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Differential Role of TLR- and RLR-Signaling in the Immune Responses to Influenza A Virus Infection and Vaccination

Shohei Koyama,*|| Ken J. Ishii,†‡§# Himanshu Kumar,* Takeshi Tanimoto,*|| Cevayir Coban,*|| Satoshi Uematsu,* Taro Kawai,* and Shizuo Akira1,∗§§###

The innate immune system recognizes influenza A virus via TLR 7 or retinoic acid-inducible gene I in a cell-type specific manner in vitro, however, physiological function(s) of the MyD88- or interferon-β promoter stimulator 1 (IPS-1)-dependent signaling pathways in antiviral responses in vivo remain unclear. In this study, we show that although either MyD88- or IPS-1-signaling pathway was sufficient to control initial antiviral responses to intranasal influenza A virus infection, mice lacking both pathways failed to show antiviral responses, resulting in increased viral load in the lung. By contrast, induction of B cells or CD4 T cells specific to the dominant hemagglutinin or nuclear protein Ags respectively, was strictly dependent on MyD88 signaling, but not IPS-1 signaling, whereas induction of nuclear protein Ag-specific CD8 T cells was not impaired in the absence of either MyD88 or IPS-1. Moreover, vaccination of TLR7- and MyD88-deficient mice with inactivated virus failed to confer protection against a lethal live virus challenge. These results strongly suggest that either the MyD88 or IPS-1 signaling pathway is sufficient for initial antiviral responses, whereas the protective adaptive immune responses to influenza A virus are governed by the TLR7-MyD88 pathway.


Protective initial immune responses to viral infection rely largely on the induction of a set of antiviral proteins, including type-I IFNs, by infected cells and/or neighboring or recruited immune cells such as dendritic cells (DC)3 (1–3). Recent evidence suggests that host immune systems recognize nucleic acids, including RNA and DNA, derived from viral genomes or generated during viral replication. This recognition triggers type-I IFN as well as other cytokines and chemokines in TLR-dependent and TLR-independent manners (4–6); the mechanisms underlying this activation are unclear, but they are thought to play an important role in both innate and adaptive immune responses to viral infection (7, 8).

Influenza A virus is one of the most important causes of respiratory tract diseases. The influenza virus belongs to the Orthomyxoviridae family and consists of negative-sense single-stranded RNA genome (9). The virus genome, as ssRNA, is recognized by plasmacytoid DC through TLR7 and its adaptor MyD88, triggering robust type-I IFN production (10, 11). In contrast, fibroblasts or myeloid DC recognize the influenza A virus via retinoic acid-inducible gene I (RIG-I), a cytosolic RNA helicase (12, 13), triggering type-I IFN production through an adaptor protein IPS-1, interferon-β promoter stimulator 1 (IPS-1) (also known as MAVS, CARDIF, and VISA) (14–17), independently of the TLR7- and MyD88-pathways. A recent report suggests that influenza A virus infection does not generate dsRNA, but rather, that the 5′-triphosphate of its ssRNA genome is recognized by RIG-I (18, 19). In contrast, TLR3, known to be a receptor for dsRNA, has been shown to play a pathological role in influenza A virus infection in vivo (20). Nevertheless, such cell-type specific innate immune recognition of influenza A virus through distinct signaling pathways has not been elucidated in vivo especially in the case of airway infection and vaccination. Furthermore, innate immunity control of the adaptive immune response by TLR- or RIG-like receptor (RLR)-dependent signaling pathways has not been clarified, whereas TLR-dependent (21) and -independent (22) adaptive immune responses to influenza A virus have been observed.

To elucidate the mechanism(s) by which TLR-dependent or RLR-dependent recognition of influenza virus infection in vivo triggers innate and adaptive immune responses, we infected various mutant mice lacking MyD88, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF), IPS-1, TLR7, TLR9, or cells derived from these mice, with influenza virus, and measured their innate immune responses, including type-I IFN induction, and their adaptive immune responses, including the production of Ag-specific CD4, CD8 T cells, and B cells.

Materials and Methods

Animals, cells, viruses, and reagents

The generation of Trif−/−, Tr9−/−, Myd88−/−, Trif−/−, and Ips-1−/− mice has been described previously (23–26). These mice were back-crossed with C57BL/6 (B6) mice (CLEA) for over eight generations. In some experiments, Myd88−/−, Trif−/−, Ips-1−/− mice, and Ips-1−/− mice have been used.

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1 Abbreviations used in this paper: DC, dendritic cell; MEF, mouse embryonic fibroblast; IPS-1, interferon-β promoter stimulator 1; TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β; HA, hemagglutinin; NP, nuclear protein; IRF3, interferon regulatory factor 3; WT, wild type; NC, New Caledonia; PR, Puerto Rican; KO, knockout; RLR, RIG-like receptor.

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Myd88−/−, double deficient mice on a 129/Ola × C57BL/6 (B6;129) background were used. Influenza A virus (A/PR/8/34 (H1N1) and A/New Caledonia/20/99 (H1N1)), recombinant hemagglutinin (HA) protein and influenza vaccine were prepared as previously described (27). Mouse embryonic fibroblasts (MEF) from Myd88−/−, Trif−/− mice, Tank-binding kinase 1 (Tbk1−/−), Ibkbκ−/−, and Irs-1−/− mice were prepared as described previously (25, 26, 28). DCs were prepared as described previously (29). In brief, bone marrow cells were cultured in DMEM supplemented with 10% FCS, 100 μg/ml M-2M, and 100 ng/ml human Flt3 ligand (PeproTech) or 10 ng/ml murine GM-CSF (PeproTech), for 7–9 days, to use as FL-DCs and GM-DCs. Spleen adherent cells were prepared as described elsewhere (30).

**Influenza virus infection and vaccination**

MEFs were infected with 1 × 10^6 (6–7) PFUs of influenza virus to achieve infection at multiplicity of infection (MOI) = 1 or 10, in serum-free medium for 1 h. Washed with PBS and cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in an incubator with 5% CO2. For influenza virus infection, mice were anesthetized and administered intranasally with 40 μl of PBS (20 μl for each nose) containing serial amount of influenza A virus described above. For influenza vaccination, mice were anesthetized and administered intranasally with 40 μl of PBS (20 μl × 2) of PBS containing 1.0 μg/mouse New Caledonia (NC) vaccine.

**Western blot analysis**

After influenza A virus infection, MEFs, and lung tissue were lysed using a lysis buffer (Cell lytic-MT; Sigma-Aldrich) in the presence of a protease inhibitor mixture (Sigma-Aldrich). Lysates were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked and analyzed by Western blot using the following specific Abs: polyclonal mouse Ab to influenza virus, anti-phospho STAT1 (Cell Signaling Technology), anti-STAT1 (Santa Cruz Biotechnology), anti-phospho interferon regulatory factor 3 (IRF3; Cell Signaling Technology). Signals were detected using a HRP-conjugated secondary Ab and LumiGlo reagent (Cell Signaling Technology).

**Plaque assay**

Virus titers were determined using a plaque assay as previously described (27). In brief, confluent monolayers of Mardin-Darby canine kidney cells were incubated with serum-free Eagle’s MEM containing serial amount of influenza virus for 1 h in an incubator with 5% CO2. The cells were first overlaid with MEM supplemented with 1 μg trypsin ml and 0.6% agar and maintained in an incubator with 5% CO2 for 2 days. They were then overlaid again with MEM supplemented with 10% FBS, 0.6% agar, and 0.012% neutral red for 1 day and the number of plaques was then counted.

**RT-PCR**

RT-PCR was performed as previously described elsewhere (31). In brief, total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies) and then 1 μg total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen Life Technologies) according to the manufacturer’s protocol. cDNA was generated by standard PCR of 28 cycles with primers as described previously (30).

**ELISA**

Cell culture supernatants and mice sera were collected and analyzed for IFN-α, IFN-β, IL-6, IL-12p40, and CXCL10 production by ELISA according to the manufacturer’s protocol. ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories; those for IL-6, IL-12p40, and CXCL10 were obtained from R&D Systems.

**Serum Ab titer**

Ninety-six well plates were coated with a recombinant hemagglutinin (HA) protein at 1 μg/ml or ultrasonicated whole virus at 5 μg/ml in a carbonate buffer (pH 9.6), and incubated overnight at 4°C. Plates were then washed with PBS containing 0.05% Tween 20. Serial dilutions of serum in PBS/Tween 20 containing 5% skim milk were applied and incubated for 2 h at room temperature. After washing, Abs were detected using goat anti-mouse total IgG, IgG1, or IgG2a conjugated to HRP (Southern Biotechnology Associates). After an additional washing step, the plates were stained with 3, 3’-5’-tetramethylbenzidine (Sigma-Aldrich) as a substrate. The reaction was stopped with 1 M H2SO4 and the absorbance was measured. The cut-off value was defined as the mean value of absorption of negative control serum plus two SDs.

**Nuclear protein (NP)-specific IFN-γ production**

Spleens were extracted 14 days after the second infection and 5 × 10^6 spleen cells were seeded on 96-well plates and then stimulated with peptides specific for NP- and I-Aβ (ARSALILGSR/VAHKSCLAPCAVYG), H-2^d (ASNENMETM; 0.1, 1, 10 μg/ml), or Con-A (5 μg/ml). Seventy-two hours later, the cell culture supernatants were collected and analyzed for the IFN-γ concentration by ELISA.

**CTL assay**

An NP-specific CTL assay was performed as previously described (32). In brief, single cell suspensions of spleen cells were prepared from mice 14 days after the second infection, and were seeded onto 24-well plates and cultured in RPMI 1640-complete medium in the presence of a peptide specific to NP (H-2^d) (1 μg/ml), for 5 days, and used as effector cells. Ten-thousand target EL4 cells pulsed with the same peptide were incubated with increasing numbers of effectors for 4 h at 37°C in 5% CO2, and then the LDH levels in cell culture supernatants were measured according to the manufacturer’s protocol (Promega). Tetramer assays for measuring NP-specific CD8 T cell precursors were performed as previously described (33). In brief, peripheral blood was collected in the presence of heparin from mouse tails 14 and 21 days after influenza infection, and incubated with H-2D^b tetramer specific to NP peptide (ASNENMETM), PE-conjugated TRBβ, allophycocyanin-conjugated CD8 and anti-CD16/32 (Fc block), for 30 min at 37°C, and washed with PBS. Pellets were then fixed with 0.5% paraformaldehyde-PBS and analyzed with FACScalibur (BD Biosciences) using CellQuest software (BD Biosciences).

**Statistical analysis**

Statistical significance (p < 0.05) between groups was determined by the Student’s t test. A survival curve was generated using Kaplan-Meier methodology and the susceptibility of mice after infection was compared using the log-rank test.

**Results**

**Requirement of distinct innate immune signaling pathways for type-I IFN induction in response to influenza A virus infection in vitro**

We initially examined the roles of three distinct innate immune receptor-mediated signaling pathways, TLR7-MyD88 (10), TLR3-TRIF (34), and RIG-I-IPS-1 (35), in innate immune recognition of influenza virus infection in vitro. Three cell types derived from wild-type (WT) and mutant mice lacking MyD88 and/or TRIF, or IPS-1 were examined: MEFs and two types of bone marrow-derived DC generated in vitro in the presence of GM-CSF (referred to as GM-DC) and Flt3-ligand (referred to as FL-DC). The mRNA expression levels of Ifnb and Cxcl10 were up-regulated in WT MEFs infected with a nonvirulent strain of influenza A virus (A/New Caledonia/20/99 (H1N1), referred to as NC) and to a lesser extent with a virulent (A/Puerto Rico/8/34 (H1N1), referred to as PR) strain, at both MOI 10 and 1 (Fig. 1 and data not shown).

These up-regulations of Ifnb and Cxcl10 mRNAs were abrogated in IPS-1 deficient MEFs as well as in TBK1 and IKKα double deficient MEFs, whereas the levels of Ifnb and Cxcl10 mRNAs in MyD88 and TRIF deficient MEFs were comparable to those in WT MEFs (Fig. 1, a–c), suggesting that IPS-1 is essential for TLR-independent, TBK1/IKKα-dependent type-I IFN production in response to both virulent and nonvirulent influenza A virus infection in MEFs. This indispensable role of IPS-1 in type-I IFN production in MEFs in response to both virulent and nonvirulent influenza A virus was confirmed at the protein level by ELISA (Fig. 1, d and e), and was well associated with phosphorylation of both IRF3 and STAT1 (Fig. 1f).

When GM-DCs, which are conventional DCs of myeloid lineage, were infected with the two strains of influenza A virus, production of both IFN-β and IFN-α was dependent on IPS-1, but...
not MyD88, TRIF, TLR7, or TLR9 (Fig. 2), similar to the observations in MEFs. By sharp contrast, FL-DCs, which contain plasmacytoid DCs, the so-called IFN-producing cells, produced comparable levels of IFN-α and IFN-β between those derived from WT and IPS-1-deficient mice, in response to both PR and NC (Fig. 3a). In contrast, FL-DCs derived from mice lacking MyD88 and TLR7, but not TRIF or TLR9, failed to produce IFN-α and IFN-β in response to either strain of influenza A viruses (Fig. 3, b and c). These data confirm previous reports (13) and suggest that type-I IFN induction by RNA viruses is mediated through distinct innate immune receptors in a cell-type specific manner.

Roles of MyD88- and IPS-1-dependent pathways in the antiviral A virus response in vivo

These results prompted us to examine the roles of these distinct recognition/signaling pathways in the innate immune response to influenza virus infection in vivo. WT mice, mice lacking MyD88 and IPS-1, and mice lacking both MyD88 and IPS-1 were infected

FIGURE 1. Innate immune activation of MEFs in response to influenza virus infection. a–c, The expression levels of Ifnb (IFN-β), Cxcl10 (IP-10), and Actb (β-actin) mRNAs in MEFs infected with influenza virus PR(A/PR/8/34) and NC (A/New Caledonia/20/99), as indicated, were assessed by RT-PCR at 6 and 24 h after infection at 10 MOI. d and e, The amount of IFN-β in the culture supernatant was measured by ELISA at 24 h and 48 h after PR and NC infection at 0.1 MOI (d), and 24 h after infection at 10 and 1 MOI (e). Error bars, SD of duplicate wells in a single experiment. f, STAT1 and IRF3 phosphorylation in WT and Ips-1−/− MEFLs was assessed by Western blotting as indicated, at various time points after NC infection. ND, Not detected.

FIGURE 2. Type I IFN production by GM-DCs in response to influenza A virus infection. IFN-β (a) and IFN-α (b) production were measured by ELISA at 24 h after PR and NC infection at 10 MOI. Error bars indicate the SD of duplicate wells in a single experiment. a, WT and Ips-1−/− GM-DCs. b, WT, Myd88−/−, and Trif−/− GM-DCs. c, WT, Tlr7−/−, and Tlr9−/− GM-DCs.
intronasally with $2 \times 10^4$ PFU of influenza A virus NC strain. Twenty-four hours after infection, whole lung mRNA expression of type-I IFNs, cytokines and chemokines were evaluated by RT-PCR. The mRNA expression levels of \textit{Ifna}, \textit{Ifnb}, and \textit{Cxcl10} were up-regulated in MyD88- or IPS-1-deficient lung to a level comparable to that seen in WT lung; however, expression of these mRNAs was diminished in MyD88 and IPS-1 doubly deficient lung (Fig. 4\textit{a}). Similarly, phosphorylation of STAT1 was detected in infected lung homogenates of WT, MyD88-, and IPS-1-deficient mice, but was totally absent in MyD88 and IPS-1 doubly deficient lung (Fig. 4\textit{b}). Serum IP-10 (CXCL10) was also increased in infected WT, MyD88-, and IPS-1-deficient mice, but was severely reduced in MyD88 and IPS-1 doubly deficient mouse sera (Fig. 4\textit{c}). Viral titers in the lung measured at 24 h and 6 days after infection were significantly higher in MyD88 and IPS-1 doubly deficient lung compared with WT, MyD88-, or IPS-1-deficient

**FIGURE 3.** Type-I IFN production by FL-DCs in response to influenza A virus infection. IFN-\(\beta\) (\textit{a}) and IFN-\(\alpha\) (\textit{b}) production were measured by ELISA 24 h after PR and NC infection at 10 MOI. Error bars, SD of duplicate wells in a single experiment. \(\textit{a}\), WT and Ips-1\(-/-\) GM-DCs. \(\textit{b}\), WT, Myd88\(-/-\), and Trif\(-/-\) GM-DCs. \(\textit{c}\), WT, Tlr7\(-/-\), and Tlr9\(-/-\) GM-DCs.

**FIGURE 4.** Innate immune response of lung to in vivo infection with influenza A virus. Mice were infected intranasally with $2 \times 10^4$ PFU/mouse NC strain and lungs were extracted 24 h after infection. \(\textit{a}\), The expression of mRNA and (\(\textit{b}\)) the STAT1 activation and control total STAT1 in the lungs of WT, Myd88\(-/-\), Ips-1\(-/-\), and Myd88\(-/-\), Ips-1\(-/-\) mice were assessed by RT-PCR and Western blotting. \(\textit{c}\), ELISA of serum IP-10. Error bars, SE of duplicate wells in a single experiment. \(\textit{d}\), The lung virus titers of WT, Myd88\(-/-\), Ips-1\(-/-\), and Myd88\(-/-\), Ips-1\(-/-\) were assessed by plaque assay at 1 day and 6 days after infection. Error bars (10% and 90%) and boxes (25% and 75%) and a horizontal line (median) are shown. *, \(p < 0.05\) vs WT control. The results shown are representative of two independent experiments.
lungs, all of which showed comparable levels of viral titers (Fig. 4d). TRIF-deficient mice infected with influenza A virus elicited expression of type-I IFN mRNAs in the lung at comparable levels to those seen in WT mice (data not shown). These data strongly suggest that the presence of either MyD88- or IPS-1-dependent innate immune signaling pathways was sufficient to trigger type-I

**FIGURE 5.** Serum Ab responses to HA Ag or whole virion of influenza A virus after infection. WT, MyD88-, TRIF-, or IPS-1-deficient mice (n = 4) were infected with either the PR (a) or NC (b) strain of influenza A virus intranasally. At various days after the first and second infections, as described in the methods, the serum titers of anti-HA IgG, IgG1, IgG2a, or anti-virion (c) were measured by ELISA. *, p < 0.05 vs WT control.
IFN production, and to control the viral load in the lung, suggesting that these two pathways each compensate for the anti-viral functions of the other.

Effect of the TLR7-MyD88 and RIG-I-IPS-1 pathways on induction of adaptive immune responses to influenza A virus infection

We further explored the roles of these two innate immune signaling pathways in the induction of adaptive immune response(s) to influenza A virus infection in vivo. WT mice or mice lacking MyD88, TRIF, or IPS-1 were infected intranasally with either 2 × 10^5 PFU of a PR strain of influenza A virus or 2 × 10^5 PFU of an NC strain of influenza A virus. Fourteen days after infection, mice were reinfected with 2 × 10^5 PFU of the same strain of virus. Serum was collected at 14 days after the first infection and 7 days after second infection, and was measured for Ab-titer specific to a protective Ag, HA. HA-specific IgG, including IgG1 and IgG2a, was detected at 14 days after initial infection and strongly enhanced after secondary infection with both PR and NC in IPS-1- and TRIF-deficient mice to a level comparable to that of WT mice (Fig. 5, a and b). However, MyD88 knockout (KO) mice had significantly lower titers of total anti-HA IgG and IgG2a than WT mice, after infection with PR and NC strains, while IgG1 level were comparable between WT and MyD88 KO mice (Fig. 5, a and b). By contrast, IPS-1-deficient mice mounted a similar response to WT mice, producing Abs of total anti-HA IgG and cognate isotypes (Fig. 5, a and b). MyD88-deficient induction of anti-HA IgG and IgG2a was abolished in TLR7-deficient but not in TLR9 deficient mice (data not shown), suggesting that these immunodominant HA-specific B cell responses are mediated by TLR7- and MyD88-dependent signaling pathways. When we measured the serum titer of IgG including IgG1 and IgG2a against whole virus, it was of note that there was no significant difference in the levels of any of the IgGs tested among the mouse groups infected with PR strain; however, the production of IgG and IgG2a was impaired in MyD88-deficient mice infected with the NC strain compared with WT or TRIF-deficient mice (Fig. 5c).

We then examined virus-specific CD4 T cell production of IFN-γ after secondary infection. When spleen cells from WT mice were infected with either the PR or NC strain of influenza A virus were stimulated with I-A^b^- and NP-specific peptide in vitro, Ag-specific and peptide dose-dependent IFN-γ secretion were observed (Fig. 6). By contrast, MyD88-deficient spleen cells, but not TRIF- or IPS-1-deficient spleen cells, produced significantly less IFN-γ in response to the NP I-A^b^- peptide, whereas the responses to Con-A were intact in all cells (Fig. 6). This MyD88-dependent CD4 T cell induction specific to the immunodominant NP Ag was abolished in TLR7-deficient mice, but not in TLR9-deficient mice (data not shown), suggesting that induction of virus-specific CD4 T cells and B cells is dependent on a TLR7-mediated, MyD88-dependent signaling pathway, not a TRIF- or IPS-1-dependent pathway.

Virus-specific CD8 T cell activation was also evaluated in terms of the frequency of their precursors, Ag-specific IFN-γ production and cytotoxicity. Virus-infected mice with sublethal dose of the virulent PR strain, were analyzed for NP-specific CTL precursors in peripheral blood. After secondary infection with the PR strain, the number of NP- and H-2b-specific CD8 T cells was measured by tetramer assay in the peripheral blood, as well as by NP- and H-2b-specific lysis by the spleen cells. Although we observed a substantial decrease in NP- and H-2b-specific lysis in MyD88 KO mice infected with the PR strain compared with other infected groups (Fig. 7), WT and MyD88- or IPS-1-deficient mice infected with the nonvirulent NC strain showed comparable CTL responses measured as the level of NP-specific CD8 T cell precursors in peripheral blood and NP-specific cytotoxicity (Fig. 7 and data not shown). Similarly, there was no significant difference in NP- and H-2b-specific lysis in TLR7 or TLR9 KO mice (data not shown). These data suggest that the CD8 T cell response to virulent or nonvirulent NC strains of the influenza A virus is not mediated by MyD88-, TRIF-, or IPS-1-mediated innate immune signaling alone.

A TLR7-mediated, MyD88-dependent signaling pathway is required for induction of the protective immune response by influenza A virus vaccination

We finally examined the role of the innate immune signaling pathway in influenza A virus vaccine-induced immunogenicity, as well as its efficacy. WT, MyD88-, or TLR7-deficient mice were immunized with an inactivated vaccine of the NC strain. After secondary immunization, mice were challenged with the virulent PR strain at a lethal dose. NC vaccine immunization protected WT mice, but...
The results shown above strongly suggest that innate immune responses to influenza A virus are not regulated by a single receptor or intracellular signaling pathway; rather, they appear to be regulated by multiple receptors and distinct signaling pathways in an orchestrated manner, at least in the case of lung infection. Consistent with previous reports including studies of other RNA viruses (13, 25, 40, 41), IPS-1 was required for MEFs and GM-DCs to trigger type-I IFN production in response to influenza A virus infection in vitro, whereas MyD88 was required for FL-DCs to trigger the same response (Fig. 1–3). However, the results obtained from mouse lung and serum infected with influenza A virus suggest that abrogation of either the MyD88- or IPS-1-dependent pathway does not affect lung mRNA expression of type-I IFNs and chemokines. Rather, the two pathways compensate for each other as their doubly deficient lung failed to up-regulate antiviral responses (Fig. 4, a–c), resulting in significantly higher virus titers than single KO mice (Fig. 4d). Thus, protective innate immune responses to influenza lung infection, characterized by induction of type-I IFNs, were dependent on either MyD88 or IPS-1. Based on the results obtained in MEFs and DCs, it is conceivable that non-immune cells in the lung, such as epithelial cells and fibroblasts, as well as myeloid cells, including macrophages and conventional DCs, may be responsible for IPS-1-dependent production of type-I IFNs. MyD88-dependent production of type-I IFNs may be due to the presence of TLR7-expressing immune cells such as plasmacytoid DCs, which are known to be a potent type-I IFN producer, or B cells, although further clarification of the cell type(s), which resides in, is or recruited to, the infected lung tissue will be needed.

In contrast to the essential role of these two distinct MyD88- and IPS-1-dependent signaling pathways in the induction of antiviral innate immune responses to influenza A virus infection, our results also suggest that MyD88 signaling, but not IPS-1 signaling, plays a critical role in the adaptive immune responses characterized by viral Ag (HA)-specific B cell production of Ab and NP-specific CD4 Th1 production of IFN-γ (Fig. 5 and 6). We initially noticed that serum titer of IgG against whole virion was dependent on MyD88 in the mice vaccinated with nonvirulent NC strain, but not in the mice immunized with virulent PR strain (Fig. 5c). The
strain-specific dependence for induction of anti-virion IgG may reflect the two contradictory previous reports (21, 22). Serum IgG titer (IgG2a, in particular) against “immunodominant” HA Ag, however, were strictly dependent on MyD88 (and TLR7, data not shown) in both NC and PR strains (Fig. 5, a and b), suggesting that MyD88-dependent signaling pathway is critical for induction of the protective IgG against “immunodominant Ag”. Such dependency of the induction of not only B cells but also CD4 T cells specific to immunodominant Ags on the MyD88-dependent pathway might be attributed to a loss of TLR7, because TLR7-deficient...
mice, but not TLR9-deficient mice, displayed an identical phenotype to that of MyD88-deficient mice (data not shown). Moreover, the protective immunity raised by the TLR7 and MyD88-dependent pathway was consistent between mice infected with either virulent or nonvirulent strains of influenza A virus, and even with those receiving inactivated (replication free) vaccine immunization (Fig. 5, 6 and 8), suggesting that TLR7-mediated recognition of a well-conserved molecular pattern in the virion, most probably genome RNA, acts as an internal adjuvant for protective adaptive immune responses. Our results also support a recent finding that TLR-mediated innate immune recognition specifically regulates the immunodominance in antimicrobial CD4 T cells in vivo (42). As the titer of IgG specific to HA alone is known to confer protection against influenza challenge (43), we emphasize the importance of the TLR7-mediated, MyD88-dependent signaling pathway in the immunogenicity of influenza vaccine, and the protective adaptive immune responses to influenza A virus for preventive as well as therapeutic applications.

The CTL response, however, seems to be regulated by an additional factor(s), which was distinct from TLR7- and MyD88-dependent B and CD4 T cell responses. We observed a reduction in the level of CTL precursors in the peripheral blood as well as spleen CTL function in MyD88- and TLR7-deficient mice infected with sublethal doses of the virulent PR strain, compared with those of WT or IPS-1-deficient mice (Fig. 7 and data not shown). It is, therefore, possible that CTL responses induced by influenza A virus infection or vaccination may play some roles in the protective immune responses to the following challenge in a MyD88-dependent manner. However, wild-type and MyD88- or IPS-1-deficient mice infected with high doses of the nonvirulent (nonlethal) NC strain elicited comparable numbers of CTL precursors in the blood and similar CTL function in the spleen (Fig. 7). This discrepancy might be due to a difference in the virus dose between PR and NC infections; alternatively, the superior ability of nonvirulent NC strains to induce host type-I IFNs and their inducible chemokines during infection may enhance NP-specific CD8 T cell induction. In fact, type-I IFNs and certain chemokines have been shown to activate CD8 T cells in an Ag independent manner: so-called bystander activation (44). In this case, the IPS-1-mediated pathway for type-I IFN induction (and/or that of other factors) may compensate for the CD8 T cell response to influenza A virus in MyD88- or TLR7-deficient mice, whereas IPS-1-deficient mice might have sufficient type-I IFNs or chemokines through the TLR7-dependent, MyD88-dependent pathway (Fig. 4). In addition, NP-specific CD8 T cells become dominant after secondary infection, because nonimmune cells rather than DC can present NP peptide (45), indicating that nonimmune cells may play a role in this MyD88-independent CD8 T cell response to influenza virus. Thus, it will be of interest to investigate the role of the IPS-1-dependent pathway without TLR/MyD88-dependent type-I IFN production in the induction of Ag-specific CD8 T cells after nonvirulent NC strain infection. Although we focus in this work on the effect of type-I IFNs, there will likely be a need to clarify the role of novel type-III IFNs (also termed as IL-28 and IL-29) in anti-viral innate and adaptive immune responses (46).

Taken together, our results demonstrate that innate immune recognition of influenza virus through TLR7/MyD88-dependent and RLR/IPS-1-dependent pathways play a pivotal role in initial anti-viral responses. Furthermore, the TLR7/MyD88 pathway, but not the RLR/IPS-1 pathway, plays an essential role in the induction of protective immune responses to the dominant Ags.

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Disclosures
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