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# Myxoma Virus Induces Type I Interferon Production in Murine Plasmacytoid Dendritic Cells via a TLR9/MyD88-, IRF5/IRF7-, and IFNAR-Dependent Pathway▽

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**Poxviruses are large DNA viruses that replicate in the cytoplasm of infected cells. Myxoma virus is a rabbit poxvirus that belongs to the *Leporipoxvirus* genus. It causes a lethal disease called myxomatosis in European rabbits but cannot sustain any detectable infection in nonlagomorphs. Vaccinia virus is a prototypal orthopoxvirus that was used as a vaccine to eradicate smallpox. Myxoma virus is nonpathogenic in mice, whereas systemic infection with vaccinia virus can be lethal even in immunocompetent mice. Plasmacytoid dendritic cells (pDCs) are potent type I interferon (IFN)-producing cells that play important roles in antiviral innate immunity. How poxviruses are sensed by pDCs to induce type I IFN production is not well understood. Here we report that infection of primary murine pDCs with myxoma virus, but not with vaccinia virus, induces IFN-α, IFN-β, tumor necrosis factor (TNF), and interleukin-12p70 (IL-12p70) production. Using pDCs derived from genetic knockout mice, we show that the myxoma virus-induced innate immune response requires the endosomal DNA sensor TLR9 and its adaptor MyD88, transcription factors IRF5 and IRF7, and the type I IFN positive-feedback loop mediated by IFNAR1. It is independent of the cytoplasmic RNA sensing pathway mediated by the mitochondrial adaptor molecule MAVS, the TLR3 adaptor TRIF, or the transcription factor IRF3. Using pharmacological inhibitors, we demonstrate that myxoma virus-induced type I IFN and IL-12p70 production in murine pDCs is also dependent on phosphatidylinositol 3-kinase (PI3K) and Akt. Furthermore, our results reveal that the N-terminal Z-DNA/RNA binding domain of vaccinia virulence factor E3, which is missing in the orthologous M029 protein expressed by myxoma virus, plays an inhibitory role in poxvirus sensing and innate cytokine production by murine pDCs.**

Plasmacytoid dendritic cells (pDCs) are potent type I interferon (IFN)-producing cells that play critical roles in antiviral immunity. Human and murine pDCs are present in the blood, bone marrow, spleen, lymph nodes, and various other tissues (2, 7, 9, 40). pDCs express endosomal localized receptors TLR7 and TLR9 to detect viral RNA and DNA. Upon ligand binding, these receptors utilize a coadaptor molecule, MyD88, to form complexes resulting in the recruitment and activation of transcription factors, leading to the production of type I IFN and proinflammatory cytokines (22).

Type I IFNs are critical for antiviral immunity. Type I IFNs induce DC maturation and immune cell recruitment to sites of infection and enhance the effector functions of NK, T, and B cells and macrophages. Through binding to the type I IFN receptor, a heterodimeric molecule consisting of IFNAR1 and IFNAR2, type I IFNs activate the JAK-Stat pathway to trigger an antiviral state. Type I IFN pathways are frequently targeted for subversion by viral immune modulators. For example, the vaccinia virus B18R gene encodes a soluble type I IFN receptor. A deletion mutant lacking B18R is attenuated in a murine intranasal infection model (44). The vaccinia virus E3L gene encodes an intracellular protein, composed of an N-terminal Z-DNA/RNA binding domain (ZBD) and a C-terminal double-stranded RNA (dsRNA) binding domain (RBD), that antagonizes host sensing of viral nucleic acids and the induction of type I IFN (11, 12, 52). The ZBD and RBD of the E3 protein are required for vaccinia pathogenesis in a murine intranasal model (6).

Myxoma virus is a rabbit-specific poxvirus that belongs to the *Leporipoxvirus* genus. It causes a rapidly disseminating lethal myxomatosis in European rabbits (*Oryctolagus cuniculus*) and a benign localized infection in its natural evolutionary host in South American rabbits (*Sylvilagus brasiliensis*) but is nonpathogenic in humans and mice. In contrast, systemic infection with vaccinia virus is lethal in mice, and even vaccination

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through skin scarification can produce severe clinical complications in immunocompromised humans. We suspect that type I IFN and proinflammatory cytokine induction by myxoma virus and vaccinia virus might be different in host immune cells from the same species. This is supported by our recent findings that infection of human primary pDCs with myxoma virus, but not vaccinia virus, induces IFN-α and tumor necrosis factor (TNF) production (H. Cao, P. Dai, H. Li, W. Wang, F. Wang, J. Yuan, J. Liu, R. C. Condit, G. McFadden, J. W. Young, S. Shuman, and L. Deng unpublished data). To understand the mechanisms through which myxoma virus is sensed in murine pDCs, we utilized primary cells derived from genetic knockout mice that have deficiencies in molecules/pathways important for viral sensing and type I IFN induction. We found that TLR9 and MyD88 are required for the induction of type I IFN by myxoma virus in murine pDCs. The transcription factors IRF5/IRF7 and IFNAR1 that mediate the type I IFN positive-feedback loop are also essential for this induction. Our results define an important pathway in sensing myxoma virus infection and the induction of type I IFN in murine pDCs. These findings have relevance in the understanding of poxvirus pathogenesis in various host species and have significant ramifications for the development of poxviruses as oncolytic viruses and vaccine vectors.

## MATERIALS AND METHODS

**Viruses and cell lines.** The WR strain of vaccinia virus was propagated, and the titers of the virus were determined in BSC40 (African green monkey kidney cells) monolayers at 37°C. The ΔE3L, E3LΔ83N, E3LΔ26C, E3LY48A viruses were kindly provided by B. L. Jacobs (Arizona State University). ΔE3L and E3LΔ26C viruses were propagated in BHK-21 (baby hamster kidney) cells, and the titers of the viruses were determined on RK13 (rabbit kidney) cells. E3LΔ83N and E3LY48A viruses were propagated, and the titers were determined on BSC40 cells. The tyrosine-to-alanine change at amino acid 48 of the E3 protein in E3LY48A virus was verified by PCR amplification of the E3 gene, followed by sequencing analysis. Recombinant myxoma virus (Lausanne strain) with a cassette expressing green fluorescent protein (GFP) under the control of a vaccinia virus synthetic early/late promoter inserted between myxoma virus genes M135R and M136R was propagated, and the titers of the virus were determined on RK13 cells. BSC40 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). BHK-21 and RK13 cells were cultured in DMEM containing 10% FBS, 0.1 mM nonessential amino acids, and 50 μg/ml gentamicin. All cells were grown at 37°C in a 5% $CO_2$ incubator.

**Mice.** Female C57B/6 mice between 6 and 10 weeks of age were purchased from the Jackson Laboratory and were used for the preparation of bone marrow-derived dendritic cells. These mice were maintained in the animal facility at the Sloan-Kettering Cancer Institute. All procedures were performed with the consent of the Institutional Animal Care and Use Committee. $MAVS^{-/-}$, $IRF3^{-/-}$, $TLR3^{-/-}$, $MyD88^{-/-}$, $TLR9^{-/-}$, $TLR7^{-/-}$, and $TRIF^{-/-}$ ($TRIF^{LPS2/LPS2}$) mice were generated in the laboratories of Zhijian Chen (University of Texas Southwestern Medical Center), Tadatsugu Taniguchi (University of Tokyo), Richard Flavell (Yale University), Shizuro Akira (Osaka University), and Bruce Beutler (Scripps Research Institute). $IFNAR1^{-/-}$ mice were provided by Eric Pamer (Sloan-Kettering Institute); the mice were purchased from B&K Universal and were backcrossed with C57B/6 mice for more than five generations. The $TLR7^{-/-}$ and $IRF5^{-/-}$ mice were maintained at the animal facilities at the Johns Hopkins University School of Medicine. The $IRF7^{-/-}$ mice were maintained at the animal facilities at the University of Massachusetts Medical School.

**Generation of bone marrow-derived dendritic cells.** The bone marrow cells from the tibiae and femurs of mice were collected by first removing muscles from the bones and then flushing the cells out using 0.5 ml U-100 insulin syringes (Becton Dickinson) with RPMI medium with 10% fetal calf serum (FCS). After centrifugation, cells were resuspended in ACK lysing buffer (Lonza) for red blood cell lysis by incubating the cells on ice for 1 to 3 min. Cells were then collected, resuspended in fresh medium, and filtered through a 40-μm cell strainer (BD Biosciences). The number of cells was counted. For the generation of fms-like tyrosine kinase-3 ligand-cultured murine bone marrow-derived dendritic cells (Flt3L-BMDCs), the bone marrow cells ($5 \times 10^6$ cells in each well of 6-well plates) were cultured in the presence of Flt3L (100 ng/ml; R&D Systems) for 7 to 9 days. Cells were fed every 2 to 3 days by replacing 50% of the old medium with fresh medium. For the generation of granulocyte-macrophage colony-stimulating factor (GM-CSF)-BMDCs, the bone marrow cells ($5 \times 10^6$ cells in each 10-cm cell culture dish) were cultured in the presence of GM-CSF (30 ng/ml, produced by the Monoclonal Antibody Core facility at the Sloan-Kettering Institute) for 10 to 12 days. Cells were fed every 2 days by replacing 50% of the old medium with fresh medium and replated every 3 to 4 days to remove adherent cells. Only nonadherent cells were used for experiments.

**RNA isolation and real-time PCR.** RNA was extracted from whole-cell lysates with an RNeasy minikit (Qiagen) and reverse transcribed with a First Strand cDNA synthesis kit (Fermentas). Quantitative real-time PCR was performed in triplicate with iQ SYBR green Supermix and an iCycler iQ thermal cycler and detection system (Bio-Rad) using gene-specific primers. Relative expression was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

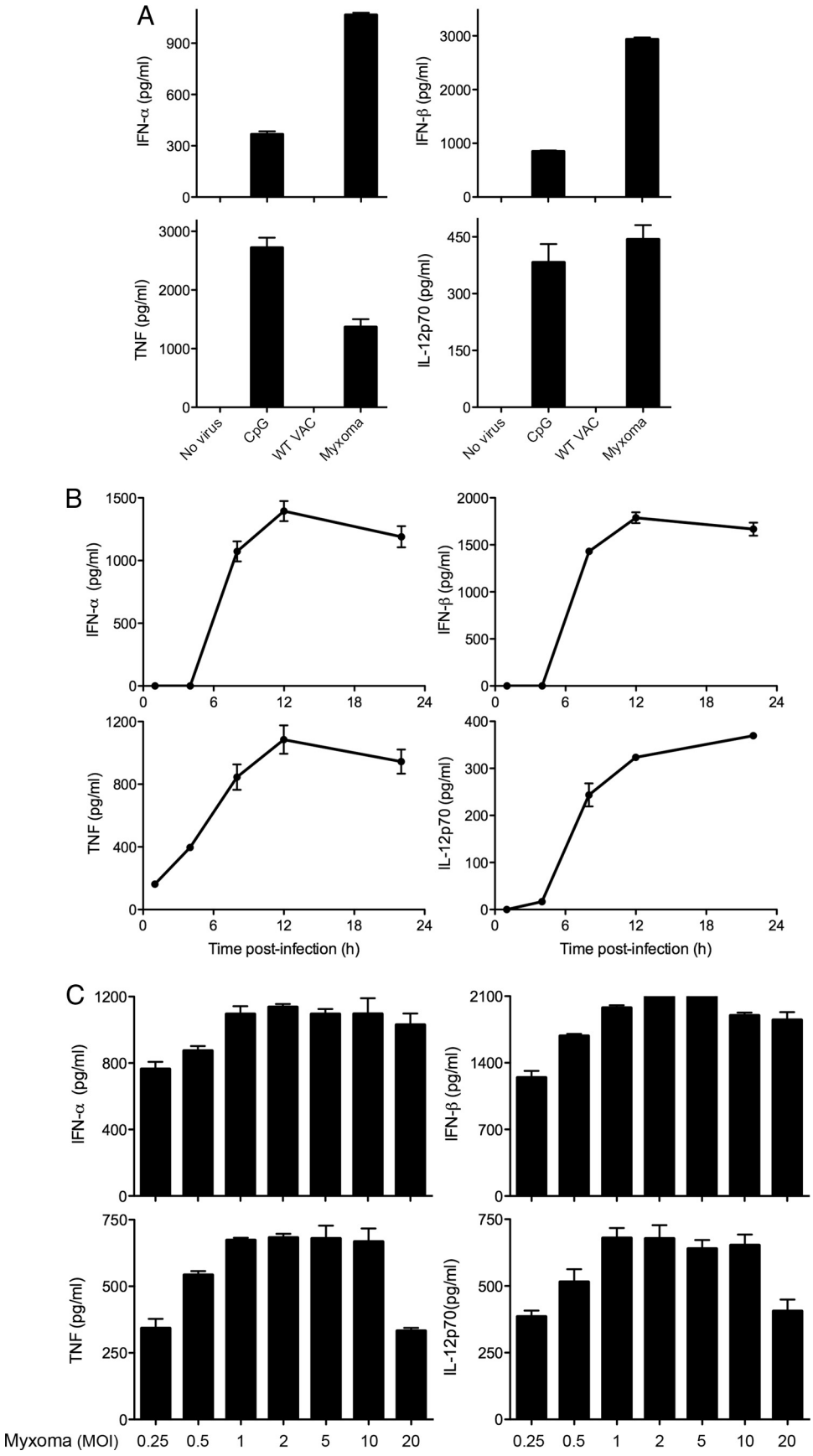
**Reagents.** The commercial sources for reagents were as follows: CpG oligodeoxynucleotide ODN2216, InvivoGen; chloroquine, wortmannin, and 3-methyladenine, Sigma-Aldrich; AKT inhibitors VIII and X, Calbiochem; murine IFN-α/β enzyme-linked immunosorbent assay (ELISA) kits, PBL Biomedical Laboratories; Flt3L, TNF, and interleukin-12p70 (IL-12p70) ELISA kits, R&D systems; anti-mouse CD11c APC, BD Pharmingen; anti-mouse B220 APC-Cy7 and anti-mouse PDCA-1 PE, Miltenyi Biotec.

**Statistics.** Student's two-tailed $t$ test was used for each pairwise comparison. The $P$ values deemed significant are indicated in the figures as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

## RESULTS

**Myxoma virus but not vaccinia virus infection of Flt3L-BMDCs induces type I IFN production.** To test whether murine pDCs respond differently to myxoma versus vaccinia virus infection, we generated Flt3L-BMDCs by culturing bone marrow cells in the presence of Flt3L for 7 to 9 days. Flt3L drives the commitment and differentiation of hematopoietic progenitors to the DC lineage (26, 32). In this culture system, we typically obtain 15 to 25% pDCs and 55 to 65% conventional DCs (cDCs), as determined by fluorescence-activated cell sorter (FACS) analysis. Flt3L-BMDCs were either treated with TLR9 agonist CpG-containing DNA or infected with wild-type (WT) vaccinia virus or myxoma virus at a multiplicity of infection (MOI) of 10. Supernatants were collected at 22 h postinfection. The levels of secreted IFN-α/β and proinflammatory cytokines were determined by ELISA. We found that CpG treatment or myxoma virus infection of Flt3L-BMDCs triggered the induction of IFN-α/β, TNF, and IL-12p70 (the biologically active form of IL-12, composed of p35 and p40 subunits [48]), whereas infection with WT vaccinia virus did not induce the secretion of these cytokines (Fig. 1A). A kinetic analysis of induced cytokine secretion was performed; this analysis revealed that IFN-α/β, TNF, and IL-12p70 were released into the supernatants at 8 h postinfection with myxoma virus. The levels of these cytokines continued to rise and reached plateaus at 12 h postinfection (Fig. 1B). To determine dose-dependent induction of cytokine secretion by myxoma virus, we infected Flt3L-BMDCs with myxoma virus at MOIs ranging from 0.25 to 20. We found that the maximum induction of cytokine secretion occurred at an MOI of 1 to 10 (Fig. 1C).

Using GFP-expressing (under the control of vaccinia p7.5 promoter) vaccinia and myxoma viruses, we estimated the efficiencies of infection based on the percentage of GFP-positive cells using FACS analysis. In GFP-vaccinia virus-infected



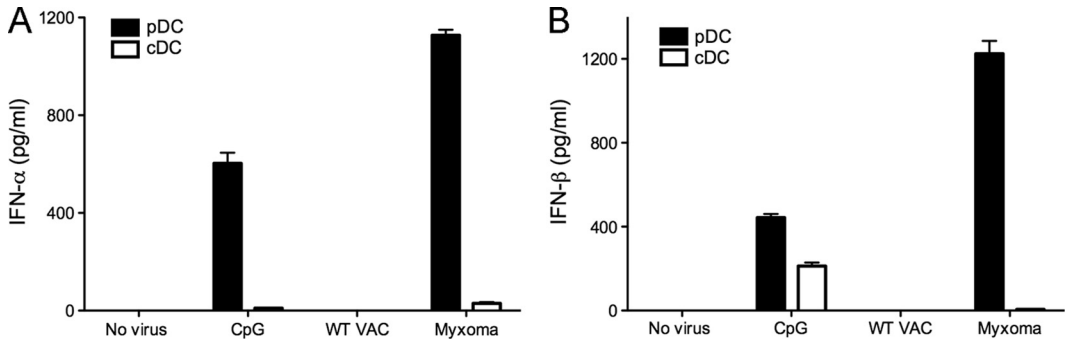



FIG. 2. Myxoma virus infection of purified murine pDCs induces type I IFN induction. Flt3L-BMDCs were sorted using FACS to purify pDCs and cDCs to high purity (~98%). pDCs ($2 \times 10^5$) ($CD11c^+$ $B220^+$ $PDCA\text{-}1^+$) and cDCs ($1 \times 10^6$) ($CD11c^+$ $B220^-$ $PDCA\text{-}1^-$) were stimulated with CpG or infected with either WT VAC or myxoma virus at an MOI of 10. Supernatants were collected at 22 h postinfection. The concentrations of IFN-α (A) and IFN-β (B) were determined by ELISA. Data are means ± SEM. The experiment was repeated at least twice, and the results of a representative are shown.

pDCs, about 50% of cells were GFP positive, whereas in GFP-myxoma virus-infected pDCs, about 20% of cells were GFP positive (data not shown). DAPI (4′,6-diamidino-2-phenylindole) staining was used to determine cell death. We observed that 8 to 10% of mock-infected cells were DAPI positive after a 24-h culture in the absence of Flt3L growth factor. In Flt3L-BMDCs infected with WT vaccinia virus at an MOI of 10 for 24 h, 20 to 25% cells were DAPI positive, whereas 25 to 30% of cells infected with myxoma virus at an MOI of 10 were DAPI positive at 24 h postinfection (data not shown).

**Myxoma virus induces type I IFN production in murine pDCs but not in cDCs.** Granulocyte-macrophage colony-stimulating factor (GM-CSF)-cultured murine bone marrow-derived dendritic cells (BMDCs) are $CD11c^+$ $B220^-$ $PDCA\text{-}1^-$ cDCs. We observed that myxoma virus infection of GM-CSF-cultured murine BMDCs did not result in the induction of type I IFN (data not shown), suggesting that the induction of type I IFN by myxoma virus in Flt3L-BMDCs was mediated by pDCs but not by cDCs. To verify that, we isolated pDCs ($CD11c^+$ $B220^+$ $PDCA\text{-}1^+$) and cDCs ($CD11c^+$ $B220^-$ $PDCA\text{-}1^-$) from Flt3L-BMDCs to a purity of greater than 98% using FACS. Purified murine pDCs ($2 \times 10^5$ cells) and cDCs ($1 \times 10^6$ cells) were treated with CpG or infected with WT vaccinia or myxoma virus. Mock treatment controls were included. Myxoma virus induced high levels of IFN-α/β in purified murine pDCs but not in cDCs (Fig. 2A and B). CpG treatment resulted in the induction of IFN-α/β in purified pDCs but only IFN-β in cDCs. WT vaccinia virus infection failed to induce type I IFN production in either pDCs or cDCs (Fig. 2A and B).

**Myxoma virus induces the expression of *IFNA4*, *IFNB*, *IL-12p35*, and *IL-12p40* genes in purified murine pDCs.** To test whether myxoma or vaccinia virus infection of pDCs alters the gene expression of type I IFN and proinflammatory cytokines, we performed quantitative real-time PCR analysis of FACS-purified pDCs infected either with WT vaccinia virus or myxoma virus at an MOI of 10 for 3 or 6 h. Mock infection control was also included. We found that myxoma virus infection increased the expression of *IFNA4* (14,000-fold), *IFNB* (2,500-fold), *IL-12p35* (16-fold), and *IL-12p40* (220-fold) at 6 h postinfection (Fig. 3A to D). In contrast, WT vaccinia virus infection leads to a much smaller induction of these cytokines, including *IFNA4* (8-fold at 3 h postinfection and 3-fold at 6 h postinfection), *IFNB* (6-fold at 3 h postinfection and 1.6-fold at 6 h postinfection), and *IL-12p35* (2.5-fold at 3 h postinfection and 3.5-fold at 6 h postinfection). No induction of gene expression was observed with *IL-12p40* (Fig. 3A to D). These results indicate that the induced secretion of type I IFN and IL-12p70 by purified pDCs in response to myxoma virus infection correlates with upregulation of gene expression. The failure of WT vaccinia virus-induced secretion of IFN and IL-12p70 is due to its inability to upregulate these genes at the transcriptional level.

**Myxoma virus induction of type I IFN and IL-12p70 is abolished in $MyD88^{-/-}$ or $TLR9^{-/-}$ murine pDCs.** pDCs utilize endosomal TLR7 and TLR9 to detect viral single-stranded RNA (ssRNA) and CpG-containing DNA. MyD88 is a coadaptor molecule that mediates TLR7 and TLR9 sensing in pDCs (13, 16, 29, 30). To test whether TLR7, TLR9, or MyD88 is involved in myxoma virus induction of type I IFN in pDCs,

FIG. 1. Innate immune responses of murine Flt3L-BMDCs to myxoma virus or vaccinia virus infections. Flt3L-BMDCs were generated from female C57B/6 mice by culturing bone marrow cells in the presence of Flt3L. (A) Cells ($1 \times 10^6$) were infected with wild-type vaccinia virus (WT VAC) or myxoma virus at a multiplicity of infection (MOI) of 10 for 1 h. A mock-infected control (no virus) was included. Alternatively, cells were stimulated with TLR9 agonist CpG 2216 (10 μg/ml). Supernatants were collected 22 h later, and the concentrations of IFN-α, IFN-β, TNF, and IL-12p70 were determined by ELISA. Data are means ± standard errors of the means (SEM). The experiment was repeated at least twice, and the results of a representative are shown. (B) Cells ($1 \times 10^6$) were infected with myxoma virus at an MOI of 10. Supernatants were collected at 1, 4, 8, 12, or 22 h postinfection. The concentrations of IFN-α, IFN-β, TNF, and IL-12p70 were determined by ELISA. Data are means ± SEM. A representative experiment is shown and repeated at least twice. (C) Cells ($1 \times 10^6$) were infected with myxoma virus at increasing MOIs ranging from 0.25 to 10. Supernatants were collected at 22 h postinfection. The concentrations of IFN-α, IFN-β, TNF, and IL-12p70 were determined by ELISA. Data are means ± SEM. The experiment was repeated once, and the results of a representative are shown.

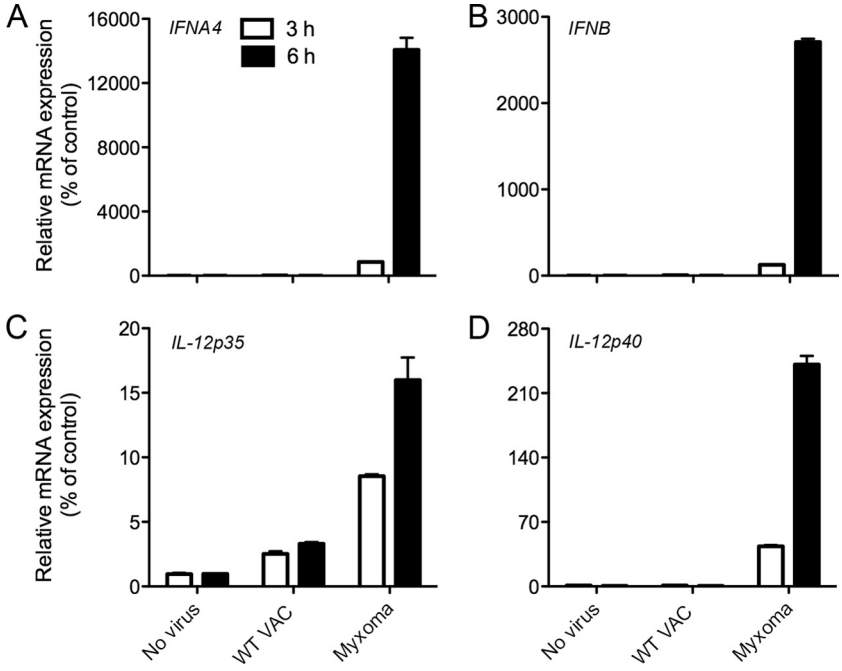



FIG. 3. Myxoma virus infection of purified murine pDCs induces the expression of *IFNA4*, *IFNB*, *IL-12p35*, and *IL-12p40* genes. FACS-sorted pDCs ($5 \times 10^5$) were infected with either WT VAC or myxoma virus at an MOI of 10. Cells were collected at 3 or 6 h postinfection. Real-time PCR analysis of *IFNA4* (A), *IFNB* (B), *IL-12p35* (C), and *IL-12p40* (D) mRNAs was performed. Data are means ± SEM. The experiment was repeated twice, and the results of a representative are shown.

we purified pDCs from Flt3L-BMDCs from MyD88$^{-/-}$, TLR9$^{-/-}$, TLR7$^{-/-}$, or WT control mice by FACS as described above. Cells were then treated with CpG or infected with myxoma virus at an MOI of 10. We found that the induction of IFN-α/β and IL-12p70 by CpG was abolished in MyD88$^{-/-}$ or TLR9$^{-/-}$ murine pDCs but not affected in TLR7$^{-/-}$ pDCs (Fig. 4A to C). Myxoma virus-induced production of IFN-α and IFN-β was also abolished in MyD88$^{-/-}$ or TLR9$^{-/-}$ murine pDCs but only modestly reduced in TLR7$^{-/-}$ pDCs (Fig. 4A and B). We found that neither IL-12p70 nor TNF induced by myxoma virus was affected in TLR7$^{-/-}$ pDCs. Taken together, these results indicate that myxoma virus infection in murine pDCs is sensed through a TLR9/MyD88-dependent pathway. In contrast, TLR7 plays a minor role.

**Myxoma virus induction of type I IFN in murine pDCs is independent of MAVS, TRIF, or IRF3.** We assessed the contributions of other known signaling pathways to the induction of type I IFN by myxoma virus. MAVS (mitochondrial antiviral signaling protein), also known as IPS-1, VISA, or Cardif, is an adaptor for cytosolic RNA sensors RIG-I and MDA5. TRIF (TIR-domain-containing adaptor-inducing beta interferon) is an adaptor for the endosomal TLR3 that senses extracellular dsRNA (1, 22). IRF3 (interferon regulatory factor 3) is an essential transcription factor for the regulation of type I IFN expression in many cell types except pDCs (18, 37). We infected Flt3L-BMDCs from MAVS$^{-/-}$, TRIF$^{-/-}$, IRF3$^{-/-}$, and WT control mice with myxoma virus at an MOI of 10. Supernatants were collected at 22 h postinfection. We found that MAVS-, TRIF-, or IRF3-deficient cells produced amounts of type I IFN similar to those of control WT pDCs in response to myxoma virus infection (Fig. 5). This result is consistent with previous findings that MAVS, TRIF, or IRF3 is not involved in type I IFN induction in pDCs (18, 43, 53).

**Transcription factors IRF5 and IRF7 are required for the induction of type I IFN in murine pDCs by myxoma virus.** The interferon regulatory factors IRF5 and IRF7 are transcription factors that are critical for host defense against viral infections (18, 54). IRF5-deficient mice had increased susceptibility to vesicular stomatitis virus (VSV) and herpes simplex virus 1 (HSV-1) infections and produced lower levels of IFN-α/β and IL-6 than WT control mice *in vivo* (54). IRF5$^{-/-}$ macrophages produced lower levels of these cytokines than WT cells in response to VSV and HSV-1 infections. However, IRF5$^{-/-}$ murine embryonic fibroblasts (MEFs) produced amounts of these cytokines similar to those of WT cells, indicating that the contribution of IRF5 to type I IFN and IL-6 production is cell type specific. IRF7$^{-/-}$ mice are highly susceptible to encephalomyocarditis virus (EMCV) and HSV-1 infections, with markedly reduced levels of IFN-α in the serum. IRF7$^{-/-}$ pDCs failed to produce IFN-α in response to TLR7 or TLR9 stimulation (18). To test whether the transcription factors IRF5 and IRF7 are required for the induction of type I IFN in murine pDCs by myxoma virus infection, we generated Flt3L-BMDCs from IRF5$^{-/-}$, IRF7$^{-/-}$, or WT control mice and purified pDCs by FACS. We then infected these cells with myxoma virus at an MOI of 10 or treated them with CpG. Supernatants were collected at 22 h postinfection, and the secreted cytokine levels were determined by ELISA. We found that type I IFN, TNF, and IL-12p70 induction by myxoma virus or CpG was abolished or significantly reduced in IRF5- or IRF7-deficient pDCs (Fig. 6A and B). These results indicate

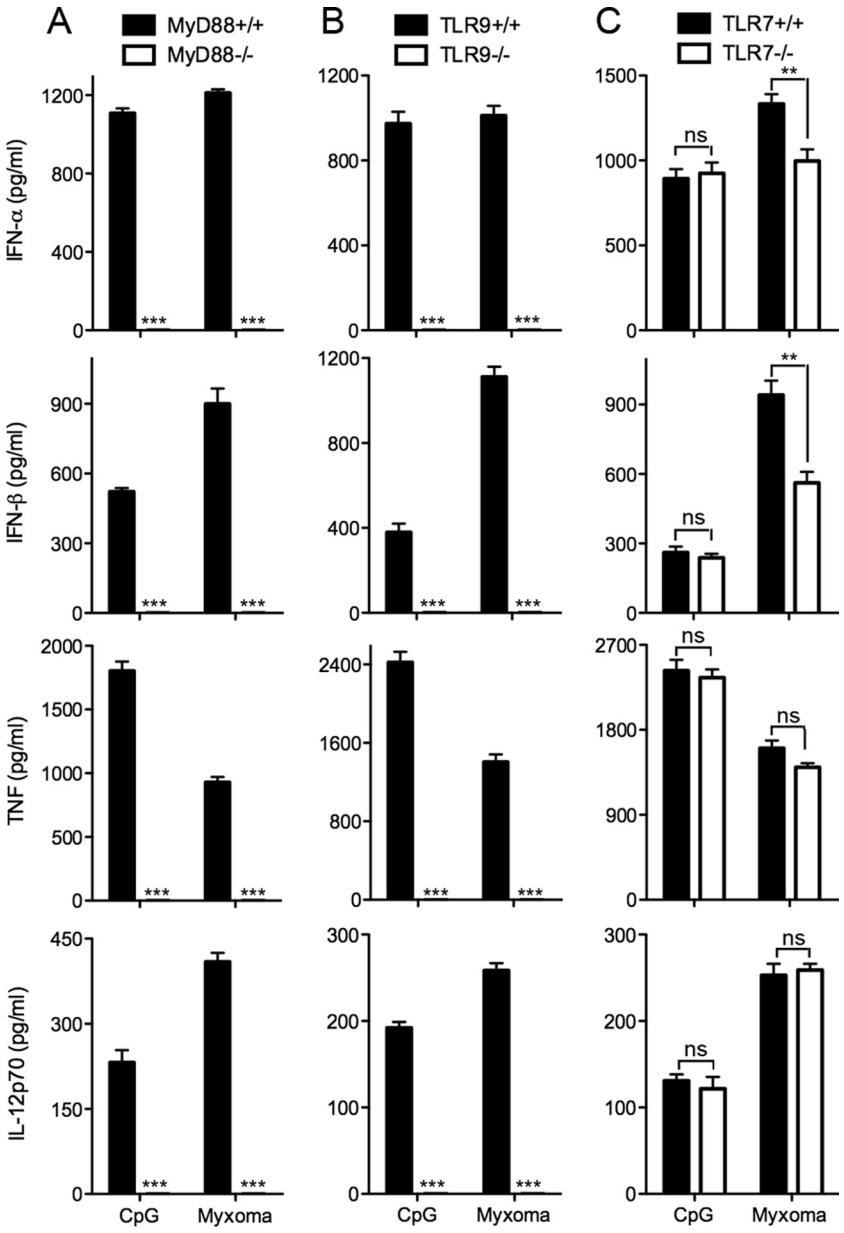



FIG. 4. Myxoma virus induction of type I IFN, TNF, and IL-12p70 in murine pDCs requires TLR9/MyD88. Purified murine pDCs were obtained using FACS from Flt3L-BMDCs generated from MyD88$^{-/-}$ (A), TLR9$^{-/-}$ (B), and TLR7$^{-/-}$ (C) mice and their age-matched WT controls. pDCs ($2 \times 10^5$) were stimulated with CpG or infected with myxoma virus at an MOI of 10. Supernatants were collected 22 h later. The concentrations of IFN-α/β, IL-12p70, and TNF were determined by ELISA. Data are means ± SEM. The combined results of three independently performed experiments are shown. **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant (comparisons were made between WT cells and various knockout cells as indicated).

that IRF5 and IRF7 are both required for the induction of the expression of IFN-α/β, TNF, and IL-12p70 in murine pDCs in response to myxoma virus infection or to CpG stimulation.

**The IFN positive-feedback loop is essential for the induction of type I IFN in murine pDCs by myxoma virus.** Type I IFNs secreted by virus-infected cells can act in an autocrine or paracrine manner through binding to the type I IFN receptor (IFNAR1) to stimulate the JAK-Stat pathway, resulting in the upregulation of IFN-responsive genes. Infection of murine embryonic fibroblasts with Newcastle disease virus (NDV) triggered upregulation of IRF7 expression, which was abolished in IFNAR1$^{-/-}$ cells (36) or in Stat1$^{-/-}$ cells (31). The induction of IFN-α by TLR9 agonist CpG was abolished in IFNAR1$^{-/-}$ pDCs in response to CpG stimulation (18). To test whether this positive-feedback loop plays a role in type I IFN induction by myxoma virus, we obtained highly purified pDCs from Flt3L-BMDC cultures generated from IFNAR1$^{-/-}$ mice and WT controls. We found that IFN-α/β, TNF, and IL-12p70 induction by myxoma virus was abolished in IFNAR1$^{-/-}$ pDCs (Fig. 6C). This shows that the positive-feedback loop mediated

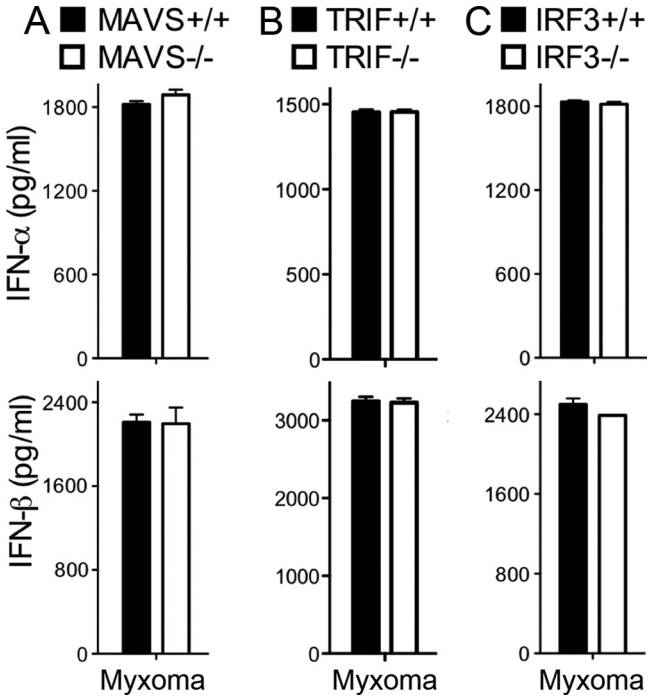



FIG. 5. Myxoma virus-induced innate immune responses in murine pDCs are independent of MAVS, TRIF, or IRF3. Flt3L-BMDCs were generated from MAVS$^{-/-}$ (A), TRIF$^{-/-}$ (B), and IRF3$^{-/-}$ (C) mice and their age-matched WT controls. FLt3L-BMDCs ($1 \times 10^6$) were infected with myxoma virus. Supernatants were collected 22 h later, and the concentrations of IFN-α/β were determined by ELISA. Data are means ± SEM. Results shown are representative of three experiments.

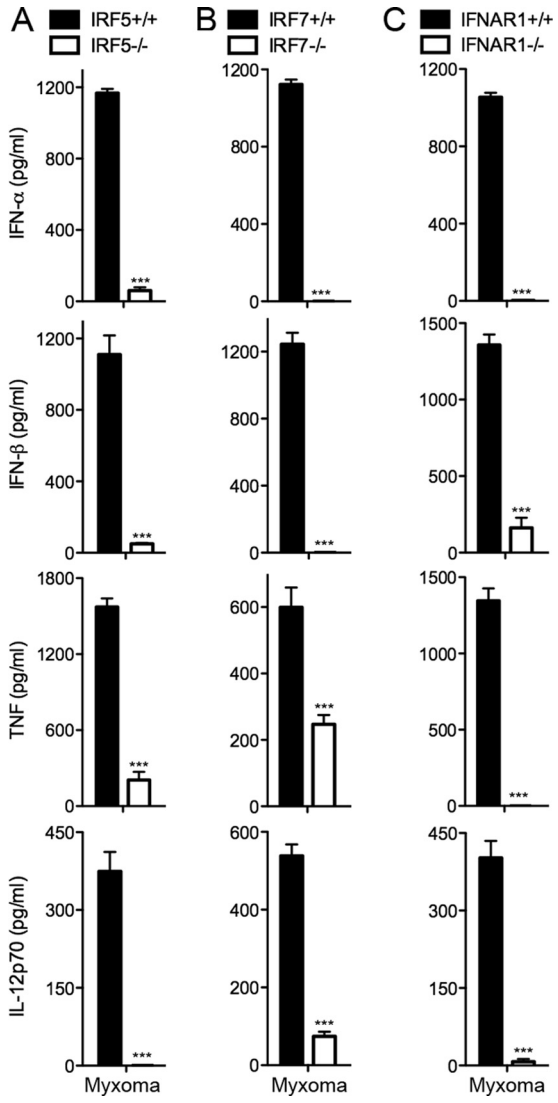



FIG. 6. The interferon regulatory factors IRF5 and IRF7 and the type I IFN positive-feedback loop mediated by IFNAR1 are required for myxoma virus-induced innate immune response in murine pDCs. Purified murine pDCs were obtained using FACS from Flt3L-BMDCs generated from IRF5$^{-/-}$ (A), IRF7$^{-/-}$ (B), and IFNAR1$^{-/-}$ (C) mice and their age-matched controls. pDCs ($2 \times 10^5$) were infected with myxoma virus at an MOI of 10. Supernatants were collected 22 h later. The concentrations of IFN-α/β, TNF, and IL-12p70 were determined by ELISA. Data are means ± SEM. The combined results of three independently performed experiments are shown. ***, $P < 0.001$ (comparisons were made between WT cells and various knockout cells as indicated).

by IFNAR1 is required for the induction of type I IFN, TNF, and IL-12p70 by myxoma virus in murine pDCs.

**Type I IFN induction in murine pDCs by myxoma virus can be blocked by pharmacological inhibitors.** Chloroquine blocks endosomal/lysosomal acidification and thereby might affect myxoma virus sensing through TLR9, which is localized in the endosomal compartment. We infected Flt3L-BMDCs with myxoma virus at an MOI of 10. At 1 h postinfection, cells were treated with chloroquine at final concentrations of 1, 10, and 100 μM. Supernatants were collected at 18 h postinfection and measured for the concentrations of IFN-α/β and IL-12p70 by ELISA. We observed a dose-dependent inhibition of IFN-α/β and IL-12p70 in the presence of chloroquine (Fig. 7A). To probe whether the PI3K/Akt signaling pathway plays a role in the induction of type I IFN by myxoma virus, we infected Flt3L-BMDCs with myxoma virus for 1 h and incubated the cells in fresh medium in the presence of wortmannin and 3-methyladenine. Wortmannin, a furanosteroid metabolite of the fungus *Penicillium funiculosum*, is a potent inhibitor of PI3Ks through covalent modification of the catalytic subunit of PI3K (34, 51). 3-Methyladenine (3-MA) also inhibits PI3K (5). To avoid the effects of the drugs on viral entry, wortmannin or 3-MA was added at 1 h postinfection. Treatment of pDCs with wortmannin or 3-MA resulted in a dose-dependent inhibition of IFN-α/β and IL-12p70 production (Fig. 7B and C). Akt (protein kinase B) is a downstream kinase of PI3K. We found that treatment with Akt inhibitor VIII and X after viral entry also blocks the induction of IFN-α/β and IL-12p70 in a dose-dependent manner (Fig. 8A to C).

**The N-terminal Z-DNA/RNA binding domain of vaccinia virus E3 inhibits the induction of IFN-β, TNF, and IL-12p70 in murine pDCs by myxoma virus.** To test whether vaccinia virus produces an inhibitor(s) to block poxvirus sensing, we performed coinfection experiments with purified murine pDCs. We found that coinfection of pDCs with myxoma virus and WT vaccinia virus or the E3LΔ83N (in which the N-terminal Z-DNA/RNA binding domain of E3 is deleted),

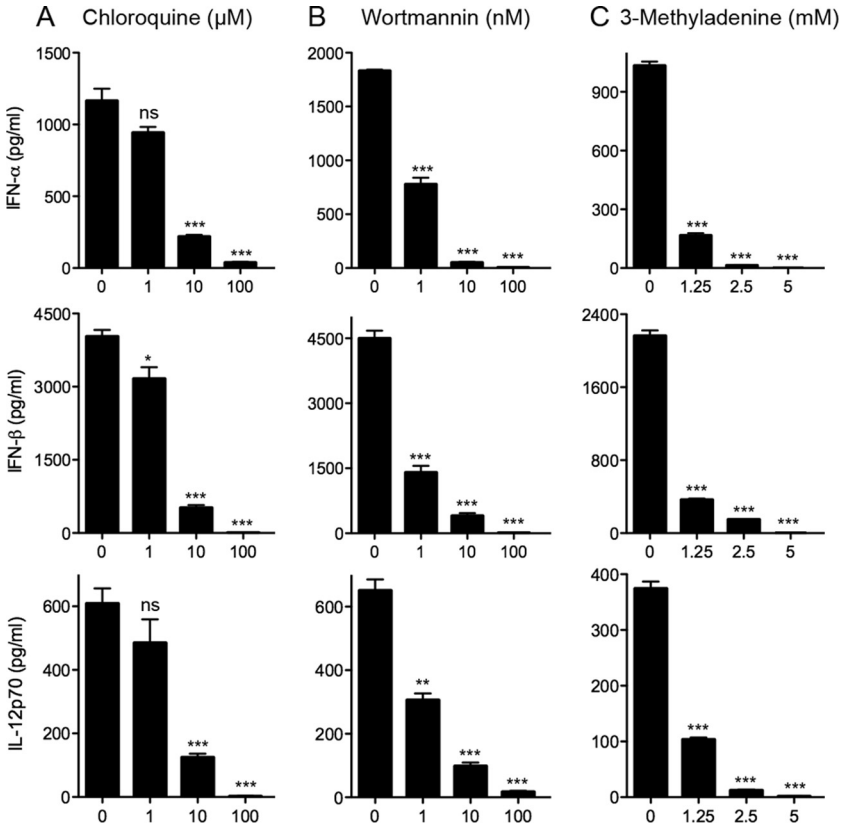



FIG. 7. Chloroquine, wortmannin, and 3-methyladenine block the induction of IFN-α/β and IL-12p70 in murine BMDCs by myxoma virus. Flt3L-BMDCs ($1 \times 10^6$) were infected with myxoma virus at an MOI of 10 for 1 h. Cells were washed and incubated in fresh medium in the presence or absence of increasing concentrations of chloroquine (A), wortmannin (B), or 3-methyladenine (C). Supernatants were collected 22 h later. The concentrations of IFN-α/β and IL-12p70 were determined by ELISA. Data are means ± SEM. The combined results of three independently performed experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant (compared with results for no drug treatment).

E3LY48A (in which a change of tyrosine residue to alanine in the Z-DNA/RNA binding domain resulting in reduced Z-DNA binding affinity), or E3LΔ26C (in which a portion of the C-terminal dsRNA binding domain is deleted) mutant abolished the induction of IFN-α. In contrast, coinfection of pDCs with myxoma virus plus ΔE3L virus (in which the full E3 gene is deleted) reduced the induction of IFN-α only by 39%. These results suggest that vaccinia virus E3 can inhibit the induction of IFN-α in murine pDCs by myxoma virus. Either the N-terminal Z-DNA/RNA binding domain or the C-terminal dsRNA binding domain suffices to block the induction of IFN-α.

A different picture emerged when we examined the production of IFN-β, TNF, and IL-12p70 in the coinfection experiments. Coinfection of murine pDCs with myxoma virus plus WT vaccinia virus dramatically reduced the induction of IFN-β, TNF, and IL-12p70. Coinfection of myxoma virus with ΔE3L or E3LΔ83N vaccinia virus only modestly reduced the induction of IFN-β, TNF, and IL-12p70. In contrast, coinfection of myxoma virus with E3LΔ26C or E3LY48A vaccinia virus produced an inhibitory effect similar to coinfection with WT vaccinia virus (Fig. 9B). These results indicate that the N-terminal Z-DNA/RNA binding domain of vaccinia virus E3 plays an inhibitory role in the induction of IFN-β, TNF, and IL-12p70 in murine pDCs in response to myxoma virus infection. We observed similar results when CpG was used in lieu of myxoma virus, indicating that the Z-DNA/RNA binding domain of vaccinia virus E3 mediates the inhibition of IFN-β, TNF, and IL-12p70 induction by a bona fide TLR9 agonist (Fig. 9A). We surmise that the Z-DNA/RNA binding domain of vaccinia virus E3 might interfere with poxviral DNA sensing and that the Z-DNA binding activity of the domain *per se* is not required for this inhibitory effect.

## DISCUSSION

The present study provides insight into how poxviruses are sensed by murine pDCs, a specialized subset of DCs that are important for type I IFN induction and antiviral immunity. We show that myxoma virus induces type I IFN, TNF, and IL-12p70 in murine pDCs and that the induction is dependent on TLR9/MyD88. The transcription factors IRF5 and IRF7 are required for this induction. Furthermore, the type I IFN feedback loop is critical for the induction, as IFNAR1-deficient pDCs fail to produce type I IFN in response to myxoma virus infection. In contrast, vaccinia virus infection of murine pDCs fails to induce type I IFN. Our results demonstrate that myxoma virus infection can be sensed in murine pDCs by endo-

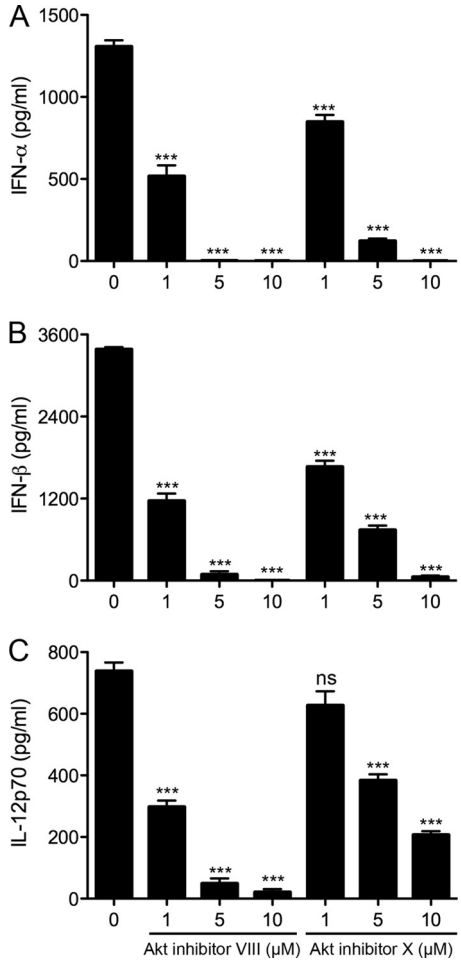



FIG. 8. Akt inhibitors block the induction of IFN-α/β and IL-12p70 in murine BMDCs by myxoma virus. Flt3L-BMDCs ($1 \times 10^6$) were infected with myxoma virus at an MOI of 10 for 1 h. Cells were washed and incubated in fresh medium in the presence or absence of increasing concentrations of Akt inhibitor VIII or X. Supernatants were collected 22 h later. The concentrations of IFN-α (A), IFN-β (B), and IL-12p70 (C) were determined by ELISA. Data are means ± SEM. The combined results of three independently performed experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant (compared with results for no drug treatment).

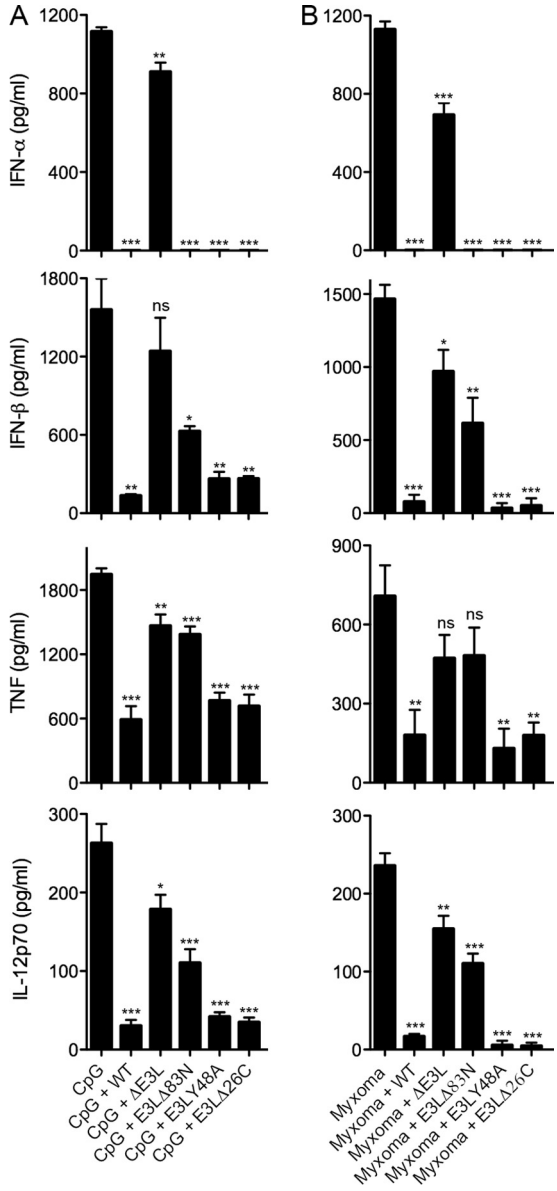



FIG. 9. The induction of IFN-α/β, TNF, and IL-12p70 in murine pDCs by CpG or myxoma virus is attenuated by vaccinia virus E3. (A) Purified murine pDCs ($2 \times 10^5$) were stimulated with CpG alone or infected with WT vaccinia virus or ΔE3L, E3LΔ83N, E3LY48A, or E3LΔ26C mutant virus followed by the addition of CpG. (B) Purified murine pDCs ($2 \times 10^5$) were infected with myxoma virus alone at an MOI of 10 or coinfected with myxoma virus and equal amounts of WT vaccinia virus or ΔE3L, E3LΔ83N, E3LY48A, or E3LΔ26C mutant virus. Supernatants were collected 22 h later. The concentrations of IFN-α, IFN-β, TNF, and IL-12p70 were determined by ELISA. Data are means ± SEM. The combined results of three independently performed experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, nonsignificant (compared with results for CpG treatment alone [A] or myxoma virus infection alone [B]).

somal TLR9 and induces type I IFN and IL-12p70 production through a PI3K/Akt-dependent signaling pathway. These findings may partly explain why myxoma virus is nonpathogenic in mice, whereas systemic infection with vaccinia virus can be lethal in either immunocompetent or immunodeficient mice.

Wang et al. (49) demonstrated that type I IFN induction by myxoma virus in primary mouse embryonic fibroblasts (MEFs) mediates cellular restriction of myxoma virus replication in these cells. Myxoma virus infection becomes permissive in cultured WT MEFs in the presence of neutralizing antibodies to type I IFNs or in IFNAR1- or Stat1-deficient MEFs. Furthermore, myxoma virus causes lethality in Stat1$^{-/-}$ mice in an intracranial infection model, highlighting the importance of the IFN-Stat1 pathway in host defense against poxvirus infection. How myxoma virus is initially sensed by primary MEFs to induce type I IFN production is currently unknown. It has been reported that myxoma virus infection in primary human macrophages induces type I IFN through a cytosolic RNA sensing pathway mediated by RIG-I (50), but the inherent diversity of operational pathogen sensors that exist between cells of dif-

ferent lineages suggests caution in extrapolating these results to other cell types.

In this study, we focused our attention on murine pDCs because of their potency in the induction of type I IFN in response to virus infection. Both human and murine pDCs utilize endosomal TLR7 and TLR9 to sense ssRNA and viral DNAs. TLR9/MyD88 is important for the induction of type I IFN in pDCs in response to herpes simplex virus 1 and 2 (HSV-1 and HSV-2) (24, 29, 17). TLR9/MyD88 is also required for the induction of cytokine responses in pDCs and NK cell activation in response to mouse cytomegalovirus (MCMV) infection, and it is essential for host defenses against MCMV infection (25, 45). Ectromelia virus (the causative agent of mousepox) activates murine pDCs through TLR9 (35). Mice with TLR9 deficiency are more susceptible to ectromelia virus infection (35). Here we show that the induction of type I IFN in murine pDCs by myxoma virus requires TLR9/MyD88. TLR7 is critical for sensing RNA viruses such as vesicular stomatitis virus and influenza virus in pDCs (13, 30). It also plays a compensatory role in host defense against MCMV *in vivo* (56). Our results demonstrate that TLR7 plays a minor role in sensing myxoma virus in murine pDCs *in vitro*.

Type I IFN is a critical mediator of antiviral innate immunity. The interferon regulator factors IRF3, IRF5, and IRF7 play important roles in the regulation of type I IFN genes. IRF3 is constitutively expressed in all cell types, whereas the constitutive expression of IRF7 is restricted to cells of lymphoid origin but can be induced in most cell types by type I IFN (3, 31). IRF3 is required for type I IFN induction triggered by TLR3/TLR4, cytosolic RNA sensing mechanisms mediated by RIG-I/MDA5/MAVS, or cytosolic DNA sensing pathway in many cell types, including cDCs, but it is not required for type I IFN induction in pDCs (8, 14, 19, 20, 42, 43, 55). It has been proposed that IRF3 plays essential roles in both early and late phases of IFN-α/β gene induction, whereas IRF7 is more important for the late induction phase (28, 37). IRF7 has been shown to form complexes with MyD88 and TRAF6 upon TLR7/TLR9 stimulation (23).

IRF5 can be activated in response to viral infections, including Newcastle disease virus, VSV, and HSV-1, but not Sendai virus (4). In the initial report on IRF5$^{-/-}$ mice by Takaoka et al. (46), IFN-α induction was not affected in the pDCs from IRF5$^{-/-}$ mice in response to CpG stimulation, whereas the induction of proinflammatory cytokines was impaired (46). Subsequent studies from the same group showed that IRF5$^{-/-}$ mice are more susceptible to VSV or HSV-1 infections. The IRF5$^{-/-}$ mice had reduced serum levels of type I IFN and IL-6 after viral infections (54). Paun et al. (33) reported that IRF5$^{-/-}$ mice are more susceptible to NDV infection, and the serum levels of IFN-α, TNF, and IL-6 were lower in IRF5$^{-/-}$ mice than in WT mice in response to NDV infection. IRF5 has also been shown to be a central mediator of TLR7 signaling (38). Here we show that both IRF5 and IRF7 are required for the induction of IFN-α/β and IL-12p70 in murine pDCs infected by myxoma virus, whereas IRF3 is dispensable for the IFN induction. This is consistent with our model that myxoma virus infection of pDCs leads to the detection of viral DNA by TLR9 and the activation of transcription factors IRF5 and IRF7 via MyD88, TRAF6, and other associated factors and results in the induction of type I IFN, TNF, and IL-12p70, which are the critical mediators of innate immunity.

The role of the type I IFN positive-feedback loop in the induction of type I IFN in pDCs in response to TLR stimulation or viral infection is not well understood. Sato et al. (36) reported that the induction of IRF7 mRNA in mouse embryonic fibroblasts in response to Newcastle disease virus (NDV) was dependent on type I IFN receptor. Marié et al. (31) reported that although an immediate-early response gene (*IFNA4*) was induced by NDV in fibroblasts in the absence of the type I IFN positive-feedback loop, secondary induction of other IFN-α subtypes was impaired in the absence of Stat1 or IFNAR. They also reported that ectopic expression of IRF7 in fibroblasts led to the induction of the secondary IFN-α subtypes. Conventional DCs produced type I IFN and IL-12p70 in response to TLR stimulation in a Stat1- and IFNAR-dependent manner (15). The type I IFN positive-feedback loop is also important for pDC activation *in vivo* and IFN induction *in vitro* in response to CpG stimulation or viral infection (2, 19). We observed that IFNAR1 is required for the induction of both IFN-α/β and IL-12p70 in pDCs by myxoma virus, supporting the important role of the IFN positive-feedback loop in the induction and sustaining of antiviral innate immune responses. Although IRF7 is expressed in pDCs, type I IFN might be necessary for the enhancement of IRF7 levels.

It is unclear how myxoma viral DNA might be sensed by endosomally localized TLRs in pDCs. Poxviruses enter host cells through fusion with the plasma membrane via a large entry/fusion complex or via a low-pH-dependent endosomal pathway and subsequent fusion with endosomal membrane to release the virion cores into the cytoplasm (39, 47). Because most of the viral entry studies were conducted with vaccinia virus in various transformed cell lines, little is known about how myxoma virus enters primary pDCs. If the basic mechanisms of viral entry are preserved among different poxviruses, we presume that myxoma virion cores are released into the cytoplasm shortly after viral entry. Some of the virion cores might then be transported to the endosomal/lysosomal compartments where viral DNAs are released and detected by TLR9. Alternatively, the cores are uncoated in the cytoplasm and some of the released viral DNA is then taken up and transported to the endosomes. Our findings that treatment of pDCs 1 h postinfection with an endosomal/lysosomal acidification inhibitor, chloroquine, blocks type I IFN and IL-12p70 induction indicate that endosomal/lysosomal processing of virions might be important for the detection of viral DNAs through TLR9. Alternatively, low pH in the endosomes/lysosomes might be important for optimal ligand and receptor interactions. It has been reported that during VSV infection of pDCs, autophagy plays an important role in the detection of cytosolic viral replication intermediates by TLR7 (27). We also observed that treatment of pDCs 1 h postinfection with the PI3K inhibitors wortmannin and 3-MA blocked the induction of type I IFN and IL-12p70. Wortmannin and 3-MA have been used widely as inhibitors of autophagy. Further investigations on the role of autophagy in poxvirus sensing in pDCs is warranted.

Vaccinia virus E3 is a key viral immunomodulator that inhibits type I IFN induction in host cells (12, 41). E3 has two distinct domains, the N-terminal Z-DNA/RNA binding do-

main and the C-terminal dsRNA binding domain. Infection of murine keratinocytes with ΔE3L or E3LΔ26C vaccinia virus but not with WT vaccinia virus or the E3LΔ83N mutant virus induces IFN-β and related cytokine and chemokine production in a MAVS/IRF3-dependent manner (12; P. Dai and L. Deng, unpublished data). This induction effect is dependent on viral DNA replication, indicating that cytosolic dsRNA produced postreplicatively is the trigger for the induction of innate immune responses but is targeted by the dsRNA binding domain of E3 (12). Here we show ΔE3L or E3LΔ26C vaccinia virus infection of murine pDCs fails to induce type I IFN, TNF, or IL-12p70 and is less capable of inhibiting type I IFN induction by myxoma virus infection or CpG stimulation than WT or E3LΔ83N vaccinia virus, implying that ΔE3L vaccinia virus infection fails to produce activators in pDCs and the Z-DNA/RNA binding domain of E3 mediates inhibition of type I IFN induction in pDCs by myxoma virus or CpG. These results indicate that both domains of vaccinia virus E3 function to block innate immune responses in a cell-type-specific fashion. Significantly, the E3 ortholog expressed by myxoma virus, M029, has an intact C-terminal dsRNA binding domain but lacks the N-terminal Z-DNA/RNA binding domain. This difference may partly explain the immune activating property of myxoma virus.

A recent study by Delaloye et al. (10) investigated the immune sensing mechanism of modified vaccinia Ankara virus (MVA) in macrophages that revealed that multiple viral sensing pathways mediate the induction of type I IFN and proinflammatory cytokine induction (10). MVA failed to induce type I IFN induction in purified murine or human pDCs but induced type I IFN and proinflammatory cytokine and chemokine secretion in cDCs (data not shown). It is possible that MVA produces an inhibitor(s) that blocks poxviral sensing in pDCs. The apparent differences in the induction of type I IFN in pDCs and cDCs between myxoma virus and MVA are interesting and warrant further investigation.

In conclusion, this study provides a molecular and genetic basis of how poxvirus sensing is mediated in pDCs. We demonstrate that induction of type I IFN and proinflammatory cytokines in murine pDCs by myxoma virus is mediated by TLR9/MyD88. In addition to IRF7, transcription factor IRF5 is also required for this induction. We also reveal an important role of the N-terminal Z-DNA/RNA binding domain of vaccinia virus E3 in attenuating poxvirus sensing and TLR9 signaling in pDCs. Our findings that drug inhibitors of PI3K/Akt signaling pathway block type I IFN and IL-12p70 induction suggest that PI3K/Akt might be involved in poxvirus sensing in pDCs. The availability of mice with the TLR and RIG-I-like receptor (RLR) sensing pathways knocked out and the feasibility of generating various primary cell types using bone marrow cells in the *in vitro* cell culture system allow us to define the role of various sensors and adaptors in innate immune responses to poxvirus infection. Once these are clearly defined, the roles of these relevant innate immune sensors in host defense against poxvirus need to be examined using *in vivo* infection models (21). These studies will aid in developing the next generations of poxvirus platforms best suited for either oncolytic virotherapy, where the suppression of innate antiviral immune pathways is often desired in order to increase the lifetime of the oncolytic virus in tumors, or vaccine agendas, where the robust induction of early innate cytokines by DCs is most beneficial in obtaining maximal immunogenicity of the desired immunogen.

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# ERRATUM

## Myxoma Virus Induces Type I Interferon Production in Murine Plasmacytoid Dendritic Cells via a TLR9/MyD88-, IRF5/IRF7-, and IFNAR-Dependent Pathway

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Volume 85, no. 20, p. 10814–10825, 2011. Page 10818, Fig. 3: Delete "(% of control)" from the *y* axis. The *y* axis represents relative mRNA expression compared with no virus control.