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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