt3-L pDC (1×10^6) were infected with VACV (black bars) or MVA (white bars) at the indicated MOI. Controls were left untreated (−) or stimulated with CpG 2216 (CpG; 10 μg/ml). At 24 h after infection, supernatant was analyzed for IFN-α by an ELISA method. (C) C57BL/6 mice were inoculated i.v. with 1×10^7 PFU of VACV (open circles) or 1×10^7 PFU of MVA (black squares) or coinfected with VACV and MVA (1×10^7 PFU of each; gray diamonds). Serum was collected at the indicated time points after infection and analyzed for IFN-α by an ELISA method ($n = 2$ to 5). (D) Flt3-L pDC were infected with MVA, VACV, or both viruses together at an MOI of 1. At 24 h after infection, supernatant was analyzed for IFN-α by an ELISA method. (E) Flt3-L pDC were infected with VACV or MVA (MOI of 1) in the presence (white bars) or absence (black bars) of AraC (40 μg/μl; upper panel), or Flt3-L pDC were infected with untreated VACV or MVA (MOI of 1) or with viruses that were irradiated with UV light (75 mJ/cm^2) prior to infection (lower panel). Control cells were left untreated (−). At 24 h after infection, supernatant was analyzed for IFN-α (left panels) and IFN-β (right panels) by an ELISA method. The data shown are representative of two to four independent experiments. Error bars indicate standard deviations from triplicate ELISA measurements. *, $P = <0.05$ and ≥ 0.01; **, $P = <0.01$ and ≥ 0.001 (unpaired two-tailed t test).

(i.v.) with 1×10^7 PFU of VACV, 1×10^7 PFU of MVA, or a mixture of both viruses, and serum samples were collected at the indicated time points after infection. Remarkably, MVA-induced IFN-α responses were completely abolished upon coinfection with VACV (Fig. 1C). Interestingly, when 1×10^5 PFU of VACV and 1×10^7 PFU of MVA were coadministered, MVA-induced IFN responses were significantly impaired but not completely abolished (data not shown). Reminiscent of these in vivo results, VACV also completely inhibited IFN-α responses after in vitro coinfection of Flt3-L pDC with MVA and VACV (Fig. 1D). Collectively, these results indicated that VACV actively inhibited MVA-induced IFN responses in vivo and in vitro.

To study whether VACV-mediated IFN inhibition was conferred by newly synthesized or preformed factors, VACV was UV irradiated prior to infection of Flt3-L pDC. Interestingly, UV-irradiated VACV induced IFN responses similar to those induced by untreated MVA (Fig. 1E, lower panels). This suggested that VACV-encoded IFN inhibitors were newly synthesized upon VACV infection. To next determine whether intermediate or late gene products accounted for the inhibition of MVA-induced IFN responses, Flt3-L pDC were VACV infected in the presence of AraC, which inhibits viral DNA replication. As under such conditions VACV still inhibited IFN responses, whereas MVA infection of AraC-treated Flt3-L pDC led to normal IFN responses, viral inhibitors were not encoded by intermediate and/or late viral genes (Fig. 1E, upper left and upper right panels) (57) but represent gene products expressed early during the viral life cycle.

VACV-encoded IFN inhibitors were sequentially lost during MVA attenuation. Considering the high number of different VACV-encoded immune modulators known so far (2, 3, 5, 37, 46), we asked whether during MVA attenuation a consecutive accumulation of mutations was associated with the stepwise loss of IFN inhibitors. To test this hypothesis, we infected Flt3-L pDC with the two MVA ancestor viruses, CVA152 and CVA386. VACV- and CVA152-infected Flt3-L pDC did not show production of either IFN-α or IFN-β. In contrast, CVA386 infection of Flt3-L pDC did not induce IFN-α but did induce IFN-β (Fig. 2A). These data suggested that VACV-mediated inhibition of IFN responses is a multistep process involving several factors that separately inhibit IFN-β and IFN-α responses. Obviously, the IFN-α-inhibitory function was depleted between passages 386 and 584, whereas the inhibitor(s) of IFN-β production was already inactivated between passages 152 and 386. In line with this, coinfection of FLt3-L pDC with MVA plus CVA152 or CVA386 inhibited MVA-induced IFN-α responses as efficiently as coinfection with VACV (Fig. 2B; compare Fig. 1D). Additionally, coinfection with VACV or CVA152, but not with CVA386, inhibited MVA-induced IFN-β (Fig. 2B).

To test whether VACV encodes secreted or intracellularly expressed IFN inhibitors, supernatants of VACV-, CVA152-, and CVA386-infected Flt3-L pDC were tested for the capacity to inhibit MVA-induced IFN responses. In brief, Flt3-L pDC were infected with the indicated virus strains for 3 h and then cells were extensively washed to eliminate free virus particles. Upon the addition of fresh medium, infected cells were incubated for another 21 h and supernatants were harvested. As verified by plaque analysis, such supernatants were devoid of free virus particles (data not shown), which was in accordance with earlier observations that VACV, CVA152, and CVA386 were unable to productively infect Flt3-L pDC (57; data not shown). Preincubation of Flt-3L pDC with supernatant pre-

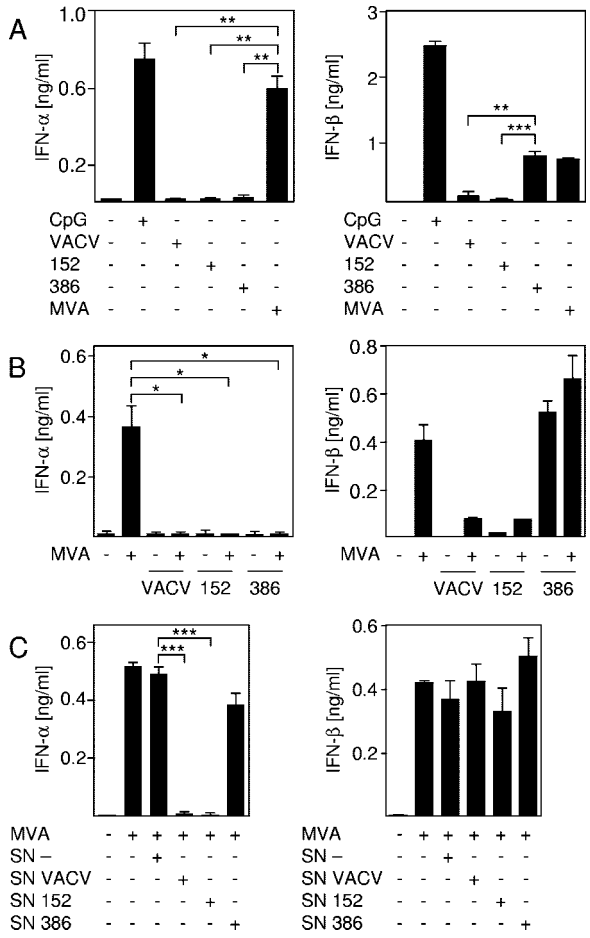

FIG. 2. VACV-encoded IFN inhibitors are secreted and intracellularly expressed factors that were lost stepwise during in vitro attenuation. (A) Flt3-L pDC were infected with VACV, MVA, and two intermediate passages, CVA152 (152) and CVA386 (386), at an MOI of 1. Controls were left untreated or stimulated with CpG 2216 (CpG; 10 μg/ml). At 24 h after infection, supernatant was analyzed for IFN-α (left panel) and IFN-β (right panel) by an ELISA method. (B) Flt3-L pDC (1×10^6) were infected with VACV, CVA152 (152), and CVA386 (386) at an MOI of 1 (left panel) or at an MOI of 10 (right panel) alone or in combination with MVA (MOI of 1). Uninfected cells served as a control. At 24 h after infection, supernatant was analyzed for IFN-α (left panel) and IFN-β (right panel) by an ELISA method. (C) Supernatants of untreated (SN −), VACV-infected (SN VACV), CVA152-infected (SN 152), and CVA386-infected (SN 386) cells were generated by infection of 1×10^6 Flt3-L pDC for 2 h at an MOI of 1. The supernatants were then discarded, and cells were washed extensively and incubated for 22 h. Freshly generated Flt3-L pDC were incubated with these supernatants for 3 h and subsequently infected with MVA (MOI of 1) for 21 h. At 24 h after treatment, supernatant was analyzed for IFN-α (left panel) and IFN-β (right panel) by an ELISA method. The data shown are representative of two or three independent experiments. Error bars indicate standard deviations from triplicate ELISA measurements. *, $P = <0.05$ and ≥ 0.01; **, $P = <0.01$ and ≥ 0.001; ***, $P = <0.001$ (unpaired two-tailed t test).

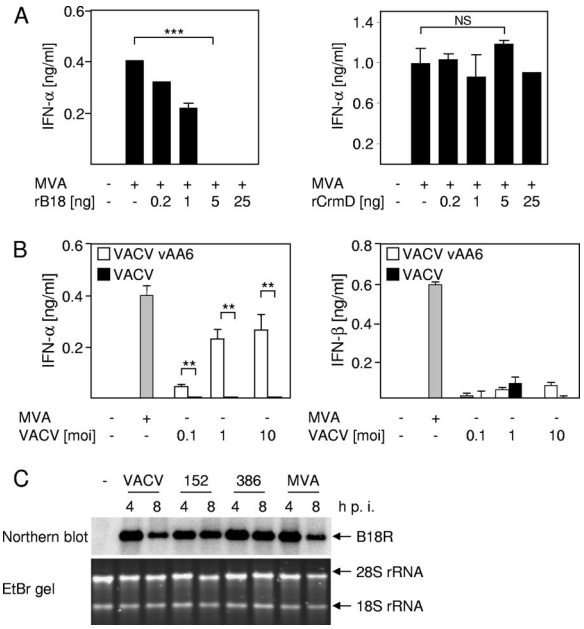

FIG. 3. B18 is one of the secreted IFN inhibitors. (A) Flt3-L pDC (1×10^6) were infected with MVA (MOI of 1) and cotreated with the indicated concentrations of rB18 protein (rB18, left diagram) or an irrelevant recombinant protein (rCrmD, right diagram). Controls were left untreated. At 24 h after infection, supernatant was analyzed for IFN-α by an ELISA method. (B) Flt3-L pDC (1×10^6) were infected with the B18R-deficient VACV variant vAA6 (white bars) or unmutated VACV (black bars) at the indicated MOI. Controls were left untreated (−) or MVA infected (MOI of 1; gray bars). At 24 h after infection, supernatant was analyzed for IFN-α (left panel) and IFN-β (right panel) by an ELISA method. (C) Northern blot analysis of B18R-specific viral transcripts. NIH 3T3 cells were infected with VACV strain Western Reserve (VACV), CVA152 (152), CVA386 (386), or MVA at an MOI of 5, and total RNA was harvested at the indicated time points after infection. Transcripts were detected with a B18R-specific riboprobe. Ethidium bromide (EtBr) staining of 28S and 18S rRNAs served as a loading control. **, $P = <0.01$ and ≥ 0.001; ***, $P = <0.001$ (unpaired two-tailed t test). NS, not significant.

pared as described above did not affect MVA-induced IFN-β responses, suggesting that VACV- and CVA152-mediated IFN-β inhibition was conferred by an intracellular immune modulator(s) (Fig. 2C, right panel). On the contrary, preincubation of Flt-3L pDC with supernatant of VACV- or CVA152-infected cells and subsequent MVA infection resulted in a complete shutoff of IFN-α, whereas supernatant of CVA386-infected cells did not show any inhibitory effect (Fig. 2C, left panel). Collectively, these results suggested that VACV and CVA152 encode an immune modulator(s) that inhibits IFN-α responses via secreted factors. IFN-β inhibition by VACV and CVA152 infection was exclusively mediated by intracellularly expressed viral proteins.

As mentioned before, VACV encodes the secreted IFN-α receptor B18, which is able to abolish IFN responses of certain cell lines by competing with the host IFN receptor (IFNAR) (6, 19, 51). To test whether B18 also plays a crucial role in the inhibition of MVA-induced IFN responses, we infected Flt3-L pDC with MVA and cotreated them with graded doses of rB18 (6, 51). Indeed, rB18 was able to inhibit MVA-induced IFN-α responses in a dose-dependent manner (Fig. 3A), whereas IFN-β production was not affected (data not shown). An irrelevant protein (rCrmD) did not inhibit MVA-induced IFN-α responses (Fig. 3A). As a control, the recombinant proteins alone were tested, and they did not induce any IFN (see Fig. 5B; data not shown). In line with these observations, infection

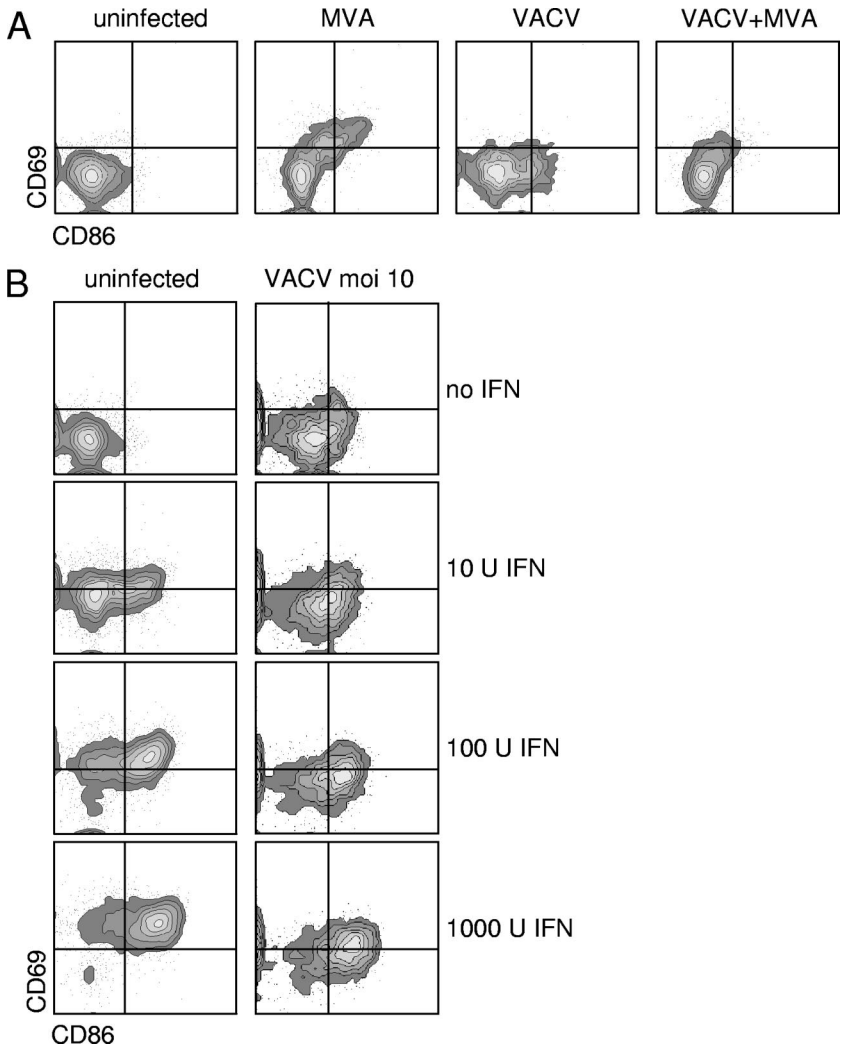

FIG. 4. VACV infection prevents maturation of DC. (A) Flt3-L pDC (1×10^6) were infected at an MOI of 1 with VACV, MVA, or a combination of both viruses. Sixteen hours later, cells were stained with anti-B220–PE–Cy5.5, anti-CD11c–allophycocyanin, anti-CD69–PE, and anti-CD86–fluorescein isothiocyanate. As a control, cells were left uninfected. Fluorescence-activated cell sorter analysis of CD69 and CD86 expression gated on $B220^+$ $CD11c^+$ pDC is shown. (B) Flt3-L pDC were left uninfected or infected with VACV at an MOI of 10 in the presence or absence of the indicated concentrations of recombinant murine IFN-α (10 to 1,000 U). Sixteen hours after infection, cells were stained as in panel A and $B220^+$ $CD11c^+$ pDC were analyzed for CD69 and CD86 expression by flow cytometry. All of the data shown are representative of two or more independent experiments.

of Flt-3L pDC with a VACV knockout mutant deficient in B18R (VACV vAA6) resulted in the induction of IFN-α responses, whereas no IFN-β was induced (Fig. 3B), further proving that B18 indeed is a critical IFN-α inhibitor. Interestingly, Northern blot analysis of NIH 3T3 cells infected with VACV, CVA152, CVA386, or MVA revealed similar levels of B18R-specific mRNA after infection with all of these viruses (Fig. 3C). Accordingly, sequence analysis of the B18R open reading frame revealed that VACV, CVA152, and CVA386 encode intact B18R sequences, whereas in MVA the B18R sequence contains a 20-nucleotide deletion causing a frameshift that resulted in a premature stop codon and a truncated and potentially inactive protein (data not shown). Collectively, these data suggest that the secreted viral inhibitor of IFN-α production B18 is expressed upon infection with VACV, CVA152, and CVA386 but is inactive in MVA.

VACV prevents DC maturation. Next we studied the impact of VACV-mediated IFN inhibition on DC maturation. As observed previously (24, 25, 57, 61), MVA infection of Flt3-L pDC cultures resulted in upregulation of the maturation markers CD69 and CD86 on $B220^+$ $CD11c^+$ pDC, whereas upon VACV infection only some CD86 induction but no CD69 induction was observed (Fig. 4A). Interestingly, upon coinfection of VACV and MVA, CD69 and CD86 induction on $B220^+$ $CD11c^+$ pDC was significantly reduced (Fig. 4A). Note that in such infection experiments overall similar results were obtained when within Flt3-L pDC cultures either $B220^+$ $CD11c^+$ pDC or $CD11c^+$ $CD11b^+$ mDC were analyzed (data not shown).

Because IFN is a potent inducer of murine DC maturation (21, 39, 52, 54), we asked whether VACV-mediated inhibition of CD69 and CD86 upregulation on DC was simply due to the

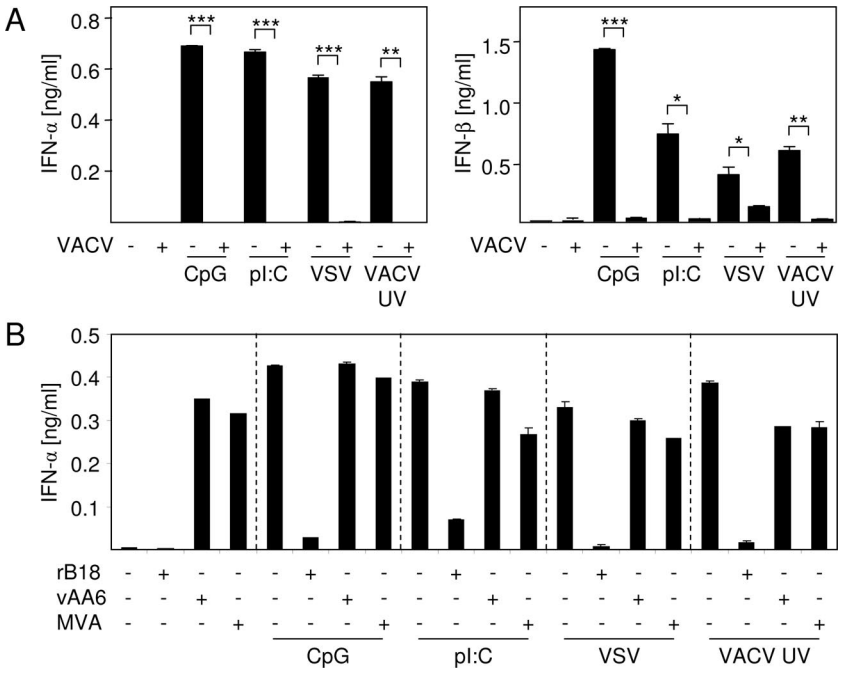

FIG. 5. VACV inhibits IFN responses induced by various different stimuli. (A) Flt3-L pDC (1×10^6) were stimulated with CpG 2216 (CpG; 10 μg/ml) or pI:C (for transfection of 2 μg of pI:C, Fugene reagent was used) or infected with VSV (MOI of 1) or UV-irradiated VACV (MOI of 1; 75 mJ/cm^2). Where indicated (+), cells were coinfected with untreated VACV (MOI of 10) 2 h prior to stimulation. At 24 h after treatment, cell-free supernatant was collected and analyzed for IFN-α (left panel) and IFN-β (right panel) production by an ELISA method. (B) Flt3-L pDC (1×10^6) were stimulated with CpG 2216 (CpG; 10 μg/ml) or pI:C (for transfection of 2 μg of pI:C, Fugene reagent was used) or infected with VSV (MOI of 1) or UV-irradiated VACV (MOI of 1; 75 mJ/cm^2). Where indicated (+), cells were cotreated with 25 ng of rB18, coinfected with MVA (MOI of 10), or coinfected with the B18R-deficient VACV variant vAA6 2 h prior to stimulation. At 24 h after treatment, cell-free supernatant was collected and analyzed for IFN-α production by an ELISA method. The data shown in panels A and B are representative of two independent experiments. Error bars indicate standard deviations from triplicate ELISA measurements. *, $P = <0.05$ and ≥ 0.01; **, $P = <0.01$ and ≥ 0.001; ***, $P = <0.001$ (unpaired two-tailed t test).

absence of IFN or whether an additional mechanism was in place. To address this question, Flt3-L cultures were VACV infected in the presence of graded concentrations of murine recombinant IFN-α. Interestingly, VACV infection impaired CD69 upregulation in DC cultures, even in the presence of 1,000 U of exogenously added recombinant murine IFN-α (Fig. 4B). These data suggest that, in addition to viral factors that down-modulate IFN responses, other viral mechanisms are in place that inhibit DC maturation.

VACV infection inhibits TLR ligand-induced IFN responses. To study whether VACV infection primarily inhibited MVA-induced IFN responses or whether IFN responses induced by other stimuli were also impaired, Flt3-L pDC cultures were first infected with VACV and then treated with the TLR9 ligand CpG 2216 or the MDA5/TLR3 ligand pI:C. Indeed, VACV-infected Flt3-L pDC cultures did not show IFN-α production upon CpG 2216 or pI:C treatment, whereas uninfected cultures secreted normal IFN-α levels (Fig. 5A). IFN-α responses induced by VSV infection, which have been shown to be triggered via TLR4, TLR7, or RIG-I (26, 33, 34), were also abrogated upon coinfection with VACV (Fig. 5A). Furthermore, IFN-α responses induced by UV-irradiated VACV were completely abolished upon coinfection with untreated VACV (Fig. 5A). IFN-β responses induced by the above-tested stimuli were also impaired upon coinfection with VACV (Fig. 5A). In line with our previous findings, rB18 protein inhibited IFN-α responses by all of the TLR ligands tested, whereas B18-deficient VACV variant vAA6 and MVA failed to inhibit TLR ligand-induced IFN-α responses (Fig. 5B). Thus, VACV-encoded IFN-α and IFN-β inhibitors impair IFN responses induced by a broad range of TLR and RIG-I-like receptor triggers.

DISCUSSION

Type I IFNs are among the most potent measures cells undertake immediately after pathogen encounter to efficiently protect neighboring cells from infection. Mice deficient in a functional IFNAR show an increased susceptibility to lethal disease after infection with a broad range of different pathogens (38, 55, 56). In this context, it is interesting that many viruses developed strategies to escape type I IFN responses (3, 37, 46). Here we demonstrated that VACV uses several mechanisms to effectively inhibit IFN-α and IFN-β responses. Additionally, VACV is able to inhibit the activation and maturation of DC.

We and others showed before that, unlike highly attenuated MVA, VACV does not induce detectable levels of systemic type I IFN upon infection of mice (53, 57) (Fig. 1). Here we showed that VACV infection impaired MVA-induced IFN-α and IFN-β responses mounted in vivo and by pDC (Fig. 1). Experiments with AraC- and UV-inactivated VACV indicated

that inhibition of type I IFNs is mediated by newly synthesized viral proteins expressed early in the viral life cycle (Fig. 1E). Analysis of two ancestor viruses of MVA indicated a stepwise loss of viral immune modulators interfering with type I IFN induction during virus attenuation. Interestingly, VACV was able to inhibit IFN-β responses by Flt3-L pDC, a property that was lost during MVA attenuation between chicken cell culture passages 152 and 386 (Fig. 2A), whereas the inhibitor(s) of IFN-α is still expressed by CVA386. These data indicate VACV-encoded immune modulators specific for both IFN-α and IFN-β. Experiments with supernatants of infected cells showed that inhibition of IFN-α can be mediated by soluble factors, whereas inhibition of IFN-β occurred exclusively via intracellularly expressed viral immune modulators (Fig. 2). Thus, we showed that VACV inhibits systemic IFN-α and IFN-β responses by a well-organized and orchestrated mechanism. However, compared to wild-type mice, mice deficient in a functional type I IFN system are highly susceptible to VACV infection (38, 55) and treatment of mice with IFNs prior to VACV infection reduces viral titers (56). These data indicate an important role for IFNs in protection against VACV infection and imply that VACV probably induces local IFN responses in vivo that promote host survival.

The most prominent candidate for a VACV-encoded IFN-α inhibitor is the soluble IFN-α/β receptor B18 (5, 51). B18 is an immunoglobulin superfamily glycoprotein with limited homology to the cellular IFNAR. It acts both in solution and when associated with the cell surface (6). We showed for the first time that B18 is able to inhibit IFN-α responses by pDC, which are potent producers of type I IFNs upon many viral infections (10–12, 20). Interestingly, in experiments with recombinant VACV in which B18R was specifically deleted (vAA6), this virus did induce IFN-α responses, excluding the presence of other soluble VACV-encoded IFN-α inhibitors. Using ancestor viruses of MVA, we showed that the function of B18 was lost between passages 152 and 386 during attenuation (Fig. 2C). The failure of MVA to produce a functional IFN-α/β decoy receptor because of fragmentation of the corresponding gene was shown before (8, 13, 29). However, sequence and Northern blot analyses (Fig. 3C; data not shown) revealed an intact open reading frame for B18R in CVA386 and the expression of B18R-specific mRNA. Moreover, analysis of the viral genome 500 nucleotides 5′ of the B18R open reading frame did not reveal any differences in CVA386 compared to CVA152 and MVA (data not shown). Nevertheless, supernatants of cells infected with CVA386 did not inhibit IFN-α responses (Fig. 2C). Thus, it is a matter for future investigations to test whether other viral factors lost between passages 152 and 386 of attenuation are needed for proper B18 expression. Even though supernatants of CVA386-infected cells did not show any inhibitory capacities (Fig. 2C), direct coinfection of cells with CVA386 and MVA completely inhibited MVA-induced IFN-α responses (Fig. 2B). These data suggest an additional intracellular IFN-α inhibitor. However, experiments with the B18R-deficient VACV vAA6 in which IFN-α responses are induced (Fig. 3B) are not in accordance with this hypothesis. Future experiments will clarify whether poxviruses encode IFN-α inhibitors other than B18. Interestingly, B18-mediated inhibition was applicable on IFN-α responses induced by a wide range of different stimuli, including CpG-containing DNA and pI:C, whereas B18R-deficient VACV variant vAA6 and MVA failed to inhibit TLR-ligand induced IFN-α responses (Fig. 5B). These data indicate a potent and universal function for B18 in the inhibition of IFN-α responses. However, the conditions for our experiments were chosen to depict strong effects mediated by B18. Minor effects of vAA6 and MVA on TLR ligand-induced IFN-α responses are a matter for future investigations.

Symons et al. showed that B18 efficiently binds mouse IFN-α, whereas it binds murine IFN-β only with low affinity (51). We consistently showed here that rB18 protein efficiently inhibited MVA-induced IFN-α responses while IFN-β responses were not affected (Fig. 3A; data not shown). In line with this, VACV strain vAA6, which is deficient in B18R, induced pDC to produce IFN-α but not IFN-β (Fig. 3B). We demonstrated that the IFN-β inhibitor is a protein newly synthesized upon DC infection that is expressed early during the viral life cycle (Fig. 1E) and inhibits IFN-β responses by acting intracellularly (Fig. 2B and C). Interestingly, CVA386-infected Flt3-L pDC did not produce IFN-α, whereas supernatants generated upon infection with this variant did not inhibit MVA-induced IFN-α. These data suggest the presence of an additional intracellular IFN-α inhibitor that is deleted after passage 386. Alternatively, one could speculate that CVA386-encoded B18 is expressed yet retained and accumulates within infected cells, where it inhibits the release of IFN-α. Collectively, our results show that B18 efficiently inhibits IFN-α responses, whereas an additional and so far unidentified inhibitor(s) accounts for IFN-β impairment.

In contrast to earlier virus passages, CVA386 carries deletion I (nonessential genes, left-hand part of the CVA genome; HindIII C fragment), deletion II (left-hand part of the CVA genome; encompassing nonessential genes M1L, M2L, and K1L), and deletion IV (right-hand part of the CVA genome; HindIII B fragment) (36). Interestingly, the K1 protein, the gene for which is absent in CVA386, was shown to inhibit the activation of NF-κB and to interfere with signaling via TLR members and IRF3 (44), which are all critical components of signaling pathways resulting in IFN-β expression (15, 39, 44). Recently, VACV-encoded intracellular virulence factor B14 was reported to interfere with NF-κB (17, 18). B14 is expressed early during the viral life cycle and was shown to down-modulate IL-1β- and TNF-α-induced NF-κB activation. Of note, within the B14R open reading frame, MVA carries three point mutations and a deletion of 18 bp, none of which is present in VACV (data obtained from the Viral Bioinformatics Resource Center at www.poxvirus.org). It is a matter for future investigations to determine if, and how, K1L or B14R contributes to the inhibition of IFN-β production by Flt3-L pDC.

It was shown previously that VACV inhibits the maturation of human DC (25), whereas UV-irradiated VACV induced upregulation of costimulatory molecules on immature murine BM-derived DC (CD80/CD86) (61). This is of relevance because type I IFNs support maturation and/or activation of DC, which is a prerequisite for the efficient induction of adaptive immunity (25, 60, 61). Here we demonstrated that VACV reduced IFN-induced maturation of DC (in terms of upregulation of CD69), even in the presence of high levels of exogenously added recombinant IFN-α (Fig. 4). Whether this inhibition is mediated by VACV-encoded proteins specifically

targeting host cell maturation or whether it is associated with the VACV-mediated overall shutdown of host cell metabolism (37, 40, 46) needs to be further investigated.

Collectively, the results of this study show that VACV-mediated inhibition of type I IFN responses by professional IFN-producing pDC is a multistep process involving secreted and intracellular inhibitors. These different viral inhibitors were sequentially lost during the attenuation of the vaccine strain MVA. We demonstrated that VACV encodes inhibitors that affect both IFN-α and IFN-β production and that these inhibitors are able to affect IFN responses that are induced upon exposure to a wide range of different stimuli. Moreover, we show that additional mechanisms are used by VACV to inhibit DC maturation upon infection. The data presented here contribute to the understanding of immune evasion strategies deployed by poxviruses and thus constitute the basis for the improvement of future poxvirus vaccination strategies.

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