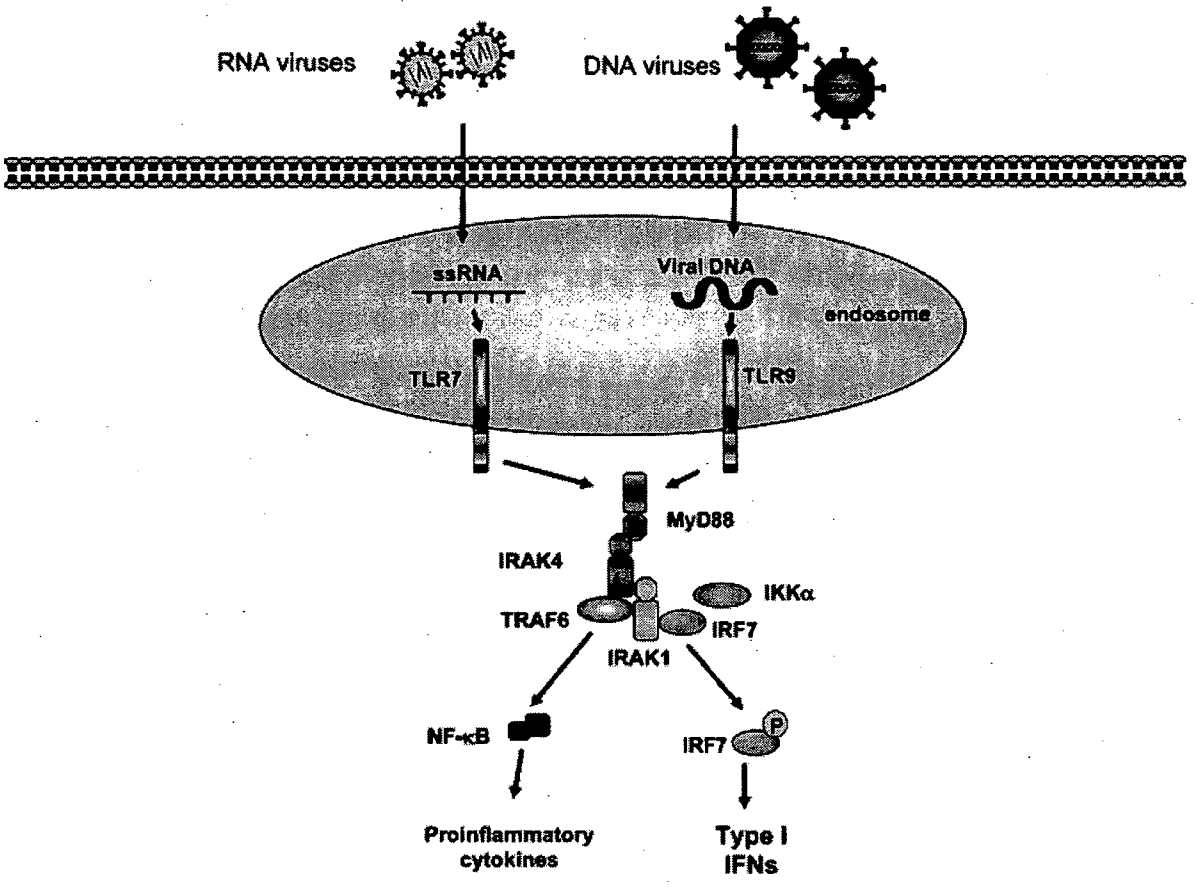

Fig. 2. TLR7 and TLR9 signaling in pDCs. ssRNA and DNA from viruses are recognized by TLR7 and TLR9, respectively. These TLRs recruit MyD88 and activate IRAK-4, IRAK-1, and TRAF6, resulting in activation of the IKK complex and nuclear translocation of NF-κB, which in turn initiates the expressions of pro-inflammatory cytokine genes. IRAK-1 and IKK-α activates IRF-7 together with TRAF3 and IKK-α, and induces nuclear translocation of IRF-7. Finally, transcription of type I IFN genes occurs. TLR, Toll-like receptor; IRF-3, interferron-regulatory factor-3; pDCs, plasmacytoid dendritic cells; TRAF6, TNF-receptor associated factor 6; IRAK-4, interleukin-1R-associated kinase-4; IKK, inhibitor of NF-κB (IκB) kinase; TNF, tumor necrosis factor.

high levels of TLR7 and TLR9, which recognize ssRNA and unmethylated DNA with CpG motifs (CpG-DNA), respectively (62–64). In pDCs, DNA viruses, including herpes simplex virus-1 (HSV-1), HSV-2, and mouse cytomegalovirus, are recognized by TLR9, while several RNA viruses, such as influenza virus, are recognized by TLR7 (62, 63, 65–67) (Fig. 2). TLR7 is also known to recognize synthetic nucleotide analogs, called imidazoquinolines (68). $TLR7^{-/-}$ and $TLR9^{-/-}$ mice did not produce type I IFNs and pro-inflammatory cytokines in response to ssRNA and CpG-DNA, respectively.

TLR7 and TLR9 signal through a TIR domain-containing adapter, myeloid differentiation factor 88 (MyD88), not through TRIF (64, 68) (Fig. 2). Upon exposure to ssRNA and CpG-DNA, MyD88 forms a complex with IL-1R-associated kinase-4 (IRAK-4), IRAK-1, TRAF3, TRAF6, IKK-α, and IRF-7, and this complex is recruited to the TLR (29, 30, 69–72). Downstream of this signaling complex, transcription factors IRF-7, and NF-κB become activated, followed by the induction of type I IFNs and cytokines.

MyD88, an adapter protein with a death domain (DD) and the TIR domain, plays essential roles in the signaling pathways of all known TLRs, with the exception of TLR3. pDCs from $MyD88^{-/-}$ mice did not produce type I IFNs and pro-inflammatory cytokines in response to TLR7 and TLR9 ligands (69). Furthermore, $MyD88^{-/-}$ pDCs showed severely impaired production of type I IFNs against ssRNA virus infection (10). On the contrary, pDCs lacking IPS-1 still exhibited normal production of type I IFNs in response to RNA viruses, indicating that the TLR system but not the RLH system plays essential roles in the recognition of viruses in pDCs (27).

IRAKs are comprised of an N-terminal DD and a C-terminal serine/threonine kinase domain. IRAKs interact with MyD88 through the DD. Analysis of $IRAK\text{-}4^{-/-}$ mice revealed that IRAK-4 was essential for the production of type I IFNs and

cytokines in response to TLR7 and TLR9 ligands (73). Recently, the IRAK-4 kinase activity was found to be essential for TLR9 responses in pDCs and other cell types (74, 75). In contrast to IRAK-4, IRAK-1 specifically controls the production of type I IFNs but not cytokines. IRAK-1 was able to phosphorylate IRF-7, which then translocated to the nucleus and initiated the expressions of type I IFN genes (71). TRAF3 and IKK-α were also responsible for the activation of IRF-7 but not NF-κB (29, 30, 72). In contrast, pDCs lacking both TBK1 and IKK-i were still capable of producing IFN-α in response to TLR9 and an RNA virus, indicating that pDCs utilize a unique signaling pathway for the production of IFNs (38).

Osteopontin was found to co-localize with MyD88 in the cytoplasm and to be required for IFN-α production in response to TLR9 stimulation (76). An ER membrane protein, UNC-93B, was identified as an essential molecule for signaling by TLR3, TLR7, and TLR9 by forward genetic screening of mice (77). Recently, an autosomal recessive mutation in UNC-93B in humans was found to result in impaired immune responses against HSV-1 encephalitis (78).

In addition to pDCs, mouse cDCs and macrophages respond to CpG-DNA and produce small amounts of IFN-β. TLR9 and MyD88 are also responsible for the recognition of CpG-DNA in cDCs. However, they utilized IRF-1, not IRF-3 or IRF-7, as the transcription factor for IFN-β mRNA expression (79, 80).

There are several possible hypotheses explaining why pDCs can secrete high levels of type I IFNs. One hypothesis is that IRF7 is expressed at a high level in pDCs, in contrast to its inducible expression in other cell types. Another hypothesis is that TLR ligands stay in the endosomal vesicles of pDCs for a longer time compared with cDCs, where the ligand is rapidly degraded by fusion to the lysosome (81). Such a long retention time can facilitate encounters between TLRs and their ligands. However, further studies are required to clarify the precise mechanisms of the high level of IFN production in pDCs.

Autophagy was recently shown to be responsible for the induction of type I IFNs in response to VSV and HSV infection. Although autophagy is an evolutionarily conserved mechanism for nutrient acquisition during starvation, it was found that autophagosomes also functioned to eliminate cytoplasmic bacteria (82). Furthermore, some ssRNA viruses, such as VSV, appeared to require live virus infection to produce type I IFNs. Autophagosomes were constitutively formed in pDCs, and pDCs lacking ATG5, a gene critical for autophagosome formation, showed severely impaired production of type I IFNs in response to VSV and HSV infection, indicating that autophagosome formation is critical for the recognition of some viruses in pDCs (83). Because TLR7 is responsible for the production of type I IFNs in response to VSV infection in pDCs, autophagosomes-containing VSV components may fuse to organelles containing TLR7.

The possible requirement of live virus infection for IFN production in pDCs remains unclear. Various viruses, including HSV and influenza, can induce type I IFN production in pDCs, even when the viruses are inactivated by ultraviolet irradiation or heat treatment. Given that endosome-localized TLR7 and TLR9 are responsible for IFN production in pDCs, it is quite simple to hypothesize that endocytosed virus particles are directly recognized by TLRs after fusion between the early endosome and the TLR-containing endosome/lysosome. Thus, it is not clear whether autophagosome-mediated recognition in pDCs is generalized to various RNA viruses.

Type I IFN-producing cells in response to viral infection

As described above, various innate immune cellular receptors recognize viruses infecting different cell types, produce type I IFNs, and evoke coordinated anti-viral responses in the body. However, most studies have been carried out by stimulating immune cells *in vitro*, and the cell types responsible for type I IFN production *in vivo* are not well understood. To clarify how the production of type I IFNs, especially IFN-α isoforms, is controlled during *in vivo* responses to virus infection, we established a reporter mouse strain that expresses green fluorescence protein (GFP) under the control of the IFN-α6 promoter (Osamu Takeuchi, Shizuo Akira, manuscript submitted). Interestingly, the expression of IFN-α6 was co-regulated with that of various IFN-α isoforms and IFN-β in NDV-infected pDCs. Examination of IFN-α6-producing cells against systemic NDV infection revealed that not only pDCs but also cDCs and macrophages contributed to the IFN-α production, although the frequency of GFP^+ cells was higher among pDCs than among the other cell types. Consistent with previous observations, $MyD88^{-/-}$ pDCs showed severely impaired GFP upregulation, whereas $IPS\text{-}1^{-/-}$ cDCs and macrophages failed to increase GFP expression (Fig. 3).

However, when the same virus was infected nasally, the contribution of pDCs to type I IFN production was diminished. Lung infection with NDV resulted in an increase in IFN-α-producing cells among alveolar macrophages and cDCs but not among pDCs. Again, the IFN-α production in alveolar macrophages and cDCs was dependent on IPS-1 (Fig. 3). pDCs started to produce IFN-α isoforms when alveolar macrophages were depleted or IPS-1 was absent, suggesting that pDCs function when the first line of defense is broken. Therefore, systemic and local RNA virus infections activate different

cell types to produce IFN-α isoforms. Although the importance of pDCs has been emphasized, these observations indicate that macrophages and cDCs play critical roles in IFN-α production, particularly during the course of local RNA virus infection.

All the IFN-α6-producing cell types identified *in vivo* using Ifna6-GFP mice were of hematopoietic origin, whereas even fibroblasts are able to express the IFN-α gene in response to viral infection *in vitro*. Thus, it will be interesting to explore whether non-hematopoietic cells contribute to anti-viral host defenses as type I IFN-producers. Furthermore, this mouse model will be useful for identifying IFN-α-producing cells in response to different virus infections through various routes.

Subversion of immune signaling by viruses

Viruses have evolved ways of suppressing the host immune responses, thereby facilitating infection. The RLH and TLR signaling pathways are targets for suppression by viral proteins, due to the importance of type I IFNs in eliminating viral infection. RIG-I-mediated recognition of triphosphate RNA was found to be suppressed by the presence of NS1 (15). In poliovirus-infected cells, MDA5 was reportedly degraded in a proteasome- and caspase-dependent manner (84). Paramyxovirus V protein was shown to interact with MDA5 and suppress IFN responses, although paramyxoviruses were recognized by RIG-1, rather than by MDA5 (85). A hepatitis C virus protease, NS3/4A, was found to cleave IPS-1 and also TRIF (25, 86, 87). This cleavage of IPS-1 dislodges it from the mitochondria, thereby blocking the activation of IRFs and NF-κB by virus infection. TLR7- and TLR9-mediated type I IFN production in pDCs was inhibited by respiratory syncytial virus and measles virus (88), while vaccinia virus A46R suppressed TLR3-mediated type I IFN responses by binding to TRIF (89, 90). In general, pathogenic viruses tend to produce quite low amounts of type I IFNs. Mutation of IFN suppressors converts the viruses to high IFN producers, and the mutated viruses lose their pathogenicity.

Type of infection	IFN-α producing cell	Receptors
Systemic	pDC	TLR
	cDC Macrophage	RLH
Respiratory	Alveolar macrophage cDC	RLH

Fig. 3. IFN-α producing cells in response to systemic and local viral infection. In response to systemic RNA virus infection, pDCs play an important role in the production of IFN-α, although cDCs and macrophages are also involved. pDCs and other cell types utilize TLRs and RLHs for inducing type I IFNs, respectively. Respiratory infection with the same virus activates alveolar macrophages and cDCs, but not pDCs, for inducing type I IFNs. TLR, Toll-like receptor; IFN, interferon; RLH, retinoic acid-inducible gene I (RIG-I)-like helicases; pDCs, plasmacytoid dendritic cells.

Role of NLRs in the recognition of viral components

NLR proteins are comprised of tripartite motifs, namely a central nucleotide-binding domain, C-terminal LRRs, and an N-terminal signaling domain that contains CARDs, a Pyrin domain, or baculovirus IAP repeats (91, 92). Cryopyrin/NALP3 contains an N-terminal Pyrin domain, and it was reported to recognize ssRNA and dsRNA in the cytoplasm. Cryopyrin/NALP3 forms a complex with an adapter protein, apoptosis-associated speck-like protein containing a caspase-activating and recruitment domain (ASC), and caspase-1. This complex is responsible for the processing of pro-IL-1β to its mature form (92). Macrophages from cryopyrin/NALP3$^{-/-}$ and ASC$^{-/-}$ mice showed severely impaired production of IL-1β in response to poly(I:C) or imidazoquinoline treatment in the presence of ATP, accompanied by defective activation of caspase-1 (93, 94). It was reported that TLR deficiency did not affect caspase-1 cleavage in response to cognate ligands. However, it remains unclear whether cryopyrin/NALP3 directly recognizes dsRNA and imidazoquinoline in the cytoplasm, because no direct associations have been reported. Furthermore, cryopyrin/NALP3 is required for caspase-1 activation following stimulation with uric acid crystals or Gram-positive bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (95–97). In addition, cell surface P2X7 ATP receptor, which induces depletion of intracellular K^+, plays an essential role in caspase-1 activation after stimulation with TLR ligands in the presence of ATP (98). These observations suggest that the caspase-1 activation induced by poly(I:C) and imidazoquinoline is not due to direct recognition of RNAs by cytoplasmic NLRs.

Perspectives

In this review article, we have described the roles of innate immune receptors that recognize viruses and their signaling pathways. Several classes of receptors play roles in the recognition of RNA virus infections. RLHs and TLRs are essential for RNA virus recognition in different cell types, and analysis of Ifna6-GFP reporter mice revealed that the types of IFN-producing cells depend on signaling by either RLHs or by

TLRs. Thus, the molecular mechanism of type I IFN production after RNA virus infection appears to be fully explained by currently known receptor systems. However, the recognition mechanism for DNA viruses still remains to be clarified. Although TLR9 is responsible for the detection of DNA viruses in pDCs, other cell types induce type I IFN production independently of the TLR system. Accumulating evidence indicates that dsDNA, in addition to RNA, is recognized in the cytoplasm in a TLR9-independent fashion. Introduction of synthetic dsDNA into cells leads to the expression of type I IFN genes in a TBK1/IKK-i-dependent manner (18). Furthermore, mice deficient in DNase II, in which accumulation of unprocessed DNA is observed in macrophages, show aberrant expression of IFN-β (99). Identification of additional receptors for the recognition of DNA viruses will be required to complete the entire picture of innate viral recognition.

Although the contributions of innate immune cells and receptors responsible for the recognition of RNA viruses are almost clarified, it is not known how these systems control the activation of acquired immune responses. There are reports showing a contribution of the TLR system to antigen-specific T-cell activation, but the roles of RLHs are not understood. Future analyses of how innate immune receptors differentially control the development of acquired immunity will be required to fully understand mammalian anti-viral immunity *in vivo*.

Overproduction of type I IFNs has been correlated with the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) (100). In this disease, anti-nuclear antibodies are frequently detected. Antibodies against nuclear products, such as dsDNA, histones, and ribonucleoproteins, can act as ligands for TLR7 and TLR9, thus implicating TLRs as a cause of SLE. However, in a spontaneous mouse SLE model, TLR9 deficiency deteriorated the disease, whereas TLR7 deficiency modestly improved the disease (101). Thus, nucleotide detectors other than TLRs are possibly involved as the cause of the disease. Future studies on the roles of RLHs and NLRs will clarify the contributions of innate immune receptors to the pathogenesis of autoimmune diseases.

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Alveolar Macrophages Are the Primary Interferon-α Producer in Pulmonary Infection with RNA Viruses

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DOI 10.1016/j.immuni.2007.07.013

SUMMARY

Type I interferons (IFNs) are critical for antiviral responses. Here we generated a knockin mouse in which green fluorescence protein (GFP) was expressed under the control of the *Ifna6* promoter. Virus-induced expression of GFP recapitulated various IFN-α subtypes. Systemic infection of the mice with Newcastle disease virus (NDV) increased GFP$^+$ plasmacytoid dendritic cells (pDCs) via the Toll-like receptor system, and GFP$^+$ conventional dendritic cells (cDCs) and macrophages via the RIG-I-like helicase system. By contrast, lung infection with NDV led to IFN-α production in alveolar macrophages (AMs) and cDCs, but not in pDCs. Specific depletion of AMs caused a marked defect in the initial viral elimination in the lung. pDCs produced IFN-α in the absence of AM-mediated viral recognition, suggesting that pDCs function when the first defense line is broken. Thus, AMs act as a type I IFN producer that is important for the initial responses to viral infection in the lung.

INTRODUCTION

The innate immune system senses viral invasion and evokes quick responses by producing various cytokines. Among them, type I interferons (IFNs) are pleiotropic cytokines essential for antiviral immune responses. They are comprised of multiple IFN-αs and single IFN-β, and other members such as IFN-ω, -ε, and -κ (Honda et al., 2006). Humans and mice have more than 13 IFN-α family members. Type I IFNs induce apoptosis of virus-infected cells and cellular resistance to viral infection, and also activate natural killer (NK) and T cells (Stetson and Medzhitov, 2006). Thus, type I IFNs have an important role not only in the innate antiviral responses, but also in the activation of the adaptive immune system.

Two innate immune receptor families, Toll-like receptors (TLRs) and RIG-I-like helicases (RLHs), have been shown to recognize viral components and induce type I IFNs (Akira et al., 2006). The TLR system senses various viral components, including double-stranded RNA (dsRNA) single-stranded RNA (ssRNA), and unmethylated DNA with CpG motifs via TLR3, TLR7, and TLR9, respectively. TLR3 triggers signaling cascades via an adaptor protein, the Toll-IL-1 receptor (TIR) domain containing adaptor-inducing IFN-β (TRIF), which activates two IκB kinase (IKK)-related kinases, TANK-binding kinase 1 (TBK1) and inducible IKK (IKK-*i*). These kinases are known to directly phosphorylate transcription factors of IFN regulatory factor 3 (IRF-3) and IRF-7 (Kawai and Akira, 2006). These transcription factors then form a dimer, translocate to the nucleus, and activate the transcription of type I IFNs and IFN-inducible genes. On the other hand, TLR7 and TLR9 activate IRF-7 via an adaptor, MyD88 (Honda et al., 2004; Kawai et al., 2004), IL-1R-associated kinase 1 (IRAK1) (Uematsu et al., 2005), and IKK-α (Hoshino et al., 2006), but not TBK1 or IKK-*i*.

The RLH family is comprised of the retinoic acid-inducible gene I (RIG-I), the melanoma differentiation-associated gene 5 (MDA5), and Lgp2 (Akira et al., 2006). RIG-I and MDA5, but not Lgp2, contain caspase-recruit domains (CARDs) in addition to a RNA helicase domain. RIG-I is responsible for detection of various RNA viruses (Kato et al., 2005; Yoneyama et al., 2004), in vitro transcribed dsRNA (Kato et al., 2006), and 5′-triphosphate RNA (Hornung et al., 2006; Pichlmair et al., 2006), whereas MDA5 recognizes picornaviruses and polyinosinic polycytidylic acid [poly (I:C)] (Kato et al., 2006). RIG-I and MDA5 activate TBK1 and IKK-*i* via a CARD domain containing IFN-β promoter stimulator-1 (IPS-1), an adaptor also known as MAVS, CARDIF, or VISA (Kawai et al., 2005; Kumar et al., 2006; Meylan et al., 2005; Seth et al., 2005; Sun et al., 2006; Xu et al., 2005).

Although various cells are reported to have the potential to produce type I IFNs when exposed to viruses in vitro,

stimulation of human peripheral blood mononuclear cells with viruses has revealed that plasmacytoid dendritic cells (pDCs), a rare subset of dendritic cells (DCs), are the major producer of type I IFNs (Cella et al., 1999; Siegal et al., 1999). Subsequently, a mouse counterpart of human pDC was identified based on the surface expression of CD11c and B220 (Asselin-Paturel et al., 2001; Bjorck, 2001; Nakano et al., 2001). It has been shown by ex vivo experiments that the TLR system is responsible for the secretion of type I IFNs in pDCs (Kato et al., 2005). pDCs produced IFN-α irrespective of the presence of type I IFN receptor (Barchet et al., 2002; Prakash et al., 2005). In contrast, conventional DCs (cDCs) and fibroblasts produce type I IFNs in response to viral infection in vitro in a RLH-dependent fashion (Diebold et al., 2003; Kato et al., 2005). These cell types produce IFN-α via type I IFN receptor-mediated positive feedback. IFN-producing killer dendritic cells (IKDCs) are also reported to produce type I IFNs (Chan et al., 2006; Taieb et al., 2006), although this has been questioned in a recent report (Vremec et al., 2007). However, the contribution of each cell type to the production of type I IFNs in vivo remains to be determined. Additionally, the in vivo role of the two abovementioned viral detector systems in the production of type I IFNs is unknown.

Currently used methods have not succeeded in monitoring the expression of type I IFNs in vivo. For instance, enzyme-linked immunosorbent assay (ELISA) is not suitable for detecting type I IFNs at a single-cell level. Although intracellular staining of IFN-α is sensitive enough to detect IFN-α expression in ex vivo experiments, it is difficult to monitor IFN-α-producing cells in vivo. Therefore, we generated a reporter mouse strain in which the coding sequence of the *Ifna6* gene was replaced by the GFP coding sequence. Comparison between GFP expression and intracellular IFN-α staining, and quantitative real-time PCR (Q-PCR) analysis for the expression of multiple IFN-α genes in GFP$^+$ cells, revealed that this reporter recapitulated the expression of various IFN-α genes. pDCs were the sole producer of IFN-α in response to TLR7 and TLR9 ligand inoculation, whereas cDCs were the main producer in response to poly (I:C), the TLR3-MDA5 ligand. In response to systemic RNA virus infection, not only pDCs, but also cDCs, macrophages, and monocytes, produced IFN-α. The TLR system was responsible for the production of IFN-α in pDCs, whereas cDCs and macrophages utilized the RLH system. When the same virus was introduced intranasally, the IFN-α-producing cells shifted from pDCs to alveolar macrophages (AMs) and cDCs. Depletion of AMs increased the virus yield after NDV infection, further supporting the role of this cell type in the control of viral infection. Production of IFNs in AMs and cDCs depended on the RLH system, whereas IPS-1 deficiency led to IFN-α production in pDCs. Our data clearly demonstrates that distinct IFN-producing cells (IPCs) are activated in a tissue-specific manner. This serves to emphasize the importance of local antiviral cells in the natural course of the response to infection.

RESULTS

Generation of *Ifna6^{gfp}* Knockin Locus and Its Validation In Vitro

To investigate which cell population or populations produce IFN-α in vivo, we generated a mouse strain in which the coding sequence of the *Ifna6* gene was replaced by the GFP coding sequence (Figures 1A and 1B). Among multiple IFN-αs, we chose IFN-α6 because IFN-α6 expression is strongly induced in response to viral infection in various cell types in vitro, as detected by Q-PCR and DNA microarray analysis (Matsui et al., 2006). Additionally, IFN-α6 was reported to be regulated solely by IRF-7, as is the case for various IFN-αs, except for IFN-α4; IFN-β and IFN-α4 are reported to be regulated by IRF-3 in addition to IRF-7 (Honda et al., 2005). In the targeted allele, the GFP coding sequence and *loxP*-flanked neomycin-resistance cassette replaced the complete sequence encoding IFN-α6. The neomycin-resistance cassette was excised in targeted embryonic stem (ES) cells in vitro by transfecting a plasmid containing the Cre recombinase coding gene. The resulting allele *Ifna6^{gfp}* produced GFP instead of IFN-α6. We used mice heterozygous for *Ifna6^{gfp}* to circumvent the interference in IFN-α6 production.

We first examined whether the *Ifna6^{gfp}* allele recapitulated IFN-α expression in vitro. Flt3L-induced bone marrow DCs (Flt3L-BMDCs) prepared from *Ifna6$^{gfp/+}$* mice were infected with Newcastle disease virus (NDV) for 6 hr, and intracellular IFN-α staining was performed (Figure 1C). Flow cytometry analysis revealed that the number of GFP-positive CD11c$^+$B220$^+$ pDCs markedly increased in infected cells, and most of the intracellular IFN-α-positive cells were also positive for GFP. Next, GFP-positive (GFP$^+$) and GFP-negative (GFP$^-$) pDCs were FACS-sorted after NDV infection. The expression of type I IFN genes was then examined by Q-PCR (Figure 1D). Expression of the *Ifna6*, *Ifna2*, *Ifna4*, and *Ifna5* genes, as well as the *Ifnb1* gene, in GFP$^+$ cells was markedly augmented, being about ten times higher than that in GFP$^-$ cells. By contrast, the expression of the *Cxcl10* gene was almost equally upregulated in both GFP$^+$ and GFP$^-$ cells. This result indicates that the expression of GFP reflects the induction of IFN-αs and IFN-β, but not the general transcriptional upregulation invoked by NDV infection. Production of IFN-α was not altered between *Ifna6$^{+/+}$* and *Ifna6$^{gfp/+}$* cells. This indicated that the heterozygous *Ifna6^{gfp}* allele does not affect IFN-α production overall (Figure 1E). Taken as a whole, these results showed that the expression of GFP in *Ifna6$^{gfp/+}$* cells is an appropriate reporter for IFN-α production in vitro.

IPCs in Response to TLR Ligand Stimulation In Vivo

We next examined the responses of *Ifna6^{gfp}* mice to TLR and RLH ligands in vivo. Systemic administration of synthetic nucleotide analogs, such as D-type CpG-oligodeoxynucleotide (CpG-ODN) (a ligand for TLR9), R-848 (TLR7), and poly (I:C) (TLR3-MDA5), are known to induce IFN-α production in vivo (Asselin-Paturel et al., 2005; Hemmi et al., 2002; Kato et al., 2006). CpG-ODN was administered in *Ifna6$^{gfp/+}$* mice and GFP expression was

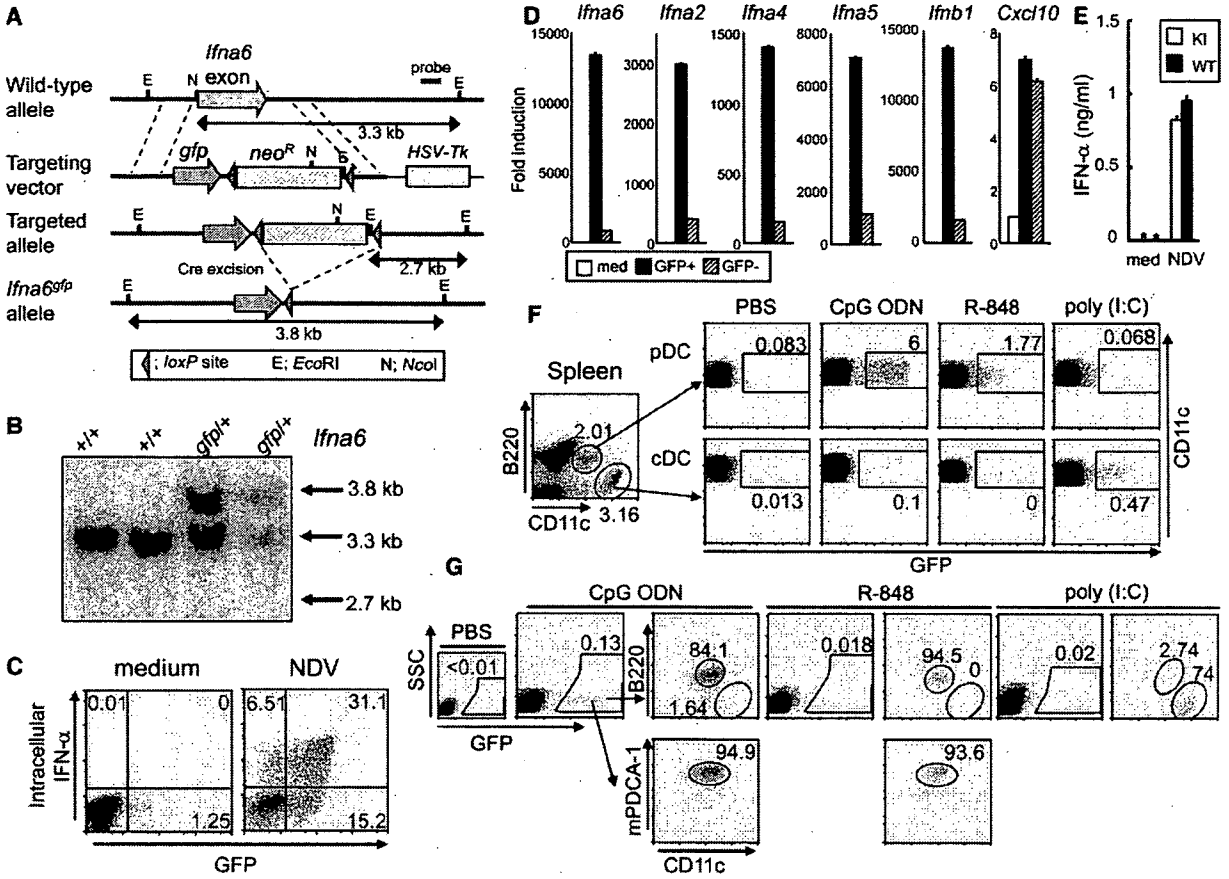

Figure 1. Generation of *Ifna6*^{gfp} Knockin Mice

(A) *Ifna6* gene and its flanking region ("wild-type allele") were used for creating the targeting vector ("targeting vector"). The resulting allele contains both *gfp* and *neo* genes ("targeted allele"). Excision of the *neo* gene by Cre recombinase resulted in the final mutated allele ("*Ifna6*^{gfp} allele") in which the *gfp* gene totally replaces the IFN-α6 coding sequence.

(B) Homologous recombination and excision of the *neo* gene was verified by Southern blot analysis.

(C) FACS plots of intracellular IFN-α (vertical) versus GFP (horizontal) staining obtained from untreated ("medium") and NDV-infected ("NDV"), Flt3L-induced, BM-derived pDCs. The data are representative of three independent experiments.

(D) GFP$^+$ cells were sorted from NDV-infected Flt3L-BMDCs, and amounts of mRNA for indicated IFN-α subtypes and other genes were quantified by quantitative real-time PCR (Q-PCR). Mean relative expressions against untreated cells are shown with standard error. Open histogram, untreated; closed, NDV-infected GFP$^+$ cells; shaded, NDV-infected GFP$^-$ cells.

(E) IFN-α concentrations in culture supernatants of NDV-infected ("NDV") or uninfected ("med") Flt3L-BMDC from *Ifna6*$^{+/+}$ and *Ifna6*$^{gfp/+}$ mice were determined by ELISA. The concentration of IFN-α is shown with standard error.

(F and G) Detection of IFN-α6-producing cells in vivo. PBS and D35/DOTAP complex (CpG) or R-848 was intravenously administered into *Ifna6*$^{gfp/+}$ mice. Four or two hours after inoculation of CpG-ODN or R-848, respectively, whole splenocytes were prepared, stained with CD11c and B220, and analyzed by FACS. pDCs and cDCs were gated and GFP expression was assessed (F). Conversely, GFP$^+$ cells were selected and analyzed for the expression of CD11c and B220 or mPDCA-1 (G). Numbers shown in each plots indicate the ratio of gated cells to total cells in the plot. The data are representative of three independent experiments.

examined in the lymphoid organs 4 hr after administration. Splenocytes most frequently contained GFP$^+$ cells, whereas cells from lymph nodes (LNs) and bone marrow (BM) were rarely GFP$^+$. In the spleen, CD11c^{int}B220$^+$ pDCs, but not CD11c^{hi} cDCs, DX5$^+$ IKDCs, or other lymphocytes, contained GFP$^+$ cells (Figure 1F and data not shown). In turn, most GFP$^+$ splenocytes expressed CD11c, B220, and mPDCA-1, indicating that pDCs are the predominant IPCs (Figure 1G). R-848 was also found to induce IFN-α production exclusively in pDCs 2 hr after inoculation (Figures 1F and 1G). Therefore, we concluded that pDCs are the sole IPC upon TLR7 and TLR9 stimulation in vivo. Of note, however, intravenous inoculation of poly (I:C) led to the increase of GFP$^+$ cDCs, but not pDCs, in the spleen (Figures 1F and 1G).

Determination of IPCs in Response to Systemic RNA Virus Infection In Vivo

FACS analysis of splenocytes from *Ifna6*$^{gfp/+}$ mice intravenously administered NDV revealed that pDCs, CD11c^{hi}B220$^-$ cDCs, and CD11c$^-$Mac1$^+$F4/80$^+$ macrophages contained GFP$^+$ cells (Figure 2A and Figure S3 in

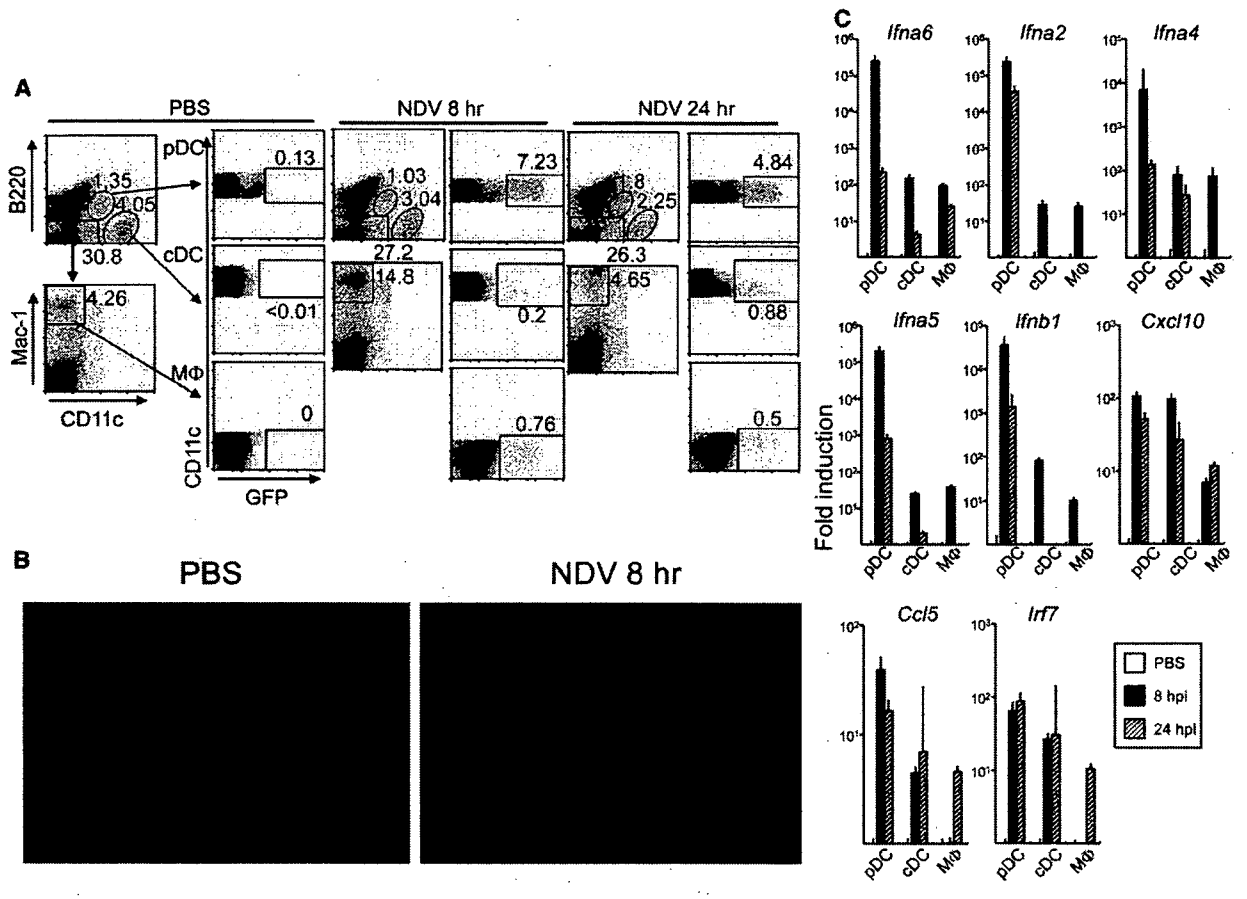

Figure 2. Identification of IPCs in Response to Systemic NDV Virus Infection

(A) NDV (1×10^7 pfu) was administered intravenously, and whole splenic cells were prepared 8 and 24 hr after infection. The expression of GFP in pDCs ($CD11c^{int}B220^+$), cDCs ($CD11c^+B220^-$), and macrophages ($Mac\text{-}1^+CD11c^-$) was analyzed. The data are representative of five independent experiments with similar results.

(B) Localization of GFP^+ cells in spleen. Cryosections of spleen from PBS- or NDV-treated mouse were stained with anti-GFP (green) and anti-MAdCAM-1 (red) as in Experimental Procedures.

(C) mRNA prepared from pDCs, cDCs, and macrophages (MΦ) obtained from NDV-infected or uninfected mice were subjected to Q-PCR. Fold induction of each mRNA type normalized to cells from PBS-treated mice is shown as mean with standard error (n = 2). Open bar, PBS; closed, 8 hr after infection; shaded, 24 hr after infection.

the Supplemental Data available with this article online). Four percent to eight percent of pDCs were GFP^+, whereas less than one percent of cDCs and macrophages were GFP^+. Nevertheless, when GFP^+ cells were collected and examined for their surface markers, cDCs and macrophages constituted more than 30% of GFP^+ cells at 8 hr after infection, indicating that these cell types occupied a substantial percentage of IFN-α producers (Figure S1A). This is because cDCs and macrophages constituted about 5% of total splenocytes, whereas around 1% of splenocytes were pDCs. Immunofluorescent microscopy revealed that GFP^+ cells localized at the marginal zone (MZ), outside of the marginal sinus stained with anti-MAdCAM-1 (Figure 2B).

Next, we sorted pDCs, cDCs, and macrophages from splenocytes of untreated or NDV-infected mice. The expression of IFN-α and chemokine genes was then determined by Q-PCR. Expression of the genes encoding IFN-α2, IFN-α4, IFN-α5, and IFN-α6 was strongly upregulated in pDCs, cDCs, and macrophages, although to a lesser extent in the latter two (Figure 2C). This result indicated that the production of IFN-α6 is representative of the production of various IFN-α subtypes, even in vivo.

In the peripheral blood, $Mac\text{-}1^+CD11c^-$ monocytes and macrophages contained GFP^+ cells at 8 hr after infection (Figure S1B). Additionally, $Mac\text{-}1^+CD11c^-$ monocytes and macrophages in the liver and BM also contained GFP^+ cells at 8 and 24 hr after injection (Figure S2). In BM, GFP^+ pDCs were also found. However, the frequency of GFP^+ cells in these organs was much lower than that in the spleen, indicating that the spleen is the organ which responds to systemic viral infection most efficiently. A vesicular stomatitis virus NCP mutant (VSV-NCP) also induced GFP expression in pDCs, cDCs, and macrophages in spleen, but hardly so in BM, LN, and liver (Figure S4 and data not shown).

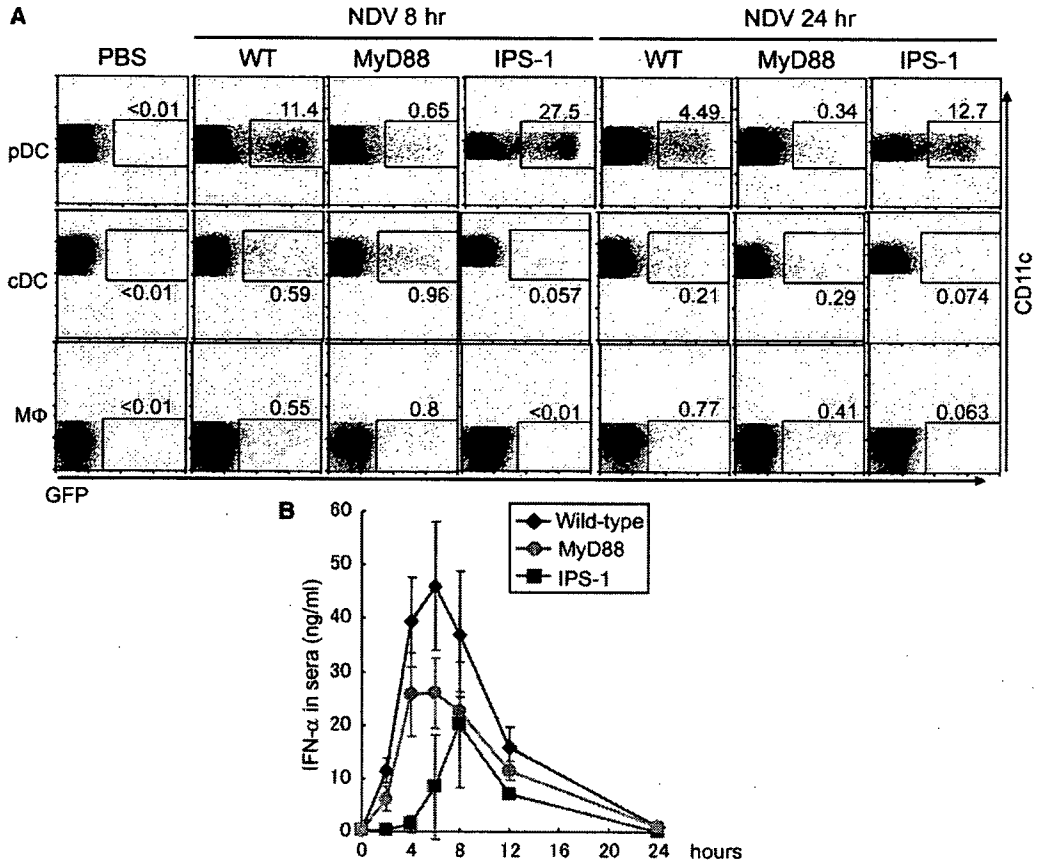

Figure 3. Differential Involvement of Signaling Pathways in IFN-α Production

(A) *Ifna6*$^{gfp/+}$ mice with wild-type ("WT"), MyD88-deficient ("MyD88"), or IPS-1-deficient ("IPS-1") backgrounds were infected with NDV and splenocytes were analyzed for the expression of GFP using FACS at the indicated time points. The data are representative of three independent experiments.

(B) IFN-α concentrations in sera from NDV-infected wild-type (diamond, green), MyD88-deficient (circle, orange), and IPS-1-deficient (rectangle, blue) mice were measured by ELISA. The mean value with standard error is shown (n = 3).

Collectively, these data indicate that upon systemic viral infection, splenic pDC, as well as cDC, monocytes, and macrophages in spleen and other organs, produced IFN-α.

Contribution of Intracellular Signaling Molecules to the Activation of IPCs In Vivo

We previously showed that the TLR and RLH pathways are important for the induction of IFN-α in pDCs and cDCs, respectively, by examining the production of IFNs in cDCs and pDCs ex vivo (Kato et al., 2005). However, it was not clear whether these pathways operate in a similar manner in vivo. When *Ifna6*$^{gfp/+}$ mice lacking MyD88 were infected with NDV, GFP$^+$ pDCs were severely decreased in number compared with those in wild-type mice (Figure 3A). In contrast, the number of GFP$^+$ cDCs and macrophages was not altered in MyD88-deficient mice, confirming that the TLR pathway mediates IFN-α induction in pDCs in vivo. In IPS-1-deficient mice, the increase of the GFP$^+$ cell number in cDCs and macrophages, but not in pDCs, was abrogated, showing that cDCs and macrophages utilize the RLH pathway in vivo (Figure 3A).

We then examined the concentration of IFN-α in the sera of NDV-infected wild-type, MyD88-deficient, and IPS-1-deficient mice. As shown in Figure 3B, production of IFN-α was partially impaired in both MyD88-deficient and IPS-1-deficient mice compared with wild-type controls. Interestingly, in the case of IPS-1-deficient mice, more severe impairment of IFN-α production was observed from 2 to 6 hr after infection than at later time points. This result showed that cells utilizing the RLH pathway, such as cDCs, monocytes, and macrophages, contribute to production of IFN-α serum at the initial stage of systemic viral infection.

Thus, consistent with former reports, cDCs and pDCs produce IFN-α via the RLH pathway and the TLR pathway, respectively.

Identification of IPCs in Response to Local Viral Infection of the Lung

In the natural course of viral infection, viruses do not directly enter the blood stream, but attack mucosal surfaces to invade the host. It remains to be clarified whether the same cell types are responsible for type I IFN production in

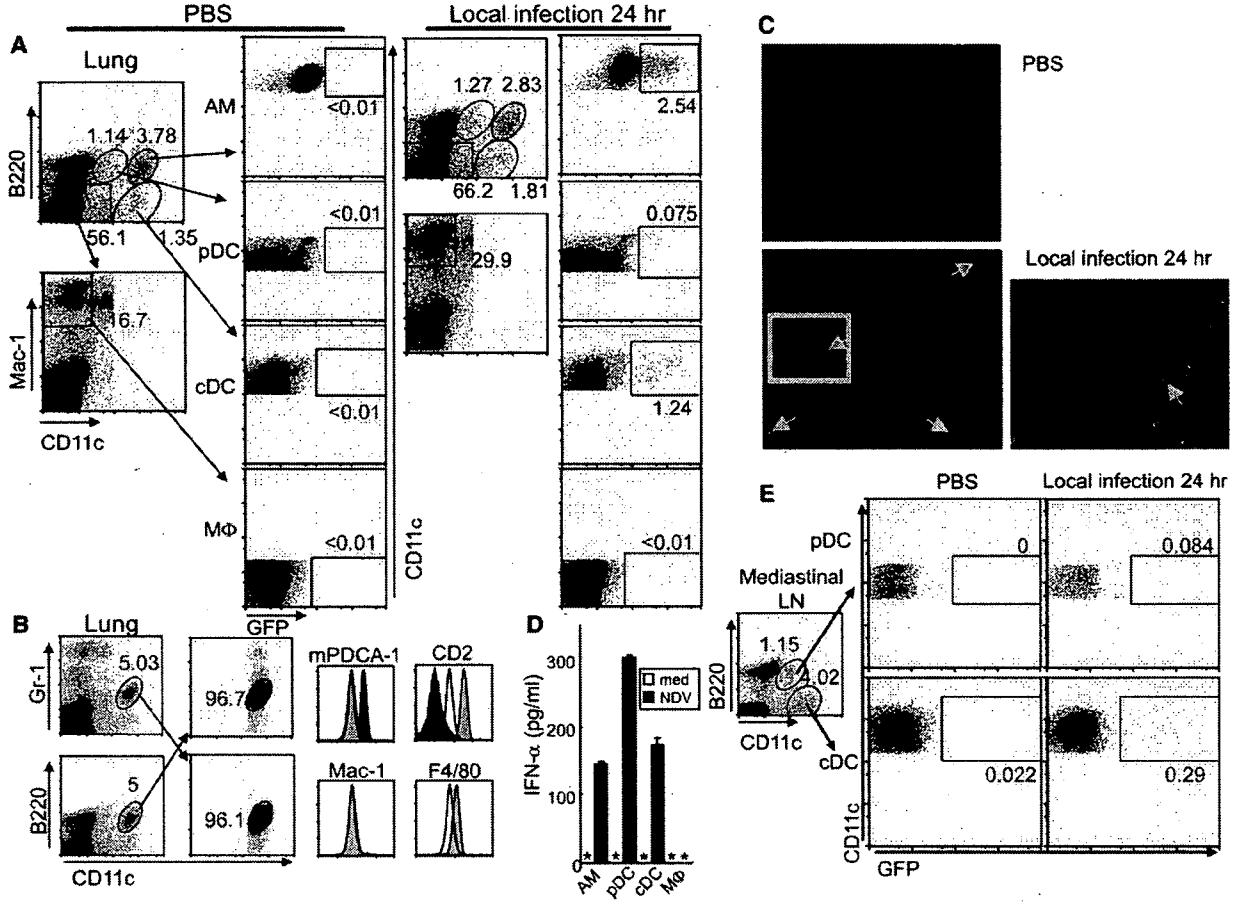

Figure 4. Identification of Alveolar Macrophages as Lung-Local IPCs

(A) *Ifna6*^{gfp} mice were intranasally administered PBS or NDV. Twenty-four hours after infection, lung cells were isolated as in Experimental Procedures and subjected to FACS analysis. AM, alveolar macrophage. Others are the same as in Figure 2A. The data are representative of four independent experiments with similar results.

(B) Lung cells from a mouse were stained and subjected to FACS analysis. In the upper two dot plots, expression of Gr-1 (vertical) versus CD11c (horizontal) is shown. In the bottom two dot plots, expression of B220 (vertical) versus CD11c (horizontal) is shown. Gated cells in the left two panels were expanded and the plots are shown in the right two panels. Histograms represent the expression of each of the surface molecules on cells gated in a B220 versus CD11c plot. Open histogram, unstained control; shaded, stained with indicated antibodies. Solid histograms in mPDCA-1 and CD2 indicate fluorescence intensities of the surface molecules on pDCs and cDCs, respectively. Data shown are representative of two mice.

(C) Lung lobes from an *Ifna6*$^{gfp/+}$ mouse infected intranasally with NDV for 24 hr were analyzed by immunofluorescence. Overlay images of phase contrast images, with DAPI-stained nucleus in blue and GFP in green, are shown. GFP$^+$ cells are indicated by yellow arrows. Upper panel, PBS-treated lung image; lower left panel, NDV-infected lung; lower right panel, magnified image of the region that corresponds to the yellow box in the lower left image.

(D) IFN-α production by sorted AMs, pDCs, cDCs, and MΦs from lung in vitro. FACS sorted cells, as described in Experimental Procedures, were infected with NDV or left uninfected. The culture supernatants were collected and subjected to ELISA at 24 hr after infection. The data shown are mean concentrations with standard errors. Asterisk, not detected.

(E) Mediastinal LNs from *Ifna6*$^{gfp/+}$ mice uninfected or intranasally infected with NDV for 24 hr were isolated and subjected to FACS analysis. The data are representative of three independent experiments.

response to systemic and local viral infection. To answer this question, we applied an intranasal infection model to *Ifna6*$^{gfp/+}$ mice. When the mice were infected intranasally with NDV, CD11c^{int}B220$^+$ pDCs did not contain a GFP$^+$ population, in contrast to the group undergoing systemic infection (Figure 4A). Also, neither Mac-1$^+$CD11c$^-$ monocytes and macrophages nor lymphocytes contained GFP$^+$ cells (Figure 4A and data not shown). cDCs showed modest but relevant increases in the induction of GFP$^+$ cells. Further, a CD11c^{hi} population was found to highly increase the number of GFP$^+$ cells (Figure 4A, AM). These CD11c$^+$GFP$^+$ cells showed high autofluorescence and the expression of B220 was comparable to that in the control staining. This indicated that this population did not express B220 (Figure S5). These cells were also negative for Mac-1, CD8a, and Gr-1, but positive for F4/80 and CD2 (LFA-2). This population was found to be identical to previously reported AMs (Figure 4B) (de Heer et al., 2004).

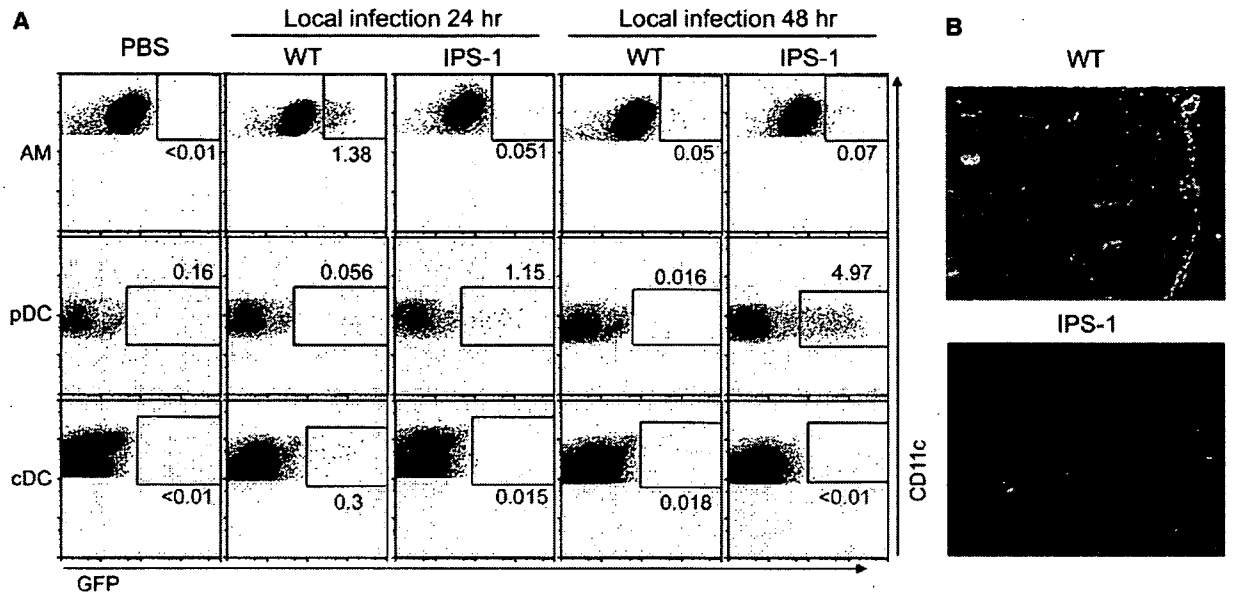

Figure 5. Effect of IPS-1 Deficiency on IFN-α Production in Lung

(A) Wild-type or IPS-1-deficient *Ifna6*$^{gfp/+}$ mice were infected intranasally with NDV, and lung cells were analyzed using FACS at the indicated time points. AMs, pDCs, and cDCs were gated as in Figure 4A. The data are representative of two independent experiments with essentially identical results.

(B) Immunofluorescence images of lung sections from wild-type mice (upper) or IPS-1-deficient *Ifna6*$^{gfp/+}$ mice (lower) are shown as in Figure 4C.

More than 80% of GFP$^+$ lung cells belonged to this cell population (Figure S5). Consistent with these observations, a histological examination revealed that most GFP$^+$ cells were localized at alveoli (Figure 4C). To verify IFN-α production from these populations, pDC, cDC, macrophage, and CD11c^{hi}CD2^{hi} AM populations were FACS-sorted and stimulated by NDV in vitro. The AMs, cDCs, and pDCs, but not Mac1$^+$CD11c$^-$ macrophages, produced comparable amounts of IFN-α in response to NDV (Figure 4D).

Lung viral infection is likely to influence mediastinal LNs. We found that cDCs contained GFP$^+$ cells in the mediastinal LN 24 hr after intranasal infection, whereas GFP$^+$ pDCs were not found (Figure 4E and data not shown). This observation suggested that virus infection in the lung induces IFN-α production in the LN by cDCs, which might have migrated from the lung.

In summary, AMs and cDCs, but not pDCs, are IFN-α-producing cells after pulmonary infection with NDV.

Role of the RLH Pathway in the Production of IFN-α in AMs

To elucidate the mechanism of virus-mediated IFN-α production in the lung, IPS-1-deficient *Ifna6*$^{gfp/+}$ mice were infected intranasally with NDV. IPS-1 deficiency abrogated induction of GFP$^+$ cells in both AMs and cDCs (Figure 5A). Interestingly, the number of GFP$^+$ pDCs was highly increased at 48 hr after infection in IPS-1-deficient mice, suggesting that the IPS-1 deficiency resulted in the failure of initial antiviral responses in AMs and cDCs, and led to massive production of IFN-α by pDCs. Further histological examination revealed that GFP$^+$ cells in IPS-1-deficient mice infected with NDV were localized in the interstitium between alveoli (Figure 5B). This result further confirmed the production of IFN-α from pDCs in the absence of IPS-1, because pDCs are localized in the interstitium (de Heer et al., 2004). In contrast, a MyD88 deficiency did not affect the frequency of GFP$^+$ AMs and cDCs (data not shown), indicating that AMs and cDCs rely on the RLH system to produce IFN-α.

Functional Role of AMs in Antiviral Responses In Vivo

Although DCs are reported to play an important role in antiviral responses, the role of AMs is unclear. To examine the role of AMs in antiviral responses, we tried to deplete AMs using liposome-encapsulated dichloromethylene bisphosphonate (Cl_2MBP-liposome). Consistent with previous reports (Thepen et al., 1989), intranasal treatment of mice with Cl_2MBP-liposome, but not PBS-liposome, led to specific depletion of the CD11c^{hi}CD2^{hi} AM population 24 hr after treatment without altering the population of cDCs, pDCs, and macrophages (Figure S6). When Cl_2MBP-liposome-treated *Ifna6*$^{gfp/+}$ mice were further infected intranasally with NDV, GFP$^+$ cells in lung pDCs increased in number, implying that impaired initial responses to NDV activated pDCs (Figure 6A). Neither the depletion of AM nor the IPS-1 deficiency markedly affected IFN-α concentration in sera 24 hr after infection, suggesting that pDCs compensated for the IFN-α production from the decreased number of AMs (Figure 6B). Histological examination of AM-depleted mice revealed that

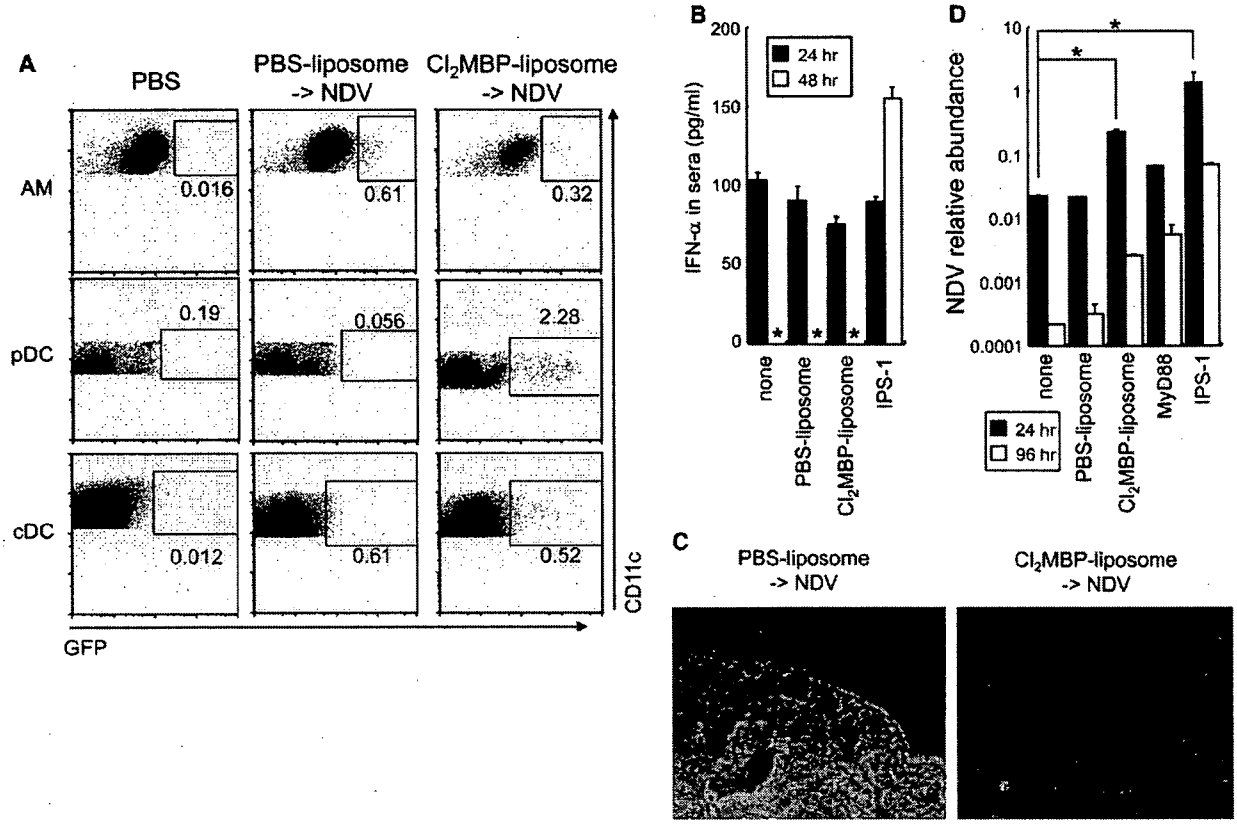

Figure 6. Effect of Depletion of AMs on Antiviral Responses in Lung

(A) AMs were depleted or left undepleted by intranasal instillation of Cl_2MBP-lipsome or PBS-liposome, respectively. Twenty-four hours after instillation, mice were infected with NDV for another twenty-four hours. Lungs were collected and GFP expression in AMs, pDCs, and cDCs was assessed using FACS.

(B) IFN-α levels in sera 24 or 48 hr after intranasal infection with NDV were measured by ELISA. Asterisks, not detected.

(C) Immunofluorescence images of lung from PBS-liposome-treated mice (left) or Cl_2MBP-lipsome-treated mice (right) are shown as in Figure 4C.

(D) The abundance of NDV in lung was quantified by Q-PCR for NDV nucleoprotein mRNA. Data shown are the mean value with standard error (n = 2). *$p < 0.05$ with two-sided Student's t test. All the data in this panel are representative of at least two independent experiments with essentially identical results.

GFP^+ cells were mainly localized in the interstitium of the lung (Figure 6C).

Next we examined the role of AMs in the clearance of infected NDV in the lung by measuring the expression of mRNA encoding NDV nucleocapsid protein with Q-PCR. The expression of NDV nucleoprotein was observed in untreated or PBS-liposome-treated mice 24 hr after infection, and decreased less than 1% from 24 hr to 96 hr after infection (Figure 6D). In contrast, the expression of the NDV nucleoprotein gene was around 10 times higher in AM-depleted mice than in control mice at 24 and 96 hr after infection, indicating that AMs play a critical role in the clearance of NDV infection. When we examined whether deficiency of MyD88 or IPS-1 influenced virus clearance, IPS-1-deficient mice exhibited a much higher NDV burden compared with MyD88-deficient mice, although MyD88-deficient mice showed a modest increase in the expression of the NDV nucleoprotein (Figure 6D). This result indicated that in the acute phase of infection, the RLH system plays a more important role in the elimination of lung viral infection than the TLR system does. These results underlined the importance of AMs in the initial elimination of invading viruses.

Subversion of AM-Mediated IFN-α Production by Wild-Type Sendai Virus

We next investigated whether other RNA viruses also induce IFN-α production in AMs. We used Sendai virus (SeV), a pathogenic virus widely used as a respiratory viral infection model in mice (Woodland et al., 2001), and VSV. When AMs and pDCs were purified from the lung and stimulated with viruses, AMs produced IFN-α in response to VSV-NCP and SeV with mutated C proteins (SeV Cm), as well as NDV (Figure 7A). However, AMs failed to produce IFN-α in response to wild-type SeV. In contrast, pDCs produced IFN-α in vitro in response to all the viruses tested. It has been shown that SeV C proteins suppress host IFN responses (Kato et al., 2007), and these results suggested that SeV inhibited IFN-α production in AMs, but not pDCs, by C proteins. When wild-type SeV was

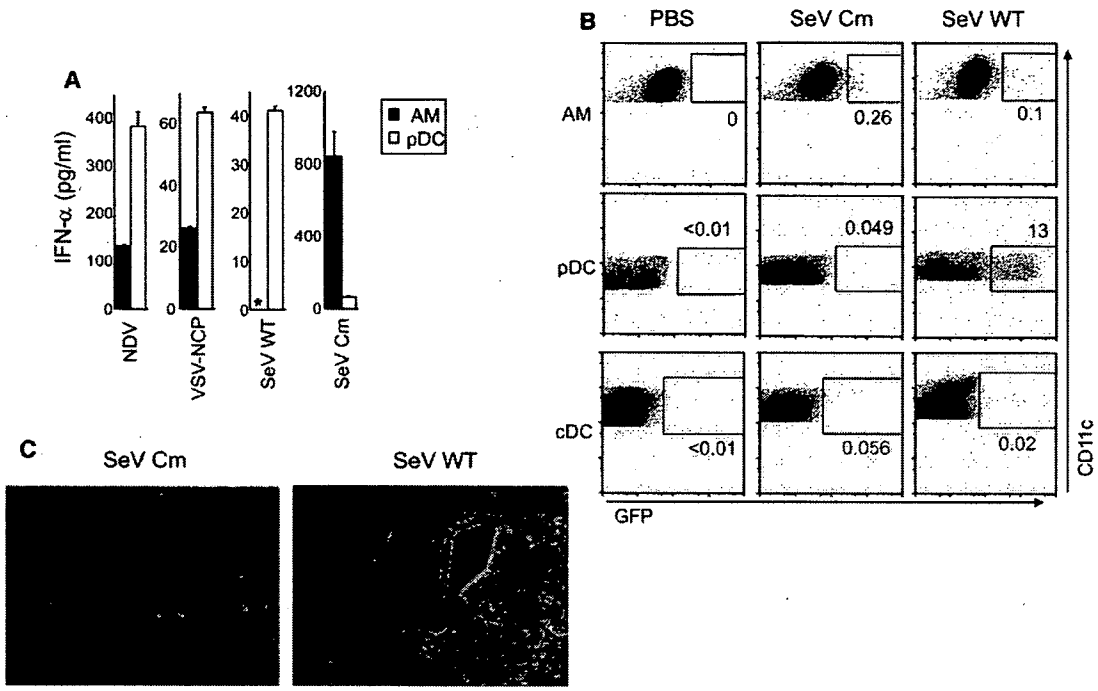

Figure 7. Subversion of AM-Mediated or cDC-Mediated Antiviral Responses, or Subversion of Both, by Sendai Virus

(A) In vitro IFN-α production by AMs and pDCs in response to various viruses. AMs and pDCs were sorted and infected in vitro for 24 hr. IFN-α concentrations in the culture supernatants were measured with ELISA. Asterisk, not detected.

(B) GFP expressions in AMs, pDCs, and cDCs were analyzed at 24 hr after intranasal infection with wild-type Sendai virus (SeV WT) or its mutant, SeV Cm.

(C) Immunofluorescence images of lung infected with SeV Cm (left panel) or SeV WT (right) are shown. The data are representative of two independent experiments with same results.

introduced intravenously, the virus induced IFN-α production from pDCs, but not from cDCs or macrophages (Figure S7).

Thus, we investigated IPCs in the lung in response to SeV Cm and wild-type SeV infection. As expected, SeV Cm, as well as NDV, induced IFN-α production from AMs, but not from pDCs (Figure 7B). In sharp contrast, the number of GFP$^+$ pDCs, but not cDCs and AMs, was highly increased in response to intranasal wild-type SeV infection, resembling the result obtained in AM-depleted and IPS-1-deficient mice (Figure 7B). This was further confirmed by immunofluorescence images of the lung infected with SeV, which showed the presence of GFP$^+$ cells in the interstitium (Figure 7C). In contrast, SeV Cm induced GFP expression in AMs. These results suggested that SeV targets AMs for suppressing IFN-α production, and that the subversion is essential for SeV virulence. Collectively, these data emphasized the importance of the first-line antiviral responses mediated by AMs.

DISCUSSION

In this study, we generated an *Ifna6^{gfp}* knockin allele in which the expression of GFP is induced under the control of the *Ifna6* promoter. Validation analysis showed that this allele recapitulated IFN-αs and IFN-β expression in response to viral infection both in vitro and in vivo. IFN-α6 is a subtype of IFN-αs whose expression is reported to be controlled by IRF-7 and induced by viral, but not bacterial, infections. In contrast, IFN-β is regulated by IRF-3 in addition to IRF-7 (Honda et al., 2005), and it is induced by both viral and bacterial components. However, an expression analysis of IFN-α2, IFN-α4, IFN-α5, and IFN-β mRNA in GFP$^+$ pDCs revealed that IFN-α6-producing cells also expressed other subtypes of IFN-α and IFN-β in response to NDV stimulation, indicating that expression of GFP recapitulates not only the expression of the *Ifna6* gene but also expressions of various IFN-α and IFN-β genes regardless of the subtypes. Thus, *Ifna6$^{gfp/+}$* mice are an appropriate reporter strain for analysis of type I IFN production, although it is not proven if type I IFNs are coregulated in response to various stimuli.

By intravenously treating *Ifna6$^{gfp/+}$* mice with NDV, we found that pDCs, cDCs, and macrophages in the spleen, monocytes and macrophages in peripheral blood, and (to a lesser degree) monocytes and macrophages in liver and BM were responsible for the production of type I IFNs. In accordance with previous observations, MyD88 was critical for the increase in GFP$^+$ pDCs, whereas IPS-1 was essential for the appearance of other GFP$^+$ cells such as cDCs and macrophages (Kato et al., 2005). It is unexpected that monocytes and macrophages consist

of a large portion of IPCs in vivo. Because the RLH system is essential for IFN-α production in this cell type, monocytes and macrophages seem to be actually infected. Impaired production of serum IFN-α in IPS-1-deficient mice also implies the importance of non-pDCs in virus-induced IFN-α production. Our data indicated that cell types other than pDCs also make a large contribution to type I IFN production during systemic viral infections.

The IPS-1-dependent pathway contributes substantially to increases in serum IFN-α at an early time point after infection with NDV, although the MyD88-dependent pathways are also critical at later time points. Accordingly, about 30% of GFP$^+$ splenocytes were macrophages and cDCs at 8 hr after NDV infection, but the ratio dropped to below 10% at 24 hr. In the spleen of NDV-infected *Ifna6^{gfp}* mice, GFP$^+$ cells were localized at the MZ. This is consistent with previous reports that used an IFN-α antibody to show that IPCs are localized at the MZ (Asselin-Paturel et al., 2005; Dalod et al., 2002). Upon systemic NDV infection, GFP$^+$ cells were almost exclusively found in the spleen, though quite a few GFP$^+$ pDCs were observed in other lymphatic organs, such as BM and LNs. VSV and SeV also induced GFP expression mostly in the spleen, indicating that the spleen is the most important organ for the production of type I IFNs in response to blood-borne viruses. Because the MZ is important for sequestering blood-borne pathogens, and mice with abnormal MZ organization failed to mount proper IFN-α production in response to viral infection (Louten et al., 2006), IPCs activated by systemic NDV infection are probably localized in the splenic MZ. pDCs are mostly localized in the T cell area and in the red pulp, but rarely localize in the MZ although they migrate to the MZ to form clusters in response to CpG-DNA stimulation (Asselin-Paturel et al., 2005). In contrast, cDCs originally localize in the MZ. Thus, it is possible that invading NDV encounters macrophages and cDCs at the MZ first, and then pDCs that have migrated to the MZ. Although further detailed analysis of localization and time-course changes needs to be addressed, this may explain the temporal differences in the contributions of IPS-1- and MyD88-dependent pathways in the production of IFN-α.

In contrast to systemic virus infection, intranasal treatment of *Ifna6^{gfp}* mice with NDV failed to increase the number of GFP$^+$ cells in pDCs. By characterizing GFP$^+$ cells upon intranasal infection, we identified AMs and cDCs, particularly AMs, as the major IFN-α producers. AMs are reported to sample and respond to microorganisms that have entered the alveolar space. They phagocytose invading bacteria and evoke inflammation by producing proinflammatory cytokines (Peters-Golden, 2004). They are also reported to phagocytose virus-infected apoptotic cells (Hashimoto et al., 2007). However, our results suggested that direct infection of AMs with viruses is required for the production of IFN-α. The role of AMs in the clearance of viral infection was also demonstrated by the consequences of the depletion of AMs. Thus, AMs function as sentinels, recognizing infectious viruses and alerting surrounding cells. AMs do not seem to migrate to mediastinal LNs even after viral infection, suggesting that type I IFNs produced by AMs activate surrounding cells in an autocrine or paracrine manner to prepare for any virus infection. Taken together, our data highlighted the importance of the initial production of IFN-α and the elimination of invading viruses by AMs.

cDCs, but not pDCs, produced IFN-α in response to local infection in mediastinal LNs. Because GFP$^+$ cDCs were also found in lung tissue, it is possible that cDCs activated in the lung tissue are migrating to mediastinal LNs. Given the function of type I IFNs in the activation of acquired immune responses (Stetson and Medzhitov, 2006), the major role of cDCs may be to activate T cells in LNs rather than function as local IPCs.

Earlier reports indicated that pDCs produce type I IFNs to help elicit the acquired immune response (Yoneyama et al., 2005), although we failed to detect GFP$^+$ pDCs in response to intranasal NDV infection in *Ifna6^{gfp}* mice. However, massive amounts of GFP$^+$ pDCs were detected either when AMs were depleted or in the absence of IPS-1. These results suggested that pDCs start to function when AM- and cDC-mediated host defense mechanisms are impaired, and that pDCs may function as the backup system to achieve robust antiviral IFN-α production.

Wild-type SeV is reported to repress host antiviral responses mainly by inhibiting type I IFN signaling via C proteins; this activity is essential for the pathogenicity of the virus (Kato et al., 2007). In vitro experiments showed that wild-type SeV did not induce IFN-α production in AMs, whereas SeV Cm highly induced IFN-α. Upon local lung infection with wild-type SeV, AMs and cDCs did not appear to contain GFP$^+$ cells, indicating that AMs and cDCs failed to produce IFN-α. This result indicates that SeV has a sophisticated mechanism or mechanisms to subvert host antiviral response by AMs and cDCs. Resembling the response in the absence of AMs, the failure in antiviral response by AMs and cDCs led to massive production of IFN-α from pDCs. Intranasal local infection of SeV Cm, however, induced IFN-α production from AMs, but not from pDCs. Thus, if SeV fails to subvert the host antiviral response due to its deficiency of C proteins, AMs will exert an effective antiviral response against SeV to control viral dissemination.

From these observations, we propose that three different IPCs in the lung, i.e. AMs, cDCs, and pDCs, are activated sequentially, but not simultaneously, for mounting antiviral immune responses. AMs act as the first-line sensor of invading viruses, and produce IFN-α at the site of infection. cDCs also initially produce IFN-α against viral infection, and have an additional role in producing IFNs in regional LNs. In contrast, pDCs are not activated until the initial defense line is broken by the viruses. It is of note that pDCs utilize the TLR system for type I IFN production, which is different from AM and cDC usage of the RLH system. Given that several virulent RNA viruses, such as SeV and influenza virus, suppress the RLH-mediated signaling pathway, type I IFN signaling pathway, or both (Kato et al., 2007; Pichlmair et al., 2006), it is tempting

to speculate that hosts have evolved two different type I IFN production systems to make it more difficult for viruses to escape the antiviral response.

This study showed that the *Ifna6*$^{gfp/+}$ mice are a quite useful tool for identifying IPCs in response to viral infection. Although we used respiratory virus infection as a model of local viral infection, as yet uncharacterized IPCs can be activated in other organs and mucosal tissues, such as intestine. Thus, this mouse model will be useful in identifing IPCs in different tissues in relation to various viral infections. Recent expansion in IFN biology has also revealed that type I IFNs are not only involved in antiviral responses, but also in responses to bacteria and autoimmune diseases (Banchereau and Pascual, 2006). For instance, overproduction of type I IFNs is reported in some autoimmune diseases, such as systemic lupus erythematosus, although the identity or identities of the type I IFN-producing cells are not clear in mouse models of the autoimmune disease. Therefore, we believe that this mouse model will benefit research in the IFN field by providing a vehicle for in vivo insights.

EXPERIMENTAL PROCEDURES

Generation of *Ifna6*^{gfp} Knockin Mice

The *Ifna6* gene and its flanking region were isolated from genomic DNA extracts of ES cells (clone GSI-I) by PCR. The targeting vector was constructed by replacing a 1.0 kb fragment encoding the entire *Ifna6* open reading frame with a neomycin-resistance gene cassette (*neo*) and a fragment encoding EGFP (*gfp*) from pEGFP-1 (Clontech). A herpes simplex virus thymidine kinase driven by PGK promoter (*HSV-Tk*) was inserted into the genomic fragment for negative selection. After the targeting vector was transfected into ES cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and Southern blotting. A plasmid that contains a gene encoding Cre recombinase was transfected into the clone and G418-sensitive colonies were selected. Clones in which excision of the *neo* gene took place were screened by Southern blotting. These clones were microinjected into C57BL/6 female mice, and heterozygous *Ifna6*$^{gfp/+}$ F1 progenies were backcrossed five times to C57BL/6 before analysis.

Mice

Mice deficient in *Myd88* and *Ips-1* have been described previously (Adachi et al., 1998; Kumar et al., 2006). All mice were bred and maintained in a specific pathogen-free facility of the Research Institute for Microbial Diseases, Osaka University, in accordance with the specifications of the Association for Assessment and Accreditation of Laboratory Animal Care. Mouse protocols were approved by Osaka University Animal Care and Use Committee.

Reagents and Viruses

All fluorochrome-conjugated and biotin-conjugated antibodies were purchased from BD PharMingen unless otherwise indicated. Flt3L was purchased from Peprotec. When administered intravenously, 5 μg of CpG-ODN D35 (Uematsu et al., 2005) was complexed to 30 μl of DOTAP transfection reagent (Boeringer-Manheim) at the final concentration of 25 μg/ml, and the conjugate was administered. R-848 has also been described (Hemmi et al., 2002), and 100 nmol of R-848 was administered intravenously. Poly (I:C) was purchased from Amersham, and 100 μg of poly (I:C) was intravenously administered. NDV and VSV-NCP were a kind gift from Dr. T. Abe and Dr. Y. Matsuura (Research Institute for Microbial Diseases, Osaka University). NDV suspended in chick allantoic fluid was administered both intravenously and intranasally at a titer of 1 × 10^7 plaque forming unit (pfu) after anesthesia. Allantoic fluid does not induce IFN-α either in vitro or in vivo by itself (data not shown). VSV-NCP was administered intravenously at a titer of 1 × 10^6 pfu. Wild-type SeV and SeV Cm were kindly provided by Dr. A. Kato (National Institute for Infectious Diseases, Japan) and administered both intravenously and intranasally at a titer of 1 × 10^7 pfu. The IFN-α ELISA kit was obtained from PBL.

Isolation of Cells from Tissues

Isolation of lung cells was performed as essentially described with some modifications (de Heer et al., 2004). Briefly, mice were sacrificed and perfused with PBS containing 10 mM EDTA from the right ventricle. Lung lobes were isolated and collagenase buffer (150 units/ml of collagenase [purchased from Wako Chemicals], 10 μg/ml of DNaseI [from Sigma], and 5% of FCS in RPMI1640 medium) was injected into the lobes using a 27G needle. The lobes were then shredded into small pieces and incubated at 37°C for 45 min. During the last 5 min, EDTA was added at 10 mM. Any remaining small pieces were dispersed by passage in and out through a 20G needle, and the suspension was passed through nylon mesh to remove debris. A single-cell suspension was prepared after RBC lysis. Cells from LNs (both inguinal and mediastinal) and liver were isolated essentially in the same manner as lung cells were.

Flow-Cytometric Analysis and Sorting

Intracellular staining of IFN-α was performed as described (Kato et al., 2005). For sorting pDCs, cDCs, and macrophages from spleen, $CD19^+$ cells and $Thy1.2^+$ cells in splenocytes were depleted by a magnetic bead sorting system using magnetic bead-conjugated anti-CD19 and anti-Thy1.2 antibodies (Miltenyi Biotech). The cells of the depleted fraction were stained with PerCP-conjugated anti-B220, APC-conjugated anti-CD11c, and FITC-conjugated anti-Mac-1 antibodies. Gates were set as in Figure 2A. For sorting AMs, a single-cell suspension of lung cells was stained with APC-conjugated anti-CD11c, FITC-conjugated anti-CD2, and PE-conjugated anti-Mac-1 antibodies. $CD11c^{hi}CD2^{hi}$Mac-1^- cells were sorted as AMs. Stained cells were sorted using a FACSArea (BD Bioscience). Sorted cells had more than 95% purity.

Immunofluorescence Staining

Isolated tissues were embedded into OCT compound (Sakura Finetechnical) and subjected to rapid cooling in *n*-hexane cooled in liquid nitrogen and stored at −80°C until further analysis. Five-micrometer thick cryosections were fixed in acetone at −30°C for 20 min, air-dried, and rehydrated in PBS and PBS containing 0.05% Tween 20 (PBST) for 1 min each before staining. Sections were incubated in 2% BSA in PBST for 30 min at room temperature. If required, streptavidin-biotin blocking was also performed using a streptavidin-biotin blocking kit (Vector laboratories). After blocking, sections were stained with anti-GFP rabbit polyclonal antibody (SantaCruz) and anti-MAdCAM-1 rat monoclonal antibody (clone MECA75). Alexa 488-conjugated anti-rabbit IgG (Invitrogen) and biotinylated anti-rat IgG (Jackson Immunoresearch) were then applied as secondary antibodies. Finally, sections were stained with Alexa 594-conjugated streptavidin (Invitrogen). The resulting stained specimens were mounted by VectaShield Mounting Medium Hard (Vector Laboratories) and observed under an Olympus IX81 microscope (Olympus). Obtained images were processed using Metamorph software.

RNA Isolation; Q-PCR

Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed using ReverTraAce (Toyobo) following the manufacturer's protocol. For sorting cells, total RNA was first purified using an RNA isolation and purification Mini Kit (QIAGEN), and 10 ng of obtained purified RNA was reverse transcribed using an Ovation Biotin System (Nugen) according to manufacturer's instructions. The resulting cDNA was subjected to Q-PCR as described (Matsui et al., 2006). Probes were purchased from Applied Biosystems. The probe for

a gene encoding NDV nucleocapsid protein has been described (Matsui et al., 2006).

Depletion of AMs with Cl_2MBP-Liposome

The preparation of liposomes was performed as described (Thepen et al., 1989) with some modifications. Briefly, cholesterol (CL, purchased from Sigma) and L-α-phosphatidylcholine (from yolk egg, Type XVI-E, Sigma) were dissolved in chloroform at concentrations of 8 and 100 mg/ml, respectively. One milliliter of CL solution was mixed with eighty-six microliters of PC solution in a two-milliliter tube. The chloroform in the mixture was evaporated in a centrifuge evaporator. Either 1 ml of PBS or Cl_2MBP (from Sigma) in PBS at a concentration of 0.25 g/ml was added into the tube and mixed well by mild vortexing for 30 min. The mixture was incubated at room temperature for 2 hr. It was then sonicated for 3 min in a waterbath sonicator and incubated for an additional 2 hr. The resulting liposomes were washed by PBS and resuspended in 200 μl of PBS. To deplete AMs in vivo, 50 μl of Cl_2MBP-liposome was administered intranasally. Twenty-four hours later, mice were subjected to further analysis.

Supplemental Data

The Supplemental Data, which consists of seven additional figures, can be found online at http://www.immunity.com/cgi/content/full/27/2/240/DC1/.

ACKNOWLEDGMENTS

We thank all colleagues in our laboratory; A. Kato, T. Abe, Y. Matsuura, T. Shioda, and E. Nakayama for providing viruses; K. Nakamura for cell sorting; P.Y. Lee for critical reading of the manuscript; M. Hashimoto for secretarial assistance; and Y. Fujiwara, M. Shiokawa, and N. Kitagaki for technical assistance.

This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology in Japan; the 21st Century Center of Excellence Program of Japan; and NIH (AI070167). The authors declare that they have no competing financial interests.

Received: March 16, 2007
Revised: May 22, 2007
Accepted: July 3, 2007
Published online: August 23, 2007

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JOURNAL OF VIROLOGY, Sept. 2007, p. 8953–8966 Vol. 81, No. 17
0022-538X/07/$08.00+0 doi:10.1128/JVI.00649-07

Hepatitis C Virus Nonstructural Protein 5A Modulates the Toll-Like Receptor-MyD88-Dependent Signaling Pathway in Macrophage Cell Lines▽

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Received 27 March 2007/Accepted 6 June 2007

Hepatitis C virus (HCV) infection induces a wide range of chronic liver injuries; however, the mechanism through which HCV evades the immune surveillance system remains obscure. Blood dendritic cells (DCs) play a pivotal role in the recognition of viral infection and the induction of innate and adaptive immune responses. Several reports suggest that HCV infection induces the dysfunction of DCs in patients with chronic hepatitis C. Toll-like receptor (TLR) has been shown to play various roles in many viral infections; however, the involvement of HCV proteins in the TLR signaling pathway has not yet been precisely elucidated. In this study, we established mouse macrophage cell lines stably expressing HCV proteins and determined the effect of HCV proteins on the TLR signaling pathways. Immune cells expressing NS3, NS3/4A, NS4B, or NS5A were found to inhibit the activation of the TLR2, TLR4, TLR7, and TLR9 signaling pathways. Various genotypes of NS5A bound to MyD88, a major adaptor molecule in TLR, inhibited the recruitment of interleukin-1 receptor-associated kinase 1 to MyD88, and impaired cytokine production in response to TLR ligands. Amino acid residues 240 to 280, previously identified as the interferon sensitivity-determining region (ISDR) in NS5A, interacted with the death domain of MyD88, and the expression of a mutant NS5A lacking the ISDR partially restored cytokine production. These results suggest that the expression of HCV proteins modulates the TLR signaling pathway in immune cells.

Hepatitis C virus (HCV) belongs to the family *Flaviviridae* and possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of approximately 3,000 amino acids. HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins. HCV infects 170 million people worldwide and frequently leads to cirrhosis and hepatocellular carcinoma (36). In over one-half of patients, acute infection evolves into a persistent carrier state, presumably due to the ability of HCV to incapacitate the activation of the host immune mechanisms. Dendritic cells (DCs) are one type of potent antigen-presenting cell in vivo and play a crucial role in the enhancement and regulation of cell-mediated immune reactions. Since DCs express various costimulatory and/or adhesion molecules, they can activate even naïve T cells in a primary response. The role of the response of HCV antigen-specific T cells in viral clearance or persistence has been investigated extensively in both humans and chimpanzees (6, 27, 48, 51). These studies suggest that acute HCV infections followed by viral clearance are associated with a high frequency of HCV-specific $CD4^+$ and $CD8^+$ T-cell responses that can persist (27, 51), while chronic HCV infections are characterized by weak and restricted $CD4^+$ and $CD8^+$ T-cell responses that are not sustained (51).

Toll-like receptors (TLRs) are membrane-bound receptors that can be activated by the binding of molecular structures conserved among families of microbes. More than 10 different TLRs have been identified to date (2). They are highly conserved among mammals and are expressed in a variety of cell types. TLR binding and stimulation by pathogen-associated molecules is followed by a cascade of intracellular events that culminate in the expression of multiple genes (2). TLR signaling is mediated primarily by the adaptor protein myeloid differentiation factor 88 (MyD88), which triggers the activation of transcription factors, such as NF-κB, that are essential for the expression of proinflammatory cytokine genes (2). This pathway also leads to the potent production of type I interferon (IFN) through the activation of IFN regulatory factor 7 (IRF7) upon stimulation of TLR7 or TLR9 (22). In contrast, Toll/interleukin-1 (IL-1) receptor homology domain-containing adaptor-inducing IFN-β (TRIF/TICAM-1) mediates the production of type I IFNs primarily through the activation of IRF3 in response to TLR3 or TLR4 stimulation (2). Type I IFN induces the maturation of DCs by increasing both the expression of costimulatory molecules such as CD80, CD86, and CD40 and antigen presentation via major histocompatibility

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▽ Published ahead of print on 13 June 2007.