Induction of type I interferon by adenovirus-encoded small RNAs

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Transduction with replication-incompetent recombinant adenovirus (Ad) vectors results in a rapid activation of innate immune responses, such as inflammatory cytokine production and subsequent tissue damage. The precise mechanisms of the innate immune responses induced by Ad vectors remain to be clarified. Possible components of Ad vectors that activate innate immune responses are the capsid protein, the viral genome (DNA), and viral transcripts. In the present study, we demonstrate that virus-associated RNAs (VA-RNAs), which are small RNAs transcribed by RNA polymerase III, induce the production of type I IFN (IFN-α and IFN-β), but they do not induce the production of inflammatory cytokines (IL-6 and IL-12), in mouse embryonic fibroblasts (MEFs) and granulocyte-macrophage colony-stimulating factor–generated bone marrow-derived dendritic cells (GM-DCs). We also show that IFN-β promoter stimulator-1 is involved in VA-RNA–dependent IFN-β production in MEFs and is partially involved in type I IFN production in GM-DCs. This study provides important insight into the mechanisms of Ad vector-triggered innate immune responses, which may lead to more advanced and rational Ad vector designs for gene therapies and vaccine applications.

innate immune response | IFN-β promoter stimulator-1 | virus-associated RNA

Replication-incompetent recombinant adenovirus (Ad) vectors are widely used for gene therapy experiments and clinical gene therapy trials. This is in great part due to the high efficiency with which such Ad vectors can transfer genes into a wide spectrum of dividing and nondividing cells in vitro and in vivo. However, Ad vector administration has in many cases been limited to local injection. One of the primary reasons for the limitation is that the systemic administration of first-generation (E1-deleted) Ad (FG-Ad) vectors triggers two types of response (i.e., adaptive and innate immune responses) (1). The adaptive immune responses against FG-Ad vectors occurs a few weeks after injection, and such responses are characterized by elevated anti-Ad antibody levels and the induction of cytotoxic T lymphocytes against Ad proteins and/or transgene products, which leads to multiorgan damage, including acute hepatic inflammation (2). To overcome this limitation, the molecular mechanisms of toxicity associated with adaptive immune responses have been extensively studied, and Ad vectors based on different species/serotypes and helper-dependent (gutted) Ad (HD-Ad) vectors devoid of almost all viral protein-coding sequences have been developed (3). Although the adaptive immune response to Ad vectors is well characterized, the mechanisms of the innate immune responses to Ad vectors remain somewhat controversial.

Induction of the antiviral innate immune response depends on the recognition of viral components by host pattern recognition receptors (PRRs). The most well-known PRRs are the Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) including lipopolysaccharides, double-stranded RNA (dsRNA), single-strand RNA (ssRNA), and unmethylated CpG DNA (reviewed in ref. 4). After the recognition of PAMPs by TLRs, all of the TLRs, with the exception of TLR3, transduce intracellular signaling through the adaptor protein MyD88, which initiates a signaling cascade leading to the activation of NF-κB and IFN regulatory factors (IRFs). We and others have reported that Ad vectors elicit the production of inflammatory cytokines such as IL-6 and IL-12 in a TLR9-dependent manner in conventional dendritic cells (cDCs) (5, 6). Zhu et al. (7) demonstrated that the Ad vector-induced production of type I IFN by cDCs is mediated by a TLR9-independent pathway, whereas the Ad vector-induced production of type I IFN by plasmacytoid DCs is mediated by a TLR9-dependent pathway. The signaling pathway for type I IFN production after Ad vector treatment is known to differ from that for inflammatory cytokine production.

In another PRR pathway, cytosolic RNAs are recognized by retinoic acid-inducible gene I (RIG-I)-like receptors, which include RIG-I and melanoma differentiation-associated gene 5 (Mda5) (8–10). RIG-I and Mda5 contain RNA helicase domains that recognize viral dsRNA (11, 12). In addition, RIG-I recognizes ssRNA containing 5′-triphosphate (13–15). RIG-I and Mda5 also contain N-terminal tandem caspase activation and recruitment domains (CARDs), which interact with the CARD domain of IFN-β promoter stimulator-1 (IPS-1, also known as MAVS, VISA, and Cardif) (16). This interaction finally activates several transcriptional factors (e.g., NF-κB, IRF3, and IRF7) and induces the production of inflammatory cytokines and type I IFN.

The possible components of FG-Ad vectors responsible for activating innate immune responses are capsid proteins, the viral genome (DNA), and viral transcripts. Comparative analysis using FG-Ad and HD-Ad vectors suggested that viral capsid proteins trigger the production of inflammatory cytokines and chemokines (17). The Ad genome possibly stimulates TLR9, leading to the production of inflammatory cytokines in certain cell types (5, 6). As regards viral transcripts, the Ad genome encodes two noncoding small RNAs, virus-associated (VA)-RNA I (major species, maximum 10^8 molecules per cell during viral replication) and VA-RNA II (minor species, maximum 5×10^6 molecules per cell during viral replication). VA-RNAs are synthesized at high levels during Ad replication (18). VA-RNAs are synthesized by RNA polymerase III, are ≈160 nucleotides long, and are structurally highly conserved. Previous studies have demonstrated that VA-RNAs induce the phosphorylation of eukaryotic initiation factor-2α via dsRNA-induced protein kinase R activation (19–21).

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However, the role played by VA-RNAs in the Ad vector-induced innate immune response has not yet been investigated.

In this report, we show that VA-RNAs activate a signaling pathway to induce the production of type I IFN but not the production of inflammatory cytokines. In addition, we examined the involvement of RIG-I, Mda5, and IPS-1 in the production of type I IFN induced by Ad vectors, and we demonstrate that Ad vectors activate the type I IFN pathway in an IPS-1–dependent as well as an IPS-1–independent manner, according to cell type.

Results

Type I IFN Production Is Largely Decreased in HD-Ad Vector–Transduced Cells. First, to examine the involvement of viral transcripts on Ad vector-triggered innate immune responses, we used FG-Ad and HD-Ad vectors, both of which express green fluorescent protein (GFP) under the control of the CMV promoter. FG-Ad vectors contain the viral genome but lack the E1 region, whereas in HD-Ad vectors all viral transcript-coding sequences have been deleted. We examined the production of type I IFN and inflammatory cytokines in mouse embryonic fibroblasts (MEFs) and granulocyte-macrophage colony-stimulating factor–generated bone marrow-derived dendritic cells (GM-DCs) (22) after transduction with FG-Ad or HD-Ad vectors. IL-6 and IL-12 are major cytokines induced by Ad vector treatment. FG-Ad vectors induced IFN-β production in MEFs from wild-type mice, whereas HD-Ad vectors failed to induce IFN-β production (Fig. 1*A*). Similarly, HD-Ad vectors mediated the decreased production of IFN-α and IFN-β in GM-DCs, as compared with FG-Ad vectors (Fig. 1*B*). Note that MEFs transduced with FG-Ad or HD-Ad vectors did not produce detectable levels of IFN-α, IL-6, and IL-12. In contrast, the HD-Ad vectors induced the same levels of IL-6 and IL-12 in GM-DCs as did the FG-Ad vectors (Fig. 1*B*). There was no significant difference in GFP expression levels and viral copy numbers in GM-DCs between FG-Ad and HD-Ad vectors (Fig. S1). These results suggest that factors other than the viral capsid protein, such as the viral genome and viral transcripts, are involved in Ad vector-induced type I IFN production.

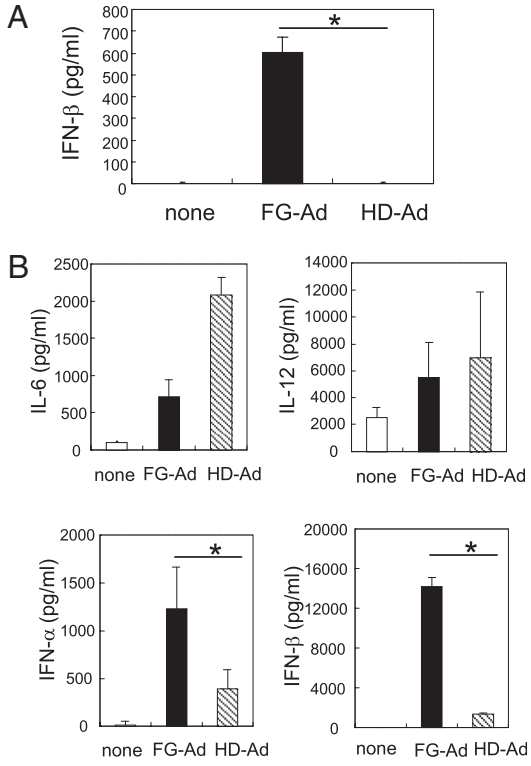


Fig. 1. Inflammatory cytokines and type I IFN production after transduction with FG-Ad and HD-Ad vectors. MEFs (*A*) and GM-DCs (*B*) were transduced with FG-Ad (Ad-GFP) or HD-Ad vectors (10,000 VP per cell) for 24 h, and the amount of IL-6, IL-12, IFN-α, and IFN-β in the culture supernatants was measured by ELISA. All data represent means ± SD ($n = 3$). *$P < 0.01$.

VA-RNAs Induce the Production of Type I IFN in MEFs and GM-DCs. To determine whether VA-RNAs play a role in type I IFN production by Ad vectors, we treated MEFs with ML-60218 (RNA polymerase III inhibitor) and then transduced the cells with FG-Ad vectors expressing luciferase (Ad-L2). IFN-β production in MEFs transduced with FG-Ad vectors was markedly reduced by ML-60218 treatment in a dose-dependent manner (Fig. 2*A*). In contrast, ML-60218 treatment had no effect on polyI:C-induced IFN-β production in MEFs (Fig. S2), suggesting that the ML-60218 effect was not due to the inhibition of IFN-β synthesis. Expression levels of VA-RNA I, a major VA-RNA, were indeed reduced by treatment with ML-60218 (Fig. 2*B*). Luciferase production in the cells remained unchanged even in the presence of ML-60218 (Fig. S3), indicating that the decreased IFN-β production had not been due to less-efficient transduction with the ML-60218 treatment. These results demonstrate that transcripts produced by RNA polymerase III are involved in type I IFN production by Ad vectors.

To analyze the molecular mechanisms of the innate immune responses caused by VA-RNAs, it is considered appropriate to use VA-RNAs–deleted Ad vectors. However, it remains difficult to generate VA-RNAs–deleted Ad vectors, because VA-RNAs play an important role in viral replication in infected cells (18). Our attempts to generate VA-RNAs–deleted Ad vectors with a deleted E1 region failed. Therefore, we prepared VA-RNAs–deleted Ad (sub720) but not Ad vectors. Sub720 is an Ad5 mutant lacking both the VA-RNA I and VA-RNA II genes but containing the E1 region (23). Viral replication of wild-type Ad and sub720 in MEFs and GM-DCs need not be taken into consideration, because even wild-type Ad does not replicate in mouse cells. Sub720 failed to induce IFN-β production in MEFs, whereas wild-type Ad induced IFN-β production (Fig. 3*A*). Similarly, GM-DCs infected with sub720 exhibited decreased production of IFN-α and IFN-β, compared with levels in cells infected with wild-type Ad (Fig. 3*B*). In contrast, wild-type Ad and sub720 yielded the same levels of IL-6 production in GM-DCs (Fig. 3*B*).

We next investigated the expression of VA-RNA I in MEFs infected with wild-type Ad. Northern blot analysis revealed that

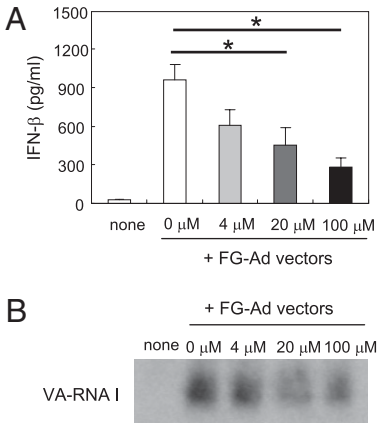


Fig. 2. RNA polymerase III inhibitor suppresses FG-Ad vector–mediated IFN-β production in MEFs. MEFs were treated with various concentration of ML-60218 for 12 h and were transduced with FG-Ad vectors (Ad-L2) (10,000 VP per cell) for 24 h. (*A*) Concentration of IFN-β in the cultured supernatants was measured by ELISA. All data are represented as means ± SD ($n = 3$). *$P < 0.01$. (*B*) Expression levels of VA-RNA I were measured by Northern blot analysis.

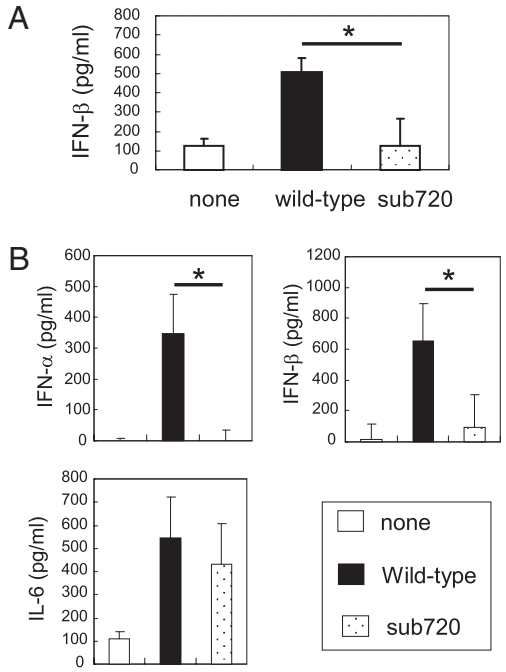


Fig. 3. Type I IFN production after transduction with wild-type Ad and sub720. MEFs (*A*) or GM-DCs (*B*) were infected with wild-type Ad (5,000 VP per cell) or sub720 (5,000 VP per cell) for 24 h, and the amount of IL-6, IFN-α, and IFN-β in the culture supernatants was measured by ELISA. $*P < 0.01$.

VA-RNA I was detectable 6 h after infection (Fig. S4*A*), and expression levels of VA-RNA I showed time-dependent increases. To examine the role of VA-RNAs on Ad-induced production of type I IFN at early time points after infection, quantitative RT-PCR analysis was performed 3 or 12 h after infection with wild-type Ad or sub720 in GM-DCs. There were no significant differences in mRNA expression levels (nearly background levels) of either IFN-α or IFN-β at 3 h with the wild-type Ad and sub720 (Fig. S4*B*). In contrast, sub720 failed to induce mRNA expression of IFN-α and IFN-β at 12 h in GM-DCs, whereas wild-type Ad strongly induced expression levels of both (Fig. S4*C*). These results indicate that VA-RNAs do activate the signaling pathways to induce type I IFN.

We also performed a flow cytometric analysis to examine the surface expression of CD86, a costimulatory molecule, on GM-DCs after infection with wild-type Ad or sub720. There was no significant difference in expression levels of CD86 between wild-type Ad and sub720 (Fig. S5), suggesting that VA-RNAs exerted no effect on DC maturation.

To further investigate whether VA-RNAs indeed play a role in type I IFN induction, we transfected MEFs with VA-RNAs expression plasmid (pAdVAntage) and then observed type I IFN production. pAdVAntage induced the production of type I IFN in MEFs, whereas pAdVAntageΔNaeI, in which the VA-RNAs–coding region in pAdVAntage is deleted, showed largely decreased production of type I IFN (Fig. S6*A*). We also examined whether VA-RNAs would elicit the expression of the IFN-stimulated genes (ISGs) 54 and ISG56. ISG54 and ISG56 are widely used as marker genes for detecting signaling by the IRF3/IRF7 signaling pathway. RT-PCR analysis revealed that pAdVAntageΔNaeI induced lower levels of ISG54 and ISG56 expression than did pAdVAntage (Fig. S6*B*). Taken together, these results indicate that VA-RNAs do induce the production of type I IFN and the expression of IFN-induced genes.

IPS-1 Is Crucial for FG-Ad Vector-Induced IFN-β Production in MEFs. To examine the involvement of TLRs, RIG-I, Mda5, and IPS-1 in Ad vector-induced type I IFN, we prepared MEFs of wild-type, MyD88-deficient, RIG-I–deficient, Mda5-deficient, and IPS-1–deficient mice and examined IFN-β production after transduction with FG-Ad vectors expressing luciferase (Ad-L2). After transduction with these FG-Ad vectors, the amount of IFN-β production was significantly increased in MEFs from wild-type mice. On the other hand, the amount of IFN-β was slightly lower in the FG-Ad vector–transduced MyD88-deficient MEFs than in wild-type MEFs (Fig. 4*A*), suggesting that IFN-β production is partially dependent on MyD88. RIG-I- and Mda5-deficient MEFs produced an amount of IFN-β relatively comparable to that of wild-type cells. On the other hand, IFN-β production in response to FG-Ad vectors was almost completely abolished in IPS-1–deficient MEFs. There was no reduction of *Cxcl10*(IP-10), *Ccl5* (RANTES), or IL-6 expression at any time point examined in IPS-1–deficient cells, whereas levels of induction of ISG54 and ISG56 in IPS-1–deficient cells were reduced compared with those of wild-type cells (Fig. S7). These results indicate that IPS-1 is crucial for the FG-Ad vector–elicited production of IFN-β and for the expression of ISGs in MEFs.

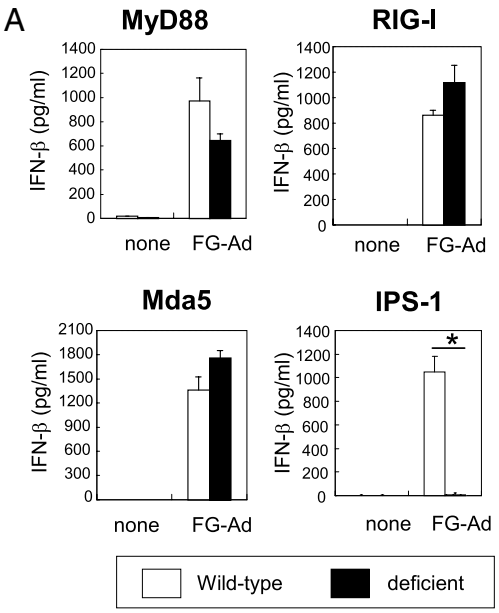
To further elucidate the signaling pathways downstream of IPS-1, we transduced wild-type and IPS-1–deficient MEFs with FG-Ad vectors and then stained the cells with anti-IRF3 antibody. IRF3 resides in the cytoplasm of nonstimulated cells. After stimulation with a pathogen, IRF3 translocates to the nucleus and regulates the expression of genes encoding IFN-β (24). Six hours after transduction, IRF3 was located in the nucleus of wild-type MEFs, whereas the nuclear translocation of IRF3 was impaired in IPS-1–deficient MEFs (Fig. 4*B*). These results indicate that the activation and nuclear translocation of IRF3 after transduction with FG-Ad vectors are dependent on IPS-1.

FG-Ad Vectors Elicit Inflammatory Cytokines and Type I IFN Production via IPS-1–Dependent and IPS-1–Independent Mechanisms in GM-DCs. To examine the role of IPS-1 in Ad vector–induced inflammatory cytokine and type I IFN production in GM-DCs, we prepared GM-DCs from the bone marrow cells of wild-type and IPS-1–deficient mice and examined the production of IL-6, IL-12, IFN-α, and IFN-β after transduction with FG-Ad vectors. FG-Ad vectors induced the production of IL-6, IL-12, IFN-α, and IFN-β in GM-DCs from wild-type mice (Fig. S8*A*). IPS-1–deficient GM-DCs produced IL-6 and IL-12 in amounts relatively comparable to those of wild-type cells. On the other hand, levels of FG-Ad vector–induced IFN-α and IFN-β production were slightly lower in IPS-1–deficient GM-DCs than in the wild-type DCs. Taken together, these results indicate that IPS-1 participates to some extent in FG-Ad vector–induced type I IFN production in GM-DCs.

We next performed a flow cytometric analysis to examine the surface expression of CD86 on GM-DCs after transduction with FG-Ad vectors. After transduction with FG-Ad vectors, the wild-type DCs showed significantly increased expression of the maturation marker CD86 (Fig. S8*B*). IPS-1–deficient GM-DCs expressed amounts of CD86 comparable to wild-type cells in response to FG-Ad vectors. The levels of CD40 and CD80 expressed at the cell surface were also similar to those of CD86. These results indicate that FG-Ad vectors induce the maturation of GM-DCs via an IPS-1–independent pathway.

Discussion

The major limitation of the use of Ad vectors as gene therapy vectors is the innate immune response, which causes inflammatory cytokine production and tissue damage. However, the precise mechanism of the Ad vector-triggered innate immune response remains to be clarified. Components of FG-Ad vectors that are thought to activate innate immune responses are the capsid protein, the viral genome (DNA), and viral transcripts. In the present study we focused on the role of Ad-encoded small RNAs, VA-RNAs. Previous studies have demonstrated that VA-RNAs induce the phosphorylation of eukaryotic initiation factor-



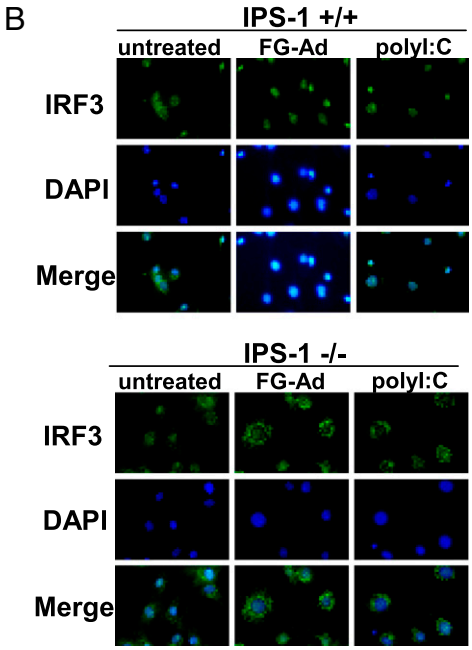


Fig. 4. IPS-1–dependent activation of MEFs after transduction with FG-Ad vectors. (*A*) MEFs from wild-type, MyD88-deficient, RIG-I–deficient, Mda5-deficient, and IPS-1–deficient mice were transduced with FG-Ad vectors (Ad-L2) (10,000 VP per cell) for 24 h, and the amount of IFN-β in the culture supernatants was measured by ELISA. All data are represented as means ± SD ($n = 3$). *$P < 0.01$. (*B*) Wild-type or IPS-1–deficient MEFs were stimulated with FG-Ad vectors (Ad-L2) (10,000 VP per cell) or polyI:C (5 μg/mL) for 6 h. Then, cells were washed and fixed, followed by staining using anti-IRF3 antibody and nuclear staining with DAPI. Cells were visualized by fluorescence microscopy.

2α via dsRNA-induced protein kinase R activation (19–21). However, the roles played by VA-RNAs in the Ad vector-induced innate immune response have not been elucidated. In the present study we found that VA-RNAs induce the production of type I IFN in MEFs and GM-DCs.

The production of type I IFN in MEFs was completely dependent on IPS-1 (Fig. 4*A*), whereas that in GM-DCs was partially dependent on IPS-1 (Fig. S8*A*). RIG-I and Mda5 are upstream activators of IPS-1 signaling. Analyses using RIG-I– or Mda5-deficient MEFs revealed that IFN-β production in these mutant cells was comparable to that in wild-type cells. There are several explanations for this phenomenon. The induction of type I IFN by Ad vectors may be mediated by both RIG-I and Mda5. Some RNA viruses, such as West Nile virus and reovirus, are recognized by both RIG-I and Mda5 (25, 26). VA-RNAs are expected to have a triphosphorylated 5′ end. The 5′ triphosphate ssRNA as well as dsRNA are recognized by RIG-I (13–15). Therefore, VA-RNAs should be recognized by RIG-I, which would in turn activate the signaling pathway for type I IFN production via IPS-1. It remains unknown how RIG-I and Mda5 recognize VA-RNAs. It is also possible that Ad vectors are recognized by laboratory of genetics and physiology-2 (LGP2), although it is not known that LGP2 itself recognizes nucleic acids and induces signaling to type I IFN. LGP2 contains RNA helicase domains that recognize viral dsRNA, although LGP2 lacks a CARD (9). LGP2 was originally identified as a negative regulator of RIG-I-like receptors (9). Recently, LGP2 was reported to be essential for type I IFN production in response to RNA virus infection and facilitate viral RNA recognition by RIG-I and Mda5 (27, 28). As other possibilities, it is possible that Ad vectors are recognized by LRRFIP1 and/or unidentified molecular sensor(s), and the signaling pathway for type I IFN production via IPS-1 might be activated. Recently, the cytosolic nucleic acid–binding protein LRRFIP1 was reported to contribute to the production of IFN-β induced by dsRNA and dsDNA in macrophages (29). Further studies are clearly needed to obtain a detailed account of the mechanisms of action of Ad vectors recognized by PRRs.

Expression of VA-RNAs was undetectable 3 h after wild-type Ad infection but began to be detectable at 6 h (Fig. S4*A*), at which point VA-RNAs would be recognized by RIG-I/Mda5 and/or unidentified molecular sensor(s) and the signaling pathway for type I IFN production via IPS-1 would be activated. Therefore, in this context, the induction of type I IFN production is thought to be delayed. This conclusion would be consistent with the observation that the production of IFN-α is delayed compared with that of IL-6 (30). In our previous study, IL-6 and IL-12 were detected in the serum within 3 h after i.v. injection of the Ad vectors (31), whereas type I IFN was undetectable even at 6 h after injection (30). Zhu et al. (32) reported a maximum time point for the detection of type I IFN production at 12 h after i.v. injection of Ad vectors. Activation of innate immune responses by viral transcripts (i.e., VA-RNAs) would be slower than that induced by viral capsid proteins and the viral genome. The production of inflammatory cytokines might be primarily mediated by viral capsid proteins or the viral genome, whereas that of type I IFN would be primarily mediated by VA-RNAs. Fejer et al. (33) reported that Ad vectors induce type I IFN and IL-6 in vivo by distinct pathways.

We and other groups have reported that cDCs produce inflammatory cytokines in a TLR9-dependent manner (5, 6). Recently, Muruve et al. (34) proposed that the NLRP3 inflammasome may recognize cytosolic dsDNA, including the Ad genome, and trigger the production of IL-1β and IL-18. Our data revealed that VA-RNAs do not induce the production of inflammatory cytokines (Fig. 3*B*). These results suggest that the Ad genome might be recognized by TLR9 and/or NLRP3 and activate signaling to inflammatory cytokines and IL-1β.

Our results show that the production of type I IFN is partially dependent on IPS-1 in GM-DCs (Fig. S8*A*), suggesting that other mechanisms than the IPS-1 signaling pathway might be involved in the Ad vector–induced type I IFN production in GM-DCs. Ad contains dsDNA in the genome. Recently, three cytosolic DNA sensor molecules, DNA-dependent activator of IFN-regulatory factors (DAI; also referred to as DLM-1/ZBP1), AIM2, and LRRFIP1, were identified (29, 35–38). However, a DAI deficiency does not substantially impair the production of type I IFN induced by cytosolic B-form DNA in certain types of primary cells of the immune system, which indicates the presence of additional

DNA sensors (39, 40). AIM2 was reported to be a cytosolic DNA sensor that activates inflammasomes and caspase-1 (35, 37, 38). The activation of inflammasomes by dsDNA leads to the proteolytic processing of pro-IL-1β, resulting in the release of IL-1β and the subsequent activation of a cascade of inflammatory cytokines and chemokines in an IL-1R–dependent manner. However, AIM2 is not involved in type I IFN induction by cytosolic DNA (41). Therefore, DAI and AIM2 are not thought to be involved in the activation of the pathway for the signaling of type I IFN by Ad vectors. The viral genome (DNA) and/or VA-RNAs might be recognized by LRRFIP1 or unidentified molecule(s).

This study demonstrated that Ad vectors stimulate innate immunity via IPS-1–dependent and IPS-1–independent pathways according to cell type (Fig. 4 and Fig. S8), suggesting that the mechanisms of Ad vector–mediated innate immune responses are more complicated than expected. Here, MEFs produced IFN-β via IPS-1–dependent pathways after transduction with Ad vectors; in contrast, IPS-1 is not essential for type I IFN production in GM-DCs. It is possible that there is cell type–specific utilization of these pathways. For example, the TLR signaling pathway is involved in Ad vector–mediated inflammatory cytokine production in DCs but has little or no corresponding role in peritoneal macrophages (6).

VA-RNAs exhibit extensive structural similarity to Epstein-Barr virus–encoded RNAs (EBERs), which are similarly small, untranslated RNAs. EBERs also induce the phosphorylation of eukaryotic initiation factor-2α via protein kinase R activation. In addition, Samanta et al. (42) have reported that EBERs are recognized by RIG-I and activate signaling to induce type I IFN. Therefore, VA-RNAs as well as EBERs should be recognized by PRRs and trigger the production of type I IFN.

The development of Ad vectors lacking VA-RNAs expression should lead to decreased stimulation of innate immune responses and therefore to more successful gene therapies. However, it is still difficult to generate VA-RNAs–deleted Ad vectors, because VA-RNAs play an important role in viral replication in infected cells (18). The development of VA-RNAs–deleted Ad vectors remains an exciting issue for future studies.

In summary, we found that VA-RNAs induce the production of type I IFN but not that of inflammatory cytokines. We also demonstrated that IPS-1 is crucial for Ad vector-induced IFN-β production in MEFs. VA-RNAs are likely to be recognized by PRRs, which in turn activates the signaling pathway for type I IFN production via IPS-1. The present study provides important insights into the mechanisms of Ad vector-triggered innate immune responses. Further examination of the intracellular pathways of Ad vector-triggered innate immune responses may lead to the development of Ad vectors with safer profiles, as well as to the generation of increasingly successful gene therapies.

Materials and Methods

Ad Vectors. The FG-Ad vector expressing luciferase or GFP (Ad-L2 and Ad-GFP, respectively) was previously constructed (43, 44). Briefly, the CMV promoter-driven luciferase gene derived from pGL3-Control (Promega) or the GFP gene derived from pEGFP-N1 (Clontech), respectively, were inserted into the E1 deletion region of the Ad genome. These viruses were grown in 293 cells by standard techniques. The HD-Ad vector encodes an expression cassette identical to that encoded by Ad-GFP but lacks all Ad sequences except for the left- and right-inverted terminal repeat and the viral packaging signal. To generate HD-Ad vectors, the CMV promoter-driven GFP expression cassette was inserted into the NotI site of pSTK129 (45), resulting in pSTK129-GFP. Then, pSTK129-GFP was cut with PmeI and the liberated viral genome was transfected into 60-mm dishes containing 116 cells expressing Cre recombinase. The HD-Ad vectors were prepared as described previously (45). Helper-virus AdNGR163, pSTK129, and 116 cells were kindly provided by Dr. Philip Ng (Baylor College, Houston, TX) (45). Helper-virus contamination of the HD-Ad vectors was assessed by quantitative real-time PCR, and the level of contamination was estimated to be less than 0.1%.

FG-Ad and HD-Ad vectors were purified with $CsCl_2$ step-gradient ultracentrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol and sorted in aliquots at −80 °C. Determination of virus particle titers (VP) was accomplished spectrophotometrically according to the method of Maizel et al. (46). The virus stocks were free of endotoxin (<0.1 endotoxin units/mL), as determined by the use of a Limulus Color KY Test (Wako Pure Chemical Industries).

VA-RNA Mutant Virus. Sub720 lacks the expression of both VA-RNA I and VA-RNA II (23). The virus stock was propagated in 293T cells. Wild-type Ad type 5 was obtained from ATCC, and mutant sub720 was kindly provided by Dr. Goran Akusjarvi (Uppsala University, Uppsala, Sweden).

Cells. The RIG-I–deficient, Mda5-deficient, and IPS-1–deficient MEFs and matched wild-type controls used here have been described previously (27, 47). The MEFs were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin. The GM-DCs were prepared as follows. Bone marrow cells were prepared from the femurs and tibiae of wild-type and IPS-1–deficient mice. These cells were cultured in RPMI 1640 containing 10% FBS and 20 ng/mL murine granulocyte-macrophage colony-stimulating factor (PeproTech). On day 8, weakly adherent cells were isolated, and 85–90% of the cells were confirmed to be $CD11c^+$ DCs by FACS analysis.

Inflammatory Cytokines and Type I IFN Measurement. MEFs were pretreated with PolIII inhibitor, ML-60218 (Calbiochem), for 12 h and then were transduced with FG-Ad vectors at 10,000 VP per cell for 24 h. GM-DCs and MEFs (5×10^5 cells per well) seeded on 24-well plates were transduced with FG-Ad vectors (10,000 VP per cell), HD-Ad vectors (10,000 VP per cell), wild-type Ad (5,000 VP per cell), or sub720 (5,000 VP per cell). After 24 h incubation, the culture supernatants were collected and analyzed for inflammatory cytokine and type I IFN levels by ELISA. ELISA kits for IL-6 and IL-12 were purchased from R&D Systems. IFN-α and IFN-β were purchased from PBL Biomedical Laboratories. ELISA was performed according to the manufacturer's instructions. Cytotoxicity was checked by alamarBlue (BioSource) staining. No cytotoxicity was observed after transduction with FG-Ad or HD-Ad vectors at 10,000 VP per cell.

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