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Viral Activation of Macrophages through TLR-Dependent and -Independent Pathways¹

Lene Malmgaard,^{2*} Jesper Melchjorsen,^{*} Andrew G. Bowie,[†] Søren C. Mogensen,^{*} and Søren R. Paludan^{*}

Induction of cytokine production is important for activation of an efficient host defense response. Macrophages constitute an important source of cytokines. In this study we have investigated the virus-cell interactions triggering induction of cytokine expression in macrophages during viral infections. We found that viral entry and viral gene products produced inside the cell are responsible for activation of induction pathways leading to IFN- $\alpha\beta$ expression, indicating that virus-cell interactions on the cell surface are not enough. Moreover, by the use of cell lines expressing dominant negative versions of TLR-associated adaptor proteins we demonstrate that Toll/IL-1 receptor domain-containing adaptor inducing IFN- β is dispensable for all virus-induced cytokine expression examined. However, a cell line expressing dominant negative MyD88 revealed the existence of distinct induction pathways because virus-induced expression of RANTES and TNF- α was totally blocked in this cell line whereas IFN- $\alpha\beta$ expression was much less affected in the absence of signaling via MyD88. In support of this, we also found that inhibitory CpG motifs, which block TLR9 signaling inhibited early HSV-2-induced TNF- α and RANTES expression dramatically whereas IFN- $\alpha\beta$ induction was only slightly affected. This suggests that virus activates macrophages through distinct pathways, of which some are dependent on TLRs signaling through MyD88, whereas others seem to be independent of TLR signaling. Finally we demonstrate that IFN- $\alpha\beta$ induction in HSV-2-infected macrophages requires a functional dsRNA-activated protein kinase molecule because cells expressing a dsRNA-dependent protein kinase version unable to bind dsRNA do not express IFN- $\alpha\beta$ on infection. *The Journal of Immunology*, 2004, 173: 6890–6898.

The innate immune response against viral infections requires a complex network of interactions between leukocytes including NK cells, dendritic cells (DCs)³ and macrophages and the cytokines/chemokines they produce. These interactions ultimately aim at resolving or limiting spread of the virus until the adaptive immune response is activated.

Macrophages are normally found in a resting state throughout the body. During viral infection, however, these cells are activated to express various cytokines, chemokines, surface markers and others (1). Activated macrophages have important roles in defense against many infections, including viral infections, as producers of cytokines and chemokines, as APCs, and as potent antimicrobial effector cells (1). The cytokines expressed by virus-activated macrophages include IL-6, IL-12, IFN- $\alpha\beta$, TNF- α , and the chemokine RANTES (CCL-5) (2–6). Among these notably IFN- $\alpha\beta$ has been

ascribed important roles in host defense against viral infections (7). For instance, mice deficient in the IFN- $\alpha\beta$ receptor are severely impaired in resistance against viruses such as vaccinia virus, vesicular stomatitis virus, and semliki forest virus (8).

Virus-cell interactions leading to activation of macrophages involve host cell pattern recognition receptors (PRRs) binding viral determinants called pathogen-associated molecular patterns (PAMPs) (9). These PRR-PAMP interactions activate downstream intracellular pathways leading to expression of proinflammatory molecules (10). Historically, viral induction pathways have been divided into those that are sensitive or insensitive toward UV treatment of the virus. The UV insensitive induction pathway can go through interactions between viral surface determinants and cell surface receptors, or it can be a result of viral factors released upon entry that interact with extracellular or intracellular receptors. This finding is exemplified by the ability of HSV glycoprotein D (gD) to bind mannose receptors inducing expression of IFN- $\alpha\beta$ in DCs (11, 12). The UV sensitive pathway is dependent on a functional viral genome (which is destroyed upon UV treatment), indicating that accumulation of viral RNA or proteins initiates this kind of induction pathway. This is exemplified by the ability of dsRNA to activate the intracellular receptor dsRNA-dependent protein kinase (PKR) thereby promoting transcription of a number of genes including IFN- $\alpha\beta$ (13–15). Other examples include viral overload of the endoplasmic reticulum and viral-induced mitochondrial oxidative stress leading to activation of NF- κ B and subsequent promotion of cytokine expression (16, 17).

Recently a new group of PRRs has been described, the TLRs. TLRs bind microbial factors at the cell surface or in endosomes and subsequently activate cytoplasmic signal transduction pathways (18). The specificity of the pathways activated by TLRs is largely determined by adaptor proteins (19, 20). All TLRs, with the possible exception of TLR3, use the adaptor protein MyD88.

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³ Abbreviations used in this paper: DC, dendritic cell; PRR, pattern recognition receptor; PAMP, pathogen-associated molecular pattern; PKR, dsRNA-dependent protein kinase; TIR, Toll/IL-1 receptor; TRIF, TIR domain-containing adaptor inducing IFN- β ; IRF, IFN regulatory factor; gD, glycoprotein D; ODN, oligonucleotide; IKK, I κ B kinase; ICP, infected cell protein; IE, immediate early; dn, dominant negative.

TLR3, in contrast, signals primarily through the alternative adaptor protein Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN- β (TRIF), whereas TLR4 signals through MyD88, TRIF, and other adaptors (10, 21, 22). Five TLRs have been associated with viral infections. dsRNA has been identified as a ligand for TLR3 (23). The downstream signaling pathway from TLR3 leads to activation of IFN regulatory factor (IRF)-3 and/or NF- κ B and subsequent expression of numerous cytokines and chemokines including IFN- α , RANTES, TNF- α , IL-6, and IL-12 (10, 23). TLR9 binds unmethylated CpG motifs from viral and bacterial DNA (24–26). This pathway goes through MyD88 leading to NF- κ B activation and expression of IFN- α , RANTES, TNF- α , and other proinflammatory factors. TLR4, which mediates the LPS-induced proinflammatory response, was shown to bind respiratory syncytial virus fusion protein leading to IL-6, IL-8, and TNF- α expression (27). TLR2 recognizes CMV and HSV particles followed by activation of NF- κ B (28, 29). Recently murine TLR7 and human TLR8 were shown to bind ssRNA located in endosomes, thereby leading to activation of NF- κ B and cytokine secretion (30, 31).

In previous studies we have described the molecular pathways leading to expression of RANTES and TNF- α during HSV infection. RANTES expression requires the transcription factors NF- κ B and IRF-3 and is dependent on a functional viral genome (3, 32). TNF- α induction, which is only partly sensitive to UV treatment of the virus, requires NF- κ B and activating transcription factor 2/Jun (6, 33). In this study we wanted to investigate the mechanisms that govern induction of IFN- α expression in macrophages during viral infections, and we also wanted to explore the virus-cell interactions that trigger activation of macrophages. We report that induction of IFN- α by HSV is dependent on viral entry and viral gene products, indicating that viral factors produced inside the cell trigger the signal transduction cascades stimulating IFN- α expression. Induction of both IFN- α and IFN- β are dependent on the transcription factors NF- κ B and IRF-3. Moreover, we demonstrate that activation of macrophages proceeds through both TLR-dependent and -independent pathways and that the dsRNA binding capacity of PKR is essential for induction of IFN- α .

Materials and Methods

Virus

The wild-type viruses used in this study were the MS strain of HSV-2 and the KOS and 17+ strains of HSV-1. The infected cell protein (ICP) 0 mutant *dl1403*, the VP16 mutant *in1814* are on a 17+ genetic background (34, 35). The mutants lacking ICP4 or ICP22, or ICP27 or glycoprotein L are on a KOS genetic background (36–39). The viruses were produced essentially as previously described (2) and quantified by titration in U2OS cells, Vero cells, and/or Vero-derived cells expressing specific viral proteins. Just before usage, virus was thawed and used as infectious virus, subjected to heat inactivation at 56°C for 30 min, or inactivated by UV light for 10 min.

Mice

Inbred, specific pathogen-free C57BL/6 mice were obtained from Taconic M&B (Ry, Denmark). Female mice were used at the age of 8–12 wk, but for individual experiments only mice born within 1 wk were used.

Cell cultures

The murine macrophage cell lines RAW264.7 and J774A.1 (American Type Culture Collection, TIB 67) were grown in DMEM with 1% glutamax I (Invitrogen Life Technologies, Taastrup, Denmark), supplemented with 5% LPS-free FCS, 200 IU/ml penicillin, and 200 mg/ml streptomycin. Peritoneal cells from C57BL/6 mice were obtained as previously described by lavage of the peritoneum with cold PBS, pH 7.4, supplemented with 2% FCS and 200 IU/ml heparin (2). After washing, the cells were counted and grown in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10 mM glutamine, 2 mM HEPES, and FCS and antibiotics as above. The J774.A1 and RAW264.7-derived cell lines were grown as the parental cell line plus 300 μ g/ml G418 (Roche Diagnostics, Mannheim,

Germany). The PKR mutant cell line RAW-PKR-M7 and empty vector control cell line RAW-pBK-CMV, kindly donated by Dr. J. A. Corbett (Washington University, St. Louis, MO), were grown in DMEM in the presence of 200 μ g/ml G418 (40).

For experiments cells were seeded in 96-well tissue culture plates to give a final concentration of 5×10^5 and 1.5×10^6 cells/ml in 200 μ l of medium for the cell lines and peritoneal cells, respectively. After indicated periods of time the supernatants were harvested for determination of expression levels of IFN- α bioactivity, RANTES protein, or TNF- α protein.

For isolation of total RNA J774A.1 cells were seeded in 2.5 or 10 cm² tissue-culture plates at a density of 0.6 or 2.5×10^6 cells/plate, respectively, and allowed to settle for 5 h. The cultures were stimulated and infected for indicated periods of time before RNA was extracted.

Stimulation with poly(I:C), stimulatory and inhibitory oligonucleotides (ODN), chloroquine, and recombinant gD

Poly(I:C), stimulatory ODN1826, and inhibitory ODN2088 (InvivoGen, San Diego, CA) were diluted in sterile H₂O or PBS, respectively, as recommended by the manufacturer. ODN1826 and ODN2088 are on a phosphorothioate backbone. Recombinant gD was obtained from Viral Therapeutics (Ithaca, NY) and chloroquine (Sigma-Aldrich, St. Louis, MO) were diluted in PBS and the pH value of the solution was adjusted before use. For experiments, dilutions were made in DMEM in the concentrations shown (see Figs. 2 and 5).

RNA extraction and RT-PCR

Total RNA was extracted, DNase treated (Ambion, Austin, TX), and analyzed by RT-PCR as previously described (4, 41). Briefly, 1–2 μ g of RNA was reverse transcribed into cDNA. For relative quantitative PCR, 5 μ l of cDNA was used as a template with primers specific for: IFN- β (sense) 5'-CAT CAA CTA TAA GCA GCT CCA-3', (antisense) 5'-TTC AAG TGG AGA GCA GTT GAG-3'; consensus primers recognizing most IFN- α subtypes (sense) 5'-ATG GCT AGR CTC TGT GCT TTC CT-3', (antisense) 5'-AGG GCT CTC CAG AYT TCT GCT CTG-3'; or HSV-2-ICP27 (sense) 5'-AGA TCG GCT ACA CGA CCG-3', (antisense) 5'-TGC CGT GCA CAT ATA AGG G-3'. Gene-specific primers for IFNs were identified from an earlier publication (42) and synthesized by DNA Technology (Aarhus, Denmark). As internal controls for random variations, β -actin primers and competitors (Ambion) were used (41). Amplifications were conducted in a thermal cycler (PTC-200; MJ Research, Waltham, MA).

IFN- α bioassay

IFN- α bioactivity in supernatants was detected by a biological assay for protection against vesicular stomatitis virus, as described earlier (41). The dilution mediating 50% protection was defined as containing 1 U/ml IFN. Specificity was determined by neutralization with two polyclonal rabbit Abs directed against IFN- α and IFN- β (PBL Biomedical Laboratories, Piscataway, NJ).

ELISA

Protein levels of RANTES and TNF- α in supernatants were measured by ELISA. The protocol for RANTES ELISA was described earlier (32). For measurement of TNF- α an ELISA duoset from R&D Systems (catalogue no. DY410; Minneapolis, MN) was used, following recommendations of the manufacturer. The concentrations of coating Ab and detection Ab were 0.8 and 0.15 mg/ml, respectively. The ELISA was able to detect TNF- α from 15 to 2000 pg/ml.

Stable transfections

The DNA plasmids used for transfections encoded dominant negative (dn) versions of IRF-3, I κ B kinase (IKK)- β , MyD88, or TRIF (17, 21, 32, 43). For transfections, the cells were seeded at a density of 5×10^6 cells/25-cm² plate and left for 24 h. The cells were transfected using LipofectAMINE (7.5 μ g of DNA and 20 μ l of LipofectAMINE; Invitrogen Life Technologies) and serum-free medium. Four hours after transfection FCS was added to 10% and the cells were incubated 24 h before application of selection with 300 μ g/ml G418. Surviving colonies were pooled to avoid single-clone abnormalities and cells were grown through three to five passages in DMEM supplemented with 5% FCS in the presence of selection.

Results

Kinetics of IFN- α expression in macrophages

Expression of IFN- α is a hallmark of virus infections, so we first wanted to compare virus-induced expression of IFN- α between

primary macrophages and the macrophage-like cell lines RAW264.7 and J774.A1. After infection with HSV-1 or HSV-2 for the indicated periods of time, supernatants were harvested for determination of IFN- $\alpha\beta$ expression. As can be seen from Fig. 1 IFN- $\alpha\beta$ bioactivity was undetectable in uninfected cells. IFN- $\alpha\beta$ became measurable after 4–6 h of infection and continued to accumulate during the period of measurements. HSV-2 generally induced a higher IFN- $\alpha\beta$ response than HSV-1, but the kinetics were very similar. In addition we infected all three cell populations with HSV-1 and HSV-2 rendered unable to replicate due to UV treatment. In both primary macrophages and macrophage cell lines these inactivated preparations of HSV were unable to induce any bioactive IFN- $\alpha\beta$ expression. This suggests that viral factors produced during the replication cycle are responsible for HSV-induced IFN- $\alpha\beta$ expression in macrophages.

Viral glycoproteins are not responsible for IFN- $\alpha\beta$ induction in macrophages

Earlier reports have shown that UV-inactivated HSV is able to induce IFN- $\alpha\beta$ (25, 44, 45), indicating that viral transcription and replication are not involved in the induction pathway. Because our results in Fig. 1 did not agree with these reports we wanted to examine this phenomenon in more detail. We looked at mRNA accumulation for IFN- α and IFN- β during early times of infections with live and UV-inactivated HSV-1 in J774.A1 cells using a semiquantitative RT-PCR. In correlation with the data in Fig. 1 we observed no mRNA for IFN- α and IFN- β in mock-infected cells and at 1 h after infection, small amounts at 2 h after infection, and high-level expression at 4 h after infection (Fig. 2A). UV-inactivated virus did not induce IFN- $\alpha\beta$ mRNA accumulation at any time point observed. These data again suggested that the HSV particle in itself is not enough to induce IFN- $\alpha\beta$ in macrophages. Instead viral factors produced inside the cells during the viral replication cycle could be involved.

To test this hypothesis further, we performed an RT-PCR analysis on RNA from J774.A1 cells infected with a mutant variant of HSV-1, incapable of entering the cells due to a mutation in glycoprotein L (HSV-1, gL86). As seen in Fig. 2, B and C, this HSV-1 mutant virus was not able to induce any IFN- $\alpha\beta$ mRNA accumulation in J774.A1 cells and no bioactivity was detected in either primary macrophages or any of the macrophage cell lines infected with HSV-1 gL86. In addition we tried to stimulate cells with

recombinant gD, also reported to induce IFN- $\alpha\beta$ (46, 47), but no induction could be observed (Fig. 2B). Together these data clearly show that the induction pathway for IFN- $\alpha\beta$ during HSV infections in macrophages does not rely on interactions between viral glycoproteins and cell surface receptors, but requires a functional viral particle infecting the cells.

Effect of UV treatment on viral infectivity, viral mRNA accumulation and IFN- $\alpha\beta$ induction

Earlier work from this lab has examined the kinetics of expression of HSV genes during infections of J774.A1 cells, showing peak expression of immediate early (IE) genes at 2–4 h after infection (3). Together with the above results we suspected accumulation of IE gene mRNA as the triggering factor inducing IFN- $\alpha\beta$ expression. We examined this hypothesis with two different approaches.

First, we examined the effect of different time periods of UV treatment on viral infectivity and accumulation of IE gene mRNA and compared it with the ability of the virus to induce IFN- $\alpha\beta$ expression. Briefly, HSV-2 was diluted to 3×10^6 pfu/ml and subjected to UV irradiation for 0.5, 1, 2, 4, and 8 min. These preparations were either 1) titrated for infectivity using a plaque assay, 2) used for infections of RAW264.7 cells for 24 h at which point supernatants were harvested and tested for expression level of bioactive IFN- $\alpha\beta$, and 3) used for infections of RAW264.7 cells for 5 h where RNA was harvested and subjected to RT-PCR using primers specific for ICP27. As shown in Fig. 3A, infectivity was reduced by 3 logs after 30 s of UV treatment and almost gone by 1 min of UV treatment. Induction of IFN- $\alpha\beta$ expression declined in parallel with infectivity, but with a delay of 1 min (Fig. 3A). Reduction of expression of the viral IE gene ICP27 occurred in parallel with reduction of infectivity (Fig. 3B).

Second, we investigated the role of IE genes in the induction of IFN- $\alpha\beta$ by using mutant HSV-1 virus deficient for the IE genes ICP0, ICP4, ICP22, and ICP27. However, supernatants from J774.A1 cells infected with these mutant viruses did not show any significant reduction in expression of IFN- $\alpha\beta$ bioactivity as compared with cells infected with wild-type virus (data not shown).

Collectively, these data support the above conclusion that viral factors produced inside the macrophage initiate the induction pathway to IFN- $\alpha\beta$ expression. However, none of the IE genes alone were responsible for IFN- $\alpha\beta$ induction.

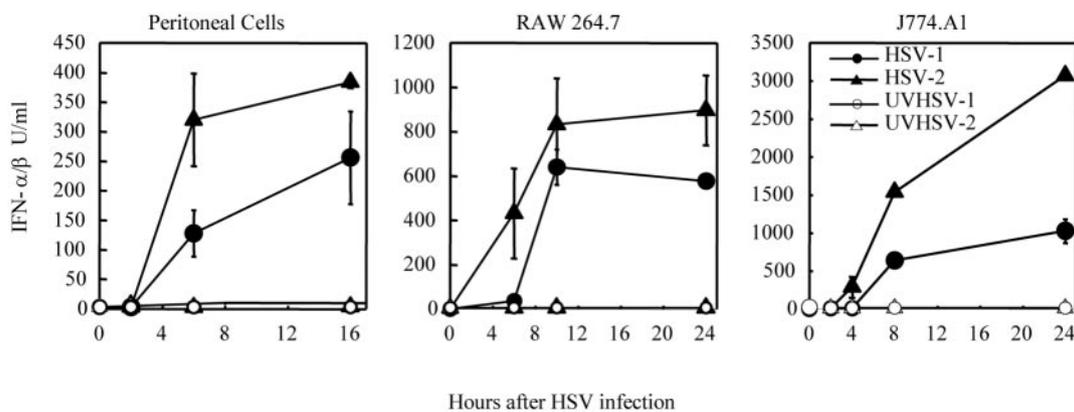


FIGURE 1. Kinetics of IFN- $\alpha\beta$ production during infections with live and UV-treated HSV in peritoneal macrophages and the macrophage cell lines RAW264.7 and J774.A1. Peritoneal macrophages were harvested from C57BL/6 mice and left overnight to settle. All cell types were infected with infectious HSV-1 or HSV-2 at a multiplicity of infection of 1.7 for peritoneal cells and of 6 for cell lines. Alternatively the cells were treated with identical doses of HSV-1 or HSV-2 subjected to UV treatment for 10 min. Supernatants were harvested at the indicated time points for measurement of IFN- $\alpha\beta$ bioactivity. Results are shown as mean of triplicates \pm SEM.

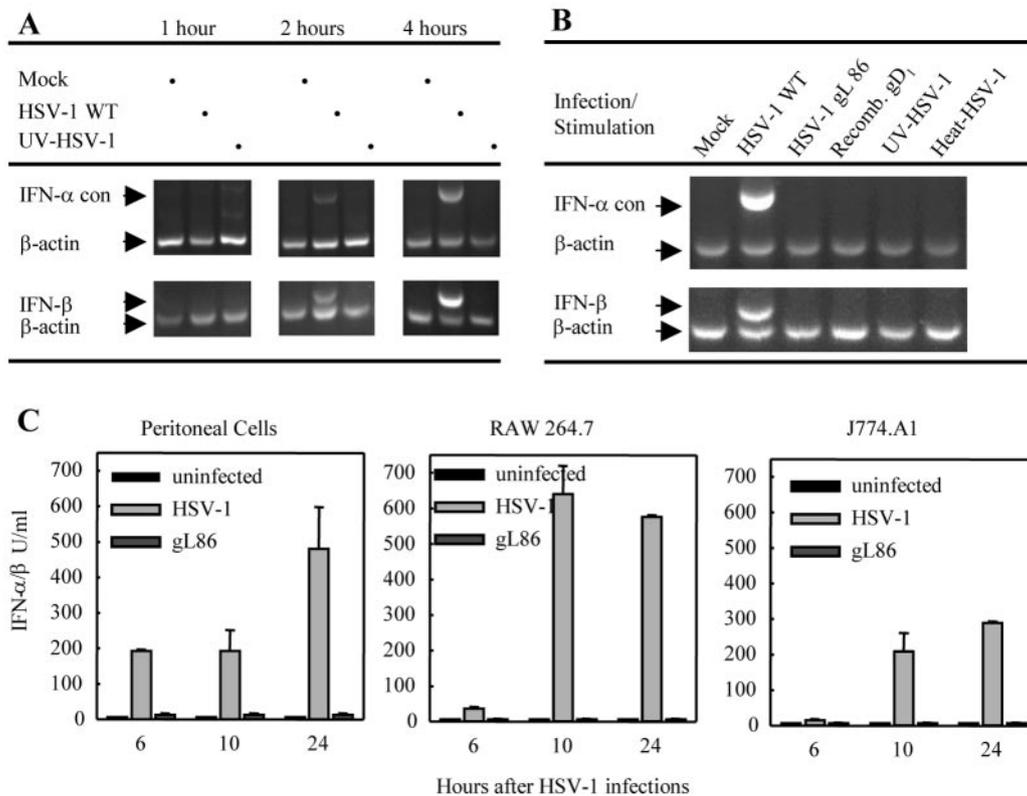


FIGURE 2. Kinetics of IFN- $\alpha\beta$ mRNA accumulation and viral requirements for induction of IFN- $\alpha\beta$. *A*, J774.A1 cells were treated with mock preparations, infectious HSV-1, or HSV-1 UV-treated for 10 min. After 1, 2, or 4 h RNA was harvested and subjected to RT-PCR with primers specific for IFN- α or IFN- β . *B*, J774.A1 cells were treated with mock, wild-type HSV-1, HSV-1 (gL86), UV-treated HSV-1, heat-treated HSV-1, or stimulated with recombinant gD (1 mg/ml). All virus preparations were used at a multiplicity of infection (m.o.i.) of 3. At 4 h posttreatment RNA was harvested and subjected to RT-PCR with primers specific for IFN- α or IFN- β . *C*, Peritoneal macrophages, J774.A1, and RAW264.7 cells were infected with wild-type HSV-1 or HSV-1 (gL86). Supernatants were harvested at the indicated time points for measurement of IFN- $\alpha\beta$ bioactivity. Results are shown as mean of triplicates \pm SEM.

Transcription factors involved in HSV-induced IFN- $\alpha\beta$ expression

It is well known that expression of IFN- β is governed by the transcription factors IRF-3, c-Jun, and NF- κ B, whereas the IFN- α mainly relies on IRF-3/IRF-7. To examine the importance of the transcription factors NF- κ B and IRF-3 in HSV-2-induced IFN- $\alpha\beta$ expression in macrophages, we transfected J774.A1 cells with an empty control vector (pcDNA3) or vectors containing dominant negative forms of IRF-3 (dnIRF-3) and dnIKK- β . After infection with HSV-2 for 16 and 24 h, RNA and supernatants were harvested from the different cell lines for examination of IFN- $\alpha\beta$ expression by RT-PCR and bioassay. As seen from Fig. 4*B*, cells expressing the dominant form of nonfunctional IKK- β were nearly 100% abrogated in their ability to express IFN- $\alpha\beta$ bioactivity after an HSV-2 infection. Cells expressing a dnIRF-3 protein also mount a significantly reduced IFN- $\alpha\beta$ response as compared with the control cells. The RT-PCR analysis revealed that transcription of both IFN- α and IFN- β was reduced in dnIKK- β and dnIRF-3 cells (Fig. 4*A*). These data demonstrate that transcription of both IFN- α and IFN- β genes are governed by IRF-3 and NF- κ B during viral infections of macrophages.

Roles of TLRs for activation of macrophages

With the aim of identifying cellular PRRs responsible for viral activation of macrophages, we now examined the role of TLRs, which have been described as receptors for pathogenic factors mediating induction of proinflammatory cytokines (18). For this pur-

pose we made stable transfections of RAW264.7 cells with plasmids encoding dominant negative versions of either of the two TLR-related adaptor proteins MyD88 and TRIF or with the empty vector pcDNA3. Poly(I:C) and CpG are known to induce cytokines through a pathway dependent on the adaptor proteins TRIF and MyD88, respectively. As a positive control for the effect of the transfections we stimulated dnTRIF and dnMyD88 cells with poly(I:C) and CpG DNA, respectively, and measured expression of RANTES after 24 h. As can be seen from Fig. 5*A*, poly(I:C) induced RANTES expression was absent in dnTRIF cells and CpG-induced RANTES expression was reduced with 80% in dnMyD88 cells. These data confirm the dominant negative effect of the transfected constructs. We next infected the cell lines with HSV-2 and measured accumulation of IFN- $\alpha\beta$, RANTES and TNF- α in the supernatants. Induction of IFN- $\alpha\beta$ in HSV-2-infected pcDNA3 cells resembled the results from unmanipulated RAW cells (Fig. 1), with maximal induction at 12 h after infection. IFN- $\alpha\beta$ expression was slightly reduced in dnMyD88 cells compared with pcDNA3 cells whereas dnTRIF cells expressed significantly higher levels at both 12 and 24 h after infection (Fig. 5*B*). HSV-2 infection induced a very strong expression of RANTES and TNF- α in pcDNA3 cells (Fig. 5, *C* and *D*). dnTRIF cells expressed RANTES and TNF- α at levels comparable to pcDNA3 cells. However, virus-induced expression of RANTES and TNF- α was reduced 70–80% in dnMyD88 cells, which was in striking contrast to the modest reduction in IFN- $\alpha\beta$ expression in this cell line. These data therefore strongly suggest that HSV-2 induces

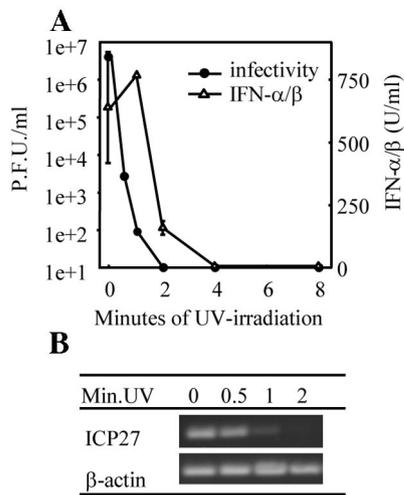


FIGURE 3. Effect of varied time periods of UV treatment of HSV-2 on viral infectivity, viral mRNA accumulation, and ability to induce IFN- $\alpha\beta$ expression in RAW264.7 cells. Preparations of HSV-2 (3×10^6 pfu/ml) were subjected to UV treatment for 0, 0.5, 1, 2, 4, and 8 min. *A*, These preparations were used in either plaque assay (*left y-axis*) or infections of RAW264.7 cells. Supernatants of infected RAW264.7 cells were harvested after 24 h and IFN- $\alpha\beta$ bioactivity was determined (*right y-axis*). *B*, Alternatively RNA was harvested from infected RAW264.7 cells after 5 h and subjected to RT-PCR with primers specific for ICP27.

expression of RANTES and TNF- α in macrophages through a pathway involving one or more TLRs that signal through the adaptor protein MyD88. In contrast, IFN- $\alpha\beta$ is induced through a pathway, which is independent of MyD88 and TRIF signaling and therefore probably independent of TLR signaling at all.

Role of TLR9 in HSV-2-induced cytokine expression

TLR9 has been described to recognize HSV DNA and hence trigger cytokine expression in plasmacytoid DCs (25). To investigate whether the MyD88-dependent induction of TNF- α and RANTES in Fig. 5, *C* and *D*, was a result of TLR9 activation, we used an ODN with an inhibitory CpG motif (ODN2088) known to specifically block TLR9 signaling (48). To confirm the activity of the inhibitory CpG, we coincubated with stimulatory CpG and measured TNF- α in RAW264.7 cells. As seen in Fig. 6*A*, inhibitory CpG did inhibit CpG-induced TNF- α expression completely, thus confirming the inhibitory activity in this system. We next treated RAW264.7 cells with HSV-2 and inhibitory CpG and measured expression of TNF- α , RANTES, and IFN- $\alpha\beta$. Whereas inhibitory CpG only had a modest effect on HSV-2-induced IFN- $\alpha\beta$ expression (Fig. 6*B*), this treatment reduced induction of RANTES and TNF- α by 65–75% at 12 h after infection (Fig. 6, *C* and *D*). However, at 24 h after infection inhibitory CpG did not affect any of the three factors dramatically (Fig. 6, *B–D*). As an alternative inhibitor of TLR9 signaling we used chloroquine, which has been reported to inhibit signaling from endosomal TLRs including TLR9 (25). We found that IFN- $\alpha\beta$ expression in HSV-2-infected RAW264.7 cells was not inhibited by chloroquine treatment, whereas early expression of RANTES was inhibited by 50–70% in the presence of chloroquine (Fig. 6, *E* and *F*). Collectively, these data indicate that TLR9 is responsible for early HSV-2-induced TNF- α and RANTES expression whereas early induction of IFN- $\alpha\beta$ mainly occurs through TLR-independent pathways. In addition, they indicate that alternative TLR9-independent induction pathways to IFN- $\alpha\beta$, RANTES, and TNF- α expression are functional at a later time point after the infection. Similar results were obtained in

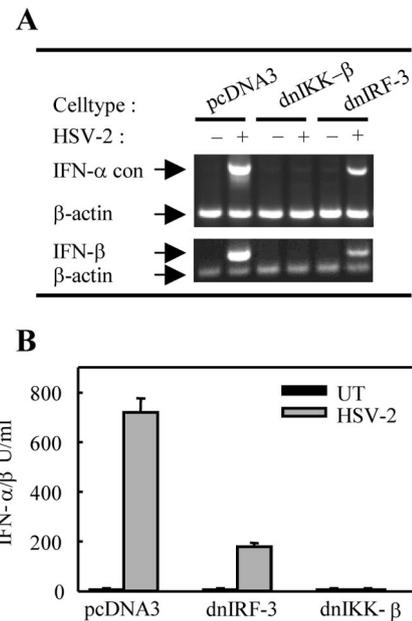


FIGURE 4. Induction of IFN- $\alpha\beta$ expression in J774.A1 cells over-expressing dominant negative versions of IRF-3 and NF- κ B. J774.A1 cells stably transfected with empty vector pcDNA3 or constructs encoding dnIKK- β or dnIRF-3 were left untreated (UT) or infected with HSV-2 at a multiplicity of infection (m.o.i.) of 6. *A*, After 16 h, RNA was harvested and subjected to RT-PCR with primers specific for IFN- α or IFN- β . *B*, After 24 h, supernatants were harvested and IFN- $\alpha\beta$ bioactivity measured. Results are shown as mean of triplicates \pm SEM.

J774.A1 cells infected with HSV-1 and treated with inhibitory CpG (data not shown). This indicates that the differential involvement of TLR9 is a general phenomenon for HSV-induced cytokine expression in macrophages

Role of PKR in induction of IFN- $\alpha\beta$

PKR is another possible PRR involved in viral activation of macrophages. To investigate the role of PKR in IFN- $\alpha\beta$ induction, we used RAW 264.7 cells transfected with empty vector (pBK-CMV) or a plasmid encoding a dnPKR construct lacking the first dsRNA binding domain, and hence unable to bind dsRNA. These cells were subsequently infected with HSV-2 and supernatants harvested at different time points after infection. Control cells expressed bioactive IFN- $\alpha\beta$ protein from 4 to 24 h after infection (Fig. 7). By contrast, the cells expressing the nonfunctional PKR protein did not express any detectable bioactive IFN- $\alpha\beta$ after HSV infections. This was also confirmed at the RNA level using RT-PCR (data not shown). These data reveal that the dsRNA-binding capacity of PKR is essential for the ability of HSV-2 to activate IFN- $\alpha\beta$ expression in macrophages.

Discussion

In this study we have investigated the induction pathway for IFN- $\alpha\beta$ expression during HSV infection of murine macrophages and the PRRs responsible for activation of cytokine expression in macrophages. We demonstrate that viral infection activates macrophages to express IFN- $\alpha\beta$, RANTES, and TNF- α , through different pathways of which some are dependent on TLR signaling whereas others are not. This implies that several distinct virus-cell interactions lead to expression of proinflammatory cytokines within the same cell type.

We define in this study an alternative pathway for activation of IFN- $\alpha\beta$ expression depending on viral entry and viral replication.

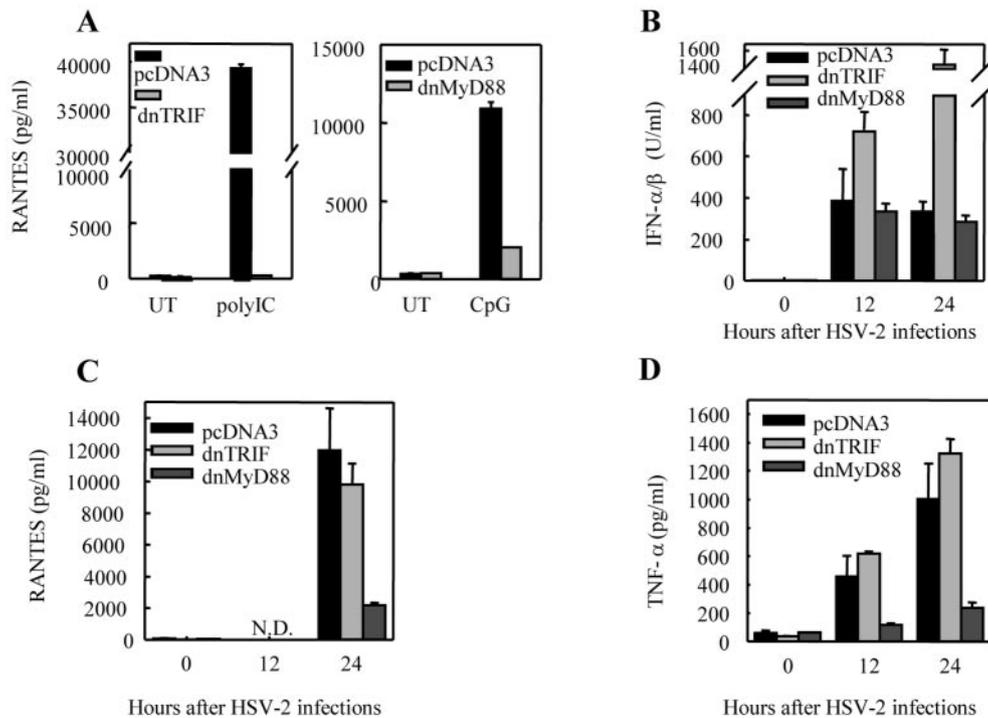


FIGURE 5. Expression of IFN- $\alpha\beta$, RANTES, and TNF- α in RAW264.7 cells transfected with dominant negative versions of MyD88 and TRIF. RAW264.7 cells were stably transfected with the empty vector pcDNA3 or constructs encoding dnMyD88 or dnTRIF. *A*, pcDNA3 and dnTRIF cells were treated with poly(I:C) (2.5 $\mu\text{g/ml}$) and pcDNA3 and dnMyD88 cells were treated with ODN1826 (1 μM). After 24 h RANTES expression was measured in the supernatants. *B–D*, pcDNA3, dnMyD88 and dnTRIF cells were infected with HSV-2 at an m.o.i. of 6. After 12 and 24 h of infection supernatants were harvested and used for determination of IFN- $\alpha\beta$ bioactivity (*B*), RANTES (*C*) or TNF- α (*D*). N.D., Not done. Results are shown as mean of triplicates \pm SEM.

This induction pathway is distinct from the previously described HSV-activated pathways that are insensitive to UV inactivation and triggered by a binding between viral glycoproteins and cellular lectins or through TLR9 (11, 25, 47). These discrepancies could reflect cell type-specific induction pathways because we have used macrophages whereas most other studies have focused on DCs (11, 25) or mixed cell populations like PBMCs (47). This implies that HSV induces IFN- $\alpha\beta$ expression in macrophages mainly through a mechanism different from that observed in plasmacytoid DCs, which is the main producer of IFN- $\alpha\beta$ during several viral infections (49–51). The inability of UV-inactivated virus to induce IFN- $\alpha\beta$ in certain cells like fibroblasts and macrophages is not a new observation and it is supported by recent studies using Newcastle disease virus, Sendai virus, and influenza virus (12, 52–54). The reason for this cell type specificity could be differential expression of cell surface receptors, or a different intrinsic ability to respond to extracellular stimuli. Another possible explanation is difference in viral doses used in our vs other studies. We have used a fairly low dose of virus and we have clear indications that viral dose is important for which induction pathway is activated (L. Malmgaard, unpublished observation).

We found that a functional viral genome is necessary for induction of IFN- $\alpha\beta$ expression and provide data suggesting a role for early virus-induced events, in triggering this response. The parallel declines in ICP27 mRNA formation and induction of IFN- $\alpha\beta$ upon UV treatment of the virus and the kinetic of IFN- $\alpha\beta$ expression suggest that IE genes could be involved (Figs. 2 and 3). We were, however, not able to define a single IE gene as responsible for induction of IFN- $\alpha\beta$. This is possibly due to redundancy, or perhaps the triggering factor is a feature common to all IE genes for example formation of dsRNA structures. Another possibility is that production of viral proteins in

the cell initiates host cell stress mechanisms that in turn triggers induction of IFN- $\alpha\beta$ expression (17).

Two recent studies have demonstrated that TLR9 recognizes HSV-2 DNA in endosomes thereby inducing IFN- $\alpha\beta$ expression (25, 26). Therefore, we wanted to explore the possible involvement of TLRs by the use of cell lines transfected with double negative versions of MyD88 and TRIF. We also included RANTES and TNF- α in our study to get a more general picture of what is triggering activation of cytokine/chemokine expression in macrophages. First we found that none of the measured cytokines were inhibited in their induction by expression of a dnTRIF. This may appear surprising because TLR3, which signals through TRIF, has been shown to be expressed at high levels on macrophages (23, 55, 56). TLR3 was also an attractive candidate because induction of all three factors is predominantly sensitive to UV-inactivation of HSV-2 that suggested involvement of intracellular viral PAMPs, such as dsRNA (3, 33). What we observed, however, was elevated IFN- $\alpha\beta$ expression in dnTRIF macrophages that might indicate that TRIF signaling also mediates induction of factors that negatively regulates the immune response. This phenomenon is observed in many other knockdown and knockout studies underscoring the complexity of removing just a single component of the immune system (57).

Secondly, we found that HSV-2-induced expression of IFN- $\alpha\beta$, RANTES, and TNF- α differ in their requirement for MyD88, with IFN- $\alpha\beta$ being mainly independent and RANTES/TNF- α being highly dependent. This suggests that RANTES and TNF- α are induced by mechanisms involving one or more TLRs that signal through MyD88. This could potentially be TLR7, TLR8, or TLR9 that are found in endosomes, and are expressed constitutively in RAW264.7 cells (56, 58). We investigated the involvement of

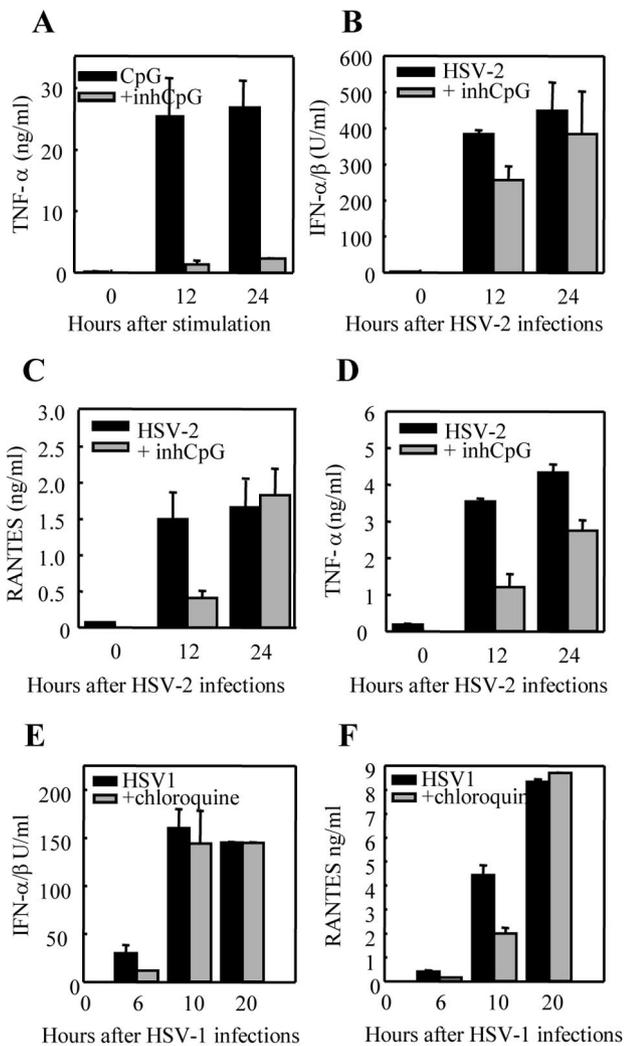


FIGURE 6. Expression of IFN- $\alpha\beta$, RANTES, and TNF- α in HSV-2-infected RAW264.7 cells treated with inhibitory CpG or chloroquine. RAW264.7 cells were either stimulated with 1 μ M CpG (ODN1826)(A) or infected with HSV-2 at a multiplicity of infection of 6 (B–F) in the presence of 3 μ M inhibitory CpG (ODN2088) (A–D) or 10 μ M chloroquine (E and F). At 12 and 24 h after infection supernatants were harvested for determination of RANTES (A, C, and F), IFN- $\alpha\beta$ (B and E) or TNF- α (D). Results are shown as mean of triplicates \pm SEM.

TLR9 through the use of inhibitory CpGs, which block activation of TLR9. We were able to block early induction of TNF- α and RANTES by inhibiting TLR9 signaling whereas this treatment had limited effect on HSV-induced IFN- $\alpha\beta$ expression. Similar results were obtained with chloroquine, which is a general inhibitor of endosomal maturation and hence TLR9 signaling. This is in correlation with the data obtained with dnMyD88 cells and suggests that TLR9 is at least one of the TLRs activated by HSV-2 but TLR9-independent pathways are also activated, especially in the late phase of the infection. This conclusion is supported by a study showing that mice lacking TLR9 or MyD88 control an HSV-2 infection equally well as wild-type mice, which indicates that other PRRs beside TLR9 are able to activate the immune system during an HSV-2 infection (26). A potential mechanism for activation of TLR9 could be that viral factors produced during the replication cycle are transported to endosomes or released from the cell and subsequently brought in contact with TLRs, which in turn induces expression of RANTES and TNF- α . Alternatively, viral infection is known to trigger expression of host cell factors such as heat-

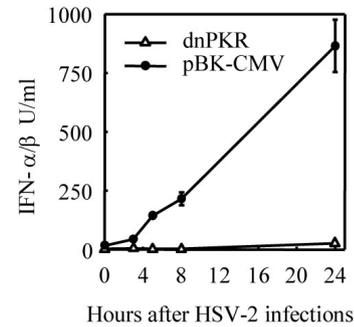


FIGURE 7. Induction of IFN- $\alpha\beta$ expression in RAW264.7 cells transfected with dominant negative version of PKR. The PKR mutant cell line RAW-PKR-M7 and empty vector control cell line RAW-pBK-CMV were infected with HSV-2 at a multiplicity of infection of 6. After the indicated periods of time supernatants were harvested for determination of IFN- $\alpha\beta$ bioactivity. Results are shown as mean of triplicates \pm SEM.

shock proteins that can activate TLRs (59). In contrast IFN- $\alpha\beta$ seems to be induced in macrophages mainly through a pathway not involving TLRs. Another possible intracellular PRR is PKR, which has been shown earlier to be involved in induction of IFN- $\alpha\beta$ by dsRNA (13). We show that PKR is indeed necessary for virus-induced IFN- $\alpha\beta$ expression in macrophages. However, we do not know whether it works as a direct signal transducer in response to dsRNA-binding (60) or rather plays a structural role in receptor-mediated signal transduction (61). Lately a new family of cytosolic pattern-recognition receptors have been described called the nucleotide-binding oligomerization domain proteins (62). Given the intracellular localization of these proteins, this class of proteins could potentially play an important role for initiation of the innate immune response during viral infections. Future studies will show whether induction of IFN- $\alpha\beta$ involve nucleotide-binding oligomerization domains.

The transcription factors responsible for activation of the IFN- β promoter have been well described and include NF- κ B, IRF-3, and c-Jun/activating transcription factor 2 (63). We confirm the observations regarding NF- κ B and IRF-3 by showing reduced accumulation of IFN- β mRNA in cells without a functional IRF-3 protein and no accumulation at all in dnIKK- β cells. In addition we found that accumulation of IFN- α mRNA was also dependent on IKK- β and to a lesser extend IRF-3. Earlier studies have shown that IRF-3, IRF-5, and IRF-7 can be involved in activation of the IFN- α promoter, whereas a dependency on NF- κ B has not been described before (64). However, the dependency on IKK- β could be a consequence of IFN- β inducing IFN- α in a positive feedback loop and therefore not a direct effect (42).

Collectively, in this study we show that HSV induces IFN- $\alpha\beta$ in macrophages by a mechanism dependent on viral entry and viral replication. The results of the present work and others point to the fact that during an infection, several distinct induction pathways and producer cells could function at the same time to ensure expression of IFN- $\alpha\beta$ and other cytokines that contribute to the antiviral response (51, 54). Moreover, we define distinct pathways for activation of macrophages that are dependent and independent of TLR signaling and show specifically that TLR9 is involved in induction of RANTES and TNF- α . This may allow the infected organism and even a single cell to detect viral infections and initiate a proinflammatory response through several different PAMP-PPR pathways.

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