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*J Immunol* 2007; 179:4711-4720; doi: 10.4049/jimmunol.179.7.4711

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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. The Journal of Immunology, 2007, 179: 4711–4720.

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) (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  Supplemental Table II, Trif-/-, and Ips-1-/- mice has been described previously (23–26). These mice were backcrossed with C57BL/6 (B6) mice (C57/BL6) for over eight generations. In some experiments, Myd88-/-, Trif-/-, Ips-1-/- mice, and Ips-1+/-,...
embryonic fibroblasts (MEF) from enza vaccine were prepared as previously described (27). Mouse donia/20/99 (H1N1), recombinant hemagglutinin (HA) protein and influ-
ground were used. Influenza A virus (A/PR/8/34 (H1N1) and A/New Cale-

For influenza virus infection, mice were anesthetized and administered

IFN-

were incubated with serum-free Eagle’s MEM containing serial amount of

SNP-CD8 T cell precursors were performed as previously described

The reaction was stopped with 1 MH 2SO4 and the absorbance was

were incubated with serum-free Eagle’s MEM containing serial amount of

were incubated with H-2Dp tetramer specific to NP (ASNNMETM), PE-conju-
gated TCR5, allophtocyanin-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).

TCRβ, 3. C57BL/6 (B6.129) back-

702 PFUs of influenza virus to achieve

influenza virus for 1 hi na nincubator with 5% CO2. The cells were first

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 Technolog-

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 ac-

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 Biotechn-
ology Associates). After an additional washing step, the plates were

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*) (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-2Dp tetramer specific to NP peptide (ASNNMETM), PE-conju-
gated TCRβ, 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-

These up-regulations 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

Nuclear protein (NP)-specific IFN-γ production

Spleens were extracted 14 days after the second infection and 5 × 10^5 spleen cells were seeded on 96-well plates and then stimulated with pep-
tides specific for NP- and I-Ab (ARSALILRGSVAHKSCLPACVYGP), H-2*a (ASNNMETM: 9.1, 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

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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.
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 anti-influenza 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 or IPS-1, and mice lacking both MyD88 and IPS-1 were infected

![FIGURE 1. Innate immune activation of MEFs in response to influenza 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−/− MEFs was assessed by Western blotting as indicated, at various time points after NC infection. ND, Not detected.](http://www.jimmunol.org/)

![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.](http://www.jimmunol.org/)
intranasally 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 Ifna, Ifnb, and 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. 4a). 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. 4b). 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. 4c). 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-β (a) and IFN-α (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. a, WT and Ips-1−/− GM-DCs. b, WT, Myd88−/−, and Trif−/− GM-DCs. 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. a, The expression of mRNA and (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. c, ELISA of serum IP-10. Error bars, SE of duplicate wells in a single experiment. d, The lung virus titers of WT, Myd88−/−, Ips-1−/−, and Myd88−/−, Ips-1−/− mice 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
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 $2 \times 10^2$ PFU of a PR strain of influenza A virus or $2 \times 10^5$ PFU of an NC strain of influenza A virus. Fourteen days after infection, mice were reinfected with $2 \times 10^5$ 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 iso-types (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 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 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-2^b^-specific CD8 T cells was measured by tetramer assay in the peripheral blood, as well as by NP- and H-2^b^-specific lysis by the spleen cells. Although we observed a substantial decrease in NP- and H-2^b^-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-2^b^-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
FIGURE 6. IFN-γ production by spleen cells specific to CD4 T cell epitope (I-A\[^b\]) peptide derived from NP after influenza A virus infection. Spleen cells from mice infected with the PR (a) or NC strain (b) of influenza A virus as described in Fig. 5 were stimulated with a peptide specific to NP and I-A\[^b\] at various concentrations, as indicated, for 72 h. The concentration of IFN-γ in the supernatant was then measured by ELISA. *, \( p < 0.05 \) vs WT control.

not MyD88- or TLR7-deficient mice, against lethal challenge with the virulent PR strain (Fig. 8). When the serum titer of anti-HA IgG, which is a well known and critical indicator for influenza vaccine efficacy, was measured just before the challenge, the anti-HA titer was significantly reduced in MyD88- and TLR7-deficient mice compared with WT mice (Fig. 8). Without vaccination, MyD88- and TLR7-deficient mice survived lethal PR infection significantly better than WT mice (S. Koyama, unpublished results). Taken together, these data suggest that a TLR7-mediated, MyD88-dependent signaling pathway plays an important role in influenza vaccine-induced immunogenicity and efficacy in vivo.

Discussion

Nucleic acid-induced innate immune responses are differentially regulated by the type of ligand nucleic acids (RNA or DNA, single- or double-stranded, short or long, backbone, nucleotide modification, and/or the sequences), the host receptors, cells that express the cognate receptor(s), or mode of cell entry (coreceptor or cofactor) (36, 37). In influenza A virus infection, accumulating evidence suggests that influenza viral RNA is immunostimulatory and can be detected by distinct host receptors: TLRs and RLRs (38, 39). Their distinct signaling pathways are mediated by their specific adaptor molecules, such as MyD88 and IPS-1, respectively, which have been shown to play an important role in the induction of antiviral responses in vitro. Influenza A viruses might initially infect stromal cells, such as epithelial cells and fibroblasts, and release their nucleic acids into the cytosol and/or nucleus. It is conceivable that viral RNA in the cytosol is detected by RIG-I, triggering robust innate immune responses including type-I IFNs and proinflammatory cytokines, through IPS-1. TLR7-mediated, MyD88-dependent innate immune activation might occur simultaneously or subsequently, when virus or infected cells are captured via endocytosis by immune cells, such as macrophages and dendritic cells. Although TLR7-mediated innate immune recognition of influenza A virus and its signaling pathway in vivo has been described previously (21, 22), the contribution of RIG-like receptors and their signaling pathway through IPS-1 to influenza A virus infection in vivo, had not been addressed.

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 is 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). 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.

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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.

Acknowledgments
We thank Dr. Kuniaki Koyama and Drs. Hiroki Kato and Osamu Takeuchi for their scientific advice and Yukiko Fujita for her excellent technical assistance. We also thank Dr. Masahito Ebina and Prof. Toshihiro Nukiwa for their generous support. This study was supported by Grants from the Ministry of Education, Culture, Sports, Science and Technology in Japan, and from the 21st Century Center of Excellence Program of Japan.

Disclosures
The authors have no financial conflict of interest.

FIGURE 8. Effect of TLR7 and MyD88 on influenza vaccination. WT, MyD88-, or TLR7-deficient mice (n = 8) were immunized with inactivated influenza A virus, and serum anti-HA Ab titer was monitored (b). Mice were then infected with a lethal live PR strain of influenza A virus (2 × 10⁴ PFU/mouse). Survival and body weight were monitored (a). *, p < 0.05 vs WT control.
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