Activation of the RIG-I Pathway during Influenza Vaccination Enhances the Germinal Center Reaction, Promotes T Follicular Helper Cell Induction, and Provides a Dose-Sparing Effect and Protective Immunity

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ABSTRACT

Pattern recognition receptors (PRR) sense certain molecular patterns uniquely expressed by pathogens. Retinoic-acid-inducible gene I (RIG-I) is a cytosolic PRR that senses viral nucleic acids and induces innate immune activation and secretion of type I interferons (IFNs). Here, using influenza vaccine antigens, we investigated the consequences of activating the RIG-I pathway for antigen-specific adaptive immune responses. We found that mice immunized with influenza vaccine antigens coadministered with 5'ppp-double-stranded RNA (dsRNA), a RIG-I ligand, developed robust levels of hemagglutination-inhibiting antibodies, enhanced germinal center reaction, and T follicular helper cell responses. In addition, RIG-I activation enhanced antibody affinity maturation and plasma cell responses in the draining lymph nodes, spleen, and bone marrow and conferred protective immunity against virus challenge. Importantly, activation of the RIG-I pathway was able to reduce the antigen requirement by 10- to 100-fold in inducing optimal influenza-specific cellular and humoral responses, including protective immunity. The effects induced by 5'ppp-dsRNA were significantly dependent on type I IFN and IPS-1 (an adapter protein downstream of the RIG-I pathway) signaling but were independent of the MyD88- and TLR3-mediated pathways. Our results show that activation of the RIG-I-like receptor pathway programs the innate immunity to achieve qualitatively and quantitatively enhanced protective cellular adaptive immune responses even at low antigen doses, and this indicates the potential utility of RIG-I ligands as molecular adjuvants for viral vaccines.

IMPORTANCE

The recently discovered RNA helicase family of RIG-I-like receptors (RLRs) is a critical component of host defense mechanisms responsible for detecting viruses and triggering innate antiviral cytokines that help control viral replication and dissemination. In this study, we show that the RLR pathway can be effectively exploited to enhance adaptive immunity and protective immune memory against viral infection. Our results show that activation of the RIG-I pathway along with influenza vaccination programs the innate immunity to induce qualitatively and quantitatively superior protective adaptive immunity against pandemic influenza viruses. More importantly, RIG-I activation at the time of vaccination allows induction of robust adaptive responses even at low vaccine antigen doses. These results highlight the potential utility of exploiting the RIG-I pathway to enhance viral-vaccine-specific immunity and have broader implications for designing better vaccines in general.

Innate immune responses not only provide the first line of defense against infectious agents, but also provide signals needed for the induction of optimal adaptive immune responses. Several cell types and receptors take part in the innate immune responses against pathogens (1). Among these, pattern recognition receptors (PRRs) are particularly specialized in recognizing pathogen-associated molecular patterns (PAMP), which are unique to microbial classes. Toll-like receptors (TLRs) are a major class of PRRs that are either expressed on cell surfaces or located in host cellular endosomes. In addition to the TLRs, several other types of PRRs expressed in the host cytoplasm have been discovered, including retinoic-acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (Nod)-like receptors (NLR) (2, 3). Members of the RLR family include RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory genetics and physiology 2 (LGP2), all of which re-

quire interferon (IFN) promoter stimulator 1 (IPS-1), a mitochondrion-associated adapter protein, in their signaling (4). RIG-I typically recognizes single- or double-stranded viral RNA molecules with a 5'-triphosphate (5'ppp) group, and engagement of RIG-I with its ligand leads to production of type I interferons (IFN-I) (5).

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We and others have previously shown RIG-I to be an essential receptor in sensing many viruses, including influenza virus (2, 6). We have also shown that activation of the RIG-I pathway induces type I IFN and panantiviral effects both *in vitro* and *in vivo* (7, 8). The role of cytokines, including type I IFN, in antiviral immunity is well known, and recent studies from our laboratory, as well others, highlight the way in which type I IFN can modulate adaptive immune responses (9–12).

Influenza infections have caused epidemics and pandemics for years, posing threats to human health, as well as economic burdens for many nations. Although vaccines remain the best means to combat these infections (13) and the inactivated influenza vaccines have been used with considerable success, drawbacks associated with their poor immunogenicity and requirement for antigens on a large scale have created a demand for newer vaccines (14–16). To maximize pandemic preparedness, there is a strong emphasis on the antigen-sparing aspect of the vaccine formulations to meet the global need. Therefore, a strategy for enhancing the immunogenicity of pandemic influenza vaccines and implementing dose-sparing methods through the use of molecules that can boost influenza vaccine-specific immunity is needed.

In the present study, we addressed whether activation of the RIG-I pathway with 5'ppp-double-stranded RNA (dsRNA), a ligand for RIG-I, leads to enhancement of both the quality and quantity of antigen-specific adaptive immune responses, including the provision of an antigen-sparing effect. We used pandemic 2009 influenza vaccine to show that RIG-I activation, even at sparing doses, can robustly enhance germinal center (GC) reactions and T follicular helper cell (T_{fh}) responses, leading to induction of long-lasting antibodies, enhanced antibody affinity, and augmented antibody-secreting cells (ASCs), including those that home to bone marrow. Furthermore, RIG-I activation also conferred protective immunity against a pandemic virus challenge at sparing antigen doses. 5'ppp-dsRNA-mediated immune enhancement was significantly dependent on type I IFN and IPS-1 signaling but independent of the MyD88 and TLR3 pathways.

MATERIALS AND METHODS

PRR ligands and antigens. The 5'ppp-dsRNA, alum, monophosphoryl lipid A (MPL-A), and imiquimod (R837) used as activators of PRRs in this study were procured from Invivogen, San Diego, CA. 5'ppp-dsRNA, a potent activator of the RIG-I receptor, is a synthetic product obtained by hybridization of one 5' triphosphate single-stranded 19-mer phosphodiester RNA (5'-pppGCAUGCGACCUCUGUUUGA-3') with its non-triphosphatase 19-mer complementary strand (3'-CGUACGUGGAGACAAACU-5'). GenJet Plus DNA In Vivo Transfection Reagent (Signagen, CA) was used to deliver 5'ppp-dsRNA. Alhydrogel 2%, referred to as alum, is an aluminum hydroxide wet gel suspension that activates the NALP3 inflammasome, which has been suggested to activate the NLRP3 pathway. MPL-A, a TLR4 agonist, is a product extracted from lipopolysaccharide (LPS) produced by the Re mutant of a rough strain of *Salmonella enterica* serovar Minnesota R595. R837, an imidazoquinoline amine analog to guanosine synthetic molecule, is known to activate TLR7.

Influenza A (2009) H1N1 inactivated monovalent split virus [A/California/07/2009(H1N1pdm09)] vaccine was purchased from the manufacturer, Sanofi Pasteur. Ovalbumin (OVA) (grade VI), used in the initial experiments, was purchased from Sigma-Aldrich.

In assessing the effects of PRR ligands on inducing antigen-specific T cell receptor (TCR) transgenic (SMARTA) CD4 T cell responses, recombinant lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) was used for immunization purposes. The LCMV glycoprotein construct was created according to the method of Eschli et al. (17) with some mod-

ifications. A codon-optimized DNA construct encoding the ectodomain of the LCMV clone 13 glycoprotein sequence (amino acids 1 to 430) was synthesized commercially (Genscript). A fibritin foldon trimerization motif was added to the C terminus of the protein, followed by a Gly-Ser-Gly linker and a V5-His tag. An Arg-to-Ala mutation was introduced at amino acid 262 to disrupt the S1P cleavage site between the GP1 and GP2 domains. The coding sequence was cloned into a lentiviral expression vector (C. Davis, unpublished data) that is a modification of the vector pNL-EGFP/CMV/WPREdU3 (18). The cytomegalovirus (CMV) promoter was replaced with a murine stem cell virus (MSCV) long terminal repeat (LTR) from plasmid MSCV-IRES-Thy1.1 (19). The Thy1.1 cassette from MSCV-IRES-Thy1.1 was placed 3' of the coding sequence and 5' of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). The vector was also modified to include sequences from a mouse immunoglobulin enhancer and a short 5' untranslated region (UTR) sequence from a mouse heavy-chain gene. The full sequence was deposited in GenBank. The final plasmid containing the LCMV glycoprotein coding sequence is referred to as pNL-sGPC. Lentiviral particles were produced by transduction of HEK293T cells with pNL-sGPC, along with plasmids pMD2.G and psPAX2 (Didier Trono; Addgene plasmids 12259 and 12260). These particles were used to infect 293A cells (Invitrogen) at a high multiplicity of infection (MOI). The cells with the highest levels of Thy1.1 expression were single-cell sorted and screened for high glycoprotein secretion. The highest-producing clone was expanded, and the soluble glycoprotein was purified from culture supernatants by immobilized metal affinity chromatography using nickel-nitrilotriacetic acid (NTA) superflow (Qiagen).

Mice, immunizations, and virus challenge. The animal experiments were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Emory University School of Medicine, and the mice were housed at the Emory University Division of Animal Resources facility. C57BL/6, MyD88^{-/-}, and TLR3^{-/-} mice on a B6 background and BALB/c mice were purchased from Jackson Laboratories. IFN-I receptor (IFN-IR)-deficient mice on a 129/SvEv background backcrossed to B6 for 14 generations were maintained in our laboratory (10). IPS-1-deficient mice on a C57BL/6 background were kindly provided by Michael Gale (University of Washington School of Medicine, Seattle, WA).

In all experiments, 8- to 12-week-old mice were injected intramuscularly (i.m.) in both quadriceps muscles at a 50- μ l dose per quadriceps with antigens (either 5, 0.5, 0.05, or 0.005 μ g of hemagglutinin [HA] equivalent influenza vaccine or 200 μ g of ovalbumin) with or without PRR activators. While 5'ppp-dsRNA, MPL, and R837 were used at concentrations of 25, 30, and 40 μ g per mouse, respectively, alum was used at a 1:1 dilution in phosphate-buffered saline (PBS) containing antigen.

For virus challenge, A/Mexico/4108/2009(H1N1)pdm09 virus stocks were prepared by inoculation of 10-day-old embryonated chicken eggs, the titer was determined, and the 50% lethal dose (LD₅₀) was calculated by using standard methods (20). Virus challenge was carried out using intranasal inoculation with virus diluted in sterile PBS. The mice received a dose of 4.5×10^6 PFU (50 LD₅₀) in a total volume of 50 μ l, with 25 μ l inoculated into each naris. After the virus challenge, the mice were monitored for weight loss and mortality for 14 days and were euthanized when weight loss reached 25%.

Antibody assays. Influenza virus antigen-specific antibody titers were determined by the endpoint titer dilution method using enzyme-linked immunosorbent assay (ELISA). Maxisorp plates (Nunc) were coated with the vaccine (5 μ g/ml) at 4°C overnight, followed by blocking with 1% casein buffer (Bio-Rad Laboratories) for 1 h. One hundred-microliter volumes of 2-fold serially diluted serum samples from immunized mice were added to each well, and the plates were incubated for 2 h at room temperature, followed by incubation with the appropriate isotype-specific antibody conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch, PA). The plates were visualized with the addition of 100 μ l of

TMB substrate (Bio-Rad Laboratories, CA), and 50 μ l of stop solution (1.0 M H_2SO_4) was then added. The absorbance was measured at 450 nm. The specific antibody titer of the serum was expressed as the reciprocal of the serum dilution that gave an A_{450} value above the cutoff, which was defined as twice the absorbance value of the unimmunized control wells run in duplicate. For quantifying serum IFN- α , a VeriKine Mouse Interferon Alpha ELISA Kit (PBL Assay Science, NJ) was used, following the manufacturer's instructions.

The avidity indices of immunized serum were measured by its resistance to 8 M urea in binding to influenza antigens, as reported previously (21, 22). Briefly, microwell plates were prepared and blocked as described above. Serum samples were diluted to give an optical density at 450 nm (OD_{450}) readout between 1.0 and 1.5 in endpoint ELISA and incubated for 2 h at room temperature. The wells were then washed three times with either PBS-Tween or 8 M urea in PBS-Tween before incubating with a secondary antibody. The plates were washed and developed with TMB as described above. The avidity index was calculated as the average urea-treated OD_{450} divided by the average PBS-Tween-treated $OD_{450} \times 100$. Sera with index values of $>50\%$ were designated high avidity, those with index values of 30 to 50% were designated intermediate avidity, and those with index values of $<30\%$ were designated low avidity.

To determine hemagglutination inhibition (HAI) titers, sera were first pretreated with DENKA receptor-destroying enzyme (Accurate Chemical and Scientific) and then inactivated at 56°C for 30 min. Two-fold serial dilutions of 25 μ l pretreated sera and positive-control sera were treated with a 25- μ l virus (8 HA units) working dilution. Samples were incubated at room temperature, 50 μ l turkey 0.5% red blood cell (RBC) suspension (Lampire Biologics, PA) was dispensed into each well, and the plates were again incubated at room temperature. The test endpoint was determined by visual inspection for an agglutination reaction: formation of a red dot indicated a positive reaction (inhibition), whereas a diffuse patch of cells indicated a negative reaction (hemagglutination). The titer was defined as the highest serum dilution at which hemagglutination was inhibited, and the antibody concentration corresponded to the reciprocal value of the titer.

ELISPOT assay for assessing ASC response. Influenza virus-specific ASCs were quantitated by enzyme-linked immunospot (ELISPOT) assay. Following immunization on days 7, 14, and 28, three mice per group were euthanized to collect spleen, draining lymph node (inguinal and dorsal), and bone marrow (femur) tissues. Single-cell suspensions were prepared as described previously (23). The monovalent influenza vaccine used for immunization was used as the capture antigen. After coating the ELISPOT plates (Millipore) overnight, the cells were incubated for 8 h and then detected using biotinylated goat anti-mouse IgG (Caltag Laboratories, CA) and HRP-conjugated avidin D (Vector Laboratories Inc., CA). Spots were developed using an AEC substrate set (BD Bioscience) and read with the ELISPOT reader.

Flow cytometry. Mice were immunized with the indicated antigens and PRR activators by the i.m. route. At serial time points postimmunization, as described in Results, the indicated tissues were collected. Single-cell preparations were stained with phenotypic and functional markers to assess the GC reaction, Tfh response, and CD4 effector functions. In all the staining panels, dead cells were excluded by staining with a LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). All antibodies were procured from BD Biosciences unless otherwise specified. To assess GC formation, cells were stained with antibodies against B220, CD4, CD95, and peanut agglutinin (PNA) (Vector Laboratories). B cells ($B220^+ CD4^-$) expressing CD95 and PNA were termed GC-B cells. To determine the Tfh response, cells were stained with antibodies against CD4, B220, CD44, CXCR5, PD-1 (BioLegend), Bcl6, and ICOS (e-Bioscience). CD4 T cells ($B220^- CD4^+ CD44^+$) expressing CXCR5, along with Bcl6, PD-1, or ICOS, were termed Tfh cells. In SMARTA transfer experiments to monitor antigen-specific CD4 T cell differentiation into the Tfh phenotype, cells were stained with the Tfh panel of antibodies as stated above, along with the antibody against the CD45.1 congenic marker. Fur-

ther, to assess the SMARTA effector functions, intracellular cytokine staining was performed using antibodies against IFN- γ , tumor necrosis factor alpha (TNF- α), and interleukin 2 (IL-2) (e-Bioscience). The cells were resuspended in either fluorescence-activated cell sorter (FACS) buffer or 2% paraformaldehyde (PFA) before acquisition on a FACS LSRII flow cytometer (BD Biosciences). Data analysis was performed using FLOWJO version 9.4.11 (Tree Star Inc.).

Statistical analysis. Data sets were analyzed using two-tailed *t* tests and/or one-way analysis of variance (ANOVA) with Prism 5.0 (GraphPad Software). *P* values of less than 0.05 were considered statistically significant. All values are reported as means and standard errors of the mean (SEM).

RESULTS

Activating the RLR pathway induces better antibody and CD4 T cell responses than activating the TLR or NLR pathway. Alum has been successfully used for many years to boost antibody responses to vaccine antigens, and recently, it has been suggested to activate the NLRP3 pathway (24). Some of the TLR agonists have also been used to boost vaccine-induced immunity (25). Recent discoveries have suggested the cytosolic RLR/RIG-I pathway is a potential alternative target for boosting vaccine-induced immunity (26, 27). 5'ppp-dsRNA is a synthetic ligand for the RIG-I receptor that, when engaged with the receptor, leads to production of type I IFN (5). In this study, we first compared the efficacy of 5'ppp-dsRNA with those of alum, MPL-A (a TLR4 ligand), and R837 (a TLR7 ligand) in inducing influenza virus-specific antibody responses. Mice were administered the 2009 pandemic monovalent influenza vaccine at a dose containing 5 μ g of HA in the presence or absence of PRR activators. A booster administration with the same formulation was given 4 weeks later. As shown in Fig. 1A, mice that received 5'ppp-dsRNA, alum, or MPL-A together with the vaccine antigen developed significantly higher antigen-specific IgG titers following primary, as well as booster, immunization than mice immunized with antigen alone or antigen plus R837. Importantly, the postbooster antibody titers in mice that received 5'ppp-dsRNA were significantly higher than in the alum-immunized mice, suggesting superior recall memory B cell responses induced by RIG-I activation. However, no significant differences were observed in the postbooster titers in mice that received either 5'ppp-dsRNA or MPL-A. Since we used a lipid-based vehicle to deliver 5'ppp-dsRNA, we also investigated possible vehicle-mediated effects on enhancing influenza virus-specific antibody responses. Although the mice given vaccine mixed in vehicle only had slightly elevated antibody titers, inclusion of 5'ppp-dsRNA induced robust enhancement of influenza virus-specific IgG titers compared to vaccine-plus-vehicle recipient mice (data not shown).

We further sought to investigate the effects of activating different PRR pathways on augmenting Tfh responses, as well as CD4 T cell effector functions, using an adaptive TCR-transgenic CD4 T cell transfer system (SMARTA CD4 T cells, which are specific to an LCMV glycoprotein-derived peptide) to allow tracking of the antigen-specific CD4 T cells. We transferred 5×10^4 LCMV-specific SMARTA CD4 T cells into naive C57BL/6 mice and immunized them with LCMV recombinant GP alone or with either RIG-I ligand (5'ppp-dsRNA), TLR-4 ligand (MPL-A), or alum. SMARTA cell expansion, Tfh differentiation ($CD4^+ CD44^+ CXCR5^+ Bcl6^+ PD-1^+ ICOS^+$), and CD4 T cell effector functions were quantified at day 7 post-GP immunization. As shown in Fig. 1B, mice administered 5'ppp-dsRNA plus GP showed strikingly

FIG 1 Activating the RIG-I pathway induces superior antibody and CD4 T cell responses compared to activation of the TLR4, TLR7, or alum/NLR pathway. (A) Mice were immunized with 5 μ g HA equivalent of 2009 pandemic influenza vaccine supplemented with 5'ppp-dsRNA (RIG-I ligand), MPL-A (TLR4 ligand), R837 (TLR7 ligand), or alum (NLRP3 ligand), with a booster administration at 4 weeks. Serum IgG titers were measured at the indicated time points postimmunization. (B to D) To investigate the effects of different PRR ligands on augmenting antigen-specific Tfh responses, as well as CD4 T cell effector functions, LCMV-specific CD4 TCR transgenic SMARTA cells were adoptively transferred into naive mice, which were then immunized intramuscularly with 10 μ g of LCMV-derived recombinant GP supplemented with the indicated adjuvants. At 7 days postimmunization, lymph nodes were evaluated for SMARTA cell expansion (B); endogenous (total) and SMARTA-Tfh frequency and counts (C); and SMARTA cells producing IL-2, IFN- γ , and TNF- α cytokines (D). The representative flow plots show cell frequencies observed in the lymph nodes, and the bar graphs adjacent to the plots show the absolute cell counts observed. Each treatment group had 3 or 4 mice, and the data from one of three independent experiments are presented. The asterisks (*, $P \leq 0.05$, and **, $P \leq 0.01$) indicate significant differences compared to mice that received vaccine or GP alone. The error bars indicate means and SEM.

greater expansion of the antigen-specific donor CD4 T cells in the draining lymph nodes than all other groups. About 3 to 10% of these expanded donor CD4 T cells (Fig. 1C, top), and endogenous CD4 T cells (Fig. 1C, bottom) indeed showed the Tfh phenotype, as confirmed by expression of the chemokine receptor CXCR5 and the transcription factor Bcl-6. The percentage of antigen-spe-

cific Tfh differentiation of these clonally expanded CD4 T cells was much higher in mice with RIG-I activation via 5'ppp-dsRNA than in those that received alum or MPL (Fig. 1C). Similar results were also obtained when the cells were stained with the alternative Tfh markers PD-1 and ICOS (data not shown). Furthermore, the frequency of polyfunctional antigen-specific CD4 T cells (expressing

FIG 2 RIG-I activation enhances influenza vaccine-specific antibody responses at sparing doses, and the effects are dependent on signaling by IFN-IR and IPS-1. (A) The optimal dose-sparing effect of 5'ppp-dsRNA was determined by prime-boost immunizing mice with different HA equivalent vaccine doses and measuring serum IgG titers at the indicated time points. (B) The dose-sparing efficacy of 5'ppp-dsRNA was compared with that of MPL-A, R-837, and alum by immunizing mice with 0.5 μ g HA of influenza vaccine with or without 5'ppp-dsRNA or with 5 μ g HA of vaccine alone, followed by a booster at 4 weeks. The recall memory IgG titers were measured. (C and D) Mice were immunized with 0.5 μ g HA of influenza vaccine with or without 5'ppp-dsRNA or with 5 μ g HA of vaccine alone, followed by a booster at 4 weeks. The total vaccine-specific serum IgG (C) and HAI antibody (D) titers were determined at the indicated time points. (E) To investigate the mechanistic dependency of 5'ppp-dsRNA-induced effects, mice deficient in IFN-IR, MyD88, TLR3, TLR4, and IPS-1 were immunized with a 5- μ g HA equivalent vaccine dose with or without 5'ppp-dsRNA, along with appropriate wild-type controls. A booster dose was given at 4 weeks, and serum IgG and HAI titers were determined at different time points postimmunization. The results shown here represent the titers observed at 2 weeks following the booster. Each treatment group had 3 or 4 mice, and the data are representative of at least three independent experiments. The asterisks (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$) indicate significant differences compared to mice that received 5- μ g HA vaccine alone, unless otherwise indicated by the intragroup comparison. The error bars indicate means $\pm$ SEM.

IL-2 and TNF- α or IL-2 and IFN- γ) induced in the presence of RLR activation was significantly higher than those activated in the presence of antigen alone or antigen plus alum or MPL-A (Fig. 1D). We also investigated the possible role of vehicle in augmenting CD4 T cell responses using the same TCR-transgenic CD4 T cell transfer system in a separate experiment. Although the expansion of transferred donor CD4 T cells in mice receiving vaccine plus vehicle, in response to GP immunization, was significantly better than in those that received vaccine alone, no significant changes were observed in either GP-specific Tfh differentiation or CD4 T cell effector functions (data not shown). Taken together, these results clearly showed that activating the RLR pathway induces robust antibody responses to vaccine and CD4 T cell responses to protein antigens compared to TLR or NLR pathway activation.

Activating the RLR pathway provides a marked dose-sparing effect and induces long-lasting antibody response. Next, we examined whether RLR pathway activation has antigen dose-sparing effects. To this end, mice were immunized with between 0.005 μ g and 5 μ g of HA of influenza vaccine in the presence of the RIG-I ligand by a prime-boost regimen, and total vaccine-specific IgG, as well as HAI antibodies, in the sera were measured. As shown in Fig. 2A, mice immunized in the presence of the RIG-I ligand showed a more robust humoral response, even at a 100-fold-lower (0.05 μ g) vaccine dose, than mice given 5 μ g HA without RLR activator. Similar effects were also observed when HAI titers in the mice that received 0.05- μ g HA vaccine were determined (data not shown). Interestingly, the recall antibody titers at 2 weeks post-booster immunization in mice that received 0.5 μ g HA with 5'ppp-dsRNA were similar to those in mice that received a 10-

times-higher dose (5 μ g HA) of vaccine with 5'ppp-dsRNA (Fig. 2A). However, this may have been due to the inhibitory effect of high levels of circulating antibodies, after the single immunization, on the booster effect in the 5- μ g HA-immunized mice, since such a negative-feedback inhibitory effect of antibodies has been noted previously (28). We further compared the dose-sparing efficacy of RIG-I pathway activation with TLR4, TLR7, and alum (NLR) pathway activation in inducing recall memory antibody responses when a sparing dose of 0.5- μ g HA vaccine was used. Mice that received 5'ppp-dsRNA and MPL-A, the RIG-I and TLR4 ligands, respectively, had significantly higher antibody levels than mice that received 5- μ g HA vaccine with no adjuvant, suggesting the dose-sparing abilities of the two molecules (Fig. 2B). While the antibody titers in mice administered 5'ppp-dsRNA and MPL-A were even significantly higher than the antibody titers in those receiving alum and R-837, interestingly, the intragroup comparison also showed that 5'ppp-dsRNA could induce significantly higher antibody titers than MPL-A. This observation suggests that RIG-I pathway activation, along with influenza vaccine, is a better inducer of memory recall antibody responses than others investigated in the present study (Fig. 2B). Furthermore, we also excluded the possible role of the vehicle used to deliver 5'ppp-dsRNA in mediating dose-sparing effects (data not shown).

To assess if activating the RLR pathway can reduce the amount of antigen required to induce optimal long-lasting immune responses, serum antibody responses were measured in immunized mice over a 6-month period. Consistent with the results shown in Fig. 2A, mice immunized with 5'ppp-dsRNA-supplemented 0.5- μ g HA vaccine had significantly (4- to 5-fold) higher IgG titers than those immunized with 5- μ g HA vaccine (Fig. 2C). Importantly, the titers remained significantly elevated for up to 6 months, indicating the presence of durable antibody responses even at sparing doses. Similar IgG antibody responses were also observed when these immunizations were repeated using BALB/c mice (data not shown). In addition, 5'ppp-dsRNA could effectively enhance HAI titers in immunized mice (Fig. 2D). A single injection of vaccine containing 0.5 μ g HA supplemented with 5'ppp-dsRNA could attain an HAI titer of 1:40 against both A/California/08/2009 and A/Mexico/4108/2009 A(H1N1)pdm09 viruses, while the vaccine containing 5 μ g HA without 5'ppp-dsRNA did not elicit detectable HAI titers at week 4. Following the booster immunization, HAI titers in mice that received 10 times less antigen with 5'ppp-dsRNA were significantly elevated by about 6-fold compared to vaccine-alone controls and remained significantly higher for a period of 6 months.

We further evaluated the efficacy of 5'ppp-dsRNA in boosting the immune response to another protein antigen, OVA. Consistent with the response to the influenza vaccine, there was a 3- to 4-fold increase in the OVA-specific IgG titers in the 5'ppp-dsRNA-immunized mice compared to controls (data not shown). Taken together, these results demonstrated that 5'ppp-dsRNA possesses an excellent dose-sparing effect and an ability to induce long-lasting antibodies to protein antigens.

5'ppp-dsRNA-mediated effects are dependent on signaling by type I interferon and IPS-1 but independent of MyD88 and TLR3 signaling. IPS-1, a mitochondrion-associated adapter protein, is required in the signaling of the RIG-I receptor leading to type I IFN production and expression of immune response genes (4). 5'ppp-dsRNA is a well-recognized ligand for the RIG-I receptor; we found that 5'ppp-dsRNA injection of mice resulted in type

I IFN production and T cell activation (data not shown). We primed and boosted mice deficient in IPS-1 or IFN-IR signaling, along with age-matched wild-type controls, with vaccine supplemented or not with 5'ppp-dsRNA. From the results shown in Fig. 2E, IPS-1-deficient mice immunized with vaccine and RIG-I ligand had substantially reduced HAI titers compared to wild-type mice during the recall response. Similarly, IFN-IR-deficient mice administered 5'ppp-dsRNA-supplemented vaccine also had significantly decreased HAI titers. However, the antibody response to vaccine alone also depended on the type I IFN signaling (Fig. 2E). We also excluded the possible involvement of other sensors of viral nucleic acids in the 5'ppp-dsRNA-mediated observed effects by immunizing mice deficient in Myd88 and TLR3 (Fig. 2E). Collectively, these findings indicated that 5'ppp-dsRNA contributes to adaptive B cell immunity via signaling through IPS-1 and, perhaps, at least in part by inducing type I interferons, the most important downstream antiviral effector molecules of innate immunity.

RIG-I activation augments GC reactions and enhances Tfh cell responses. Germinal centers are distinct anatomical sites within the lymphoid organs where antigen-primed B cells undergo somatic hypermutation and affinity maturation leading to generation of long-lived high-affinity ASCs, as well as formation of a memory pool (29). Since RIG-I activation induced elevated and durable antigen-specific antibodies, even at sparing doses, we evaluated its effects in augmenting GC development. Mice were primed and boosted with 5 or 0.5 μ g HA with or without 5'ppp-dsRNA. GC-B cell frequencies in draining lymph nodes, as determined by the B cells expressing PNA and Fas (CD95) markers, at days 7, 14, and 28 post-primary immunization, as well as 5 days postbooster (day 35), were measured. As shown in Fig. 3A, first, vaccine with 5'ppp-dsRNA significantly augmented GC-B cell frequencies by 2- to 4-fold compared to the vaccine-alone group. Second, immunization with a dose of 5 μ g HA together with RIG-I ligand was found to elicit a much greater GC reaction than a 0.5- μ g HA dose with RIG-I ligand during the primary response. However, GC-B cell frequencies in the low-dose-immunized mice together with RIG-I ligand were significantly higher than in high-dose antigen-immunized mice without RIG-I ligand. Finally, following the booster, the GC-B cell frequencies in the mice that received RIG-I ligand and antigen were 2- to 3-fold higher in lymph nodes than those in antigen-alone controls. Moreover, these frequencies were comparable between the mice that received sparing doses of 0.5 μ g of HA antigen with RIG-I ligand and the mice that received 10-times-higher vaccine doses with RIG-I ligand. Furthermore, we also found an increased GC-B cell frequency in the lymph nodes of mice given 5'ppp-dsRNA when OVA was used as an immunizing protein (data not shown).

Durable and high-affinity antibody responses characteristic of GC-derived long-lived plasma and memory cells are dependent on help from specialized Tfh cells (30). Our observation of 5'ppp-dsRNA-induced robust GC reactions prompted us to assess the kinetics of the Tfh response, as determined by the CD4 T cells expressing CXCR5, PD-1, and Bcl6 markers, in the draining lymph nodes of immunized mice. Consistent with GC formation, at day 7 postimmunization, we found robust Tfh responses in mice that received RIG-I ligand with either high or low doses of vaccine compared to those in mice that received antigen alone (Fig. 3B). Although the Tfh frequency in mice vaccinated with antigen plus 5'ppp-dsRNA steadily declined after day 7, the re-

FIG 3 5'ppp-dsRNA enhances germinal center reactions and follicular helper T cell response kinetics. (A) Mice were administered either a 5- or 0.5- μ g HA dose of the pandemic influenza vaccine with or without 5'ppp-dsRNA, with a booster given at day 30. The frequencies of B cells (B220⁺ CD4⁺) expressing PNA and Fas (CD95) in the draining lymph nodes of immunized mice were quantified at the indicated time points. (B) The frequencies of CD4 T cells (negative for B220) expressing CXCR5 and PD-1 in the lymph nodes of immunized mice were also measured at different time points, as indicated. The FACS plots show representative results observed on day 7 (primary response) and day 35 (recall response), while the graphs depict the kinetics of the responses. The representative flow plots show cell frequencies observed in the lymph node. Each treatment group had 3 or 4 mice, and the data represent one of three independent experiments. The asterisks (*, $P \leq 0.05$, and **, $P \leq 0.01$) indicate significant differences compared to mice that received 5- μ g HA vaccine alone. The error bars indicate means $\pm$ SEM.

sponse remained consistently higher than that of the vaccine-alone group at all time points evaluated after primary immunization. In addition, consistent with the GC response following booster immunization, the Tfh frequencies in the lymph nodes of

mice immunized with 0.5 μ g HA plus RIG-I ligand were at comparable levels to those observed in mice that received 10-times-higher doses of vaccine along with RIG-I ligand. This finding is consistent with our observation that postbooster antibody titers in

mice given the sparing dose of vaccine plus RIG-I ligand were comparable to those in mice immunized with high-dose antigen plus RIG-I ligand (Fig. 2A). Furthermore, we also confirmed that the increase in GC and Tfh frequencies in the mice given vaccine supplemented with RIG-I ligand is indeed a vaccine-specific response by injecting some of the naive mice with 5'ppp-dsRNA alone in one of the triplicate experiments (data not shown). Therefore, in summary, these results suggest that heightened antibody responses induced by 5'ppp-dsRNA are a result of enhanced GC formation and increased differentiation of CD4⁺ T cells into Tfh cells.

RIG-I activation enhances influenza vaccine-specific plasma cell responses and generation of high-affinity antibodies. Following immunization, antigen-experienced naive B cells in the lymphoid organs differentiate into short-lived plasma/antibody-secreting cells (ASCs) to produce antibodies rapidly. A portion of B cells that undergo the germinal center reaction differentiate into memory B cells and long-lived plasma cells that migrate to bone marrow secreting high-affinity antibodies (29). Maintenance of long-lasting IgG and HAI antibodies in the sera of mice that received RIG-I ligand and antigen led us to evaluate the ASC kinetic response postimmunization. Based on the results shown in Fig. 4A, we made three observations. First, the ASC response in mice immunized with antigen alone was found to be very poor. Second, while the high-dose (5- μ g HA) vaccine, together with RIG-I ligand, induced a robust ASC response by day 7 in lymph nodes, a low dose (0.5 μ g) with RIG-I ligand had delayed kinetics, with the peak response occurring at day 14 and declining thereafter. In the spleen, although the response was less than that of lymph nodes, similar ASC kinetics were observed (data not shown). Finally, plasma cells were seen in the bone marrow as early as 2 weeks postimmunization, and the ASC numbers showed an increasing trend beyond 28 days post-single immunization, possibly suggesting the longevity of these cells. Collectively, these results show that RIG-I activation induces robust levels of antigen-specific ASC response in tissues, including bone marrow-homing plasma cells, even at sparing antigen doses.

Affinity maturation is an important step in the GC reaction that influences the production of high-affinity antibodies in response to immunization (31). Therefore, we sought to investigate further the effects of 5'ppp-dsRNA in inducing antibodies with higher avidity, which is a measure of the overall strength of binding of an antigen with many antigenic determinants and multivalent antibodies. We measured the avidity of sera collected from mice at 4 weeks post-primary injection, as well as at 2 weeks following the booster. As shown in Fig. 4B, the avidity indices for sera from mice that received RIG-I ligand along with the antigen were markedly higher (>50%) than for sera from vaccine-alone controls during primary, as well as booster, responses. This observation suggested that addition of 5'ppp-dsRNA to the vaccine formulation could result in the production of high-avidity IgG antibodies. Importantly, the avidity indices of sera from mice immunized with 5'ppp-dsRNA-supplemented 0.5- μ g HA vaccine were substantially elevated (from 51 to 90%) following the booster. This observation possibly supports the presence of higher HAI titers following the booster (Fig. 2A) in these mice. However, the avidity indices following the booster of sera from mice immunized with 5'ppp-dsRNA plus 5- μ g HA vaccine remained comparable to the indices of sera collected during the primary response. Collectively, these results showed that 5'ppp-dsRNA has the abil-

ity, not only to augment the antigen-specific ASC response, but also to induce high-affinity antibodies.

RIG-I activation induces protective immune responses in mice against influenza virus challenge. Although it is critical for an adjuvant molecule to enhance the immunogenicity of a vaccine antigen, such enhanced immunogenicity may not always be associated with protection against disease (32). Therefore, we next examined the protective efficacy of the immune response induced by 5'ppp-dsRNA plus pandemic influenza vaccine against challenge with a lethal dose of virus. Mice were immunized with either 5 μ g HA or a sparing dose (0.5 μ g HA) of the vaccine with or without 5'ppp-dsRNA, and protection in response to a single immunization, as well as during the recall response, was assessed.

First, as shown in Fig. 5A, while all the unimmunized mice, as well as those that received 5- μ g and 0.5- μ g HA vaccine alone, succumbed to infection by day 9 postchallenge, all mice that received either 5 μ g or 0.5 μ g HA together with 5'ppp-dsRNA survived lethal virus challenge (50 LD₅₀), although these mice lost about 10 to 15% of their body weight at around days 4 and 5. This transient weight loss is likely accounted for by the high virus challenge. These observations indicated the ability of 5'ppp-dsRNA to generate strong protective immune responses against a lethal influenza virus challenge in these mice. Protected mice were found to develop significantly higher HAI titers at day 7 postchallenge (data not shown).

Next, we challenged mice that received vaccine with or without 5'ppp-dsRNA 5 months following the booster to evaluate the durability of the protective immunity. As shown in Fig. 5B, all the unimmunized mice, as well as those receiving 0.5- μ g HA vaccine, succumbed to infection by day 9 postchallenge. While mice immunized with 5- μ g HA vaccine alone had a survival rate of 60%, those administered 5- μ g and 0.5- μ g HA vaccine with RIG-I ligand had survival rates of 80% and 100%, respectively. This observation demonstrated the protective efficacy when the vaccine was given with 5'ppp-dsRNA. Furthermore, the weight loss in the 5- μ g HA-alone group was much higher than in those mice that received HA plus 5'ppp-dsRNA. From this experiment, it can be inferred that a 0.5- μ g HA vaccine dose given with 5'ppp-dsRNA had protective ability superior to that of the dose that was 10 times higher, since all the mice in the former group survived and lost less weight than the latter group of mice. This finding can be supported by the presence of higher IgG and HAI titers in these mice (Fig. 2A), and further, we found that the mice that received vaccine with RIG-I ligand developed significantly higher HAI titers against A/Mexico/4108/2009 (H1N1pdm09) virus at day 7 postchallenge (data not shown). Collectively, our data demonstrated that 5'ppp-dsRNA not only augments B and T cell responses, but also confers protective immunity even at sparing vaccine antigen doses. It is also likely that the protective efficacy of 5'ppp-dsRNA observed in our study depended not only on its ability to augment B and Tfh cell responses, but also on inducing polyfunctional IFN- γ -producing antigen-specific CD4 T cells (Fig. 1D) that can exert antiviral cytolytic activity and that are considered essential in influenza immunity (33).

DISCUSSION

The innate immune component, apart from being the front line of host defense against microbial pathogens, also initiates the induction of adaptive immune responses against microbial pathogens (1). The recent discovery of PRRs has expanded our understand-

FIG 4 RIG-I activation enhances influenza vaccine-specific ASC responses and induces generation of high-affinity antibodies. (A) Mice were immunized with 5 and 0.5 μ g HA of 2009 H1N1 pandemic influenza vaccine with or without 5' ppp-dsRNA, followed by a booster at 4 weeks. Following single immunization, the primary ASC kinetic response was evaluated in the draining lymph nodes and bone marrow of immunized mice at the indicated time points. (B) The affinities of antibodies collected at 4 weeks following single immunization, as well as those collected at 6 weeks (2 weeks postbooster), were measured by calculating their avidity indices as described in Materials and Methods. The antibodies whose index values were $\geq 50\%$ (dashed line) were considered to possess high affinity. Each treatment group had 3 or 4 mice, and the data represent 1 of 2 or 3 independent experiments. The asterisks (*, $P \leq 0.05$, and **, $P \leq 0.01$) indicate significant differences compared to mice that received 5- μ g HA vaccine alone unless otherwise specified. The error bars indicate means $\pm$ SEM.

ing of the cellular and molecular nature of innate immunity and its impact on adaptive immunity, which has allowed the exploitation of PRR pathways to design and develop several molecules to boost protective immunity (34, 35). However, in this context, a recently discovered RLR pathway that comprises a cytosolic class of receptors, such as RIG-I, specialized in sensing viral nucleic acids (36), has not been well explored, particularly the effects of RIG-I path-

way activation in reducing vaccine antigen doses in the induction of optimal antiviral humoral and protective response, as well as enhancement of B and T cell responses. We have shown previously that activation of the RIG-I pathway induces panantiviral effects both *in vitro* and *in vivo* (7, 37). In the present study, we show that the activation of the RIG-I (RLR) pathway in the presence of an antigen offers dose-sparing effects and induced enhanced germi-

FIG 5 RIG-I activation induces protective immune responses in mice against virus challenge. Mice (5 per group) were immunized with 5 or 0.5 µg of HA with or without 5'ppp-dsRNA and challenged with 50 LD₅₀ of the A/Mex/4108/2009 pandemic virus strain at 4 weeks post-single immunization (A) or at 5 months postbooster (B). The mice were monitored for mortality and weight loss during a 14-day observation period. The error bars indicate means $\pm$ SEM.

nal center reaction, follicular T helper cells, humoral immune responses, and protective immunity. We used 5'ppp-dsRNA, a synthetic molecule known to activate the RIG-I pathway (5), along with 2009 H1N1 pandemic monovalent influenza vaccine antigens, as well as chicken OVA antigen, to assess immune responses, including protective immunity against influenza virus.

For decades, alum has been successfully used to boost antibody responses to viral-vaccine antigens. Although its precise mechanism of action at the cellular and molecular levels is not clear, alum has been recently suggested to activate the NLR pathway (24). Several molecules that activate the TLR pathway have also been used to boost antibody and T cell responses (25). Therefore, we compared the relative efficacies of activating the RIG-I, NLR, and TLR pathways in inducing influenza virus-specific antibody responses. Although the activation of the NLR, TLR, or RIG-I pathway could markedly augment the antigen-specific antibody response, the postbooster antibody titers in mice that received 5'ppp-dsRNA, a RIG-I ligand, were significantly higher than those in the alum-immunized mice, suggesting superior recall memory B cell responses induced by RIG-I activation. More importantly, the recall memory antibody response in mice administered sparing doses of 0.5-µg HA vaccine along with the RIG ligand were significantly higher than in those that received MPL-A, R-837, and alum, the TLR4, TLR7, and NLR pathway ligands, respectively (Fig. 2B). The potent ability of RIG-I activation to induce type I IFN production (reference 38 and data not shown) suggests that the type I IFN signaling in memory B and CD4 T cells is vital in the rapid and robust differentiation of memory B cells into ASCs (9, 39, 40). We found that IFN-IR-deficient mice administered 5'ppp-dsRNA-supplemented vaccine had markedly decreased HAI titers. However, the antibody response to vaccine

alone also depended in part on the type I IFN signaling (Fig. 2D). This is likely because of the presence of viral RNA in the vaccine used in this study; we have previously shown that the HAI antibody response induced by pandemic influenza vaccine alone depends, at least in part, on signaling by TLR7, a receptor that senses viral nucleic acids (41). Interestingly, in partial support of this idea, mice deficient in MyD88 (an adapter molecule downstream of TLR7 signaling) in the present study, when immunized with vaccine alone, had reduced HAI titers, though not statistically significant, compared to their wild-type controls. Mechanistically, RIG-I pathway activation leading to type I IFN production requires the IPS-1 adapter molecule (38). Therefore, we further confirmed that, in addition to partial dependency on type I IFN signaling, RIG-I agonist-mediated effects were largely dependent on signaling by IPS-1. Since the viral RNA can also be sensed by receptors other than RIG-I, such as TLR3 and TLR7, and since MyD88 is an essential adapter molecule in the signaling of most TLRs, including TLR7 (42), we also confirmed that the effects mediated by 5'ppp-dsRNA were independent of MyD88 and TLR3 signaling.

Although the use of several TLR ligands can circumvent the issues of poor immunogenicity associated with certain inactivated vaccines, such as influenza vaccines (43), ensuring the availability of sufficient vaccine to protect the global population in pandemic influenza outbreaks is very challenging, given the limited production capacity (44). A sizable reduction in the vaccine dose will overcome this bottleneck. Our preclinical data suggest the possibility of reducing the antigenic dose of vaccines by about 10 to 100 times (equivalent to a dose as small as 50 ng of HA) compared to the conventional dose (4 to 5 µg of HA) using RIG-I ligands that produce an excellent dose-sparing effect. We found that mice im-

munized with sparing doses of vaccine antigens with RIG-I ligand had long-lasting IgG and HAI antibodies sustained at significantly higher levels and were protected against a lethal pandemic virus challenge. Recently, two other studies also suggested the use of RIG-I agonists in boosting influenza immunity (27, 45). However, the abilities of these molecules to offer antigen-sparing effects, induction of HAI antibodies, and protection against pandemic influenza virus challenge were not investigated in these studies. More importantly, an analysis of RIG-I activation effects in enhancing antigen-specific B cell and CD4 T cell responses was also not addressed in detail.

Innate immune pathway activation resulting in effective priming of the adaptive immune component is critical in vaccine-induced immunity. Recent reports show that the critical cellular players in humoral immunity are the GC-B and CD4⁺ Tfh cells. Within the lymphoid organs, GC reaction allows B cells to undergo somatic hypermutation and affinity maturation, resulting in the generation of long-lived high-affinity ASCs and a memory pool (46). Importantly, the GC-driven activities depend on help from specialized CD4 Tfh cells (30). In this context, it was evident in our study that the RIG-I activation could markedly augment GC-B and Tfh cell responses in the vaccinated mice following a single immunization. Importantly, the booster immunization that included 5'ppp-dsRNA in the vaccine could further markedly heighten the GC and Tfh responses, even at sparing doses, suggesting that the magnitude of the GC response was positively linked to the size of the Tfh cell pool and that the Tfh cells are critical for GC formation (46, 47). Using an LCMV-specific CD4 T cell transgenic system, we also found that activating the RIG-I pathway can lead to significantly greater expansion of antigen-specific CD4 T cells, their differentiation into a Tfh cell population, and increased polyfunctionality of CD4 T cells compared to TLR (MPL-A) and NLR (alum) activation pathways. Consistent with the GC and Tfh responses, the influenza virus-specific plasma cell (ASC) response in tissues was substantially augmented. Importantly, the migration and residency of long-lived plasma cells in bone marrow was seen as early as 2 weeks postimmunization, and their numbers appeared to increase beyond 28 days post-single immunization. Furthermore, the higher avidity indices of sera from mice administered RIG-I ligand suggested that RIG-I activation may be required for the affinity maturation process, an important step in the GC reaction (29).

Collectively, our data indicate that activating the RIG-I pathway at the time of vaccination can robustly enhance GC-B, Tfh, and plasma cell responses, leading to induction of long-lasting high-affinity antibodies and protective immunity. To our knowledge, this is the first report to demonstrate the effectiveness of RIG-I pathway activation, not only in priming the innate responses, but also, importantly, in shaping the adaptive immune component through augmenting vaccine antigen-specific B and T cell responses. Therefore, natural ligands or small molecules that activate the RIG-I pathway have potential utility as the next-generation molecular adjuvants for vaccines against viral diseases, as well as, perhaps, for cancer.

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