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INFECTIOUS DISEASE

Plasmacytoid Dendritic Cells Delineate Immunogenicity of Influenza Vaccine Subtypes

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A variety of different vaccine types are available for H1N1 influenza A virus infections; however, their immunological mechanisms of action remain unclear. Here, we show that plasmacytoid dendritic cells (pDCs) and type I interferon (IFN)–mediated signaling delineate the immunogenicity of live attenuated virus, inactivated whole-virus (WV), and split virus vaccines. Although Toll-like receptor 7 acted as the adjuvant receptor for the immunogenicity of both live virus and WV vaccines, the requirement for type I IFN production by pDCs for the immunogenicity of the vaccines was restricted to WV. A split vaccine commonly used in humans failed to immunize naïve mice, but a pDC-activating adjuvant could restore immunogenicity. In blood from human adults, however, split vaccine alone could recall memory T cell responses, underscoring the importance of this adjuvant pathway for primary, but not secondary, vaccination.

INTRODUCTION

Vaccination is considered to be the best prophylaxis for influenza virus infection (*1*). There are three different types of influenza virus vaccines: live attenuated influenza virus (LAIV), formalin-inactivated whole-virus (WV) vaccine, and ether-treated hemagglutinin (HA) antigen–enriched virion-free "split" virus (SV) or "subunit" virus (SU) vaccine (*2*). Among them, SV and SU are the most commonly used in clinics because there are fewer reactogenicities, although LAIV and WV have been shown to have superior immunogenicity, especially in children (*3*–*5*). The immunogenicity and efficacy of these vaccines can be affected by host factors, such as age and immunological status [such as immunodeficiency (*6*–*8*)], and viral factors, including the antigenic mismatch between the vaccines and the circulating virus strains (*9*). However, the exact mechanisms used by the three types of vaccine compositions to achieve immunogenicity and how these mechanisms differ are not fully understood.

It is known that most, if not all, successful vaccines that induce strong protection against pathogens contain adjuvant components that activate the innate immune system via specific receptors, including Toll-like receptors (TLRs), retinoic acid–inducible gene-I (RIG-I)–like receptors (RLRs), and Nod-like receptors (NLRs) (*10*–*12*). These receptors are expressed by antigen-presenting cells, such as dendritic cells (DCs), which mediate the subsequent adaptive immune response by releasing specific cytokines, such as interferons (IFNs) and interleukins (ILs), and activating antigen-specific T and B cells. The nature of the adjuvant and the specific receptors that are activated on DCs can determine the type of immune response that is generated (*10*–*12*). Recent studies suggest that TLR7 and a certain NLR that activates the inflammasome—a collection of components that spur the innate immune system—are involved in controlling adaptive immune responses to influenza A virus infection (*13*, *14*). Our previous work has characterized a key role for TLR signaling in the immune response to inactivated WV vaccination (*13*); however, no comprehensive study directly compares the role of TLRs, NLRs, and RLRs in either live virus or inactivated WV vaccinations (*15*). Here, we decipher the mechanism(s) by which the various virus preparations drive development of immunogenicity.

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RESULTS

Inactivated WV requires TLR7-mediated, but not RLR- or NLR-mediated, immune activation for its immunogenicity

We first examined whether an inactivated WV preparation of the A/New Caledonia/20/1999 (NC) (H1N1) strain can immunize mice lacking TLR7, IPS-1 (IFN-β promoter stimulator–1; an adaptor protein for RIG-I–mediated type I IFN production), and ASC (apoptotic speck protein; containing a caspase recruitment domain, an adaptor molecule required for NLRP3 inflammasome activation). All three of these genes have been shown to be involved in innate immune recognition of live influenza virus infection (*14*, *16*–*19*).

We immunized TLR7-deficient or IPS-1–deficient mice intranasally (i.n.) with WV of NC H1N1 twice at a 2-week interval. One week after the second injection, the vaccination-induced, virus-specific B cell and $CD4^+$ T cell adaptive immune responses were analyzed. TLR7-deficient mice showed virtually no virus-specific immunoglobulin A (IgA) in bronchoalveolar lavage fluid (BALF), no virus-specific IgG in serum (Fig. 1A), and no IFN-γ production from $CD4^+$ T cells specific to a viral nucleoprotein (NP) antigen epitope ($NP_{260–283}$ specific to $I\text{-}A^b$) (Fig. 1B), whereas IPS-1–deficient mice mounted comparable levels of these responses to wild-type mice (Fig. 1, A and B). Consistently, the immunized TLR7-deficient mice were not protected against a lethal challenge with the A/Puerto Rico/8/34 (PR) (H1N1) strain, whereas the immunized IPS-1–deficient mice were protected to a level comparable to vaccinated wild-type mice (Fig. 1C). We

obtained similar results with intramuscular (i.m.) immunization of these mice, with respect to total IgG titer and IgG2a titer (fig. S1). In contrast to i.n. immunization, i.m. immunization failed to elicit BALF IgA in either wild-type or TLR7-deficient mice. There was an enhanced serum IgG1 response in TLR7-deficient mice relative to wild-type mice, but this response failed to protect the TLR7-deficient mice from lethal challenge with PR H1N1 (fig. S1, A and C). Thus, the inactivated WV vaccine requires TLR7-mediated, but not RIG-I–mediated, IPS-1 for its immunogenicity, which is consistent with findings for the live virus vaccination (*13*).

We also immunized ASC-deficient mice with WV (NC H1N1) because a role for the ASC inflammasome in the adaptive immune response to influenza virus infection is controversial (*14*, *19*). ASC-deficient mice did not show any defects in their adaptive immune responses to WV immunization relative to wild-type mice (Fig. 1, D and E). Notably, ASC was not involved in the immunogenicity of live virus immunization, except for serum IgG1 production, whereas $CD8^+$ T cell responses were not observed with WV immunization (fig. S2). Immunized wild-type and ASC-deficient mice were similarly protected against lethal PR H1N1 virus challenge relative to naïve wild-type mice (Fig. 1F). Therefore, ASC inflammasome activation was not essential for the induction of virus-specific B and CD4 T cell responses to WV.

The type I IFN receptor is important for the immunogenic response to inactivated WV vaccine but not to the live virus

Because both the inactivated WV and the live virus require TLR7 for their immunogenicity, we sought to identify the downstream effector molecule(s) involved. Type I IFNs, such as IFN-α and IFN-β, are known to have potent adjuvant activity (*20*, *21*), and a recent study showed that WV immunization substantially up-regulated the expression of IFN-inducible genes, such as *Cxcl10* (*22*). Therefore, as a systemic indicator of type I IFN responses, we examined the amounts of the cytokine CXCL10 in the sera of mice 24 hours after vaccination. The induction of CXCL10 was significantly reduced in TLR7-deficient mice relative to wild-type, IPS-1–deficient, and ASC-deficient mice (Fig. 2A). Similar results were obtained for messenger RNA (mRNA) analyses of IFN-β and CXCL10 in the lung (fig. S3A), suggesting that type I IFNs might be dominant effector molecules in this TLR7-dependent system. To test this hypothesis directly, we i.n. immunized mice deficient in the IFN-α and IFN-β receptor 2 (IFNAR2) with WV as in Fig. 1. IFNAR2-deficient mice failed to induce virus-specific antibodies (including BALF IgA and serum IgG) and $CD4^+$ T cell responses (Fig. 2B and fig. S3, B and C) relative to the heterozygous IFNAR2 knockout mice. As a result, mortality was increased and a significantly larger body weight loss was observed after lethal PR H1N1 challenge in the IFNAR2-deficient mice (Fig. 2C). Naïve wild-type and IFNAR2-deficient mice showed similar susceptibilities to PR H1N1 challenge, consistent with a previous study (*23*). Similar results were also observed after i.m. immunization (fig. S3, D and E). When naïve IFNAR2-deficient mice were immunized with live virus, there was no alteration of the virus-specific serum IgG and IFN-γ secretion by virus NP antigen-specific $CD4^+$ T cells ($NP_{260–283}$ specific to $I\text{-}A^b$) and $CD8^+$ T cells ($NP_{366–374}$ specific to $H\text{-}2D^b$) (fig. S3, F to I), consistent with a previous study (*23*). Together, these results suggest that TLR7, but not RLRs or NLRs, is required for immunogenicity of inactivated as well as live influenza virus vaccination. In addition, the type I IFN receptor–mediated signaling pathway was critical for the immunogenic response to WV but not to the live virus.

Live virus and inactivated WV vaccines induce type I IFNs through distinct DC types

DCs are a critical component of the innate immune system that recognize vaccines and mediate the adaptive immune response. There are different subtypes of DCs that can be divided loosely into conventional myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), which express different receptors and secrete different cytokines on activation. To characterize in more detail the immune response triggered by the WV vaccine and live virus, we stimulated two types of bone marrow–derived DCs—Flt3

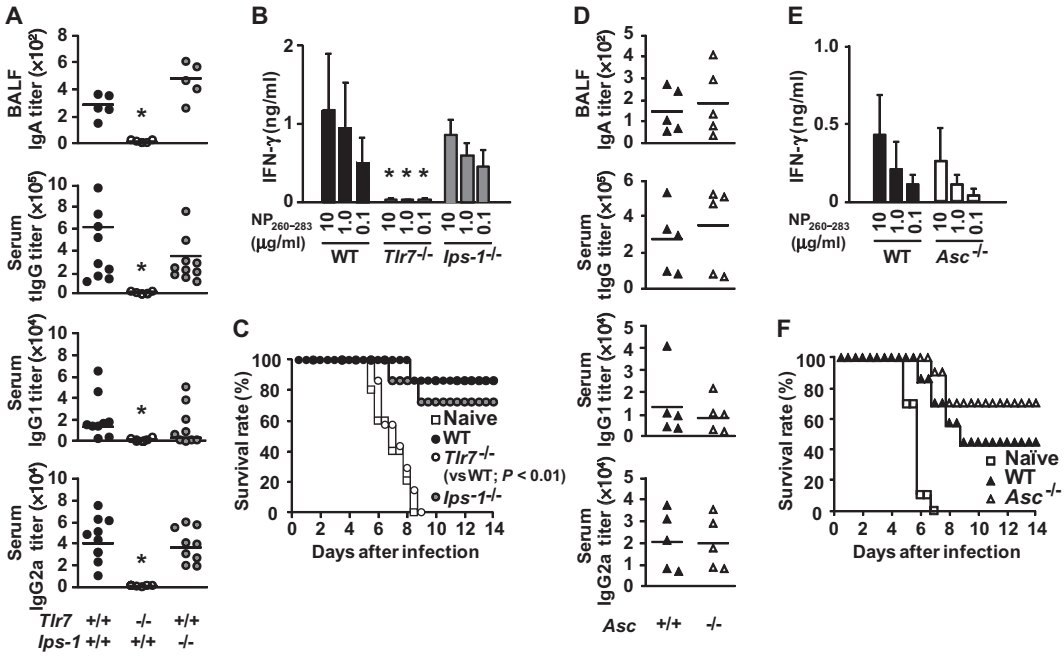

Fig. 1. TLR7-dependent, but not IPS-1–dependent or ASC-dependent, signaling is required for the induction of protective immune responses by inactivated WV. (**A** to **C**) Wild-type (WT), *Tlr7*-deficient, and *Ips-1*–deficient mice ($n = 9$ per group) were i.n. vaccinated with WV of NC (1.5 μg per mouse) twice, with a 2-week interval. One week after the second vaccination, we measured titers of antigen-specific mucosal (BALF) IgA and serum total IgG (tIgG), IgG1, and IgG2a (A) and IFN-γ production by $CD4^+$ T cells (B) by ELISA as described in Materials and Methods. *$P < 0.05$ versus WT mice. (C) Vaccinated mice ($n = 9$ per group) were challenged with $10 \times LD_{50}$ (median lethal dose) (2×10^4 PFU per mouse) of lethal influenza virus PR, and their survival was monitored. *$P < 0.05$ versus vaccinated WT mice. (**D** to **F**) WT and *Asc*-deficient mice ($n = 5$, each group) were similarly vaccinated, and their antigen-specific antibody responses (D), IFN-γ production by $CD4^+$ T cells (E), and survival (WT, $n = 7$; *Asc*-deficient, $n = 10$; naïve, $n = 10$) (F) were determined by ELISA as described in Materials and Methods. Each bar represents the mean (A and D) or mean ± SD (B and E). These results are representative of two independent experiments.

ligand–generated DCs (FL-DCs; which contain pDCs) and granulocyte-macrophage colony-stimulating factor (GM-CSF)–generated DCs (GM-DCs; which contain conventional DCs but no pDCs)—with the live virus or WV and then measured their IFN-β production by enzyme-linked immunosorbent assay (ELISA). The live virus strongly stimulated FL-DCs to produce IFN-β in a TLR7-dependent manner, whereas production by GM-DCs was in a TLR7-independent manner (Fig. 2D), consistent with our previous study (*13*). In contrast, WV-activated FL-DCs, but not GM-DCs, produced IFN-β, a process entirely dependent on TLR7 signaling (Fig. 2D), indicating that there is a clear distinction between the live virus and WV in terms of their abilities to activate DCs to secrete type I IFNs.

To confirm these in vitro observations in vivo, we next depleted pDCs in vivo to examine the role of these cells in the induction of adaptive immune responses to live virus and WV, as pDCs are known to play a key role in bridging the innate and adaptive immune responses (*24*). Wild-type mice were treated intravenously with an antibody to mPDCA-1 (*25*) and then immunized 24 hours later with the live virus or WV. Depletion of pDCs was confirmed in the spleen (fig. S4). After live virus vaccination, the concentrations of mRNAs derived from genes involved in induction of the adaptive immune response, specifically *Ifnb*, *Cxcl10*, *Il6*, and *Ccl2*, were clearly elevated in the lung irrespective of treatment with antibody to mPDCA-1 (Fig. 2E). In contrast, these transcriptional responses were severely impaired after WV vaccination in pDC-depleted mice relative to isotype control antibody–treated mice (Fig. 2E). Serum CXCL10 was also reduced in pDC-depleted mice treated with WV but not the live virus (Fig. 2F). Thus, both live virus and WV induced type I IFNs predominantly through pDCs in vivo; however, WV was dominantly recognized by pDCs, whereas live virus could also stimulate other cell types to activate innate immune responses.

Although it was previously reported that pDC activation is not essential for the induction of adaptive immune responses in live influenza virus infection (*26*, *27*), pDC depletion specifically rendered the inactivated WV nonimmunogenic, as measured by virus-specific IgG concentrations in serum (Fig. 3A). In these experiments, mice were treated with an antibody to mPDCA-1 twice at both the primary and the secondary vaccinations. To further examine the more detailed role of pDCs in the primary and/or secondary vaccinations, we treated mice with an antibody to mPDCA-1 at either the primary or the secondary (boost) vaccination. Virus-specific mucosal IgA, serum IgG (Fig. 3B), and $CD4^+$ T cell IFN-γ (Fig. 3C) were significantly impaired when pDCs were depleted in the primary, but not in the secondary, vaccination with the inactivated WV. Thus, pDC activation is essential for inducing B cell and $CD4^+$ T cell responses to the inactivated WV during primary, but not secondary, vaccination. By sharp contrast, pDC activation at priming was not required for inducing B cell and $CD4^+$ T cell responses with the live virus.

WV-loaded pDCs were sufficient to transfer immunogenicity to naïve mice, which requires intrinsic as well as extrinsic type I IFN signaling

To further examine the role of pDCs, we performed cell transfer experiments. FL-DCs from wild-type mice were separated into two populations, namely, a B220 (CD45R)–enriched population containing pDCs and a B220-depleted population containing virtually no pDCs (as indicated in Fig. 3D). The cell populations were pulsed with WV and injected intravenously into wild-type mice. The virus-specific IgG concentrations elicited by the B220-enriched FL-DCs were significantly higher than those elicited by the B220-depleted population (Fig. 3D). In addition, when we transferred B220-enriched FL-DCs derived from the IFNAR2-deficient mice (lacking type I IFN) into wild-type mice or vice versa, virus-specific IgG induction was significantly impaired in both cases (Fig. 3E). We also confirmed that IFNAR2-deficient FL-DCs secreted significantly less type I IFN relative to that of FL-DCs from the heterozygous IFNAR2 knockout mice (fig. S5A). When we tested TLR7 deficiency with

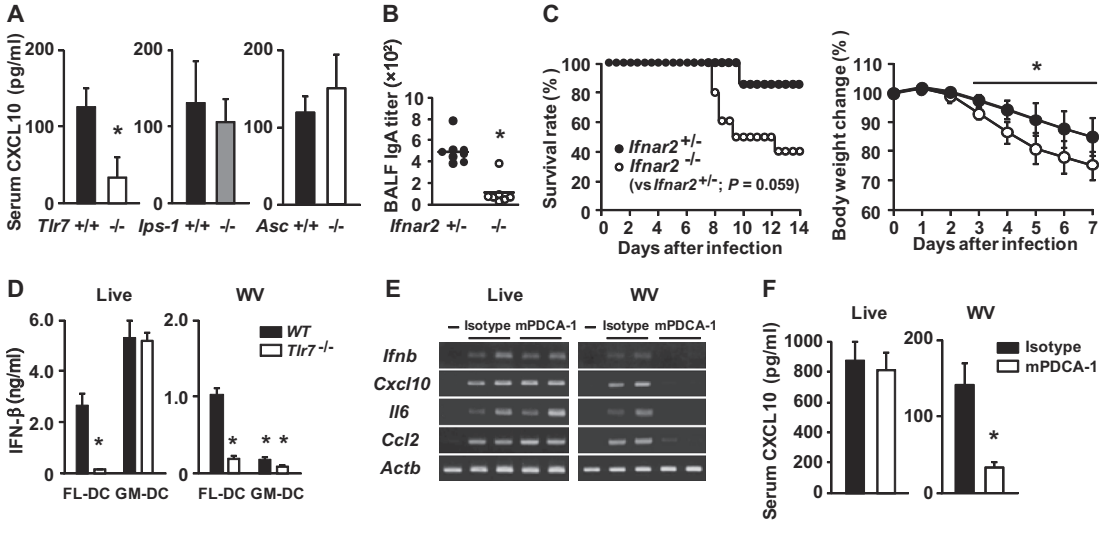

Fig. 2. Critical roles of type I IFN receptor–mediated signaling and pDC activation in inducing adaptive immune responses to the inactivated WV vaccine but not to the live virus. (**A**) *Tlr7*-deficient, *Ips-1*–deficient, *Asc*-deficient, and control mice ($n = 3$ per group) were i.n. vaccinated with WV (3.0 μg per mouse), and CXCL10 production in sera was measured by ELISA after 24 hours. *$P < 0.05$ versus control mice. (**B** and **C**) Control ($Ifnar2^{+/-}$) and type I IFN receptor–deficient ($Ifnar2^{-/-}$) mice ($n = 8$, each group) were i.n. vaccinated with WV (1.5 μg per mouse) as in Fig. 1, and BALF IgA (B) was measured by ELISA. *$P < 0.05$ versus control mice. The mice were then infected with lethal influenza PR at $10 \times LD_{50}$, and their survival and body weight (C) were monitored. *$P < 0.05$ versus control mice. (**D**) Type I IFN production by FL-DCs and GM-DCs from WT and *Tlr7*-deficient mice in response to the live virus [multiplicity of infection (MOI) = 10] and WV (5 μg/ml). IFN-β production was measured by ELISA 24 hours after the stimulation. *$P < 0.05$ versus WT FL-DC. (**E** and **F**) Innate immune responses to WV in pDC-depleted mice. Mice were treated with an antibody to mPDCA-1 24 hours before inoculation and then i.n. challenged with the live virus (1×10^5 PFU per mouse) or WV vaccine (5 μg per mouse). The expression of IFN-β, CXCL10, IL-6, and CCL2 mRNA in the lungs (E) and CXCL10 in sera (F) 24 hours after vaccination was measured by RT-PCR and ELISA. *$P < 0.05$ versus control mice. These results are representative of at least two independent experiments.

the same approach, we observed a substantial TLR7 signaling dependency in these FL-DC transfer experiments (fig. S5B). These results suggest that type I IFN–mediated signaling in pDCs, as well as in the recipient as yet unidentified cell type(s), is indispensable for eliciting the adaptive immune response to WV.

Split vaccine does not protect naïve mice, but immunogenicity can be improved with a pDC-activating adjuvant while it recalls memory T cells in human adult blood

Currently, the most widely used influenza vaccines in many countries comprise SV or SU, which mainly consist of purified protein antigens such as HA and neuraminidase. As mentioned earlier, vaccination of mice with SV led to significantly lower production of type I IFNs and related chemokines, such as CXCL10, at both the mRNA and the protein levels in the lung and serum, respectively (Fig. 4, A and B). SV also failed to activate DCs to produce type I IFNs in vitro (fig. S6A). These data suggest that the intrinsic TLR7 ligand (that is, viral genomic RNA) was lost during the SV production process. In support of this idea, removal of the RNA content from WV by ribonuclease treatment significantly decreased the TLR7-mediated type I IFN production by pDCs (fig. S6B). The reduced immunostimulatory activity of SV was associated with its diminished immunogenicity. When naïve mice were immunized with SV at the same dose, adjusted to the HA content (fig. S6C), as WV, the HA-specific IgG and CD4 T cell responses were significantly lower than those elicited by WV (Fig. 4, C and D). Together, these data strongly suggest that SV loses its built-in TLR7 adjuvant (viral genome RNA) during purification of WV, consistent with a recent study for the H5N1 virus (*28*).

Our results thus far raise the possibility of improving SV immunogenicity by adding a pDC-activating TLR ligand. Because pDCs express both TLR7 and TLR9, we examined whether addition of a TLR9 ligand to the "adjuvant-lost" split vaccine would replace the natural TLR7-mediated pDC activation. We used a second-generation TLR9 ligand of CpG DNA complexed with β-(1→3)-D-glucan, namely, schizophyllan (SPG) (*29*). This new TLR9 ligand is more potent and durable than naked CpG DNA, and it still retains the TLR9 ligand activity. Mice were i.n. immunized with WV, SV, or SV plus the SPG-CpG DNA conjugate (SV+SPG-CpG) and then evaluated for their adaptive immune responses. The SV+SPG-CpG induced robust type I IFN responses independently of TLR7 (Fig. 4, A and B). Correspondingly, the SV+SPG-CpG successfully enhanced HA-specific B cell and $CD4^+$ T cell responses to levels comparable to those from WV immunization of wild-type mice (Fig. 4, C and D). Immunization of TLR9-deficient mice provided further evidence that the responses induced by the SV+SPG-CpG were dependent on TLR9 but not on TLR7 (fig. S7A).

The protective efficacies of these three types of vaccines were also examined in mice. WV conferred protection against lethal PR H1N1 virus challenge in a TLR7-dependent and TLR9-independent manner, whereas the SV+SPG-CpG provided protection in a TLR9-dependent and TLR7-independent manner (Fig. 4E and fig. S7B). Notably, the original SV failed to provide protection against lethal PR H1N1 virus challenge in any of the groups of mice examined (Fig. 4E and fig. S7B). We also confirmed that the restored protective effect of the SPG-CpG adjuvant was mediated by type I IFN responses because IFNAR2-deficient mice failed to mount virus-specific B and T cell responses (fig. S7, C and D) and demonstrated no improved protection against infection (fig. S7E).

Although the usefulness of SV vaccination in the healthy adult human population has been recognized in many studies, our results are somewhat contradictory. Therefore, we tested the relevance of these observations in a human system. Human peripheral blood mononuclear cells (PBMCs) from healthy volunteers were stimulated with H1N1 live virus, inactivated H1N1 WV, and H1N1 SV for 24 hours, and then IFN-α and IFN-γ secretion was measured by ELISA. Consistent with the mice data, IFN-α was secreted with live virus and WV but not with SV stimulation (Fig. 4F). Depletion of pDCs with BDCA4 microbeads revealed that this IFN-α secretion was totally dependent on pDC in WV and partially in live virus stimulation (Fig. 4F). On the

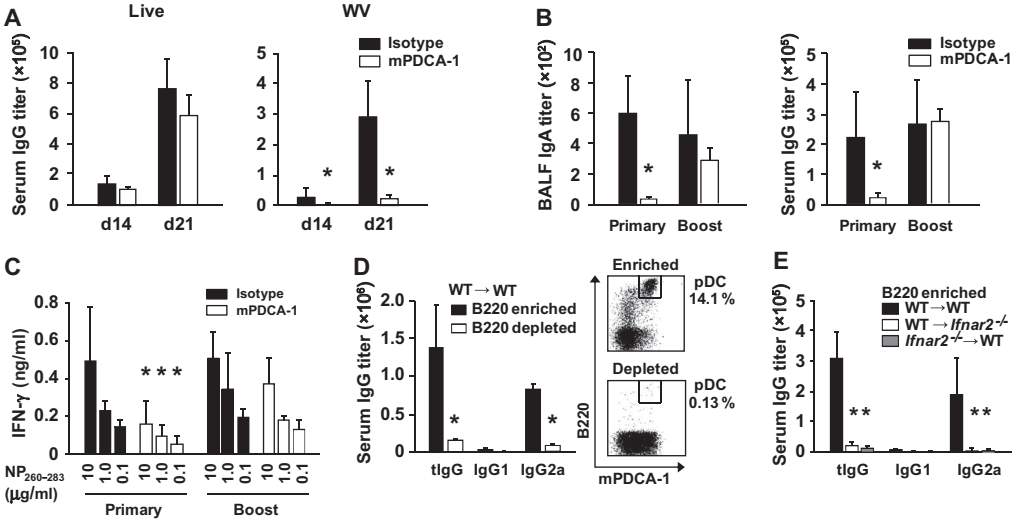

Fig. 3. Immunogenicity of the inactivated WV, but not of the live virus, depends on pDC activation at the primary vaccination. (**A**) The adaptive immune responses to WV (i.n.) were monitored in pDC-depleted mice ($n = 3$ per group). Mice were injected with an antibody to mPDCA-1 or isotype control antibody 24 hours before each vaccination, and serum IgG concentrations were measured by ELISA at the indicated times. *$P < 0.05$ versus control mice. (**B** and **C**) To determine whether pDCs are more important for priming or boosting after vaccination with inactivated WV, we treated one group of mice ($n = 3$) only with an antibody to mPDCA-1 before the primary vaccination (priming) and another group ($n = 3$) was treated only before the secondary vaccination (boosting). BALF IgA and serum IgG 1 week after the boost (B) and IFN-γ production from influenza-specific $CD4^+$ T cells (C) were measured by ELISA. *$P < 0.05$ versus control mice. (**D** and **E**) FL-DCs were separated into $B220^+$ and $B220^-$ DCs by MACS. Each DC was pulsed with WV in vitro, and $B220^+$ or $B220^-$ cells (5×10^5) were intravenously injected ($n = 3$ per group). Serum IgG was measured 2 weeks after the injection in WT mice. *$P < 0.05$ versus B220-enriched cells. (D) $B220^+$ cells (1×10^5) from WT and *Ifnar2*-deficient mice were intravenously transferred into untreated WT and *Ifnar2*-deficient mice ($n = 3$ per group), and serum IgG was measured by ELISA. *$P < 0.05$ versus WT to WT. (E) These results are representative of at least two independent experiments.

other hand, both WV and SV induced IFN-γ secretion comparably in the PBMC preparations even when the SV made from the swine-origin H1N1 A/California/04/2009 strain was used. Results obtained after $CD4^+$ and $CD8^+$ T cell depletion of the PBMC preparations revealed that virus-specific IFN-γ secretion was produced mainly by $CD4^+$ T cells in live virus and WV stimulation (Fig. 4G). These results suggest that SV could efficiently stimulate memory T cell responses without type I IFNs in a naturally (or seasonally) influenza virus–exposed human population.

DISCUSSION

Although TLR7 and certain NLRs have been shown previously to be involved in the induction of adaptive immune responses to influenza A virus infection (*13*, *14*), the current work represents a comprehensive study that directly compares the functions of TLRs, NLRs, and RLRs in the immunogenicity and efficacy of influenza inactivated WV vaccinations (Fig. 1 and fig. S1). We identified pDCs as an innate immune cell and type I IFNs as humoral factors that are essential for the immunogenicity of the inactivated WV vaccine (Figs. 2 and 3). Although our results demonstrate an essential role for pDCs in inactivated WV vaccination, other studies have identified a redundant role for pDCs in antiviral responses to live virus vaccination such as influenza virus (*26*, *27*). In addition, although TLR7 is expressed in a variety of cell types, including B cells and macrophages, our results strongly suggest an essential role for pDCs in mediating TLR7-induced innate and adaptive immune responses to inactivated influenza WV vaccination but not to live virus vaccination.

The critical role of pDCs in vaccine priming, but not in boosting, is apparent from results of the pDC depletion study, in which pDCs were removed from mice before vaccination with inactivated WV (Fig. 3, A to C). These findings parallel our in vitro data, in which the pDC-containing FL-DC preparation, but not the mDC-dominant GM-DC preparation, responded to inactivated WV to produce type I IFNs (Fig. 2D); however, these data do not necessarily exclude the involvement of other antigen-presenting cells for either priming or boosting. For example, pDCs pulsed with inactivated WV in vitro can prime naïve mice (Fig. 3D), but the type I IFNs produced by these pDCs were required not only for stimulating the pDCs themselves but also for priming as yet unidentified cell types of the recipient mouse (Fig. 3E). This suggests that cross talk exists between pDCs and the other as yet unidentified cells via type I IFN–mediated signaling. In addition, it will be of interest to examine whether other DCs, such as mDCs, are involved in this intercellular cross talk in such a way that pDCs can transfer the flu antigens to mDCs or that pDCs can present antigens directly to T cells and/or B cells. We note the distinct regulation of type I IFN induction by inactivated WV vaccination, which differed from IFN induction by live virus vaccination. Indeed, although type I IFNs were induced by both inactivated and live virus vaccinations, inactivated WV vaccines activated only TLR7 on pDCs,

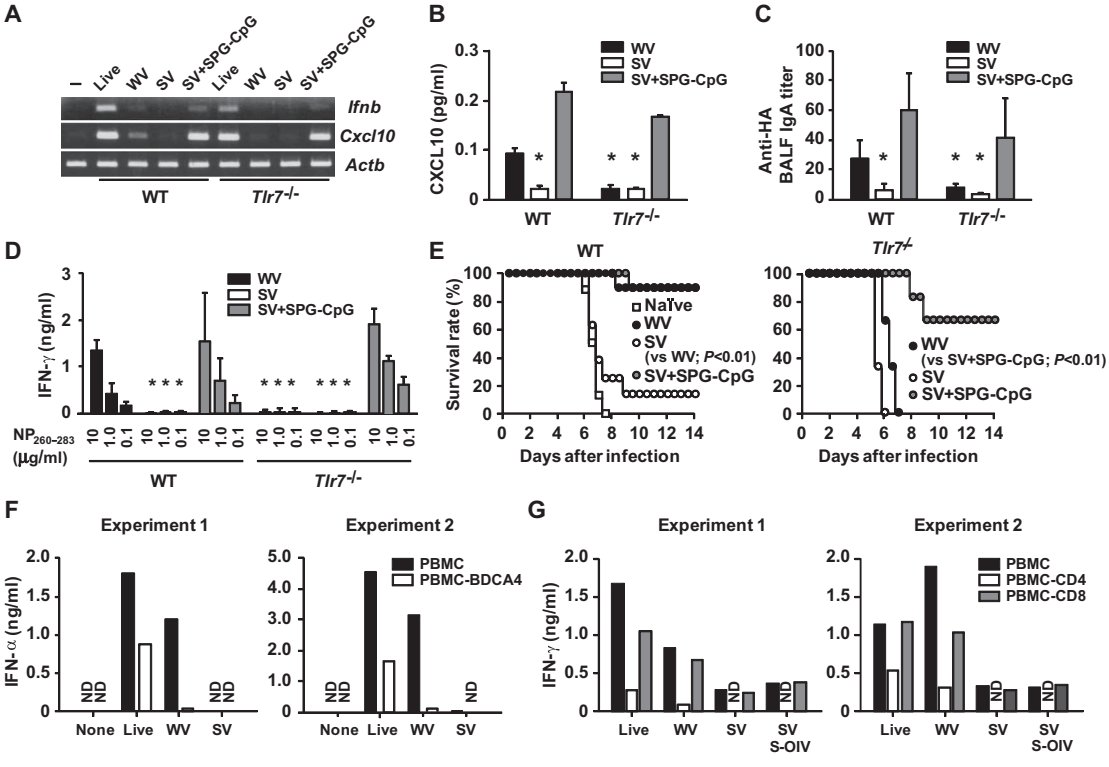

Fig. 4. Immunogenicity differences between WV and SV depend on type I IFN production via pDC activation in naïve hosts but not in primed hosts. (**A** and **B**) WT and *Tlr7*-deficient mice were i.n. vaccinated with three different vaccines as described in Materials and Methods. After 24 hours, IFN-β and CXCL10 expression in the lungs (A) and CXCL10 production in the sera (B) was measured by RT-PCR and ELISA. **P* < 0.05 versus WT mice immunized with WV. (**C** and **D**) To compare the immunogenicities of the three vaccines, we i.n. vaccinated WT ($n = 9$) and *Tlr7*-deficient ($n = 6$) mice as described in Fig. 1 and determined antigen (HA)–specific BALF IgA, serum total IgG (C), and IFN-γ production by $CD4^+$ T cells (D) by ELISA. **P* < 0.05 versus WT mice immunized with WV. (**E**) Vaccinated mice were challenged with the PR strain at $10 \times LD_{50}$, and their survival rates were determined as described in Materials and Methods. **P* < 0.05 versus WT mice immunized with WV or control mice. These results are representative of at least two independent experiments. (**F** and **G**) IFN-α and IFN-γ production in response to inactivated influenza vaccines in human PBMCs. PBMCs from healthy volunteers were stimulated with live NC virus, WV, and SV. Total PBMCs and pDC-depleted PBMCs (PBMC-BDCA4) were stimulated with live NC virus (0.01 MOI), WV (1.0 μg/ml), and SV (0.5 μg/ml), and IFN-α production was measured by ELISA (F). Total PBMCs and CD4- or CD8-depleted PBMCs were stimulated with WV and SV of NC (10 and 5 μg/ml, respectively) and SV of swine-origin influenza virus (SV S-OIV) (5 μg/ml). IFN-γ production was measured by ELISA (G). These results are from 2 representatives of 10 volunteers.

whereas live virus activated both TLR7 on pDCs and other TLR7-independent pathways in the other cells, possibly mDCs (Fig. 2, D and E). Therefore, although TLR7, pDCs, and type I IFNs all were essential for inactivated WV vaccination, pDCs and type I IFNs were not essential for live virus vaccination.

The adaptor ASC is a critical component of NLRP3 inflammasome (*30*). In contrast to type I IFN responses, ASC-dependent inflammasome activation has been shown to play a critical role in the survival of the mice challenged with live influenza virus (*14*, *18*, *19*). However, the requirement for inflammasome activation to induce influenza-specific adaptive immune responses has been controversial (*14*, *19*). Our data indicate that ASC-dependent inflammasome activation is dispensable for inducing adaptive immune responses to WV and live virus, except for IgG1 production in live virus vaccination (Fig. 1 and fig. S2). Concurrent analysis comparing three innate immune signaling pathways, TLR, NLR, and RLR, enabled us to elucidate that the TLR-dependent pathway dominantly controlled the T helper 1–type protective immunity elicited by WV and live virus vaccination.

Although SV, which is now used as the first choice for influenza vaccination in many countries, was not protective in naïve mice, its decreased immunogenicity was fully restored by adding a new TLR9 ligand that stimulates pDCs to secrete type I IFNs (Fig. 4, A to E, and fig. S7). These data above further support the notion that pDC activation and their type I IFN production play a critical role in the induction of inactivated influenza vaccine immunogenicity in naïve hosts. These results might explain in part the well-known fact that the efficacy of adjuvant-less SV is lower in young children than in adults (*7*), in which SV is simply boosting the memory T and/or B cell responses. This is further supported by our results obtained using human PBMCs (Fig. 4, F and G), which suggest that most human adults have virus-specific $CD4^+$ T cells that produce IFN-γ in response not only to seasonal flu viruses but also to the novel swine H1N1 virus. Our results also indicate that memory T cells react to both internal proteins, such as those in SV, and a wide spectrum of influenza virus surface antigens, such as those on swine-origin H1N1 (*31*, *32*) and H5N1 (*33*). The age distribution of the affected population in swine-origin H1N1 and H5N1 infections, which was limited to the young, might reflect the importance of memory T cells established by recurrent exposure to seasonal influenza live viruses and vaccines (*34*, *35*)

LAIVs activate both influenza-specific IgA-secreting B cells and cytotoxic $CD8^+$ T cells (*36*), which provides certain advantages over inactivated vaccines including WV and SV. Although WV is now unavailable for seasonal influenza, it is cost-effective and can induce heterosubtypic protection not only against a challenge by H1N1 (Fig. 1C and fig. S1C) but also against H5N1 (*37*, *38*), as with LAIV (*39*). In addition, recent progress in manufacturing techniques could reduce the adverse event rate in i.m. WV immunization (*37*, *38*) to yield results that are quite different from those of past clinical trials (*3*, *40*). An i.n. WV immunization may produce a sufficient combination of efficacy, safety, and utility for both seasonal and pre-pandemic vaccines (*41*–*45*).

Together, analysis of the molecular and cellular mechanisms of different influenza vaccines provides useful information for improving vaccine immunogenicity and efficacy, as well as for choosing an appropriate form of influenza vaccine with a rational safety approach.

MATERIALS AND METHODS

Animals, cells, viruses, and reagents

The generation of *Tlr7*-, *Ips-1*–, *Ifnar2*-, and *Tlr9*-deficient mice, either on a 129/Ola × C57/BL6 or on a C57/BL6 background, has been described previously (*13*, *46*). ASC-deficient mice were a gift from V. M. Dixit (*47*).

All animal experiments were performed in accordance with the institutional guidelines for the Osaka University animal facility.

Purified influenza viruses, H1N1 (PR and NC), a recombinant HA protein of PR, and both inactivated WV and split vaccines of NC were prepared as previously described (*48*). Both types of vaccines were derived from the NC strain. Briefly, the viruses were purified from allantoic fluid by filtration (0.45 μm) followed by sedimentation through a linear sucrose gradient. For formalin-inactivated WV vaccines, purified viruses were treated with 0.1 to 0.2% formalin at 4°C for a week. For the ether-split vaccines (SV), the viruses were mixed with an equal volume of ether and then incubated for 30 min at room temperature with stirring. The mixture was centrifuged (3000 rpm, 15 min), and the aqueous phase was collected and evaporated. CpG DNA forming a triple helix with SPG, a natural polysaccharide composed of β-(1→3)-D-glucan, was used as the second-generation TLR9 ligand as previously described (*29*, *49*, *50*). DCs were prepared as described previously. Briefly, bone marrow cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 mM 2-mercaptoethanol, and human Flt3 ligand (100 ng/ml) (PeproTech) or murine GM-CSF (10 ng/ml) (PeproTech) for 7 to 9 days to use as FL-DCs and GM-DCs.

Influenza virus infection and vaccination

For influenza virus infection or vaccination, mice were anesthetized and administered i.n. with 30 μl of phosphate-buffered saline (PBS) (15 μl for each nares) containing serial amount of influenza NC viruses and vaccines. Mice were infected with 1×10^5 to 2×10^5 plaque-forming units (PFU) of virus per mouse or vaccinated with WV (1.5 to 3.0 μg per mouse) or SV (0.75 μg per mouse) with or without SPG-CpG (30 μg per mouse) twice at a 2-week interval. For the analysis of protection, mice were infected with the indicated doses of lethal PR strain.

Measurement of innate immune responses

Reverse transcription polymerase chain reaction (RT-PCR) was performed to measure mRNA expression levels of type I IFNs, cytokines, and chemokines using the RNA of the stimulated cells as previously described (*13*). Protein concentrations of IFN-α, IFN-β, and CXCL10 in the culture supernatants of the stimulated cells were measured using ELISA kits (IFN-α and IFN-β, PBL Biomedical Laboratories; CXCL10, R&D Systems).

Plasmacytoid DC depletion and cell transfer

Plasmacytoid DCs were depleted by intravenous injection of antibody to mPDCA-1 (500 μg) (Miltenyi Biotec) 24 hours before live virus infection or inactivated WV vaccination.

FL-DCs were separated into two populations, B220-enriched and B220-depleted population, by B220 antibody MACS microbeads (Miltenyi Biotec) according to the manufacturer's protocol to obtain B220-enriched FL-DC. Each cell population was incubated with WV (5 to 10 μg/ml) for 3 hours, and 1×10^5 to 5×10^5 cells per mouse

were injected intravenously into each type of mice. Immunological assays were performed 2 weeks after injection.

Confirmation of pDC depletion in spleen by flow cytometric analysis

After Fc blocking with an antibody to CD16/32, isolated spleen cells were stained with fluorescein isothiocyanate (FITC)–conjugated antibody to CD11c, phycoerythrin (PE)–conjugated antibody to CD45R/B220, and allophycocyanin-conjugated antibody to mPDCA-1 (Miltenyi Biotec) for 30 min at room temperature and washed with PBS containing 1% bovine serum albumin. Just before fluorescence-activated cell sorting (FACS) analysis using FACSCalibur and CellQuest software (BD Biosciences), 7-aminoactinomycin D (BD Biosciences) was added.

Measurement of antigen-specific T and B cell responses

After two i.n. vaccinations, B cell–mediated humoral responses were measured as immunoglobulin production by ELISA using goat antibody to mouse total IgG, IgG1, IgG2a, and IgA conjugated to horseradish peroxidase (Southern Biotech) as previously described (*1*). T cell–mediated cellular responses were monitored by measuring $NP_{260–283}$/I-A^b–specific or $NP_{366–374}$/H-$2D^b$–specific IFN-γ secretion of splenocytes and the frequency and cytotoxicity of H-$2D^b$–specific CD8 T cells as described previously (*13*).

Preparation of human PBMCs for cytokine analysis

PBMCs were obtained from 10 healthy adult volunteers (30 to 50 years old, 6 males and 4 females). All of the experiments using human PBMCs were approved by the Institutional Review Board of the Research Institute for Microbial Diseases, Osaka University. Cells were purified from heparinized blood by density centrifugation using Ficoll-Paque Plus (Amersham). Human pDCs, CD4, or CD8 T cells were depleted with BDCA4 and CD4 or CD8 antibody MACS microbeads (Miltenyi Biotec), respectively, according to the manufacturer's protocol. Plasmacytoid DC depletion was confirmed by FACS analysis staining with FITC-conjugated antibody to BDCA2 and PE-conjugated antibody to CD123 (Miltenyi Biotec). PBMCs or pDC-depleted PBMCs (1×10^6 to 2×10^6 cells) were stimulated with each influenza vaccine at the indicated concentration. Twenty-four hours later, IFN-α and IFN-γ (R&D Systems) were measured in supernatants by ELISA according to their manufacturers' protocol.

Statistical analysis

Statistical significance ($P < 0.05$) between groups was determined using 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.

SUPPLEMENTARY MATERIAL

www.sciencetranslationalmedicine.org/cgi/content/full/2/25/25ra24/DC1

Materials and Methods

Fig. S1. TLR7-dependent, but not IPS-1–dependent, signaling is required for the induction of protective immune responses with inactivated WV vaccine by i.m. immunization.

Fig. S2. ASC-dependent inflammasome activation was dispensable for adaptive immune response to influenza virus infection, except for systemic IgG1 production.

Fig. S3. Type I IFN receptor–mediated signaling was indispensable for adaptive immune response to WV but not to the live virus.

Fig. S4. Plasmacytoid DC depletion by mPDCA-1 antibody was confirmed in spleen.

Fig. S5. Type I IFN interaction between pDCs and other immune cells was required for WV vaccine immunogenicity.

Fig. S6. Different manner of type I IFN response to WV vaccine and split vaccine is dependent on the presence of the viral genome RNA.

Fig. S7. Indispensable role of type I IFN–mediated signaling in vaccination with split vaccine plus SPG-CpG.

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