JVI Journals.ASM.org

Innate Immune Response Induced by Baculovirus Attenuates Transgene Expression in Mammalian Cells

Chikako Ono,[a] Akinori Ninomiya,[a] Satomi Yamamoto,[a] Takayuki Abe,[a]* Xiauyu Wen,[a]* Takasuke Fukuhara,[a] Miwa Sasai,[b] Masahiro Yamamoto,[b] Tatsuya Saitoh,[c] Takashi Satoh,[c] Taro Kawai,[c]* Ken J. Ishii,[d] Shizuo Akira,[c] Toru Okamoto,[a] Yoshiharu Matsuura[a]

Department of Molecular Virology[a] and Department of Immunoparasitology,[b] Research Institute for Microbial Diseases, Osaka University, Osaka, Japan; Laboratory of Host Defense[c] and Laboratory of Vaccine Science,[d] WPI Immunology Frontier Research Center, Osaka University, Osaka, Japan

The baculovirus *Autographa californica* nucleopolyhedrovirus (AcNPV) has been widely used to achieve a high level of foreign gene expression in insect cells, as well as for efficient gene transduction into mammalian cells without any replication. In addition to permitting efficient gene delivery, baculovirus has been shown to induce host innate immune responses in various mammalian cells and in mice. In this study, we examined the effects of the innate immune responses on gene expression by recombinant baculoviruses in cultured cells. The reporter gene expression in IRF3-deficient mouse embryonic fibroblasts (MEFs) infected with the recombinant baculovirus was shown to be enhanced in accordance with the suppression of beta interferon (IFN-β) production. Furthermore, efficient gene transduction by the recombinant baculovirus was achieved in MEFs deficient for stimulator of interferon genes (STING), TANK binding kinase 1 (TBK1), IFN regulatory factor 3 (IRF3), or IFN-β promoter stimulator 1 (IPS-1), but not in those deficient for IRF7, MyD88, or Z-DNA binding protein 1 (ZBP1)/DAI. Enhancement of gene expression by the recombinant baculovirus was also observed in human hepatoma cell lines replicating hepatitis C virus (HCV), in which innate immunity was impaired by the cleavage of IPS-1 by the viral protease. In addition, infection with the recombinant baculovirus expressing the BH3-only protein, BIM_S, a potent inducer of apoptosis, resulted in a selective cell death in the HCV replicon cells. These results indicate that innate immune responses induced by infection with baculovirus attenuate transgene expression, and this characteristic might be useful for a selective gene transduction into cells with impaired innate immunity arising from infection with various viruses.

The baculovirus *Autographa californica* nucleopolyhedrovirus (AcNPV) is an enveloped, double-stranded-DNA (dsDNA) virus that is primarily pathogenic to insects. Although AcNPV has long been used as an efficient gene expression vector in insect cells (1, 2), recombinant baculoviruses (rBVs) have also been shown to be capable of entering into various mammalian cells without any replication and of expressing foreign genes under the control of mammalian promoters (3–7). Therefore, baculovirus is now recognized as a useful viral vector not only for abundant gene expression in insect cells but also for gene delivery into mammalian cells. Recent studies suggest that dynamin- and clathrin-dependent endocytosis, macropinocytosis, and cholesterol in the plasma membrane play crucial roles in the internalization of baculovirus; however, the mechanisms of entry of baculovirus into insect and mammalian cells have not been well characterized yet. In contrast to the efficient transgene expression *in vitro* by the recombinant baculoviruses, the *in vivo* gene delivery is still unsatisfactory. Several obstacles, such as serum complement and the acute inflammatory response induced by inoculation of baculovirus, might be implicated in the inactivation of viral particles (8–12). *In vivo* foreign gene expression has been achieved by introducing the baculovirus vectors into rabbit endothelial cells lining the artery through collar-mediated delivery (13), mouse skeletal muscle cells in the quadriceps by intramuscular injection (14), neural or choroid plexus cells in the rodent brain by intracranial injection (15, 16), mouse retinal pigment epithelial cells following subretinal injection (17), and the cerebral cortex and testis of mice by direct inoculation (4). The level of foreign gene expression has been no more than satisfactory, mainly due to the brief duration of transgene expression *in vivo* following local administration. In addition to achieving gene delivery of recombinant baculovirus vectors, the wild-type (WT) baculovirus has also been shown to stimulate host antiviral immune responses in mammalian cell lines (8–10, 18–23). These observations suggest possible disadvantages to use of the baculovirus for sustained transduction of gene expression *in vivo*. Moreover, AcNPV was shown to possess a strong adjuvant activity to promote humoral and cellular immune responses against coadministered antigens, maturation of dendritic cells (DCs), and production of proinflammatory cytokines, chemokines, and type I interferons (IFNs) (24). We have previously shown that baculovirus induced the expression of proinflammatory cytokines and type I IFNs through the Toll-like receptor (TLR)-dependent and -independent pathway in mouse embryonic fibroblasts (MEFs), respectively (19). However, the precise mechanisms of the innate immune response and their influence

Received 17 October 2013 **Accepted** 2 December 2013

Published ahead of print 11 December 2013

Address correspondence to Yoshiharu Matsuura, matsuura@biken.osaka-u.ac.jp.

* Present address: Takayuki Abe, Department of Medicine, University of Miami School of Medicine, Miami, Florida, USA; Xiauyu Wen, Department of Hepatology, The First Hospital of Jilin University, Changchun, Jilin Province, China; Taro Kawai, Laboratory of Molecular Immunobiology, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Nara, Japan.

C.O. and A.N. contributed equally to this article.

doi:10.1128/JVI.03055-13

on the transgene expression by recombinant baculovirus vectors in mammalian cells remain unclear.

Induction of type I IFN by pathogens is crucial for innate immunity, and it is mediated by the activation of pattern recognition receptors (PRRs), such as TLRs and retinoic-acid-inducible protein I (RIG-I)-like receptors (RLRs), including RIG-I and melanoma differentiation-associated gene 5 (MDA5) (25). The type I IFN induction is primarily controlled at the gene transcriptional level, and a family of transcription factors, IFN regulatory factors (IRFs), plays a pivotal role in this regulation (26). IRF3 and IRF7 are now known to be essential for the RIG-I, MDA5, and TLR-mediated type I IFN production pathway. IRF3 is induced primarily by a response to initiate beta interferon (IFN-β) production, whereas IRF7 is induced by IFN-β and participates in the late phase of IFN-α induction (26). All TLRs, except for TLR3, activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the TRIF-dependent pathway. A novel TLR-independent cytosolic surveillance system for transfected dsDNA that elicits type I IFN induction through the TANK binding kinase 1 (TBK1)/IκB kinase-related kinase (IKKi)-IRF3 pathway has been discovered (27, 28). Recently, a cytoplasmic recognition receptor, ZBP (Z-DNA binding protein 1), was shown to be activated by dsDNA from a variety of sources and to produce type I IFN through an IRF3 pathway and probably through an IRF7 pathway (29). More recently, it has been shown that an endoplasmic reticulum (ER)-associated multiple transmembrane protein, termed "stimulator of interferon genes" (STING; also referred to as TMEM173, MPYS, MITA, and ERIS) is an essential innate immune signaling adaptor involved in the cytosolic sensing of dsDNA ligands in the TBK1/IRF3 axis (30–33).

In this study, we examined the mechanisms of induction of the innate immune response and their influence on the transgene expression in MEFs upon inoculation with the recombinant baculovirus. The efficiency of transgene expression by the recombinant baculovirus was enhanced in IRF3$^{-/-}$ MEFs compared to the wild-type, IRF7$^{-/-}$, or MyD88$^{-/-}$ MEFs. These results are consistent with the abrogation of IFN-β production via the STING/TBK1/IRF3 pathway. Importantly, IFN-β promoter stimulator 1 (IPS-1), which is an adaptor molecule of the RLR signaling pathway, was also suggested to be involved in the efficient gene transduction by baculovirus. This was consistent with the enhancement of transgene expression by baculovirus in human liver cell lines replicating hepatitis C virus (HCV), in which innate immunity was impaired by the cleavage of IPS-1 by the viral protease. Finally, infection with a recombinant baculovirus expressing BIM$_S$, a potent inducer of apoptosis, selectively induced cell death in cells replicating HCV. Collectively, these results suggest that an innate immune signaling pathway attenuates the transgene expression in mammalian cells by recombinant baculovirus.

MATERIALS AND METHODS

Mice and cells. All animal experiments were conducted in accordance with the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan. C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). MyD88-, IRF3-, IRF7-, or IPS-1-deficient mice were described previously (19). TBK1$^{+/-}$ IKKβ$^{+/-}$ (WT) and TBK1$^{-/-}$ IKKβ$^{+/-}$ (TBK1$^{-/-}$) MEFs or ZBP1-deficient MEFs were derived from mice that were established as described previously (34, 35). STING-deficient MEFs were provided by G. Barber. MEFs from the deficient mice were obtained from day-12.5 to -13.5 embryos and maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. In some cases, MEFs were immortalized by a lentivirus carrying the simian virus 40 (SV40) large T antigen. Huh7OK1 cells, which exhibit high susceptibility to propagation of cell culture-adapted HCV of the genotype 2a JFH1 strain (HCVcc) (36), Huh7 cells, and HCV subgenomic RNA replicon (SGR)-cured cells were maintained in DMEM supplemented with 10% FCS. Huh7 cells harboring SGR of genotype 1b (Con1 strain) (37), 1a (RMT strain) (provided by M. Kohara), and 2a (JFH1 strain) were cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan). The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Plasmids, reagents, and viruses. The cDNA fragments encoding murine IRF3 and IPS-1 were amplified by reverse transcription (RT)-PCR from total RNA of MEFs and cloned into a retroviral vector, pMIH (provided by D. Huang), and the resulting plasmids were designated pMIH FLAG-IRF3 and pMIH FLAG-IPS-1, respectively. Retroviral vectors for expressing short hairpin RNA (shRNA) targeting human IRF3, IPS-1, or STING were purchased from TaKaRa Bio (Shiga, Japan). The pFG EF BIM$_S$2A GFP and pFG EF BIM$_S$4E 2A GFP constructs were generated by PCR from pEF BIM$_S$2A GFP and pEF BIM$_S$4E 2A GFP, respectively, and cloned into pFastBac VSVG (Invitrogen, Tokyo, Japan) for the construction of recombinant baculoviruses by using an In-Fusion cloning kit (TaKaRa Bio). All PCR products were confirmed by sequencing by using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Tokyo, Japan). The generation of recombinant vesicular stomatitis virus (VSV) possessing a luciferase gene, VSV-luc, was described previously (38). The recombinant baculoviruses were generated by a Bac-to-Bac system (Invitrogen) following the manufacturer's protocol. For efficient gene transduction to MEFs, we utilized the baculovirus possessing the cDNA of VSV envelope G protein (VSVG) under the control of the polyhedrin promoter and bearing VSVG on the virion surface (3, 4). Recombinant baculoviruses carrying cDNAs of luciferase and green fluorescence protein (GFP) under the CAG promoter consist of chicken β-actin promoter (39), and those carrying cDNAs of BIM$_S$ and BIM$_S$4E under the human elongation factor (EF) promoter (40) were designated rBV-luc, rBV-GFP, rBV-BIM$_S$, and rBV-BIM$_S$4E, respectively. These recombinant baculoviruses and wild-type baculovirus (AcNPV C6 strain) were propagated in *Spodoptera frugiperda* (Sf-9) cells in Sf-900II insect medium (Invitrogen) supplemented with 10% FCS and purified as previously described (18). The infectious titers of recombinant baculovirus are expressed in PFU (41). The retroviruses expressing FLAG-IRF3, FLAG-IPS-1, or shRNAs against human IRF3, IPS-1, or STING were generated by PlatE cells as described previously (42). The culture supernatants containing retroviruses were passed through a 0.45-μm-pore filter, inoculated into MEFs, and incubated with hygromycin (50 μg/ml), and then stable cell lines were established. The VSV variants GLPLF and NCP12.1, derived from Indiana strains, were provided by M. Whitt. Human recombinant IFN-α was purchased from PBL Biomedical Laboratories (New Brunswick, NJ).

Antibodies. Antibodies to GFP (polyclonal), FLAG tag (M2), mouse IPS-1 (no. 3013), TBK1/NAK1 (no. 4983), HCV NS5A (HCM-131-5), calnexin (sc-70481), and β-actin (A8481) were purchased from Clontech (Mountain View, CA), Sigma, Cell Signaling (Tokyo, Japan), Austral Biologicals (San Ramon, CA), and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. The BIM antibody (rat monoclonal antibody 3C5) was a gift from A. Strasser (43).

Immunoblotting. Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and suspended in 0.4 ml lysis buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 1% glycerol, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals, Mannheim, Germany). Cell lysates were incubated for 30 min at 4°C and centrifuged at 14,000 × *g* for 15 min at 4°C. The proteins were boiled in 20 μl of sample buffer, subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). The

membranes were then blocked with Tris-buffered saline containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 0.05% Tween 20, and 5% skim milk and incubated with primary antibody at room temperature for 1 h and then with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The immune complexes and cell lysates were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Immunofluorescence assay. MEFs were infected with recombinant baculovirus expressing luciferase (rBV-luc) at a multiplicity of infection (MOI) of 100 and fixed with methanol-acetone (1:1) for 10 min at 6 h postinfection after being washed twice with PBS. Cells were incubated for 1 h at 4°C with 1 μg/ml of primary antibody in PBS containing 10% FCS (PBSF) and then incubated at room temperature for 1 h with 1 μg/ml of Alexa Fluor-conjugated secondary antibody (Invitrogen) after three washes with PBSF. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). After an extensive wash with PBSF, the samples were examined with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Reporter assays. Cells were inoculated with rBV-luc or VSV-luc, and the relative light units (RLU) of luciferase were determined by using the Promega luciferase assay system (Promega, Madison, WI) at 24 h postinfection according to a protocol provided by the manufacturer. The expression of GFP in various cells inoculated with rBV-GFP was examined by fluorescence microscopy (Olympus).

Production of cytokines. MEFs prepared from each of the deficient mouse strains were seeded into 96-well plates at a concentration of 2×10^4 cells/well and infected with rBV-GFP at an MOI of 100, and then the production of IFN-β in the culture supernatants was determined at 24 h postinfection by enzyme-linked immunosorbent assay (ELISA) kits purchased from PBL Biomedical Laboratories (Piscataway, NJ).

Real-time PCR. Baculoviral genomes attached on the cell surface were quantified by SYBR green-based quantitative PCR (qPCR) using a virus-specific primer as described previously (44). In brief, MEFs derived from WT and IRF3$^{-/-}$ mice were inoculated with rBV-GFP at an MOI of 100, incubated at 4°C for 30 min, and washed three times with PBS. The total DNA was prepared from cells by using QIAamp DNA minikit (Qiagen, Valencia, CA), and the amounts of baculovirus genome in the total DNA (20 ng) were determined by using Platinum SYBR green qPCR SuperMix UDG (Invitrogen) using GP64-specific primers and normalized with GAPDH (glyceraldehyde-3-phosphate dehydrogenase) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). To determine the gene expression, total RNA was prepared from cells by using an RNeasy minikit (Qiagen, Valencia, CA), and the first-strand cDNA was synthesized by using a ReverTra Ace (Toyobo, Osaka, Japan) and oligo(dT)$_{20}$ primer. The mouse IFN-β, IFN-γ-induced protein 10 (IP-10), GFP, and GAPDH genes were amplified using the primer pairs 5′-ACACCAGCCTGGCTTCCATC-3′ and 5′-TTGGAGCTGGAGCTGCTTATAGTTG-3′, 5′-TGAATCCGGAATCTAAGACCATCAA-3′ and 5′-AGGACTAGCCATCCACTGGGTAAAG-3′, 5′-ACACCAGCCTGGCTTCCATC-3′ and 5′-TTGGAGCTGGAGCTGCTTATAGTTG-3′, and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, respectively. The human IP-10, ISG15, and interleukin-8 (IL-8) genes were amplified using the primer pairs 5′-GGCCATCAAGAATTTACTGAAAGCA-3′ and 5′-TCTGTGTGGTCCATCCTTGGAA-3′, 5′-AGCGAACTCATCTTTGCCAGTACA-3′ and 5′-CAGCTCTGACACCGACATGGA-3′, and 5′-ACACTGCGCCAACACAGAAATTA-3′ and 5′-TTTGCTTGAAGTTTCACTGGCATC-3′, respectively. The amount of each cDNA was determined by using Platinum SYBR green qPCR SuperMix UDG (Invitrogen). Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). The human IRF-3, IPS-1, and STING genes were amplified by using the TaqMan probes (IRF3, Hs01547283_m1; IPS-1, Hs00920075_m1; STING, Hs00736958). The amount of each cDNA was determined by using TaqMan Fast advanced master mix (Applied Biosystems). Fluorescent signals were analyzed by a ViiA7 PCR system (Applied Biosystems). The expression of mRNA of each of the cytokines was normalized with that of GAPDH mRNA.

Cell viability. Cells in the culture supernatants and adherent cells detached from the plates by trypsin were collected by centrifugation at 1,500 rpm for 5 min. Cell pellets were resuspended in 0.25 ml of PBS containing 2% FCS and 100 μg/ml of propidium iodide (PI), and cell viability was determined by flow cytometry using FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. Results are expressed as means ± standard deviations (SD). The significance of differences in the means was determined by Student's *t* test.

RESULTS

IRF3-dependent innate immune response suppresses transgene expression by recombinant baculovirus in MEFs. We have previously shown that baculovirus induced proinflammatory cytokines and type I IFNs in MEFs through the TLR-dependent and TLR-independent pathways, respectively (19). Although the recombinant baculovirus was shown to be capable of internalizing into mammalian cells without any replication, the precise mechanisms of induction of an innate immune response that induces suppression of the transgene expression in MEFs remain unclear. To elucidate the involvement of innate immunity in the influence of gene transduction by recombinant baculovirus, MEFs derived from wild-type and IRF3-, IRF7-, or MyD88-deficient mice were inoculated with rBV-GFP possessing the GFP genes under the control of the CAG promoter, and the transgene expression was evaluated by microscopic observation. Although GFP expression in the MEFs derived from wild-type, MyD88-, and IRF7-deficient mice was low, IRF3-deficient cells exhibited efficient GFP expression upon infection with rBV-GFP (Fig. 1A). The finding that the transgene expression was not enhanced by rBV-GFP in MyD88-deficient MEFs suggests that TLR-mediated innate immunity—except for that involving the TLR3-dependent pathway—was not involved in the suppression of transgene expression by recombinant baculovirus in MEFs. In addition, there was no difference in the amounts of viral particles attached to the cell surface between wild-type and IRF3-deficient MEFs (Fig. 1B). Immunoblotting and quantitative RT-PCR analyses also confirmed the enhancement of gene transduction in IRF3-deficient MEFs upon infection with rBV-GFP (Fig. 1C, left panel, and D). Furthermore, the production of IFN-β and IP-10 in the IRF3-deficient MEFs decreased significantly compared with that in the wild-type cells (Fig. 1C, middle and right panels), suggesting that the enhancement of GFP expression in IRF3-deficient MEFs was due to the suppression of innate immune responses. To further confirm that the transgene expression was enhanced by the recombinant baculovirus through the suppression of IRF-3, FLAG-IRF3 was exogenously expressed in IRF3-deficient MEFs by a retroviral vector (Fig. 2A). *trans*-complementation of FLAG-IRF3 in the IRF3-deficient MEFs induced suppression of the luciferase expression by rBV-luc (Fig. 2B). Furthermore, confocal microscopy observation revealed that exogenous expression of FLAG-IRF3 in the IRF3-deficient MEFs was translocated into the nucleus upon infection with rBV-luc (Fig. 2C), suggesting that the FLAG-IRF3 exogenously expressed in the IRF3-deficient MEFs was functional. Collectively, these results suggest that the innate immune response through the IRF3-dependent pathway attenuates the transgene expression by recombinant baculovirus in MEFs.

Type I IFN production through the STING/TBK1/IRF3 axis is

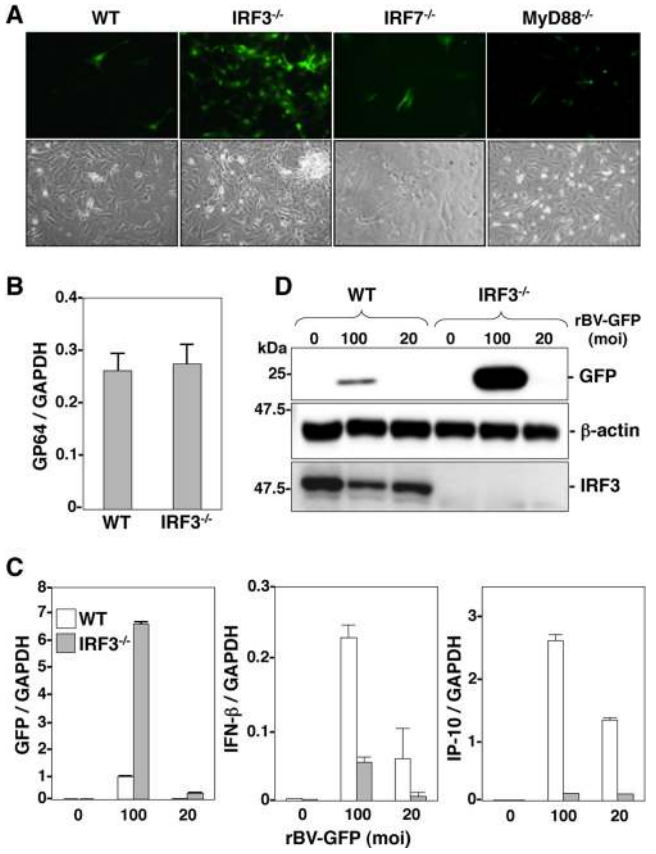

FIG 1 Efficient gene transduction by recombinant baculovirus in the IRF3$^{-/-}$ MEFs. (A) MEFs derived from wild-type (WT) and IRF3$^{-/-}$, IRF7$^{-/-}$, or MyD88$^{-/-}$ mice were inoculated with rBV-GFP (MOI of 100). At 24 h after inoculation, GFP signal was determined by microscopic observation after fixation in 4% paraformaldehyde. (B) MEFs derived from WT and IRF3$^{-/-}$ mice were inoculated with rBV-GFP at an MOI of 100, incubated at 4°C for 30 min, and washed three times with PBS. The total cellular DNA was extracted, and the amounts of baculovirus genome were quantified by real-time PCR. Data represent means ± SD from 2 independent experiments. (C) MEFs derived from WT and IRF3$^{-/-}$ mice were inoculated with 2 doses of rBV-GFP (MOI of 100 or 20). At 24 h after inoculation, total RNA was extracted, and the expression of GFP, IFN-β, and IP-10 mRNAs was determined by real-time PCR. (D) MEFs derived from WT and IRF3-deficient mice were inoculated with 2 doses of rBV-GFP (MOI of 100 and 20). At 24 h after inoculation, cell extracts were subjected to SDS-PAGE and immunoblotted with antibodies against GFP, IRF3, or β-actin, respectively.

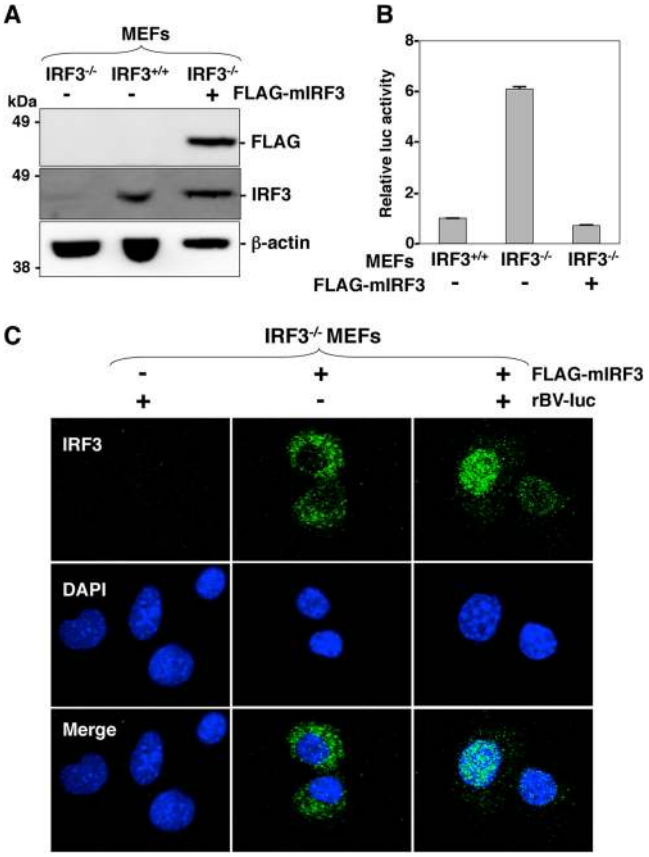

FIG 2 IRF3-dependent enhancement of gene transduction by recombinant baculovirus. (A) Cell extracts of MEFs derived from WT or IRF3$^{-/-}$ mice and FLAG-mIRF3-transduced IRF3$^{-/-}$ MEFs were subjected to SDS-PAGE and immunoblotted with antibodies against FLAG, IRF3, or β-actin, respectively. (B) MEFs derived from WT or IRF3$^{-/-}$ mice and FLAG-mIRF3-transduced IRF3-deficient MEFs were inoculated with rBV-luc at an MOI of 100. At 24 h after inoculation, the luciferase activity was determined. Data represent the means ± SD from 3 independent experiments. (C) MEFs were inoculated with rBV-luc at an MOI of 100. At 6 h after inoculation, cells were fixed in 50% methanol–50% acetone for 10 min. IRF3 (green) was stained with the appropriate antibodies, followed by staining with Alexa Fluor 488-conjugated secondary antibodies. Nuclei were stained by DAPI.

involved in the suppression of transgene expression by recombinant baculovirus in MEFs. A TLR-independent cytosolic surveillance system against exogenous dsDNA that induces type I IFN production through a TANK binding kinase 1 (TBK1)/IκB kinase-related kinase (Ikki)/IRF3 pathway has been identified (27, 28). To determine the involvement of the TLR-independent cytosolic sensor to detect exogenous dsDNA on the transgene expression by recombinant baculovirus, we examined the involvement of TBK1 in the induction of the innate immune response in MEFs upon infection with rBV-GFP. Immunofluorescence, immunoblotting, and quantitative RT-PCR analyses revealed that TBK1-deficient MEFs exhibited enhancement of the transgene expression by rBV-GFP, as seen in the IRF3-deficient MEFs (Fig. 3A, B, and C, left panel). In addition, significant reductions in IFN-β and IP-10 production were observed in the TBK1-deficient MEFs compared with the levels in the wild-type cells (Fig. 3C, middle and right panels), suggesting that the enhancement of the transgene expression in the TBK1-deficient MEFs was due to suppression of the innate immune response. Recently, the ER-associated membrane protein STING was identified as a candidate DNA sensor for production of type I IFN in response to virus infection (30–33, 45). On the other hand, the Z-DNA binding protein 1 (ZBP1; also referred to as DAI/DLM-1) has also been reported as a candidate DNA sensor (29), while the involvement of these DNA sensors in the type I IFN production is still controversial (35). We have previously shown that the induction of an innate immune response upon infection with baculovirus was dependent on viral DNA (19). To examine the roles of STING and ZBP1 in the induction of innate immune responses that lead to the suppression of transgene expression by the recombinant baculovirus, STING- or ZBP1-deficient MEFs were inoculated with either rBV-GFP or rBV-luc. As shown in Fig. 4A and B, STING-deficient MEFs exhibited the enhancement of GFP and luciferase expression upon

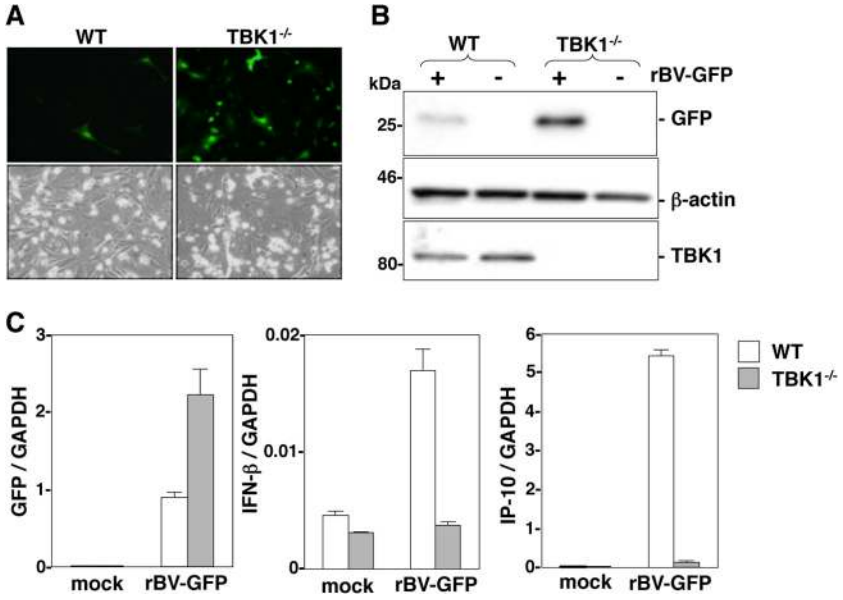

FIG 3 Involvement of TBK1 in efficient transgene expression by recombinant baculovirus. (A) MEFs derived from TBK1$^{+/-}$ IKKβ$^{+/-}$ (WT) and TBK1$^{-/-}$ IKKβ$^{+/-}$ (TBK1$^{-/-}$) mice were inoculated with rBV-GFP (MOI of 100). At 24 h after inoculation, the GFP signal was determined by microscopic observation after fixation in 4% paraformaldehyde. (B) MEFs derived from WT and TBK1$^{-/-}$ mice were inoculated with rBV-GFP at an MOI of 100. At 24 h after inoculation, cell extracts were subjected to SDS-PAGE and immunoblotted with antibodies against GFP, TBK1, or β-actin, respectively. (C) MEFs derived from WT and TBK1-deficient mice were inoculated with rBV-GFP (MOI of 100). At 24 h after inoculation, total RNA was extracted, and the expression of GFP, IFN-β, and IP-10 mRNA was determined by real-time PCR. Data from the real-time PCR were normalized to the amount of GAPDH mRNA.

inoculation with rBV-GFP and rBV-luc, respectively, while there was no such enhancement in ZBP1-deficient MEFs. STING-deficient MEFs, but not ZBP1-deficient MEFs, exhibited a significant reduction of IFN-β production upon infection with rBV-GFP (Fig. 4C). It has previously been shown that STING is dynamically translocated from the ER to the perinuclear region in cells stimulated with exogenous DNA ligands or infected with DNA virus (30, 46). We confirmed that endogenous STING was accumulated in the perinuclear region upon infection with AcNPV in MEFs (Fig. 4D), suggesting that baculovirus DNA participates in the induction of innate immune response through the translocation of STING to the perinuclear region. These results suggest that recombinant baculovirus induces type I IFN production through the STING/TBK1/IRF3 axis in MEFs, and those signaling pathways may be involved in the suppression of transgene expression by recombinant baculoviruses.

RLR signaling pathways participate in the suppression of transgene expression in MEFs by recombinant baculovirus. RIG-I and MDA5 have been identified as TLR-independent cytoplasmic RNA detectors and have been shown to induce type I IFN production through an adaptor molecule, IPS-1 (also referred as MAVS/VISA/Cardif), that localizes in mitochondria (25). We have previously shown that AcNPV produced substantial amounts of the inflammatory cytokines, chemokines, and type I IFNs in IPS-1-deficient MEFs in spite of a slight reduction in IFN-β and IL-6 (19). Recently, it was shown that the DNA virus is also capable of stimulating the RLR signaling pathways to induce type I IFNs through the participation of an adaptor molecule, IPS-1 (47, 48). To determine the involvement of RLR signaling pathways in the transgene expression by recombinant baculovirus, IRF3-, TBK1-, IPS-1-, or ZBP1-deficient MEFs were inoculated with rBV-luc. Interestingly, not only MEFs deficient in IRF3 or TBK1 but also those deficient in IPS-1 showed significant enhancement of luciferase expression (Fig. 5A). In addition, IPS-1-deficient MEFs exhibited enhancement of GFP expression and a significant reduction of IFN-β and IP-10 production compared with those in wild-type cells upon infection with rBV-GFP, as seen in IRF3-deficient MEFs (Fig. 5B and C). These results suggest that the RLR signaling pathway also participates in the suppression of the gene transduction by recombinant baculovirus in MEFs. To confirm the enhancement of the transgene expression by the recombinant baculovirus through the suppression of IPS-1, FLAG-IPS-1 was exogenously expressed in IPS-1-deficient MEFs by a retroviral vector (Fig. 5D). *trans*-complementation of FLAG-IPS-1 in the IPS-1-deficient MEFs induced suppression of luciferase expression by rBV-luc (Fig. 5E). These results suggest that an IPS-1-dependent RLR-signaling pathway also participates in the suppression of the transgene expression by recombinant baculovirus in MEFs.

RLR signaling pathways also participate in the suppression of transgene expression in human hepatoma cells by recombinant baculovirus. The NS3/4A protease of HCV participates not only in the processing of the precursor polyprotein of HCV but also in the circumvention of the induction of the host innate immune response through the cleavage of adaptor molecules, such as TRIF and IPS-1, in the human hepatoma cell lines (49). Therefore, we hypothesized that transgene expression by recombinant baculovirus might be enhanced in the HCV replicon cells, in which the innate immune response is impaired by the cleavage of IPS-1 by HCV NS3/4A protease. Reporter gene expression by infection with rBV-luc and VSV-luc was higher in the replicon cells harboring the HCV genome derived from genotypes 1a, 1b, or 2a than in those in which the viral RNA (genotype 1b) was eliminated by the treatment with IFN-α (cured) (Fig. 6A). The reporter gene expres-

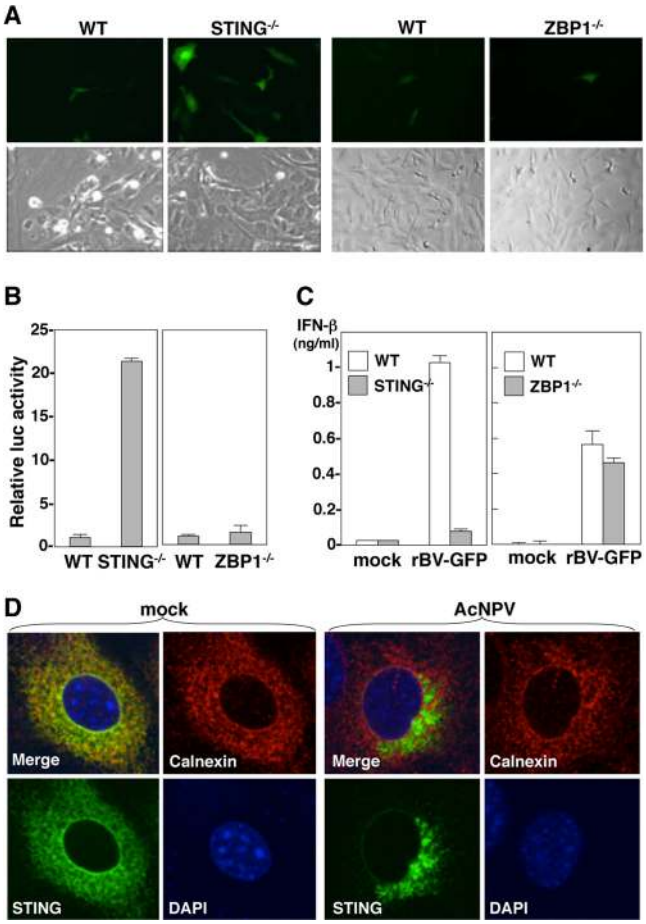

FIG 4 Efficient gene transduction by recombinant baculovirus vectors through the STING/TBK1/IRF3 axis in MEFs. (A) MEFs derived from wild-type (WT) and STING- or ZBP1-deficient mice were inoculated with rBV-GFP (MOI of 100). At 24 h after inoculation, the GFP signal was determined by microscopic observation after fixation in 4% paraformaldehyde. (B and C) MEFs derived from WT and STING- or ZBP1-deficient mice were inoculated with rBV-luc (MOI of 100) or rBV-GFP (MOI of 100). At 24 h after inoculation, the luciferase activity of cell lysates was determined (B), and the production of IFN-β in the culture supernatant was determined by sandwich ELISA (C). (D) The MEFs were inoculated with wild-type baculovirus (AcNPV) (MOI of 100). At 6 h after inoculation, cells were fixed in 50% methanol–50% acetone for 10 min. STING (green) and ER-calnexin (red) were stained with the appropriate antibodies, followed by staining with Alexa Fluor 488- or Alexa Fluor 555-conjugated second antibodies, respectively. Nuclei were stained by DAPI.

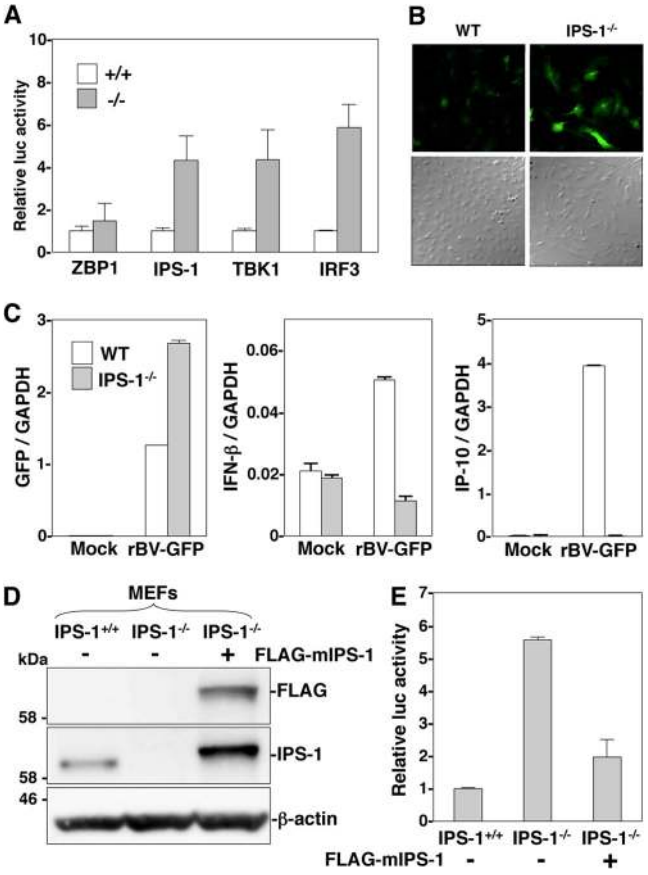

FIG 5 RLR signaling pathways participate in the suppression of gene transduction in MEFs upon infection with recombinant baculovirus. (A) MEFs derived from wild-type (WT) and IPS-1-, TBK1-, ZBP1-, or IRF3-deficient mice were inoculated with rBV-luc at an MOI of 100. At 24 h after inoculation, the luciferase activity was determined. Data represent means ± SD from 3 independent experiments. (B) MEFs derived from WT and IPS-$1^{-/-}$ mice were inoculated with rBV-GFP at an MOI of 100. At 24 h after inoculation, GFP expression was detected by microscopic observation after fixation in 4% paraformaldehyde. (C) MEFs derived from WT or IPS-1-deficient mice were inoculated with rBV-GFP at an MOI of 100. At 24 h after inoculation, total RNA was extracted, and the expression of GFP, IFN-β, and IP-10 mRNAs was determined by real-time PCR. Data from the real-time PCR were normalized to the amount of GAPDH mRNA. (D) Cell extracts of MEFs derived from WT or IPS-$1^{-/-}$ mice and FLAG-mIPS-1-transduced IPS-$1^{-/-}$ MEFs were subjected to SDS-PAGE and immunoblotted with antibodies against FLAG, IPS-1, or β-actin, respectively. (E) MEFs derived from WT or IPS-$1^{-/-}$ mice and FLAG-mIPS-1-transduced IPS-1-deficient MEFs were inoculated with rBV-luc at an MOI of 100. At 24 h after inoculation, the luciferase activity was determined. Data represent means ± SD from 3 independent experiments.

sion was increased in a dose-dependent manner upon infection with rBV-luc of the HCV replicon cells of genotype 1b (Fig. 6B). Although levels of production of IL-8 in cells infected with either rBV-luc or VSV-GFP were comparable, no induction of IP-10 and ISG15 was observed in cells infected with rBV-luc, in contrast to those infected with VSV-GFP (Fig. 6C). These data suggest that IP-10 and ISG15 are not involved in efficient gene transduction by rBV-Luc. Furthermore, the enhancement of reporter gene expression in cells infected with HCVcc was canceled by the elimination of viral replication by the treatment with IFN-α (Fig. 6D), suggesting that an IPS-1-dependent RLR-signaling pathway is involved in suppression of the transgene expression by recombinant baculovirus in human hepatoma cells, as seen in MEFs. To further confirm that IPS-1 is implicated in the suppression of the transgene expression by recombinant baculovirus, we established IPS-1, IRF3, or STING knockdown Huh7 cell lines by using shRNA (Fig. 6E, left). The highest enhancement of luciferase expression by rBV-luc was achieved in Huh7 cells with knockdown of STING, followed by those with knockdown of IPS-1 and IRF3 (Fig. 6E, right), suggesting that IPS-1 also participates in the suppression of transgene expression by recombinant baculovirus in human hepatoma cells in a manner similar to its participation in MEFs.

Induction of apoptosis in cells replicating HCV RNA by a recombinant baculovirus expressing a proapoptotic protein. The results in this study suggest that recombinant baculovirus is capable of achieving an enhanced transgene expression in cells

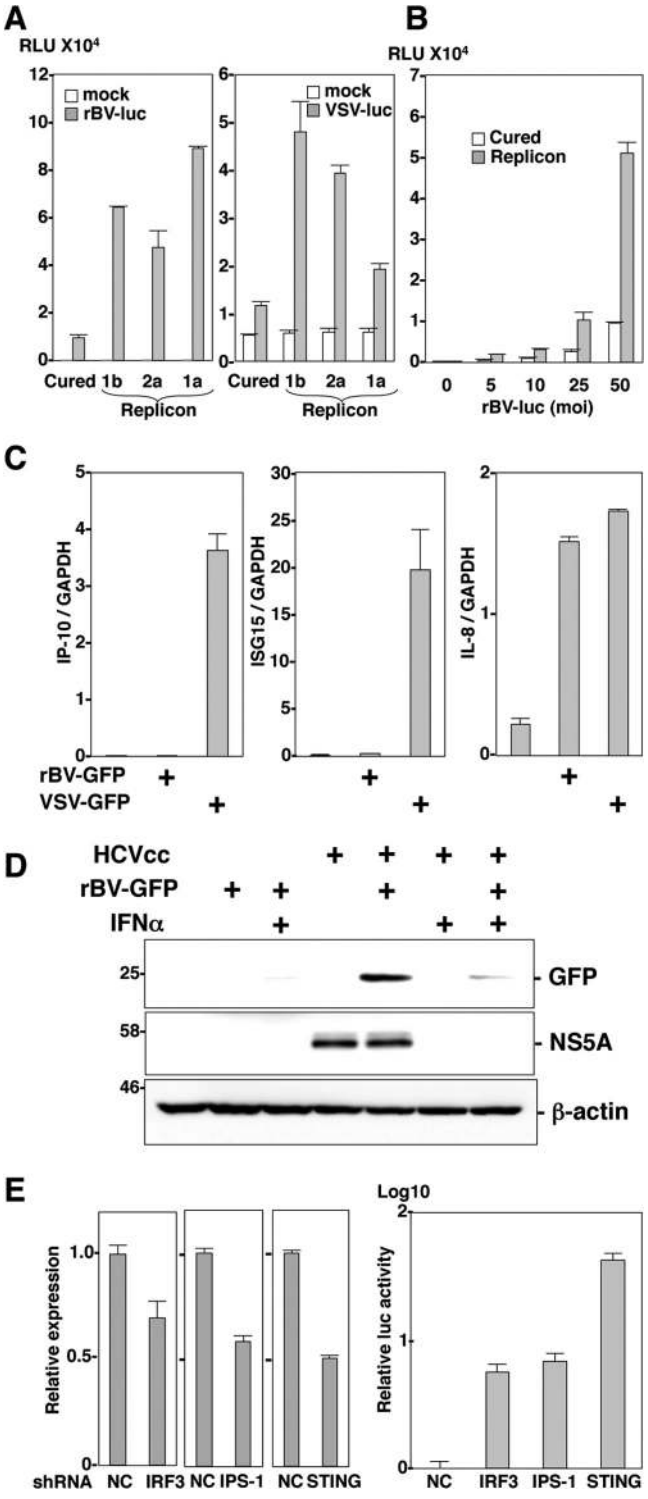

FIG 6 Efficient gene transduction by recombinant baculovirus in HCV replicon-harboring cells. (A) Huh7OK1 cells (cured) and the HCV replicon-harboring cells derived from genotype 1a (RMT strain), 1b (con1 strain), and 2a (JFH1 strain) were inoculated with rBV-luc (MOI of 100) or VSV-luc at an MOI of 5 and (B) with rBV-luc at MOI of 5, 10, 25, and 50. At 24 h after inoculation, the luciferase activity of cell lysates was determined. (C) Huh7 cells were inoculated with rBV-GFP (MOI of 100) or VSV-GFP (NCP mutant) at an MOI of 0.05. At 24 h after inoculation, total RNA was extracted, and the expression of IP-10, ISG15, and IL-8 mRNAs was determined by real-time PCR. Data from the real-time PCR were normalized to the amount of GAPDH mRNA. (D) Huh7 cells infected with HCVcc at an MOI of 1 and incubated for 72 h were inoculated with rBV-GFP (MOI of 100) in the presence or absence of human recombinant IFN-α (rIFN-α) (100 U/ml). At 24 h after inoculation, the cell extracts were subjected to SDS-PAGE and immunoblotted with antibodies against GFP, NS5A, or β-actin, respectively. (E) Relative luciferase activity in Huh7 cells with IRF3, IPS-1, or STING knocked down. Cells were inoculated with rBV-luc at an MOI of 100. At 24 h after inoculation, the luciferase activity of cell lysates was determined (right panel). Luciferase activity is normalized to control shNC cells, and data represent the means ± SD from 2 independent experiments. Total RNA was extracted, and the expression of IRF3, IPS-1, or STING mRNA was determined by real-time PCR (left panel). Data from the real-time PCR were normalized to the amount of GAPDH mRNA.

with an impaired host innate immune response. Therefore, we thought that it might be feasible to eliminate cells replicating the HCV genome by infection with a recombinant baculovirus encoding one of the proapoptotic Bcl-2 family proteins, BIM_S, which induces apoptosis through activation of Bax and Bak. We generated two recombinant baculoviruses, rBV-BIM_S and rBV-BIM_S4E, carrying cDNAs of either BIM_s or BIM_s4E (an inactive mutant of BIM_s), 2A peptides of *Thosea asigna* virus, to express multiple proteins as a polyprotein that dissociates into each protein upon translation (50), and enhanced GFP under the control of the EF promoter (Fig. 7A), and exogenous expression of both BIM_S and BIM_S4E was detected in the HCV replicon cells infected with the recombinants in the presence of an apoptosis inhibitor (Fig. 7B). HCV replicon cells infected with rBV-BIM_S, but not with rBV-BIM_S4E, exhibited an efficient cell death (Fig. 7C), and expression of rBV-BIM_S induces cell death at 24 h postinfection in the replicon cells but not in the cured cells (Fig. 7D). Furthermore, cell death induced in HCV replicon cells by the infection with rBV-BIM_S was inhibited by the treatment with a caspase inhibitor, qVD-Oph (Fig. 7E), suggesting that infection with rBV-BIM_S selectively induced apoptosis in cells replicating HCV RNA through the activation of Bcl-2 family proteins. These results suggest that infection with rBV-BIM_S might eliminate HCV through the induction of cell death by the enhanced expression of the Bcl-2 family protein in HCV-infected cells in which innate immunity is impaired by the NS3/4A protease.

DISCUSSION

Viral vectors have great advantages for efficient and sustained gene delivery to target cells, but the induction of immune responses against viral components and products, including viral proteins and nucleic acids, is a great concern (51). Production of proinflammatory cytokines and type I IFNs upon delivery of the viral vectors may abrogate transgene expression and induce cytotoxicity to the target cells and tissues (52–54). In fact, it has been shown that the removal of viral elements responsible for replication and pathogenicity, together with the provision of a large-capacity space to accommodate foreign genes and broader cell tropism by bearing envelope proteins that have high affinity to various tissues, makes the viral vectors amenable for use in gene therapy (3–7, 55–57).

Recently, the application of baculovirus vectors to gene therapy has also been shown to have several advantages: (i) low cytotoxicity in mammalian cells even at a high multiplicity of infection, (ii) inherent inability to replicate in mammalian cells to induce pathogenicity, and (iii) the absence of preexisting antibodies. In addition to the efficient gene delivery into mammalian cells,

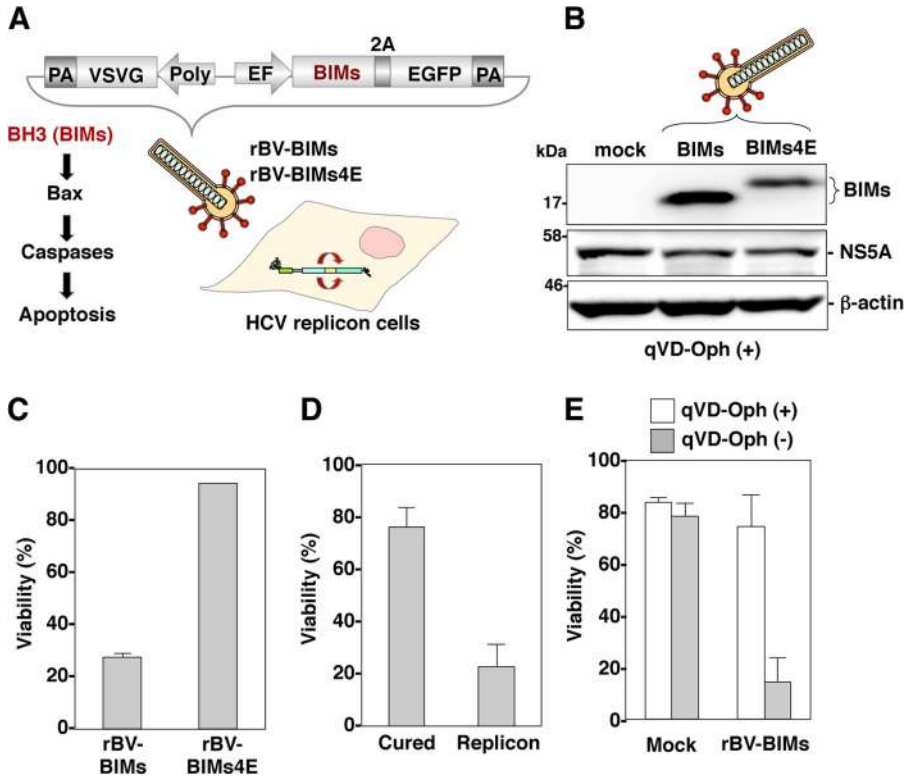

FIG 7 Induction of apoptosis in cells replicating HCV RNA by a recombinant baculovirus expressing a proapoptotic protein. (A) Structure of recombinant baculoviruses, rBV-BIM_S, and rBV-BIM_S4E carrying cDNAs of either BIM_S or BIM_S4E 2A peptides, and enhanced GFP under the control of the EF promoter. The apoptosis pathway was induced by BIM_S. (B) HCV replicon cells were infected with either rBV-BIM_S or rBV-BIM_S4E at an MOI of 500 in the presence of the caspase inhibitor qVD-Oph (20 μM). Cells were subjected to immunoblotting by appropriate antibodies. (C) HCV replicon cells were infected with either rBV-BIM_S or rBV-BIM_S4E at an MOI of 500, and cell viability was determined by PI exclusion at 24 h postinfection. (D) HCV replicon cells and cured cells were infected with rBV-BIM_S at an MOI of 500, and cell viability was determined by PI exclusion at 24 h postinfection. (E) HCV replicon cells and cured cells were infected with rBV-BIM_S at an MOI of 500 in the presence or absence of 20 μM qVD-Oph, and cell viability was determined by PI exclusion at 24 h postinfection. Data represent means ± SD from 2 independent experiments.

the baculovirus has been shown to stimulate a host innate immune response in mammalian cells and to confer protection from lethal virus infection in mice (8, 10, 18, 19). Furthermore, it has been reported that the baculovirus possesses a strong adjuvant activity to promote humoral and cellular immune responses (24). Although these characteristics might be disadvantageous for the transgene expression, the baculovirus may have potency for use as a vaccine vector possessing adjuvant activity. Recombinant baculoviruses bearing the chimeric GP64 with *Plasmodium berghei* circumsporozoites (58, 59), *Plasmodium falciparum* circumsporozoites (60), *Toxoplasma gondii* (61), rinderpest virus (62), foot-and-mouth disease virus (63), pseudorabies virus (64), and avian influenza virus (65) have been shown to elicit an efficient antibody response. These results suggest that recombinant and pseudotyped baculoviruses encoding or transiently bearing foreign epitopes may induce immune responses in the antigen-presenting cells. Furthermore, gene delivery by using recombinant baculoviruses bearing specific ligands of interest may elicit more potent immunogenicity (66–68).

In this study, we have shown that reporter gene induction by recombinant baculovirus was enhanced in STING-, TBK1-, or IRF3-deficient MEFs. Moreover, an endogenous STING was translocated to the perinuclear region upon infection with recombinant baculovirus, suggesting that STING plays a crucial role in the induction of the innate immune response via baculovirus infection. Although a direct interaction of viral and synthetic DNAs with STING is not known, baculoviral DNA may be involved in the STING-associated signaling complex, including TBK1 and IRF3. Baculovirus has a circular dsDNA that contains bioactive CpG motif sequences, and the immune responses induced by the baculovirus DNA produce inflammatory cytokines and type I IFNs through TLR-dependent and -independent pathways, respectively (18, 19). Previously, Zhang et al. reported that the helicase DDX41, which is a member of the DExD/H-box helicase superfamily, acts as a cytosolic DNA sensor in immunocompetent cells (69). However, the involvement of DDX41 in type I IFN production in other cell types has not been demonstrated. Recently, Chen and colleagues have identified a cyclic GMP-AMP (cGAMP) synthase (cGAS) as a candidate for a cytosolic DNA sensor (70, 71). cGAMP produced by cGAS works as an endogenous second messenger and activates STING in cells stimulated by a broad species of DNAs. Although further studies are needed, it might be feasible to speculate that cGAS participates in the baculovirus-mediated innate immune response in a cell-type-specific manner.

IPS-$1^{-/-}$ MEFs exhibit enhanced gene transduction by recombinant baculovirus, which is consistent with the report that murine gammaherpesvirus induces cytokine production in an IPS-1-

dependent manner (72), suggesting the involvement of the RLR pathways in the innate immune responses against DNA viruses. Although the evidence is not yet conclusive, Choi et al. suggested that IPS-1 may exclusively participate in a branch of the B-DNA/RLR/IRF3 signaling pathway (47). On the other hand, the small RNAs encoded by adenovirus (73) and the cytosolic DNA-dependent RNA polymerase III (74) are suggested to be involved in the RLR signaling pathway (75). Furthermore, transcripts of several early viral genes were detected in the baculovirus-transduced mammalian cells (76, 77). Collectively, these data suggested that viral genomes and RNA transcripts of baculovirus and cytosolic DNA-dependent RNA polymerase III may participate in the IPS-1-dependent IFN-β production in cells infected with recombinant baculovirus. Recently, it has been shown that HCV infection suppresses not only IPS-1-dependent IFN signaling by NS3/4A but also STING-mediated IFN response by NS4B (78, 79). Therefore, suppression of STING by HCV replication may participate in the efficient gene transduction in the HCV replicon cells by baculovirus.

Chronic infection with HCV is a major risk factor for liver diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (80). Newly developed directly acting antivirals targeting NS3/4A protease, NS5A, and NS5B polymerase have achieved a sustained virological response in over 80% of individuals infected with HCV, but drug-resistant breakthrough viruses have been reported to emerge during the treatment (81). In this study, we have raised the possibility of selective elimination of HCV-infected cells by the enhanced gene transduction of recombinant baculoviruses expressing proapoptotic Bcl-2 family proteins. Thus, our notion indicates that combinations of baculovirus-mediated gene therapy with current chemotherapy would be useful methods for eliminating HCV.

In conclusion, we have demonstrated that baculovirus induces innate immune responses that attenuate transgene expression. Recently, it was reported that baculovirus vectors exhibited an effective transgene expression in pluripotent stem cells, without elicitation of immune response, apoptosis, or pluripotency differentiation (82), supporting our notion that the induction of an acute immune response attenuates transgene expression by baculovirus. Although further studies are needed to clarify the induction of immune responses in more detail, these characteristics of baculovirus might be advantageous for vaccine vehicles possessing adjuvant activity and vectors capable of selective gene transduction into cells with impaired innate immunity due to infection with various viruses.

ACKNOWLEDGMENTS

We gratefully acknowledge M. Tomiyama for secretarial work and M. Ishibashi and Y. Sugiyama for technical assistance. We also thank D. C. S. Huang, R. Bartenschlager, M. Kohara, G. Barber, T. Wakita, and M. Whitt for providing experimental materials.

This work was supported in part by grants-in-aid from the Japanese Ministry of Health, Labor, and Welfare (Research on Hepatitis), the Japanese Ministry of Education, Culture, Sports, Science, and Technology, a basic research grant from the National Institutes of Health (NIH AI070167-06A1), the Japan Society for the Promotion of Science through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program), the Uehara Memorial Foundation, the Naito Foundation, and the Takeda Science Foundation.

REFERENCES

1. **Luckow VA, Summers MD.** 1988. Signals important for high-level expression of foreign genes in Autographa californica nuclear polyhedrosis virus expression vectors. Virology **167:**56–71. http://dx.doi.org/10.1016/0042-6822(88)90054-2.
2. **Matsuura Y, Possee RD, Overton HA, Bishop DH.** 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. J. Gen. Virol. **68:**1233–1250. http://dx.doi.org/10.1099/0022-1317-68-5-1233.
3. **Tani H, Nishijima M, Ushijima H, Miyamura T, Matsuura Y.** 2001. Characterization of cell-surface determinants important for baculovirus infection. Virology **279:**343–353. http://dx.doi.org/10.1006/viro.2000.0699.
4. **Tani H, Limn CK, Yap CC, Onishi M, Nozaki M, Nishimune Y, Okahashi N, Kitagawa Y, Watanabe R, Mochizuki R, Moriishi K, Matsuura Y.** 2003. In vitro and in vivo gene delivery by recombinant baculoviruses. J. Virol. **77:**9799–9808. http://dx.doi.org/10.1128/JVI.77.18.9799-9808.2003.
5. **Hofmann C, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M.** 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. U. S. A. **92:**10099–10103. http://dx.doi.org/10.1073/pnas.92.22.10099.
6. **Boyce FM, Bucher NL.** 1996. Baculovirus-mediated gene transfer into mammalian cells. Proc. Natl. Acad. Sci. U. S. A. **93:**2348–2352. http://dx.doi.org/10.1073/pnas.93.6.2348.
7. **Shoji I, Aizaki H, Tani H, Ishii K, Chiba T, Saito I, Miyamura T, Matsuura Y.** 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. **78:**2657–2664.
8. **Abe T, Takahashi H, Hamazaki H, Miyano-Kurosaki N, Matsuura Y, Takaku H.** 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J. Immunol. **171:**1133–1139. http://www.jimmunol.org/content/171/3/1133.long.
9. **Beck NB, Sidhu JS, Omiecinski CJ.** 2000. Baculovirus vectors repress phenobarbital-mediated gene induction and stimulate cytokine expression in primary cultures of rat hepatocytes. Gene Ther. **7:**1274–1283. http://dx.doi.org/10.1038/sj.gt.3301246.
10. **Gronowski AM, Hilbert DM, Sheehan KC, Garotta G, Schreiber RD.** 1999. Baculovirus stimulates antiviral effects in mammalian cells. J. Virol. **73:**9944–9951.
11. **Hofmann C, Strauss M.** 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. **5:**531–536. http://dx.doi.org/10.1038/sj.gt.3300607.
12. **Sandig V, Hofmann C, Steinert S, Jennings G, Schlag P, Strauss M.** 1996. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum. Gene Ther. **7:**1937–1945. http://dx.doi.org/10.1089/hum.1996.7.16-1937.
13. **Airenne KJ, Hiltunen MO, Turunen MP, Turunen AM, Laitinen OH, Kulomaa MS, Yla-Herttuala S.** 2000. Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene Ther. **7:**1499–1504. http://dx.doi.org/10.1038/sj.gt.3301269.
14. **Pieroni L, Maione D, La Monica N.** 2001. In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum. Gene Ther. **12:**871–881. http://dx.doi.org/10.1089/104303401750195845.
15. **Lehtolainen P, Tyynela K, Kannasto J, Airenne KJ, Yla-Herttuala S.** 2002. Baculoviruses exhibit restricted cell type specificity in rat brain: a comparison of baculovirus- and adenovirus-mediated intracerebral gene transfer in vivo. Gene Ther. **9:**1693–1699. http://dx.doi.org/10.1038/sj.gt.3301854.
16. **Sarkis C, Serguera C, Petres S, Buchet D, Ridet JL, Edelman L, Mallet J.** 2000. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc. Natl. Acad. Sci. U. S. A. **97:**14638–14643. http://dx.doi.org/10.1073/pnas.260472897.
17. **Haeseleer F, Imanishi Y, Saperstein DA, Palczewski K.** 2001. Gene transfer mediated by recombinant baculovirus into mouse eye. Invest. Ophthalmol. Vis. Sci. **42:**3294–3300. http://www.iovs.org/content/42/13/3294.long.
18. **Abe T, Hemmi H, Miyamoto H, Moriishi K, Tamura S, Takaku H, Akira S, Matsuura Y.** 2005. Involvement of the Toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. J. Virol. **79:**2847–2858. http://dx.doi.org/10.1128/JVI.79.5.2847-2858.2005.
19. **Abe T, Kaname Y, Wen X, Tani H, Moriishi K, Uematsu S, Takeuchi O,**

Ishii KJ, Kawai T, Akira S, Matsuura Y. 2009. Baculovirus induces type I interferon production through Toll-like receptor-dependent and -independent pathways in a cell-type-specific manner. J. Virol. **83:**7629–7640. http://dx.doi.org/10.1128/JVI.00679-09.
20. **Boulaire J, Zhao Y, Wang S.** 2009. Gene expression profiling to define host response to baculoviral transduction in the brain. J. Neurochem. **109:**1203–1214. http://dx.doi.org/10.1111/j.1471-4159.2009.06015.x.
21. **Chen GY, Shiah HC, Su HJ, Chen CY, Chuang YJ, Lo WH, Huang JL, Chuang CK, Hwang SM, Hu YC.** 2009. Baculovirus transduction of mesenchymal stem cells triggers the Toll-like receptor 3 pathway. J. Virol. **83:**10548–10556. http://dx.doi.org/10.1128/JVI.01250-09.
22. **Han Y, Niu M, An L, Li W.** 2009. Upregulation of proinflammatory cytokines and NO production in BV-activated avian macrophage-like cell line (HD11) requires MAPK and NF-kappaB pathways. Int. Immunopharmacol. **9:**817–823. http://dx.doi.org/10.1016/j.intimp.2009.03.008.
23. **Wilson S, Baird M, Ward VK.** 2008. Delivery of vaccine peptides by rapid conjugation to baculovirus particles. Vaccine **26:**2451–2456. http://dx.doi.org/10.1016/j.vaccine.2008.03.027.
24. **Hervas-Stubbs S, Rueda P, Lopez L, Leclerc C.** 2007. Insect baculoviruses strongly potentiate adaptive immune responses by inducing type I IFN. J. Immunol. **178:**2361–2369. http://www.jimmunol.org/content/178/4/2361.long.
25. **Kawai T, Akira S.** 2006. Innate immune recognition of viral infection. Nat. Immunol. **7:**131–137. http://dx.doi.org/10.1038/ni1303.
26. **Honda K, Taniguchi T.** 2006. IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat. Rev. Immunol. **6:**644–658. http://dx.doi.org/10.1038/nri1900.
27. **Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, Ludwig H, Sutter G, Suzuki K, Hemmi H, Sato S, Yamamoto M, Uematsu S, Kawai T, Takeuchi O, Akira S.** 2006. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. Nat. Immunol. **7:**40–48. http://dx.doi.org/10.1038/ni1282.
28. **Stetson DB, Medzhitov R.** 2006. Recognition of cytosolic DNA activates an IRF3-dependent innate immune response. Immunity **24:**93–103. http://dx.doi.org/10.1016/j.immuni.2005.12.003.
29. **Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, Ohba Y, Taniguchi T.** 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature **448:**501–505. http://dx.doi.org/10.1038/nature06013.
30. **Ishikawa H, Barber GN.** 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature **455:**674–678. http://dx.doi.org/10.1038/nature07317.
31. **Jin L, Waterman PM, Jonscher KR, Short CM, Reisdorph NA, Cambier JC.** 2008. MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals. Mol. Cell. Biol. **28:**5014–5026. http://dx.doi.org/10.1128/MCB.00640-08.
32. **Sun W, Li Y, Chen L, Chen H, You F, Zhou X, Zhou Y, Zhai Z, Chen D, Jiang Z.** 2009. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. Proc. Natl. Acad. Sci. U. S. A. **106:**8653–8658. http://dx.doi.org/10.1073/pnas.0900850106.
33. **Zhong B, Yang Y, Li S, Wang YY, Li Y, Diao F, Lei C, He X, Zhang L, Tien P, Shu HB.** 2008. The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. Immunity **29:**538–550. http://dx.doi.org/10.1016/j.immuni.2008.09.003.
34. **Matsui K, Kumagai Y, Kato H, Sato S, Kawagoe T, Uematsu S, Takeuchi O, Akira S.** 2006. Cutting edge: role of TANK-binding kinase 1 and inducible IkappaB kinase in IFN responses against viruses in innate immune cells. J. Immunol. **177:**5785–5789. http://www.jimmunol.org/content/177/9/5785.long.
35. **Ishii KJ, Kawagoe T, Koyama S, Matsui K, Kumar H, Kawai T, Uematsu S, Takeuchi O, Takeshita F, Coban C, Akira S.** 2008. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature **451:**725–729. http://dx.doi.org/10.1038/nature06537.
36. **Okamoto T, Omori H, Kaname Y, Abe T, Nishimura Y, Suzuki T, Miyamura T, Yoshimori T, Moriishi K, Matsuura Y.** 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. J. Virol. **82:**3480–3489. http://dx.doi.org/10.1128/JVI.02253-07.
37. **Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R.** 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science **285:**110–113. http://dx.doi.org/10.1126/science.285.5424.110.
38. **Tani H, Komoda Y, Matsuo E, Suzuki K, Hamamoto I, Yamashita T, Moriishi K, Fujiyama K, Kanto T, Hayashi N, Owsianka A, Patel AH, Whitt MA, Matsuura Y.** 2007. Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins. J. Virol. **81:**8601–8612. http://dx.doi.org/10.1128/JVI.00608-07.
39. **Niwa H, Yamamura K, Miyazaki J.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199. http://dx.doi.org/10.1016/0378-1119(91)90434-D.
40. **Mizushima S, Nagata S.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. **18:**5322. http://dx.doi.org/10.1093/nar/18.17.5322.
41. **Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796. http://dx.doi.org/10.1038/nm1268.
42. **Morita S, Kojima T, Kitamura T.** 2000. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. **7:**1063–1066. http://dx.doi.org/10.1038/sj.gt.3301206.
43. **O'Reilly LA, Cullen L, Visvader J, Lindeman GJ, Print C, Bath ML, Huang DC, Strasser A.** 2000. The proapoptotic BH3-only protein bim is expressed in hematopoietic, epithelial, neuronal, and germ cells. Am. J. Pathol. **157:**449–461. http://dx.doi.org/10.1016/S0002-9440(10)64557-9.
44. **Kataoka C, Kaname Y, Taguwa S, Abe T, Fukuhara T, Tani H, Moriishi K, Matsuura Y.** 2012. Baculovirus GP64-mediated entry into mammalian cells. J. Virol. **86:**2610–2620. http://dx.doi.org/10.1128/JVI.06704-11.
45. **Abe T, Harashima A, Xia T, Konno H, Konno K, Morales A, Ahn J, Gutman D, Barber GN.** 2013. STING recognition of cytoplasmic DNA instigates cellular defense. Mol. Cell **50:**5–15. http://dx.doi.org/10.1016/j.molcel.2013.01.039.
46. **Saitoh T, Fujita N, Hayashi T, Takahara K, Satoh T, Lee H, Matsunaga K, Kageyama S, Omori H, Noda T, Yamamoto N, Kawai T, Ishii K, Takeuchi O, Yoshimori T, Akira S.** 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. Proc. Natl. Acad. Sci. U. S. A. **106:**20842–20846. http://dx.doi.org/10.1073/pnas.0911267106.
47. **Choi MK, Wang Z, Ban T, Yanai H, Lu Y, Koshiba R, Nakaima Y, Hangai S, Savitsky D, Nakasato M, Negishi H, Takeuchi O, Honda K, Akira S, Tamura T, Taniguchi T.** 2009. A selective contribution of the RIG-I-like receptor pathway to type I interferon responses activated by cytosolic DNA. Proc. Natl. Acad. Sci. U. S. A. **106:**17870–17875. http://dx.doi.org/10.1073/pnas.0909545106.
48. **Kumar H, Kawai T, Kato H, Sato S, Takahashi K, Coban C, Yamamoto M, Uematsu S, Ishii KJ, Takeuchi O, Akira S.** 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. J. Exp. Med. **203:**1795–1803. http://dx.doi.org/10.1084/jem.20060792.
49. **Lemon SM.** 2010. Induction and evasion of innate antiviral responses by hepatitis C virus. J. Biol. Chem. **285:**22741–22747. http://dx.doi.org/10.1074/jbc.R109.099556.
50. **Correia-de-Sa P, Timoteo MA, Ribeiro JA.** 2001. Synergism between A(2A)-adenosine receptor activation and vasoactive intestinal peptide to facilitate [3H]-acetylcholine release from the rat motor nerve terminals. Neurosci. Lett. **309:**101–104. http://dx.doi.org/10.1016/S0304-3940(01)02030-4.
51. **Nayak S, Herzog RW.** 2010. Progress and prospects: immune responses to viral vectors. Gene Ther. **17:**295–304. http://dx.doi.org/10.1038/gt.2009.148.
52. **Olschowka JA, Bowers WJ, Hurley SD, Mastrangelo MA, Federoff HJ.** 2003. Helper-free HSV-1 amplicons elicit a markedly less robust innate immune response in the CNS. Mol. Ther. **7:**218–227. http://dx.doi.org/10.1016/S1525-0016(02)00036-9.
53. **Sung RS, Qin L, Bromberg JS.** 2001. TNFalpha and IFNgamma induced by innate anti-adenoviral immune responses inhibit adenovirus-mediated transgene expression. Mol. Ther. **3:**757–767. http://dx.doi.org/10.1006/mthe.2001.0318.
54. **Worgall S, Wolff G, Falck-Pedersen E, Crystal RG.** 1997. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. Hum. Gene Ther. **8:**37–44. http://dx.doi.org/10.1089/hum.1997.8.1-37.
55. **Volkman LE, Goldsmith PA.** 1983. In vitro survey of Autographa californica nuclear polyhedrosis virus interaction with nontarget vertebrate host cells. Appl. Environ. Microbiol. **45:**1085–1093.
56. **Carbonell LF, Klowden MJ, Miller LK.** 1985. Baculovirus-mediated

expression of bacterial genes in dipteran and mammalian cells. J. Virol. **56:**153–160.
57. **Chen CY, Lin CY, Chen GY, Hu YC.** 2011. Baculovirus as a gene delivery vector: recent understandings of molecular alterations in transduced cells and latest applications. Biotechnol. Adv. **29:**618–631. http://dx.doi.org/10.1016/j.biotechadv.2011.04.004.
58. **Yoshida S, Araki H, Yokomine T.** 2010. Baculovirus-based nasal drop vaccine confers complete protection against malaria by natural boosting of vaccine-induced antibodies in mice. Infect. Immun. **78:**595–602. http://dx.doi.org/10.1128/IAI.00877-09.
59. **Yoshida S, Kondoh D, Arai E, Matsuoka H, Seki C, Tanaka T, Okada M, Ishii A.** 2003. Baculovirus virions displaying Plasmodium berghei circumsporozoite protein protect mice against malaria sporozoite infection. Virology **316:**161–170. http://dx.doi.org/10.1016/j.virol.2003.08.003.
60. **Strauss R, Huser A, Ni S, Tuve S, Kiviat N, Sow PS, Hofmann C, Lieber A.** 2007. Baculovirus-based vaccination vectors allow for efficient induction of immune responses against Plasmodium falciparum circumsporozoite protein. Mol. Ther. **15:**193–202. http://dx.doi.org/10.1038/sj.mt.6300008.
61. **Fang R, Feng H, Nie H, Wang L, Tu P, Song Q, Zhou Y, Zhao J.** 2010. Construction and immunogenicity of pseudotype baculovirus expressing Toxoplasma gondii SAG1 protein in BALB/c mice model. Vaccine **28:**1803–1807. http://dx.doi.org/10.1016/j.vaccine.2009.12.005.
62. **Rahman MM, Shaila MS, Gopinathan KP.** 2003. Baculovirus display of fusion protein of Peste des petits ruminants virus and hemagglutination protein of Rinderpest virus and immunogenicity of the displayed proteins in mouse model. Virology **317:**36–49. http://dx.doi.org/10.1016/j.virol.2003.08.022.
63. **Tami C, Peralta A, Barbieri R, Berinstein A, Carrillo E, Taboga O.** 2004. Immunological properties of FMDV-gP64 fusion proteins expressed on SF9 cell and baculovirus surfaces. Vaccine **23:**840–845. http://dx.doi.org/10.1016/j.vaccine.2004.03.070.
64. **Grabowska AK, Lipinska AD, Rohde J, Szewczyk B, Bienkowska-Szewczyk K, Rziha HJ.** 2009. New baculovirus recombinants expressing Pseudorabies virus (PRV) glycoproteins protect mice against lethal challenge infection. Vaccine **27:**3584–3591. http://dx.doi.org/10.1016/j.vaccine.2009.03.067.
65. **Yang DG, Chung YC, Lai YK, Lai CW, Liu HJ, Hu YC.** 2007. Avian influenza virus hemagglutinin display on baculovirus envelope: cytoplasmic domain affects virus properties and vaccine potential. Mol. Ther. **15:**989–996. http://dx.doi.org/10.1038/mt.sj.6300131.
66. **Kitagawa Y, Tani H, Limn CK, Matsunaga TM, Moriishi K, Matsuura Y.** 2005. Ligand-directed gene targeting to mammalian cells by pseudotype baculoviruses. J. Virol. **79:**3639–3652. http://dx.doi.org/10.1128/JVI.79.6.3639-3652.2005.
67. **Lung O, Westenberg M, Vlak JM, Zuidema D, Blissard GW.** 2002. Pseudotyping Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV): F proteins from group II NPVs are functionally analogous to AcMNPV GP64. J. Virol. **76:**5729–5736. http://dx.doi.org/10.1128/JVI.76.11.5729-5736.2002.
68. **Mangor JT, Monsma SA, Johnson MC, Blissard GW.** 2001. A GP64-null baculovirus pseudotyped with vesicular stomatitis virus G protein. J. Virol. **75:**2544–2556. http://dx.doi.org/10.1128/JVI.75.6.2544-2556.2001.
69. **Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu YJ.** 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat. Immunol. **12:**959–965. http://dx.doi.org/10.1038/ni.2091.
70. **Sun L, Wu J, Du F, Chen X, Chen ZJ.** 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. Science **339:**786–791. http://dx.doi.org/10.1126/science.1232458.
71. **Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ.** 2013. Cyclic GMP-AMP is an endogenous second messenger in innate immune signaling by cytosolic DNA. Science **339:**826–830. http://dx.doi.org/10.1126/science.1229963.
72. **Dong X, Feng P.** 2011. Murine gamma herpesvirus 68 hijacks MAVS and IKKbeta to abrogate NFkappaB activation and antiviral cytokine production. PLoS Pathog. **7:**e1002336. http://dx.doi.org/10.1371/journal.ppat.1002336.
73. **Sano M, Kato Y, Taira K.** 2006. Sequence-specific interference by small RNAs derived from adenovirus VAI RNA. FEBS Lett. **580:**1553–1564. http://dx.doi.org/10.1016/j.febslet.2006.01.085.
74. **Chiu YH, Macmillan JB, Chen ZJ.** 2009. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell **138:**576–591. http://dx.doi.org/10.1016/j.cell.2009.06.015.
75. **Yamaguchi T, Kawabata K, Kouyama E, Ishii KJ, Katayama K, Suzuki T, Kurachi S, Sakurai F, Akira S, Mizuguchi H.** 2010. Induction of type I interferon by adenovirus-encoded small RNAs. Proc. Natl. Acad. Sci. U. S. A. **107:**17286–17291. http://dx.doi.org/10.1073/pnas.1009823107.
76. **Kenoutis C, Efrose RC, Swevers L, Lavdas AA, Gaitanou M, Matsas R, Iatrou K.** 2006. Baculovirus-mediated gene delivery into mammalian cells does not alter their transcriptional and differentiating potential but is accompanied by early viral gene expression. J. Virol. **80:**4135–4146. http://dx.doi.org/10.1128/JVI.80.8.4135-4146.2006.
77. **Fujita R, Matsuyama T, Yamagishi J, Sahara K, Asano S, Bando H.** 2006. Expression of Autographa californica multiple nucleopolyhedrovirus genes in mammalian cells and upregulation of the host beta-actin gene. J. Virol. **80:**2390–2395. http://dx.doi.org/10.1128/JVI.80.5.2390-2395.2006.
78. **Nitta S, Sakamoto N, Nakagawa M, Kakinuma S, Mishima K, Kusano-Kitazume A, Kiyohashi K, Murakawa M, Nishimura-Sakurai Y, Azuma S, Tasaka-Fujita M, Asahina Y, Yoneyama M, Fujita T, Watanabe M.** 2013. Hepatitis C virus NS4B protein targets STING and abrogates RIG-I-mediated type I interferon-dependent innate immunity. Hepatology **57:**46–58. http://dx.doi.org/10.1002/hep.26017.
79. **Ding Q, Cao X, Lu J, Huang B, Liu YJ, Kato N, Shu HB, Zhong J.** 2013. Hepatitis C virus NS4B blocks the interaction of STING and TBK1 to evade host innate immunity. J. Hepatol. **59:**52–58. http://dx.doi.org/10.1016/j.jhep.2013.03.019.
80. **Maasoumy B, Wedemeyer H.** 2012. Natural history of acute and chronic hepatitis C. Best Pract. Res. Clin. Gastroenterol. **26:**401–412. http://dx.doi.org/10.1016/j.bpg.2012.09.009.
81. **Sarrazin C, Zeuzem S.** 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology **138:**447–462. http://dx.doi.org/10.1053/j.gastro.2009.11.055.
82. **Chen GY, Hwang SM, Su HJ, Kuo CY, Luo WY, Lo KW, Huang CC, Chen CL, Yu SH, Hu YC.** 2012. Defective antiviral responses of induced pluripotent stem cells to baculoviral vector transduction. J. Virol. **86:**8041–8049. http://dx.doi.org/10.1128/JVI.00808-12.