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Activation of Autophagy by α-Herpesviruses in Myeloid Cells Is Mediated by Cytoplasmic Viral DNA through a Mechanism Dependent on Stimulator of IFN Genes

Simon B. Rasmussen,* Kristy A. Horan,* Christian K. Holm,* Amanda J. Stranks,† Thomas C. Mettenleiter,‡ A. Katharina Simon,† Søren B. Jensen,* Frazer J. Rixon,§ Bin He,¶ and Søren R. Paludan*

Autophagy has been established as a player in host defense against viruses. The mechanisms by which the host induces autophagy during infection are diverse. In the case of HSV type 1 (HSV-1), dsRNA-dependent protein kinase is essential for induction of autophagy in fibroblasts through phosphorylation of eukaryotic initiation factor 2α (eIF2α). HSV-1 counteracts autophagy via ICP34.5, which dephosphorylates eIF2α and inhibits Beclin 1. Investigation of autophagy during HSV-1 infection has largely been conducted in permissive cells, but recent work suggests the existence of a eIF2α-independent autophagy-inducing pathway in nonpermissive cells. To clarify and further characterize the existence of a novel autophagy-inducing pathway in nonpermissive cells, we examined different HSV and cellular components in murine myeloid cells for their role in autophagy. We demonstrate that HSV-1–induced autophagy does not correlate with phosphorylation of eIF2α, is independent of functional dsRNA-dependent protein kinase, and is not antagonized by ICP34.5. Autophagy was activated independent of viral gene expression, but required viral entry. Importantly, we found that the presence of genomic DNA in the virion was essential for induction of autophagy and, conversely, that transfection of HSV-derived DNA induced microtubule-associated protein 1 L chain II formation, a marker of autophagy. This occurred through a mechanism dependent on stimulator of IFN genes, an essential component for the IFN response to intracellular DNA. Finally, we observed that HSV-1 DNA was present in the cytosol devoid of capsid material following HSV-1 infection of dendritic cells. Thus, our data suggest that HSV-1 genomic DNA induces autophagy in nonpermissive cells in a stimulator of IFN gene-dependent manner. ***The Journal of Immunology*****, 2011, 187: 5268–5276.**

Macroautophagy (termed autophagy in this work) is a highly conserved vacuolar, degradation, and recycling pathway. Autophagy involves formation of so-called autophagosomes, in which a double membrane (the isolation membrane) encircles intracellular components, followed by degradation of the sequestered material by fusion with lysosomes. Autophagy has long been known to play important roles in, for example, cell death, starvation, and cellular development, but is now also appreciated to be induced during infections and to be important for host defense against pathogens (1–3). Autophagy has traditionally been viewed as a nonspecific degradation mechanism, but in recent years it has become clear that the process, at least in some cases, is selective, for example, in the targeting of invading viruses and bacteria (4–6).

In the last few years, autophagy has been linked to both the innate and the adaptive immune response. Three of the main antiviral pathways are as follows: 1) simple engulfment of the virion, resulting in degradation, thus limiting viral accumulation (4, 7); 2) connection to the adaptive immune response by translocation of endogenous Ags from the cytosol to the MHC class I and class II, thereby leading to activation of T cells (8–11); 3) promotion of the proinflammatory response, by engulfment and delivery of viral components to endosomal TLRs, resulting in, for example, type I IFN induction (12). In addition to classical autophagy, individual autophagy-related genes (ATGs) have also been demonstrated to regulate the innate immune response. The ATG5–ATG12 conjugate has been found to directly interact with the cytosolic RNA sensor retinoic acid-inducible gene I and its adaptor molecule mitochondrial anti-viral signaling protein (MAVS), resulting in decreased type I IFN induction (13). Also, ATG9a, but not ATG7, is involved in negative regulation of stimulator of IFN genes (STING), a transmembrane protein essential for type I IFN and proinflammatory cytokine induction mediated by cytosolic DNA

*Department of Biomedicine, University of Aarhus, DK-8000 Aarhus C, Denmark; †Human Immunology Unit, Nuffield Department of Clinical Medicine, Weatherall Institute of Molecular Medicine, Oxford OX3 9DS, United Kingdom; ‡Institute of Molecular Biology, Friedrich-Loeffler Institute, Federal Research Institute for Animal Health, 17493 Greifswald-Insel Riems, Germany; §Centre for Virus Research, Medical Research Council-University of Glasgow, Glasgow G61 1QH, United Kingdom; and ¶Department of Microbiology and Immunology, College of Medicine, University of Illinois at Chicago, Chicago, IL 60612

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Address correspondence and reprint requests to Dr. Søren R. Paludan, Department of Biomedicine, University of Aarhus, Bartholin Building, University Park, DK-8000 Aarhus C, Denmark. E-mail address: srp@microbiology.au.dk

Abbreviations used in this article: ATG, autophagy-related gene; BM-DC, bone marrow-derived dendritic cell; BMM, bone marrow-derived macrophage; dnPKR, dominant-negative dsRNA-dependent protein kinase; DPI, diphenyleneiodonium chloride; eIF2α, eukaryotic initiation factor 2α; L-particle, light particle; LC3, microtubule-associated protein 1 L chain 3; LNAC, *N*-acetyl-L-cysteine; 3-MA, 3-methyladenine; MAVS, mitochondrial anti-viral signaling protein; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; p.i., postinfection; PKR, dsRNA-dependent protein kinase; PRR, pattern recognition receptor; PrV, Pseudorabies virus; ROS, reactive oxygen species; STING, stimulator of IFN gene; VSV, vesicular stomatitis virus; wt, wild type.

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receptors of which several have been linked to HSV recognition (14, 15).

Conversely, many viruses, primarily of the *Herpesviridae* family, have evolved evasion strategies to suppress autophagic defense, such as targeting the autophagy protein Beclin 1 (11, 16, 17). The importance of autophagy is also illustrated by the observation that autophagy-deficient mice infected with HSV-2 and Sindbis virus, and *Drosophila* infected with vesicular stomatitis virus (VSV) exhibit increased lethality (4, 10, 18).

The α-herpesvirus HSV-1 is a ubiquitous human dsDNA virus replicating in epithelial cells and establishing lifelong latency in sensory neurons. HSV-1 is known to first induce and subsequently block autophagy in murine fibroblast and neurons (7, 19). It has been demonstrated that dsRNA-dependent protein kinase (PKR), via phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α), is essential for HSV-1–activated autophagy (19, 20). The HSV-1 neurovirulence protein ICP34.5 blocks autophagy by recruiting the host phosphatase PP1α to dephosphorylate eIF2α, and by inhibiting Beclin 1 (16, 21). A HSV-1 mutant lacking ICP34.5 is unable to block autophagy stimulation and autophagic degradation of virions in permissive cells, and is less neurovirulent in mice (7, 16, 19). The late HSV-1 protein Us11 directly binds PKR or PACT, an activator of PKR, thus antagonizing the PKR/eIF2α pathway (22, 23). Unlike what has been observed in fibroblasts and neurons, English et al. (9) have recently shown that wild-type HSV-1 is able to induce autophagy in macrophages in an apparently eIF2α-independent manner. Hence, there are data to suggest the existence of an alternative HSV-1 autophagy-stimulating pathway in nonpermissive myeloid cells.

In this study, we have used bone marrow-derived dendritic cells (BM-DCs) and the macrophage-like cell line RAW267.4 to demonstrate that HSV-1 induces autophagy, as measured by microtubule-associated protein 1 L chain 3 (LC3) foci and LC3 II accumulation, in myeloid cells in a PKR/eIF2α-independent manner. Instead, we identify a novel mechanism in which cytosolic viral DNA triggers autophagy in a mechanism dependent on STING. Furthermore, we demonstrate that blockage of autophagy by both a chemical and genetic approach reduces IFN-β and TNF-α induction during HSV-1 infection. Thus, autophagy can now be included in the list of cellular innate immunological processes activated by cytosolic DNA in a STING-dependent mechanism.

Materials and Methods

Reagents

DMEM and RPMI 1640 (both from BioWhittaker) were supplemented with antibiotics (penicillin and streptomycin) and LPS-free FCS (BioWhittaker). GM-CSF was purchased from R&D Systems. Pam_3Csk_4 and ODN1826 were obtained from InvivoGen. *N*-acetyl-L-cysteine (LNAC) and DPI were from Calbiochem. The 3-methyladenine (3-MA), DAPI, and poly L-lysine were from Sigma-Aldrich. Lipofectamine 2000, ProLong Gold, G418, and rabbit Alexa Fluor 568- and anti-mouse Alexa Flour 647-labeled secondary Abs were from Invitrogen. Abs used were mouse anti-VP5 (3B6; Virusys) and rabbit anti-LC3 (PM036; MBL), anti-eIF2α (phosphor-Ser^{52}) (KAP-CP131-E), anti-Beclin 1 (3738; Cell Signaling Technology), and anti-GAPDH (FL-335; Santa Cruz Biotechnology).

Mice

Inbred, specific pathogen-free mice used in this study were 8- to 12-wk-old female C57BL/6J, TLR2/9$^{-/-}$, MAVS$^{-/-}$, conditionally hematological *Atg7* knockout Vav-iCre; Atg7$^{Flox/Flox}$ and Vav-iCre^{-}; Atg7$^{Flox/Flox}$ control mice (24); and a mutant mouse strain, *Goldenticket* (*Gt*), that harbors a point mutation in STING (STING$^{Gt/Gt}$), which fails to produce detectable protein (25). All knockout mice strains were on a C57BL/6J background. C57BL/6J and TLR2/9$^{-/-}$ mice were bred at Taconic M&B. Bones from MAVS$^{-/-}$ mice were provided by Z. J. Chen, University of Southwestern Medical Centre (Dallas, TX).

Cells

BM-DCs were obtained, as described elsewhere (26). Bone marrow-derived macrophages (BMMs) were obtained as BM-DCs, except 10 ng/ml GM-CSF was used for differentiation, and adherent cells were harvested. For isolation of whole-cell extracts and total RNA, BM-DCs and BMMs were seeded at a density of 2×10^6 cells/well in a 12-well tissue culture plate, and for confocal microscopy BM-DCs were seeded on coverslips in a 24-well tissue culture plate at a cell concentration of 8×10^5 cells/well. To improve cell adherence, coverslips were left in poly L-lysine in PBS (0.1 mg/ml) for 1 h at 37°C before use. After seeding, the cells were left overnight at 37°C and 5% CO_2 to allow cells to settle prior to treatment. RAW 267.4 cells stably transfected with either the empty vector pBK-CMV or PKR-M7, expressing PKR mutant M7, which lacks the first dsRNA-binding domain, in the pBK-CMV vector (both provided by J.A. Corbett, St. Louis University School of Medicine) (27) were grown in DMEM with 10% FCS, supplemented with penicillin (200 IU/ml) and streptomycin (200 μg/ml), and for selection G418 (400 μg/ml). For isolation of whole-cell extracts, RAW267.4-pBK-CMV and RAW267.4-PKR-M7 were cultured at a cell concentration of 2.5×10^6 cells/well in a 6-well tissue culture plate and left overnight at 37°C and 5% CO_2.

Viruses

The wild-type (wt) viruses used in this study were the F and KOS strains of HSV-1 (where nothing else is mentioned, HSV-1 strain KOS has been used). The mutants ΔICP34.5 and ΔUL15ExII (ΔUL15) are on an F genetic background, whereas mutants lacking gB (ΔgB) and gH (ΔgH) are on a KOS genetic background. ΔICP34.5 was constructed by restoration of the tk gene in KY0234 (28). Other viruses used were Pseudorabies virus (PrV) strain Kaplan (29) and VSV Indiana strain. Inactivated HSV-1 was obtained by UV treatment for 3 min, as previously described (30). In all experiments, the control was either mock or untreated. A marginal induction of LC3 II formation was observed in mock-treated cells as compared with untreated cells. Light particles (L-particles) were isolated from HSV-1 ARΔUL36-infected cells, essentially as described elsewhere (31).

DNA oligonucleotide transfection

Transfection of BM-DCs with HSV-1 60mer oligonucleotide (sequence: 5′-TAAGACACGATGCGATAAAATCTGTTTGTAAAATTTATTAAGGGTACAAATTGCCCTAGC-3′; DNA Technology) was achieved using Lipofectamine 2000 at 2 μg/ml, as recommended by the manufacturer.

Fluorescent in situ hybridization

Fluorescent in situ hybridization with FITC-labeled oligonucleotide probe (1 ng/μl) and stained with anti-VP5 (1:200 dilution) was carried out, as described elsewhere (32).

Confocal microscopy

Cells grown on glass coverslips were fixed for 15 min in 4% paraformaldehyde in PBS and were made permeable in 0.1% Triton X-100 for 90 s. Coverslips were preincubated in PBS with 1% BSA for 15–30 min and stained for 1 h at room temperature with primary Abs (1:200 dilution) and for 1 h with Alexa Fluor 568- and Alexa Flour 647-labeled secondary Abs (1:500 dilution) in PBS with 1% BSA. Next, coverslips were incubated with DAPI (5 mg/ml) for 2 min and mounted in ProLong Gold. Images were obtained with a Zeiss LSM 710 scanning confocal microscope.

Autophagy assays

Evaluation of autophagy by fluorescence microscopy was performed by immunofluorescence of LC3, a marker of autophagy, using LC3 Ab to detect LC3 foci. Quantitation of autophagy was done based on the percentage of cells with more than two LC3 dots, thereby excluding the low background level of LC3 foci that most cells exhibit. A minimum of 100 cells per sample was counted. In evaluating autophagy by Western blotting, levels of lipidated LC3 II, the only reliable protein marker for completed autophagosomes, were determined (33). LC3 I (not always visible) and LC3 II were segregated by 4–20% gradient SDS-PAGE. For identification of LC3 II, whole-cell extracts from wt mouse embryo fibroblasts (MEFs) and Atg5$^{-/-}$ MEFs, the latter incapable of forming LC3 II, were loaded in wells next to samples in all experiments in this work. For quantification of LC3 II, computer-assisted densitometry (ImageJ) was used to quantify the bands that were captured on a Luminescent Image Analyzer (FUJI-FILM), and, after normalization to GAPDH, the results were depicted as change compared with control.

Western blotting

Nuclear extracts were denatured in XT Sample Buffer and XT Reducing Agent (Bio-Rad), heated to 90°C for 10 min, and subjected to SDS-PAGE (Bio-Rad). The proteins were blotted onto a polyvinylidene difluoride membrane and blocked for 30 min with TBS (10 mM Tris, 140 mM NaCl) supplemented with 0.05% Tween 20 and 5% skim milk powder. Primary Abs were added for overnight incubation at 4°C. The membrane was washed four times for 10 min each in washing buffer (TBS with 0.05% Tween 20), followed by incubation for 50 min at room temperature with secondary polyclonal HRP-conjugated Ab. The membrane was washed, as described above, and the HRP-conjugated Ab (Dako) was visualized by using ECL.

Quantitative RT-PCR

Total RNA was extracted with the High Pure RNA isolation kit (Roche Applied Science), according to the recommendations of the manufacturer. Murine IFN-β mRNA was quantified by real-time PCR with TaqMan one-step RT-PCR kit (Applied Biosystems). β-actin plus TNF-α mRNA was quantified by Brilliant II SYBR Green QRT-PCR Mix Kit (Stratagene; primers, 5′-TAGCACCATGAAGATCAAGAT-3′ [forward] and 5′-CCGATCCACACAGAGTACTT-3′ [reverse]). All protocols were performed according to manufacturers' recommendations.

Reproducibility of data

The results shown in this work are derived from data that are representative for the results obtained. For each series of experiments, two to four independent repetitions were performed.

Results

Autophagy is induced in BM-DCs during HSV-1 infection

Recently, English et al. (9) have shown that HSV-1 can induce autophagy in macrophages. To investigate whether autophagy can be observed in other myeloid cells, BM-DCs were examined for formation of the autophagy marker LC3 II following HSV-1 infection. BM-DCs were infected with HSV-1 for 1, 2, 4, and 6 h, and LC3 II accumulation was analyzed by Western blotting. For identification of LC3 II, whole-cell extracts from wt MEFs and Atg5$^{-/-}$ MEFs, the latter incapable of forming LC3 II, were loaded in wells next to samples in all experiments in this work (data not shown). We observed an increase in LC3 II levels starting from 2–4 h postinfection (Fig. 1*A*). To establish further the ability of HSV-1 to induce autophagy in BM-DCs, infected cells were stained with anti-LC3 Ab and foci indicating autophagosomes were visualized by confocal micoscopy (Fig. 1*B*, 1*C*). Fig. 1*A–C* shows that LC3 II formation and the number of LC3 foci increased in response to HSV-1 in BM-DCs. To investigate whether the stimulation of autophagy in BM-DCs is specific for HSV-1, we also infected BM-DCs with another α-herpesvirus, PrV. As seen in Fig. 1*D*, PrV also activated autophagy in BM-DCs as measured by formation of LC3 II. These data show that HSV-1 and PrV infection stimulate autophagy in BM-DCs.

HSV-1–induced autophagy does not correlate with increased phosphorylation of eIF2α and is independent of ICP34.5 and PKR

To establish whether HSV-1–activated autophagy in BM-DCs is a result of increased phosphorylation of eIF2α, and thus affected by ICP34.5, p-eIF2α levels in whole-cell extracts, from cells infected with HSV-1 ICP34.5 mutant (ΔICP34.5) or two wt strains of HSV-1, KOS and F, were determined by Western blotting. We observed an increase in LC3 II formation following infection with all viruses, but an increase in p-eIF2α levels was determined only following HSV-1 ΔICP34.5 infection, whereas p-eIF2α levels reduced following infection with the two wt strains of HSV-1 (Fig. 2*A*, 2*B*). This reduction and increase of p-eIF2α in BM-DCs infected with wt HSV-1 and HSV-1 ΔICP34.5, respectively, started 2–4 h postinfection (Fig. 2*C*, 2*D*). Interestingly, HSV-1 ΔICP34.5-induced LC3 II formation did not reach a level higher than that observed in cells infected with wt viruses expressing ICP34.5. As shown in Fig. 2*E* and 2*F*, the levels of p-eIF2α in cells infected for 24 h with the ΔICP34.5 mutant were decreased to a level comparable to that in wt-infected cells. In addition, at 24 h, the amount of LC3 II in both wt- and mutant virus-infected BM-DCs had peaked and was lower than at 6 h. The macrophage-like cell line, RAW264.7, also exhibited LC3 II formation independently of p-eIF2α (Fig. 2*G*, 2*H*). These data demonstrate there is no correlation between p-eIF2α and LC3 II level during HSV1 infection, thus suggesting that induction of autophagy takes place in a p-eIF2α- and possibly PKR-independent manner. To examine the role of PKR in HSV-1–mediated induction of autophagy

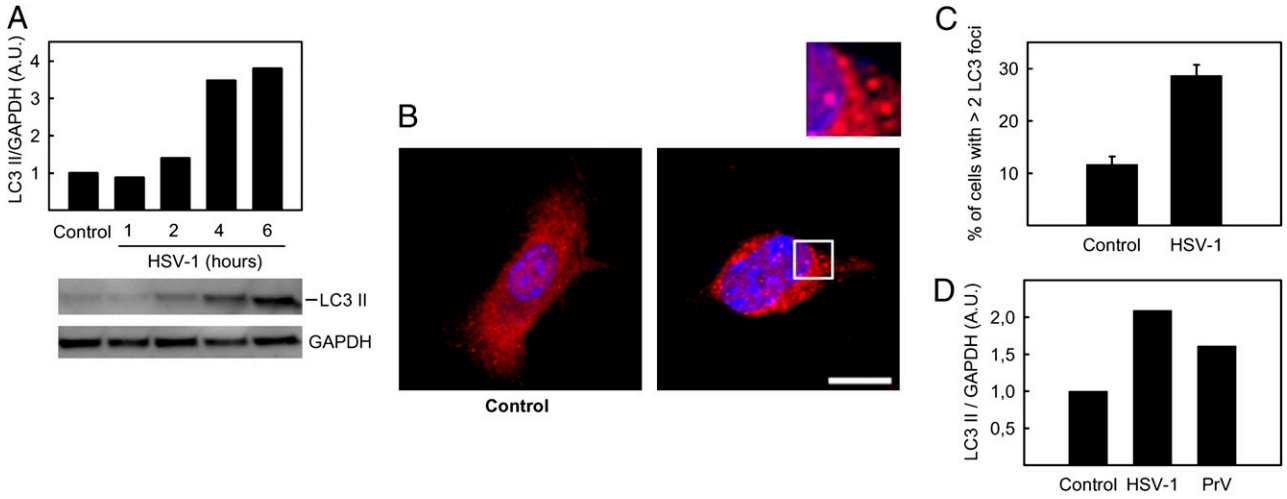

FIGURE 1. Induction of autophagy in BM-DCs during HSV-1 infection. *A* and *D*, BM-DCs were infected with HSV-1 or PrV (both with a multiplicity of infection [MOI] of 3). Whole-cell extracts were harvested at 6 h or at indicated time points, and LC3 II and GAPDH protein levels were measured by Western blotting. LC3 II was normalized to GAPDH and shown as bar charts. Western blots representing *A* are shown. *B*, Confocal microscopy of BM-DCs grown on coverslips and infected with HSV-1 (3 MOI) for 6 h, then fixed and stained with Ab to LC3 (red) and with DAPI (blue) for visualization of nuclei. *Inset*, Magnification of area outlined in main image. Images are a maximum projection of a *z*-stack. Scale bar, 10 μm. *C*, Percentage of BM-DCs stained with anti-LC3 with more than two LC3 dots, counted by confocal microscopy. The data shown represent mean ± SD from three independent experiments. A.U., arbitrary unit.

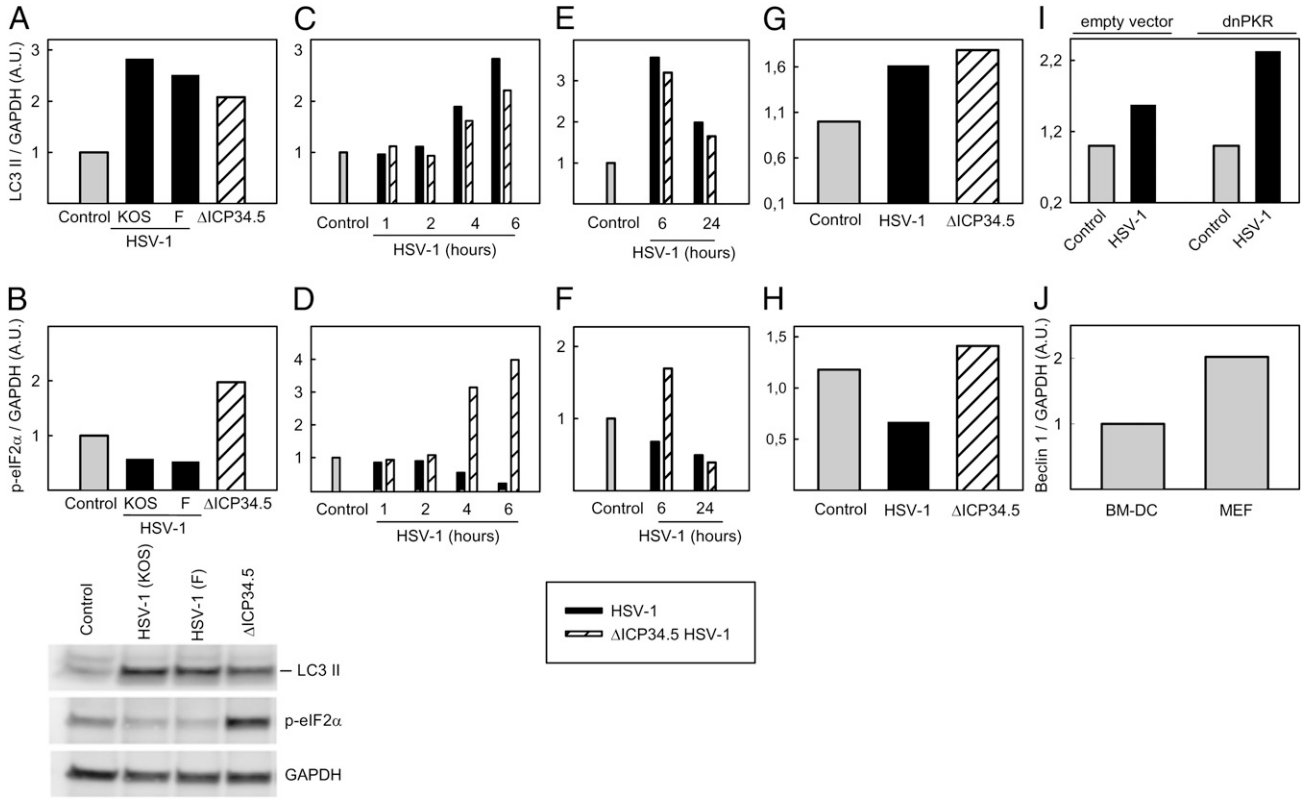

FIGURE 2. The influence of viral ICP34.5, functional PKR, level of Beclin 1, and phosphorylation of eIF2α on induction of autophagy. BM-DCs (*A–F*), RAW264.7 cells (*G–I*) stably transfected with an empty plasmid (pBK-CMV), or dnPKR (PKR-M7) were infected with HSV-1 strain KOS (*A–I*), HSV-1 strain F (*A*, *B*), or ΔICP34.5 HSV-1 strain F (*A*, *B*, *G*, *H*). For all viruses, a MOI of 3 was used. Whole-cell extracts were harvested at 6 h or at indicated time points, and LC3 II (*A*, *C*, *E*, *G*, *I*), p-eIF2α (*B*, *D*, *F*, *H*), and GAPDH (*A–I*) protein levels were measured by Western blotting. LC3 II and p-eIF2α were normalized to GAPDH and shown as bar charts. Western blots representing *A* and *B* are shown. *J*, Whole-cell extracts were harvested from BM-DCs and MEFs, and Beclin 1 protein levels were measured by Western blots, normalized to GAPDH, and shown as bar charts. A.U., arbitrary unit.

in myeloid cells, a RAW264.7 cell line expressing dominant-negative PKR (dnPKR) was used (27). LC3 II formation was unaltered in dnPKR-expressing RAW264.7 cells as compared with cells stably transfected with an empty vector (Fig. 2*I*), further indicating that HSV-1 stimulates autophagy in myeloid cells by a PKR/eIF2α-independent pathway. Our observations may indicate cell-type specificity in activation of autophagy by HSV-1. For instance, in neurons, ICP34.5 was found to inhibit Beclin 1, thereby preventing autophagy (16). To test whether the levels of Beclin 1 are particularly high in myeloid cells, we compared the level of this protein in BM-DCs and MEFs. As shown in Fig. 2*J*, the levels of Beclin 1 were lower in BM-DCs than in MEFs. Collectively, HSV-1 induces autophagy in myeloid cells independent of the phosphorylation status of eIFaα and PKR.

HSV-induced autophagy is independent of TLRs and reactive oxygen species

As observed, HSV-1–activated autophagy in BM-DCs appears to take place in a PKR/eIF2α-independent manner, suggesting a role for one or more alternative receptors. One such receptor could be a member of the TLR family, which previously has been shown to induce autophagy upon binding to pathogen-associated molecular patterns (34, 35). TLR2 and TLR9 are two pattern recognition receptors (PRRs) involved in the innate immune defense against HSV (36, 37). To determine whether TLR2 or TLR9 can induce autophagy, BM-DCs were treated with Pam_3Csk_4 (TLR1/2 ligand) or ODN1826 (TLR9 ligand), and levels of LC3 II were determined. As seen in Fig. 3*A*, a robust induction of LC3 II was observed only following treatment with ODN1826. However, although activation of TLR9 is capable of inducing autophagy in BM-DCs, this receptor seems not to be involved in HSV-1–stimulated autophagy, because LC3 II formation in TLR2/9$^{-/-}$ BM-DCs was comparable to what was observed in wt cells (Fig. 3*B*).

Reactive oxygen species (ROS) are linked to induction of autophagy and are generated during HSV-1 infection (38, 39). To evaluate whether ROS are involved in HSV-1–activated autophagy in myeloid cells, we treated BM-DCs with the antioxidants diphenyleneiodonium chloride (DPI) (Fig. 3*D*) or LNAC prior to HSV-1 infection and then analyzed LC3 II levels (Fig. 4*D*). We have recently demonstrated that HSV induces ROS production in macrophages, and this is abrogated by LNAC treatment (40). LC3 II formation was not influenced by pretreatment with either DPI or LNAC as compared with cells that received vehicle as pretreatment. Collectively, these data show that HSV-1–stimulated autophagy is independent of TLR2 and TLR9 and proceeds through a mechanism independent of ROS.

HSV-1–induced autophagy is independent of viral replication, but dependent on viral entry, the presence of cytosolic viral DNA, and STING

To examine the existence of a potential novel autophagy-inducing pathway in nonpermissive cells, we looked at the dependence on different viral components on HSV-1–induced autophagy in BM-DCs. First, we examined the role of HSV-1 replication, which results in the production of dsRNA (41). As seen in Fig. 4*A*, UV-inactivated HSV-1 was able to induce a level of LC3 II even higher than seen in cells infected with untreated virus. Thus, replicative products are apparently not necessary for autophagy

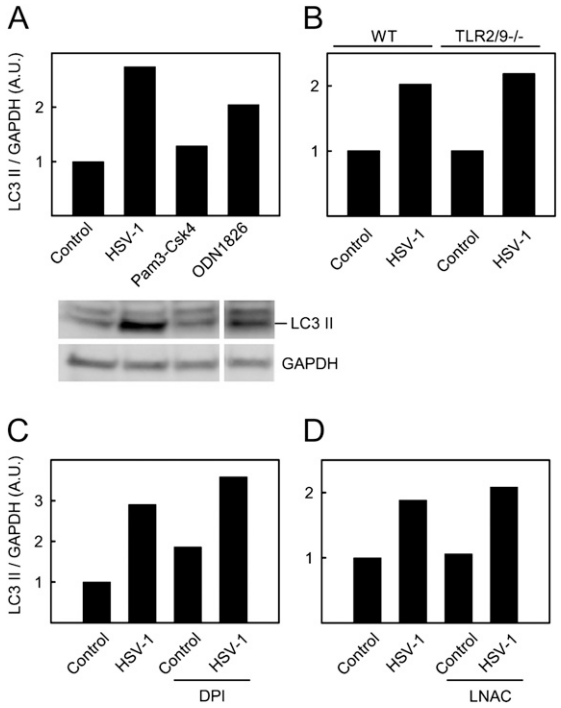

FIGURE 3. Role of TLR2/9 and ROS on HSV-1–induced autophagy. Wt BM-DCs (*A*, *C*, *D*) and tlr2/9$^{-/-}$ BM-DCs (*B*) were infected with HSV-1 (MOI of 3; 6 h), treated with Pam_3Csk_4 (0.1 μg/ml) or ODN1826 (1 μM) for 1 h (*A*), or pretreated as described with DPI (3.7 μM) (*C*) or LNAC (6.4 mM) (*D*) prior to HSV-1 infection. Whole-cell extracts were harvested, and LC3 II and GAPDH protein levels were measured by Western blotting. LC3 II was normalized to GAPDH and shown as bar charts. Western blots representing *A* are shown. A.U., arbitrary unit.

induction. Next, we investigated whether viral entry or binding to the cell membrane is necessary for HSV-1–stimulated autophagy. BM-DCs infected with the entry-incompetent gH- or gB-deficient HSV-1 (ΔgH and ΔgB), both capable of binding to cells, were unable to induce autophagy, thus demonstrating that viral entry is vital in activation of autophagy (Fig. 4*B*) (42).

To determine whether the autophagy-inducing factor of HSV-1 is located in the nucleocapsid and/or genome, we infected BM-DCs with HSV-1 L-particles, which are composed mainly of envelope and tegument proteins, lacking genome and nucleocapsid (43). L-particles were unable to stimulate LC3 II formation, indicating that the autophagy-inducing component is located in the nucleocapsid or the genome (Fig. 4*C*). To further identify the viral component responsible for autophagy induction, we infected BM-DCs with the HSV-1 UL15 mutant (ΔUL15), which retains the nucleocapsid, but largely fails to package viral DNA into the virion (44). Infection of BM-DCs with this virus mutant did not activate autophagy, indicating that the HSV-1 genome could be the autophagy-inducing component (Fig. 4*D*). This was supported by the observation that transfection of a 60-bp dsDNA oligonucleotide derived from the HSV-1 genome (HSV-1 60mer) activated LC3 II formation, as seen in Fig. 4*E*. Induction of autophagy by the 60mer oligonucleotide appears to take place in a eIF2α-independent way, because we did not observe an increase in the phosphorylation of this subunit, supporting the existence of a novel PKR/eIF2α-independent α-herpesvirus–driven activation of autophagy (Fig. 4*F*).

To examine whether STING played any role in HSV-1 DNA-activated autophagy, we first determined the dependency on this protein in the induction of IFN-β mRNA in STING-deficient BM-DCs during HSV-1 infection or transfection of HSV-1 60mer DNA. As seen in Fig. 4*G*, the induction of IFN-β was completely abrogated in cells lacking STING. Unlike HSV-1, the RNA virus VSV induced IFN-β mRNA in a completely STING-independent manner (Fig. 4*H*). Next, we looked at the ability of STING-deficient BM-DCs to induce LC3 II formation. As seen in Fig. 4*I*, LC3 II was not formed following either HSV-1 infection or HSV-1 60mer DNA transfection in cells lacking STING. Together, these results demonstrate that HSV-1–stimulated autophagy in BM-DCs takes place in a replication-independent, but entry- and viral genome-dependent manner. In addition, we found that a HSV-1 60mer transfected into the cytosol activates autophagy, and that activation of autophagy by either transfected DNA or HSV-1 takes place in a STING-dependent mechanism. Cumulatively, these data support a role for viral DNA in the cytosol as a novel mediator of autophagy during HSV-1 infection, and also provides evidence for STING being essential for this process.

The HSV genome is released from the nucleocapsid to the cytosol during infection

Recent studies have demonstrated the presence of HSV-1 DNA in the cytosol of macrophages (32). To examine whether HSV-1 DNA can also be observed in the cytoplasm of DCs, we used a specifically labeled oligonucleotide complementary to HSV-1 DNA. As seen in Fig. 5, viral DNA and nucleocapsid protein VP5 colocalize at 2 h postinfection (p.i.), whereas at 4 h p.i., viral DNA is separated from VP5. This finding suggests that HSV-1 DNA is released from the viral capsid between 2 and 4 h p.i. and is present in the cytosol in an accessible form. Interestingly, there seems to be a temporal overlap between the release of HSV-1 genome and the onset of autophagy.

Inhibition of autophagy leads to decreased IFN-β induction during HSV-1 infection

Autophagy has been linked to detection of viral pathogen-associated molecular patterns by PRRs, resulting in type I IFN induction (12). Given the STING-dependent nature of the autophagy response, we wanted to investigate whether autophagy was essential for type I IFN production during infection with α-herpesviruses, BM-DCs were treated with 3-MA, a commonly used inhibitor of autophagosome formation, and IFN-β mRNA was monitored following infection. As seen in Fig. 6*A* and 6*B*, mRNA levels for IFN-β decreased in 3-MA–treated HSV-1- and PrV-, but not VSV-infected cells as compared with control cells. This difference in the ability of 3-MA to inhibit virus-induced IFN-β induction could be a consequence of different PRRs involved in the recognition of the investigated viruses. As was seen in Fig. 4*G* and 4*H*, HSV-1 induces IFN-β in a STING-dependent manner, whereas VSV drives this response through different pathways. VSV has been shown to be detected by a nonendosomal pathway involving MAVS, an adaptor protein in the cytosolic antiviral retinoic acid-inducible gene-like receptor pathway (45, 46). This was supported by our observation in BMMs, in which MAVS was essential for induction of IFN-β in response to VSV (Fig. 6*C*). To confirm the data with 3-MA, we used BM-DCs generated from Vav-iCre; Atg7$^{Flox/Flox}$ (hereafter called Vav-ATG7$^{-/-}$) mice that do not express the essential autophagy gene ATG7 in the hematopoetic system were first examined for their ability to form LC3 II in response to HSV-1 infection. As seen in Fig. 6*D*, basal and HSV-induced LC3 II formation was observed only in wt cells expressing ATG7 (Vav-iCre^{-}; Atg7$^{Flox/Flox}$). Next, we examined the dependency on ATG7 for induction of IFN-β mRNA in response to HSV-1. Interestingly, BM-DCs deficient in ATG7 exhibited a reduced ability to express IFN-β compared with wt cells (Fig. 6*E*). As seen in Fig. 6*F*, the induction of TNF-α mRNA was

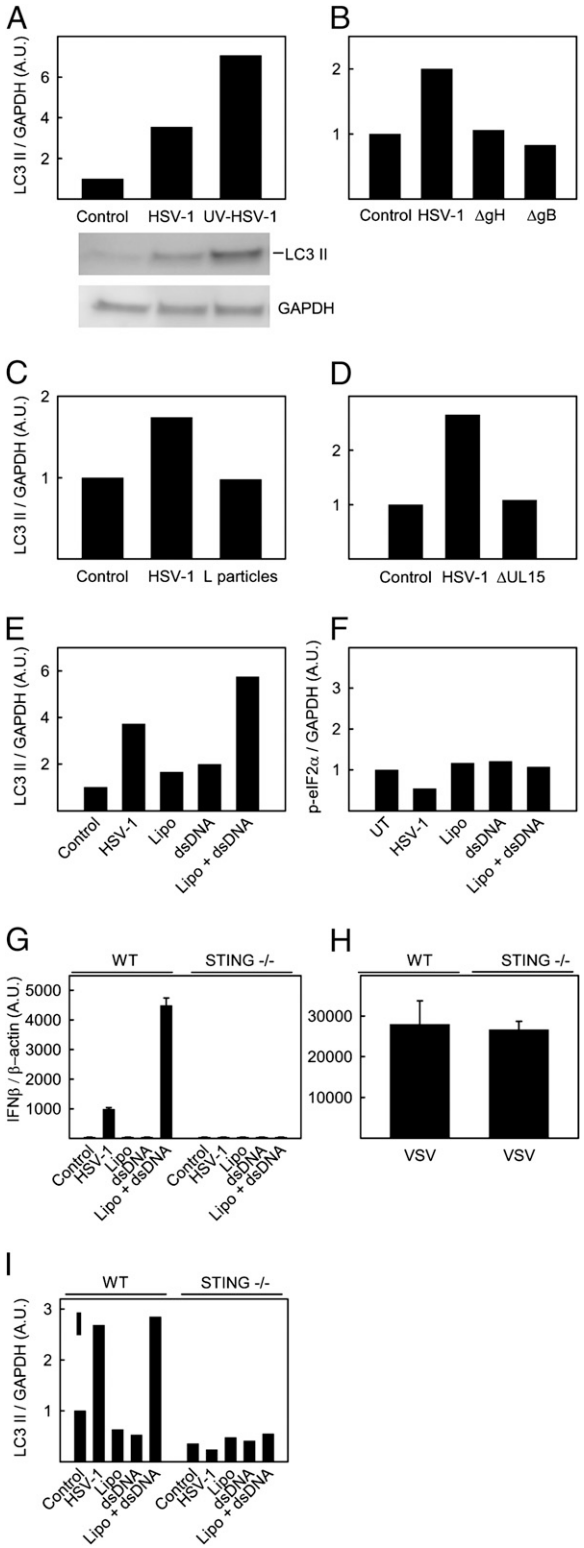

FIGURE 4. Role for viral replication, entry, viral genome, and STING in HSV-1–induced autophagy. Wt BM-DCs (*A–I*) and STING-deficient BM-DCs (*G–I*) were infected with HSV-1 (*A–G*, *I*), UV-inactivated HSV-1 (*A*), entry-deficient ΔgB or ΔgH HSV-1 (*B*), HSV-1 L-particles (*C*), ΔUL15 HSV-1 (*D*), VSV (1.6 MOI) (*H*), or transfected with HSV 60mer DNA (*E–G*, *I*). A MOI of 3 was used for infectious HSV-1, and for ΔgB and ΔgH HSV-1, HSV-1 L-particles, and ΔUL15 HSV-1, viral material corresponding to MOI 3 for the infectious virus was used. Whole-cell extracts were harvested 6 h posttreatment, and LC3 II (*A–E*, *I*), eIF2α (*F*), and GAPDH (*A–F*, *I*) protein levels were measured by Western blotting or total RNA was extracted 6 h postinfection (*G*, *H*) and expression of IFN-β mRNA was detected by RT-PCR, normalized to β-actin mRNA, and shown as means of duplicates ± SD. LC3 II (*A–E*) and p-eIF2α (*F*) were normalized to GAPDH and shown as bar charts. Western blots representing *A* are shown. A.U., arbitrary unit.

also reduced in cells lacking ATG7. Collectively, these results strongly suggest a role for autophagy (or autophagy-related proteins) in STING-dependent induction of IFN-β expression in response to viral DNA recognition.

Discussion

Previous studies have shown that HSV-1 induces autophagy in fibroblasts and neurons in a PKR/eIF2α-dependent manner, and that this induction is suppressed by ICP34.5-mediating dephosphorylation of p-eIF2α and inhibition of Beclin 1 (7, 16, 19, 21). Recently, this universal dependency on eIF2α in HSV-1–activated autophagy has been disputed by English et al. (9), showing that uninhibited autophagy is induced in a macrophage cell line following HSV-1 infection in an apparently eIF2α-independent manner. This observation led us to investigate whether α-herpesviruses activate autophagy in nonpermissive cells in an alternative way, which the virus is unable to block. In this study, we demonstrate that HSV-1 indeed does activate autophagy in myeloid cells, represented by BM-DCs and RAW264.7 cells, as measured by fluorescence microscopy of LC3 foci and Western blot of LC3 II. We present data supporting that HSV-1–mediated stimulation of autophagy is independent of the classical PKR/eIF2α pathway, but dependent on the presence of viral genome in the cytosol and STING, a vital component of the cytosolic DNA sensor system. Hence, our results indicate the existence of a novel autophagy-inducing mechanism mediated by cytosolic viral DNA.

Autophagosomes formed during HSV-1 infection of macrophages display two distinct morphologies. In addition to the conventional and well-characterized double-membrane structure, a second type of autophagosome with four-layered membranes has been observed, originating from a coiling of the inner and outer nuclear membrane, but otherwise exhibiting normal macroautophagy behavior (9). In our study, we did not observe LC3 foci in obvious proximity of the nuclear membrane, but this has to be definitively determined by electron microscopy, in which the number of membranes can be visualized.

In addition to HSV-1, PrV was also found to increase LC3 II formation, suggesting activation of autophagy in myeloid cells as a general phenomenon followed α-herpesvirus infection. The observed HSV-1–mediated autophagy induction was independent of eIF2α and ICP34.5 because both wt and ΔICP34.5 HSV-1 led to equal levels of LC3 II, starting at 2–4 h p.i., despite eIF2α being dephosphorylated following infection with the wt virus and strongly phosphorylated following infection with the ICP34.5 mutant. Strengthening the hypothesis of eIF2α-independent induction of autophagy following HSV-1 infection, we also observed the formation of LC3 II to be independent of PKR. This independence of PKR was confirmed by the efficiency of UV-inactivated HSV-1 to stimulate autophagy. UV treatment obstructs replication of HSV-1, a process essential for PKR activation (47).

As previously outlined, ICP34.5 not only blocks autophagy by dephosphorylating eIF2α, but also by inhibiting Beclin 1 (16). Beclin 1 works in both the early steps of autophagy initiation, upstream of LC3 II formation and downstream of LC3 II formation in the maturation of the autophagosome (48). Some viruses inhibit maturation of the autophagosomes, thereby avoiding de-

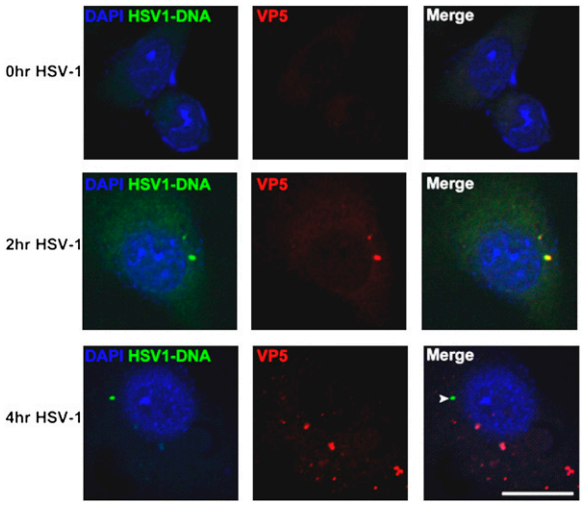

FIGURE 5. Translocation of the HSV genome from nucleocapsid to cytosol during infection. Confocal microscopy of BM-DCs grown on coverslips and infected with HSV-1 (MOI of 5). Cells were fixed at indicated time points and stained with Ab to viral capsid protein VP5 (red), DAPI (blue) for visualization of nuclei, and fluorescent in situ hybridization for HSV genome (green). Scale bar, 10 μm.

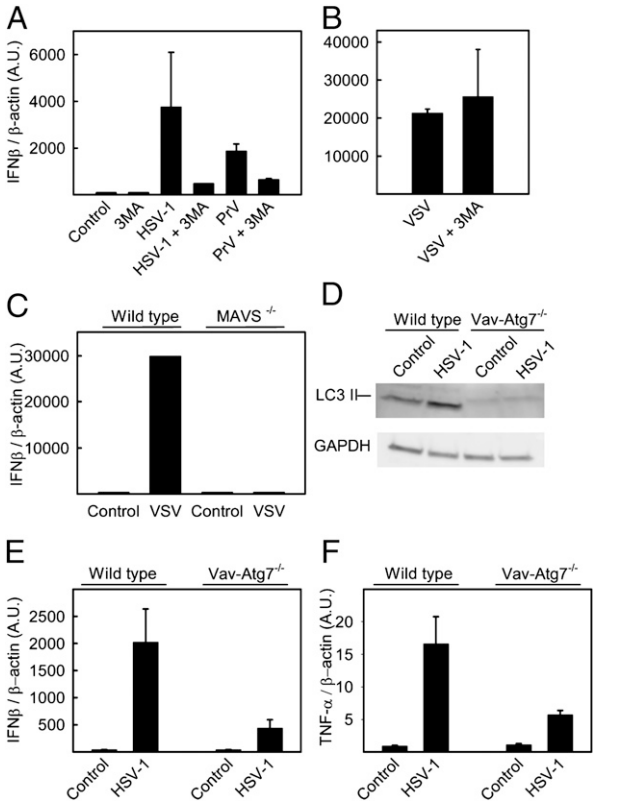

FIGURE 6. The role of autophagy in induction of IFN-β during HSV-1 infection. *A*, BM-DCs were pretreated for 20 min with 3-MA (10 mM) or left untreated and infected with HSV-1 (MOI of 3), PrV (3 MOI), or VSV (1.6 MOI) (*B*). *C*, The wt and MAVS$^{-/-}$ BMMs were infected with VSV (1.6 MOI) and wild-type (Vav-iCre^{-}; Atg7$^{Flox/Flox}$) and Vav-ATG7$^{-/-}$ (Vav-iCre; Atg7$^{Flox/Flox}$) BM-DCs were infected with HSV-1 (*D–F*). *A–C*, *E*, and *F*, Total RNA was extracted 6 h postinfection. Expression of IFN-β and TNF-α mRNA was detected by RT-PCR and normalized to β-actin mRNA. The data are shown as means of duplicates ± SD. *D*, Whole-cell extracts were harvested 6 h posttreatment, and LC3 II and GAPDH (*A–F*, *I*) protein levels were measured by Western blotting. A.U., arbitrary unit.

gradation of the sequestered material (49). We do not believe our findings are due to virus-mediated inhibition of autophagosomal turnover, because the process, including the STING dependence, can be mimicked by synthetic DNA in the absence of viral material.

Oxidative stress caused by ROS is linked to both HSV-1 infection and induction of autophagy (38, 39). Moreover, ROS has recently been demonstrated to play important roles in signaling downstream of RNA- and DNA-sensing PRRs (40, 50). We inhibited ROS by DPI, an inhibitor of flavin-containing proteins essential in many ROS-generating pathways, and the general antioxidant LNAC. Treatment with these inhibitors did not affect LC3 II formation, suggesting the ROS pathway not to be vital in DNA-stimulated autophagy during HSV-1 infection in BM-DCs.

In this study, we aimed to identify the viral component(s) responsible for HSV-1–mediated autophagy. HSV-1 binding, especially of gD, has previously been found to activate intracellular signaling, and thus might be involved (51). Using entry-defective HSV-1 lacking gB or gH, we found that attachment of the virus in itself did not activate LC3 II formation. Our data also showed that HSV-1–induced autophagy is independent of viral replication, suggesting that de novo-produced product or intermediate, such as dsRNA, is responsible for the process. Using the genome-deficient L-particles and ΔUL15 HSV-1, we found that presence of viral genomic DNA is essential for induction of autophagy. The dependence on viral DNA was supported by the finding that transfection of synthetic dsDNA into DCs increased LC3 lipidation. Further supporting the notion that HSV-1 can induce autophagy in a PKR/eIF2α-independent manner, we observed no increase in p-eIF2α following DNA transfection. Importantly, using confocal microscopy, we also observed HSV-1 DNA in the cytosol separated from the viral capsid protein VP5 at 4 h p.i. The process of viral DNA release seems to take place from 2 h p.i., because at this time point HSV-1 DNA and capsid were observed to colocalize. Interestingly, we were not able to observe LC3 II formation in STING$^{-/-}$ BM-DCs following either HSV-1 infection or transfection of dsDNA. HSV is known to be sensed by a range of cytosolic DNA receptors resulting in type I IFN induction (32, 52–55). All cytosolic DNA sensors are believed to signal though STING because this protein is essential for cytoplasmic DNA activation of the IFN regulatory factor 3 pathway, which is vital for the type I IFN response (56). In addition to type I IFN induction, cytosolic DNA also activates the inflammasome, resulting in IL-1β and IL-18 production (57–59). How STING is involved in autophagy still has to be elucidated, but given its function as a transmembrane protein involved in membrane dynamics during HSV-1 infection (56), it appears likely that STING is involved in integration of DNA sensing with membrane reorganization. Also, the involvement of STING in interaction between the autophagy and IFN pathways needs further clarification. In this respect, it is interesting that in addition to STING, TBK1 is also involved in both autophagy and IFN responses (5). Other adaptor molecules of the PRRs, including MyD88 and Toll/IL-1R domain-containing adaptor-inducing IFN-β, involved in TLR signaling, have been demonstrated to positively regulate Beclin 1 and thereby promote autophagy (35).

With our present findings, and together with a recent publication by Preston and colleagues (60), autophagy can now be added to the list of cellular innate immunological processes activated by cytosolic DNA via a mechanism dependent on STING. The cellular DNA sensor(s) mediating autophagy remains to be identified.

The data presented on a role for autophagy in the STING-dependent IFN response to α-herpesvirus infection in myeloid

cells included a dual approach using both chemical inhibition and gene deletion. Treatment with 3-MA resulted in reduced IFN-β mRNA expression p.i. with HSV-1 and PrV, but not VSV. In addition, ATG7-deficient DCs also exhibited reduced IFN-β and TNF-α expression during HSV-1 infection. These data, together with a previous report showing that HSV-1–induced IFN-α expression is reduced in plasmacytoid DCs unable to initiate autophagy (12), strongly indicate an essential role for autophagy or specific proteins of the autophagy machinery in activation of the cytokine response to HSV-1 infection. Because both 3-MA treatment and ATG7 deficiency result in reduced HSV-induced IFN-β expression, these authors favor the idea that the actual autophagocytic process is essential for STING-dependent IFN responses after DNA recognition. For instance, it is possible that DNA recognition leads to assembly of a signaling complex on autophagosomes, and that STING plays a role in the membrane dynamics required to bring this complex together at this location.

It has recently been reported by Preston and colleagues (60) that transfection of human CMV DNA into human foreskin fibroblasts induces autophagy. Using HSV-1 and PrV, we demonstrate in this study that α-herpesviruses also induce autophagy through their DNA, and further demonstrate that capsid-free viral genomic DNA is present in the cytoplasm p.i. with a kinetics correlating with activation of autophagy. Importantly, we demonstrate that STING is essential for induction of autophagy by HSV. Together with the work by Preston and colleagues (60), this report establishes cytoplasmic DNA as a potent inducer of autophagy during herpesvirus infections.

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Disclosures

The authors have no financial conflicts of interest.

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