MyD88 Plays an Essential Role in Inducing B Cells Capable of Differentiating into Antibody-Secreting Cells after Vaccination[▽]

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We investigated the roles of MyD88, an innate adaptor signaling molecule, in inducing protective humoral immunity after vaccination with influenza virus-like particles (VLPs). MyD88 knockout C57BL/6 mice (MyD88^{-/-} mice) vaccinated with influenza VLPs showed significant defects in inducing IgG2a/c isotype antibodies and in generating splenic recall memory B cell responses and antibody-secreting plasma cells in the bone marrow. The protective efficacy of influenza VLP vaccination was lower in MyD88^{-/-} mice than in the wild-type mice. Our findings indicate that MyD88-mediated innate signaling pathways are important for effectively inducing primary and boost immune responses, T helper type 1 isotype-switched antibodies, and gamma interferon (IFN- γ)-secreting T cell responses. In particular, the results in this study demonstrated for the first time that MyD88-mediated immune activation is likely an essential pathway for effective generation of long-lived antibody-secreting plasma cells and highly protective immunity after vaccination with influenza VLPs. This study provides insight into mechanisms by which recombinant viral vaccines induce protective immunity via the MyD88-mediated innate immune signaling pathway.

Influenza is a highly contagious respiratory disease resulting in widespread morbidity and mortality. Vaccination is the most cost-effective measure for preventing influenza. Influenza virus-like particles (VLPs) have been demonstrated to induce protective immunity against seasonal and pandemic potential influenza viruses even with a single vaccination (30, 33). However, their mechanisms for inducing adaptive humoral immune responses and protective immunity are not well understood.

Toll-like receptors (TLRs) are expressed in innate immune cells, recognize certain molecular patterns of pathogens, and are reported to be important for initiating innate and adaptive immune responses (14). B cells of the adaptive immune system also express TLRs (10). MyD88 (myeloid differentiation gene 88) is a common adaptor signaling molecule for TLRs (except for TLR3). MyD88 gene knockout (MyD88^{-/-}) mice were shown to induce lower levels of IgG1 and no detectable levels of IgG2a/c isotype antibodies after immunization with ovalbumin administered with lipopolysaccharide (LPS) or Freund adjuvants (27, 31). Stimulation of B cells with the TLR9 agonist CpG DNA induces antibody isotype switching (11, 16). In contrast, other studies reported that mice with a deficiency in

TLR signals induce comparable levels of antibodies specific to hapten conjugates (trinitrophenol-hemocyanin) in the presence of different types of adjuvant (alum, complete/incomplete Freund's adjuvant, or Ribi) (7). Also, B cell knockout (μ MT) mice that received MyD88-deficient B cells induced levels of nitrophenylacetyl (NP) hapten-specific antibodies similar to those in mice that received wild-type (WT) B cells (25).

Live virus infections were demonstrated to induce TLR-dependent and TLR-independent adaptive immune responses. The influenza virus single-strand RNA (ssRNA) genome is recognized by TLR7 and its adaptor MyD88, inducing type-1 IFN production (5, 13, 23). The 5' triphosphate of its ssRNA genome is recognized by retinoic acid-inducible protein 1 (RIG-1), independently of TLR7 and MyD88 (15). Sendai or influenza virus infections induced dendritic cell maturation and adaptive immunity independent of MyD88 and TLRs (23). However, infection of MyD88^{-/-} mice with influenza virus showed a lower level of IgG2a isotype antibody responses (20). Also, MyD88 was required for long-term but not short-term maintenance of humoral immunity to mouse polyomavirus infection (9), indicating that different types of adaptive immune responses may have differential requirements for TLR/MyD88 signaling pathways.

One of the main goals of vaccination is to induce long-lived memory B cells and plasma cells, which provide long-lasting protective immunity. Being located in lymphoid organs, memory B cells mediate rapid recall responses to infection by quickly dividing and differentiating into antibody-secreting plasma cells that eventually traffic to the bone marrow (4, 32). Thus, long-lived plasma cells reside mainly in the bone marrow

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constitutively producing antibodies. The role of the innate immune system in generating memory B cell phenotypes and long-lived plasma cells is not well understood.

Understanding the recognition of vaccines by the innate immune system in inducing protective immunity will be important for uncovering the mechanisms for how vaccines work and for developing more effective vaccines. In contrast to live-virus infection studies, the roles of MyD88 in generating isotype-switched antibodies, memory B cells, antibody-secreting plasma cells, and protective immunity after vaccination are largely not understood. Influenza VLPs provide protective immunity in the absence of adjuvants, indicating that they are an effective vaccine candidate that is potentially applicable to humans. In this study, we have investigated the potential roles of MyD88 innate immune signaling in inducing adaptive humoral immune responses and protective immunity after vaccination with influenza VLPs. We found that MyD88^{-/-} mice were defective in inducing effective humoral immunity in several aspects, including IgG2a/c, IgG2b, and IgG3 isotype antibodies, antibody secreting cells in the spleen, and antibody secreting plasma cells in bone marrow as well as in inducing protective immunity.

MATERIALS AND METHODS

Mice, cells, and virus. The wild-type C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN). MyD88 gene-deficient (MyD88^{-/-}) mice were generated and maintained as described previously (1). The CD4 knockout (CD4^{-/-}) mice were obtained from Jackson Laboratory (Bar Harbor, ME). SF9 insect cells (Invitrogen, Carlsbad, CA) for the production of recombinant baculoviruses (rBVs) and influenza VLPs were cultured in SF900-II serum-free medium at 27°C. Madin-Darby canine kidney (MDCK) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM). Influenza H1N1 A/PR/8/34 virus was kindly provided by Huan H. Nguyen.

Preparation of influenza VLPs. Influenza VLPs were prepared as described previously (28). Briefly, SF9 insect cells were coinfectd with rBVs expressing M1 and hemagglutinin (HA) derived from the strain of A/PR/8/34 H1N1 virus. Culture supernatants containing influenza VLPs were harvested at 3 days postinfection and purified by low-speed centrifugation (2,000 × *g* for 20 min at 4°C) to remove cells. Influenza VLPs in the supernatant were pelleted by ultracentrifugation (100,000 × *g* for 60 min). The precipitated particles were resuspended in phosphate-buffered saline (PBS) at 4°C overnight and further purified through a 20%-30%-60% discontinuous sucrose gradient at 100,000 × *g* for 1 h at 4°C. The VLP bands were collected and analyzed using Western blot and hemagglutination activity (HAA) assays. The content of HA in VLPs was estimated to be approximately 10% of total VLP proteins (28).

Immunization, viral challenge infection, and protective efficacy of serum transfer. Mice were housed in the animal facility of Emory University, and all mouse experiments were carried out following an approved Institutional Animal Care and Use Committee (IACUC) protocol. Wild-type and MyD88^{-/-} mice (8 to 10 weeks old) were intramuscularly or intranasally immunized with influenza VLPs at weeks 0 and 4. For intramuscular immunization, influenza VLPs (3 µg protein) were suspended in 100 µl PBS and were injected intramuscularly on the upper quadriceps muscles of mice. For intranasal immunization, the same dose of influenza VLPs (3 µg protein) was suspended in 50 µl of PBS and administered slowly with a pipette tip to an anesthetized mouse drop by drop to both nares. To assess the protective efficacy of vaccination, mice were intranasally challenged with mouse-adapted A/PR/8/34 virus. Challenge doses were 6 or 12 times the 50% mouse lethal dose (LD₅₀). Unimmunized naïve mice were included as a negative-control group. Mice were observed daily to monitor changes in body weight and to record death. Mice were euthanized if their body weight loss exceeded the 25% IACUC endpoint to minimize suffering. To determine the protective efficacy of immune sera, immune sera from vaccinated mice were 4-fold diluted, mixed with a lethal dose of A/PR/8/34 virus (3 LD₅₀), and then used to infect naïve mice as described previously (34, 35). Body weight changes and survival rates were monitored daily for 14 days.

Serum antibody responses. Influenza virus-specific total-IgG antibodies and different isotypes (IgM, IgG1, IgG2a/c, IgG2b, and IgG3) were determined using

standard methods as described previously (28, 29). Briefly, 4 µg of inactivated influenza A/PR/8/34 virus was used as an antigen on 96-well microtiter plates (Nunc, Rochester, NY) in coating buffer (0.1 M sodium carbonate, pH 9.5) at 4°C overnight. Serially diluted serum samples were added and incubated for 1 h at 37°C, then horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgM, IgG1, IgG2a/c, IgG2b, and IgG3 were used as secondary antibodies, and *O*-phenylenediamine was used as a substrate. Of note, the C57BL/6 mouse strain does not have the gene for IgG2a, but rather the IgG2c isotype (8). IgG2a antibody levels reported for this strain of mice may be detecting IgG2c by the use of IgG2a cross-reactive secondary antibody (13). In the present study, both anti-IgG2a and IgG2c antibodies were used to represent IgG2a/c isotypes. Purified IgG and IgM antibodies were used as a standard to determine the relative antibody concentrations in immune sera from optical spectrophotometer readings at 450 nm (optical density at 450 nm [OD₄₅₀]). Alternatively, relative units are presented as direct readings of OD₄₅₀ values. For determination of hemagglutination inhibition (HAI) titers, serum samples were collected at 2 weeks after intranasal boost immunization. HAI titers of immune sera against influenza A/PR8 virus were determined by standard methods using 4 HA units of virus as described previously (29).

Flow cytometry. Spleen and bone marrow cells were analyzed by fluorescence-activated cell sorting (FACS) to determine germinal and plasma cells after immunization of MyD88-deficient or wild-type mice. To prepare splenocytes, spleen tissues were incubated with DNase I (100 µg per ml; Sigma) and type IV collagenase (2 mg/ml; Worthington) for 30 min at 37°C, minced through frosted glass plates, and then passed through a cell strainer (40 µm; BD, Franklin Lakes, NJ). Bone marrow cells were obtained by flushing the cavities of both femoral and tibial bones with media following the procedure previously described (18). The single-cell suspensions were stained with fluorescence-conjugated antibodies specific to cell phenotypes (peanut agglutinin [PNA]-fluorescein isothiocyanate [FITC], B220-PerCP, CD95 Fas-phycoerythrin [PE], CD138 [syndecan-1]-allophycocyanin [APC]). Lymphocytes were gated according to their sizes and granularity defined in the forward light scatter (FSC) and side light scatter (SSC) plot and sorted based on their phenotypic profiles. Cell acquisition was performed with a dual-laser flow cytometer (LSR-II; BD Biosciences, Mountain View, CA), and the data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR).

ELISPOT assays. To determine virus- or vaccine-specific antibody secreting cell spots, multiscreen 96-well plates (Millipore, Billerica, MA) were coated with A/PR8 virus or VLPs (4 µg/ml) overnight. Spleen or bone marrow cells (0.3 × 10⁶ cells/well) isolated from MyD88-deficient or wild-type mice were cultured on the plate for 24 or 36 h. After a short time of culture, the plates were washed with PBS and further incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or IgM antibodies. Antibody secreting spots were developed with stable DAB (3,3'-diaminobenzidine). The number of antibody secreting cell spots was counted using an ELISPOT reader (BioSys USA, Miami, FL).

Similarly, to determine T cell immune responses, multiscreen 96-well plates were coated with capture antibodies against mouse IFN-γ and IL-4 cytokines (3 µg/ml in coating buffer; BD Biosciences, San Diego, CA). Splenocytes (0.3 × 10⁶ cells/well) were cultured on the plate with 100 µl of RPMI 1640 media with 10% FBS with A/PR8 VLPs (2 µg/ml) as a stimulator. After 36 h of incubation, the plates were washed with PBS and further incubated with biotinylated rat anti-mouse IFN-γ or IL-4 (1 µg/ml), and HRP-conjugated streptavidin solution was added to each well for 3 h at room temperature (RT). The number of cytokine-secreting cell spots was counted using an ELISPOT reader.

Virus-specific antibody-secreting cell responses. Spleen and bone marrow (0.3 × 10⁶ cells/well) cells were incubated for 24 or 36 h in the 96-well culture plates coated with inactivated A/PR virus or VLPs (2 µg/ml) for *in vitro* antigenic stimulation. Levels of antibodies specific to A/PR/8/34 virus in the culture plates were determined by enzyme-linked immunosorbent assay (ELISA). For *in vitro* cultures of B lymphocytes, spleen cells were separated using the magnetic cell sorting system (MACS) based on specific cell surface marker antibodies conjugated to magnetic beads (Miltenyi Biotec). For helper T cell and B cell separation, CD4 and CD43 antibody-coated beads were used, respectively. The purity of each subpopulation was found to be approximately 90%. B220⁺ B cells (CD43 MACS-negative fraction) from naïve, vaccinated MyD88, or WT mice were cultured at 2 × 10⁶ cells per ml for 7 days in the presence of CD4 T helper cells (2 × 10⁶ cells per ml). Levels of antibodies specific to A/PR/8/34 virus in culture supernatants were determined by ELISA.

Lung viral titers. Lung viral titers were performed using a plaque assay in MDCK cells as previously described (28). Briefly, infected mice were euthanized at day 4 postchallenge. Lungs were harvested and homogenized in 1 ml of PBS. Serially diluted lung extracts were added to the 6-well plates containing confluent MDCK cell monolayers. After 1-h incubation, the medium in the culture plates

was replaced with an overlay medium containing diethylaminoethyl (DEAE) dextran, nonessential amino acid, glutamine, and trypsin and incubated for 2 or 3 days. The cells were then fixed with 0.25% glutaraldehyde and stained with 1% crystal violet. The limit of virus detection was 50 particle forming units (PFU) per 1 ml of lung homogenates.

Statistics. All parameters were recorded for individuals within all groups. A two-tailed paired Student's *t* test was performed when comparing two groups of animals. A value of *P* < 0.05 was considered statistically significant.

RESULTS

Antibody responses in MyD88^{-/-} mice after vaccination. Although MyD88 is known to play a central role in mediating innate immune responses, its role in inducing adaptive immunity after vaccination is not well studied. To better understand the possible role of MyD88 in inducing antibody responses, MyD88-deficient (MyD88^{-/-}) mice or C57BL/6 immunocompetent wild-type (WT) mice (*n* = 10) were intramuscularly immunized with 3 μ g influenza VLPs (A/PR8) at weeks 0 and 4 (Fig. 1A and B). After the first immunization of MyD88^{-/-} mice with influenza VLPs, IgG and IgM antibody responses specific to A/PR8 virus were observed to be lower than those in WT mice. Boost immunization of MyD88^{-/-} mice induced only a marginal, 2-fold increase in IgG and IgM antibody responses, whereas WT mice showed 7- and 2-fold increases in IgG and IgM antibody responses, respectively, after boost. These results indicate that MyD88^{-/-} mice have a significant defect in inducing boost IgG antibody immune responses.

Previous studies suggested that MyD88 is important for isotype switching of antibodies (27, 31). However, antibody isotypes in MyD88^{-/-} mice after vaccination are not well known. We determined the pattern of antibody isotypes specific to A/PR8 virus in boost immune sera of MyD88^{-/-} mice since it might provide informative insight into the types of immune responses induced in MyD88^{-/-} mice (Fig. 1C). The levels of IgG1 isotype antibody were moderately lower in MyD88^{-/-} mice than in WT mice, but there was no statistical significance. Interestingly, IgG2a/c, IgG2b, and IgG3 antibody isotype levels were significantly lower in MyD88^{-/-} mice than in WT mice. In particular, MyD88^{-/-} mice showed a severe defect in inducing IgG2a/c isotype antibodies compared to other isotypes, indicating that MyD88^{-/-} mice are defective in inducing Th1 isotype antibodies after influenza VLP vaccination.

Splenic B cells capable of secreting antibodies are defective in MyD88^{-/-} mice after vaccination. Since MyD88^{-/-} mice were less responsive in inducing IgG antibody to boost immunization with influenza VLPs, we analyzed phenotypes of B cells in MyD88^{-/-} mice in comparison with WT mice by flow cytometry. There were no significant differences in total live B cells between MyD88^{-/-} and WT mice (Fig. 2A and B). Although the MyD88^{-/-} mice showed a trend of lower levels of germinal center phenotypic B cells (B220⁺PNA⁺CD95 Fas⁺), there was no statistically significant difference between MyD88^{-/-} and WT mice (Fig. 2C). Therefore, there was no defect in terms of total B cells and germinal center phenotypic B cells in MyD88^{-/-} mice after vaccination.

To determine recall antibody-secreting B cell responses, splenic B cells capable of secreting antibodies specific to VLP vaccine antigen at day 7 after boost immunization of MyD88^{-/-} or WT mice with A/PR8 VLPs were analyzed by ELISPOT after a short time culture of 24 h (Fig. 3A). Sub-

FIG. 1. Magnitude and isotype profiles of serum antibody responses to intramuscular immunization with influenza VLPs in MyD88-deficient or wild-type mice. MyD88-deficient mice or C57BL/6 immunocompetent wild-type mice (*n* = 10) were immunized intramuscularly at week 0 and boosted at week 4 with 3 μ g/mouse of influenza VLPs. Virus-specific total IgG antibody (A); virus-specific total IgM antibody (B). Total IgG and IgM antibodies specific to A/PR8 virus are shown as concentrations (ng/ml). Prime, serum antibody at 2 weeks after prime immunization; Boost, serum antibody at 2 weeks after boost immunization; Naïve, unimmunized mice. (C) Isotypes of antibodies after boost. A/PR8 virus-specific isotype antibodies were determined using goat anti-mouse isotype-specific antibodies and OD values at 450 nm are shown with diluted sera (200 $\times$). MyD88^{-/-}, MyD88 knockout mice; WT, wild type mice. Asterisks indicate significances between MyD88^{-/-} and WT groups (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.005, Student's 2-tailed *t* test).

stantial numbers of vaccine-specific antibody-secreting cell spots were observed in WT splenocytes (Fig. 3B). However, MyD88^{-/-} splenocytes showed significantly lower numbers of vaccine-specific antibody-secreting cell spots than those from WT mice (Fig. 3A and B). To determine the antibody-secreting cells even in the absence of antigenic stimulation, *in vitro* culture supernatants were collected at 24 or 36 h and vaccine antigen-specific antibody levels were determined by ELISA. Splenocytes from WT mice, but not from MyD88^{-/-} mice, showed significant levels of VLP antigen-specific antibodies that were detected in culture supernatants without vaccine antigen stimulation during culture (Fig. 3C). These results indicate that MyD88-deficient mice have a defect in generating vaccine antigen-specific antibody-secreting cells after vaccination with influenza VLPs.

FIG. 2. Flow cytometry quantification of splenic germinal center phenotypes. (A) A representative flow cytometry profile of germinal center phenotypes of spleen cells. (i) Germinal center phenotypic gating of WT spleen cells. (ii) Germinal center phenotypic gating of MyD88 spleen cells. Spleen cells were collected from WT or MyD88^{-/-} mice ($n = 6$) at day 7 post-boost vaccination and stained with fluorescence-conjugated antibodies specific to cell markers (B220, CD95 Fas, and PNA). B220⁺ B lymphocytes were gated; germinal center phenotypic cells (B220⁺PNA⁺Fas⁺) are presented as percentages at top of rectangles. (B) Total splenic B cells. Average percentages of B220⁺ B cells out of total live lymphocytes are presented ($n = 6$). (C) Average percentages of germinal center phenotypic cells (B220⁺PNA⁺Fas⁺) out of total live B lymphocytes are presented ($n = 6$). Bars indicate standard deviations. WT, spleen cells from WT mice; MyD88, spleen cells from MyD88^{-/-} mice.

FIG. 3. Quantification of antibody-secreting cells by ELISPOT and *in vitro* antibody production. (A) A representative picture of ELISPOT wells to quantify antibody-secreting cell spots. MyD88^{-/-}, MyD88^{-/-} spleen cells (0.3×10^6 cells/well); WT, WT spleen cells (0.3×10^6 cells/well). (B) IgG ELISPOT of spleen cells. Spleen single-cell suspensions (0.3×10^6 cells/well) were cultured on precoated ELISPOT plates with A/PR8 influenza VLPs ($4 \mu\text{g/ml}$) and incubated for 24 h before spot development. (C) *In vitro* antibody production. Spleen cells were cultured on 96-well plates (0.3×10^6 cells/well) in the absence of antigenic stimulation. Culture supernatants were harvested after 24 or 36 h of cultures and A/PR8 influenza VLP-specific IgG antibodies were determined by ELISA. For panels B and C: Naïve, spleen cells from unvaccinated mice; MyD88, spleen cells from MyD88^{-/-} mice; WT, spleen cells from WT mice. Spleen cells were collected at day 7 post-boost vaccination of mice with A/PR8 influenza VLP vaccines. Average percentages of B220⁺ B cells out of total live lymphocytes are presented ($n = 6$). Asterisks indicate significances between MyD88^{-/-} and WT groups (**, $P < 0.01$; ***, $P < 0.005$, Student's 2-tailed t test).

Bone marrow cells capable of secreting antibodies are defective in MyD88^{-/-} mice. Since a defect in antibody-secreting cell responses was observed with splenocytes from MyD88^{-/-} mice, we analyzed antibody-secreting bone marrow plasma cells (Fig. 4). Plasma cell phenotypes (B220^{-/low}CD138⁺) in bone marrow were analyzed by flow cytometry (Fig. 4A). A slightly lower level of plasma cells was observed with bone marrow cells from MyD88^{-/-} mice than with those from WT mice (Fig. 4B). VLP vaccine antigen-specific IgG and IgM antibody-secreting cells in bone marrow were determined at day 7 after boost immunization of MyD88^{-/-} or WT mice with A/PR8 VLPs and were analyzed by ELISPOT after a short time culture of 24 h (Fig. 4C and D). Numbers of vaccine antigen-specific IgG and IgM antibody-secreting cell spots were approximately 4- and 2-fold lower, respectively, in MyD88^{-/-} bone marrow cells than in WT bone marrow cells. These results suggest that MyD88^{-/-} mice are not effective in generating antibody-secreting plasma cells after vaccination.

T-cell immune responses in MyD88^{-/-} mice after vaccination. It is also important to determine T-cell immune responses in MyD88^{-/-} mice after vaccination. At day 7 postboost, spleen cells were prepared and cultured on ELISPOT plates coated with cytokine-capturing antibodies in media containing A/PR8 VLPs as an antigenic stimulator (Fig. 5). Interleukin-4 (IL-4)-secreting cell spots were displayed at similar and substantial levels in splenocytes from both MyD88^{-/-} and WT mice (Fig. 5A). However, numbers of IFN- γ -secreting cell spots from MyD88^{-/-} splenocytes were significantly lower than those from WT splenocytes (Fig. 5B). These results suggest that MyD88^{-/-} mice are likely to have a defect in inducing T helper type 1 (Th1)-like immune responses, which is consis-

FIG. 4. Flow cytometry quantification of bone marrow plasma cell phenotypes and antibody ELISPOT. (A) A representative flow cytometry profile of bone marrow plasma cell phenotypes (B220^{low}CD138⁺). (i) Plasma cell phenotypic gating of WT bone marrow cells. (ii) Plasma cell phenotypic gating of MyD88^{-/-} bone marrow cells. Bone marrow cells were collected from WT or MyD88^{-/-} mice ($n = 6$) at day 7 post-boost vaccination and stained with fluorescence-conjugated antibodies specific to cell phenotypes (B220, CD138). Bone marrow plasma phenotypic cells (B220^{low}CD138⁺) are presented as percentages at top of rectangles. (B) Percentages of bone marrow plasma cell phenotypes. Average percentages of plasma phenotypic cells (B220^{low}CD138⁺) out of total live bone marrow cells are presented ($n = 6$). (C) Bone marrow IgG ELISPOT. (D) Bone marrow IgM ELISPOT. For panels B and C, bone marrows were collected at day 7 post-boost vaccination of mice ($n = 6$). Bone marrow single-cell suspensions (0.3×10^6 cells/well) were cultured on precoated ELISPOT plates with A/PR8 influenza VLPs (4 μ g/ml) and incubated for 24 h before spot development. IgG and IgM ELISPOTs were developed using HRP-conjugated goat anti-mouse IgG and IgM antibodies, respectively. Bars indicate standard deviations. Asterisks indicate significances between MyD88^{-/-} and WT groups (*, $P < 0.05$; ***, $P < 0.005$, Student's 2-tailed t test).

tent with the pattern of antibody isotypes observed with MyD88-deficient mice after vaccination.

Intramuscularly vaccinated MyD88^{-/-} mice are less effective in controlling viral replication. To determine protective efficacy, MyD88-deficient and wild-type mice ($n = 10$) were challenged with a lethal dose of live A/PR8 influenza virus via an intranasal route ($6 \times \text{LD}_{50}$) at 12 weeks after prime boost intramuscular immunization (Fig. 6). Mice were monitored

FIG. 5. Cellular immune responses. (A) IL-4 ELISPOT. (B) IFN- γ ELISPOT. The cellular immune responses were assessed using splenocytes collected at day 7 post-boost vaccination of mice with influenza A/PR8 VLPs ($n = 6$). Splenic cells from unvaccinated naïve, vaccinated MyD88^{-/-}, or WT mice were stimulated with A/PR8 VLPs on ELISPOT plates for 36 h and cytokine forming cell spots were determined by ELISPOT assay. Bars indicate standard deviations. Asterisks indicate significances between MyD88^{-/-} and WT groups (***, $P < 0.005$, Student's 2-tailed t test).

daily for body weight changes and survival rates. Naïve mice showed severe loss in body weight over 25% and died or had to be euthanized by day 5 postinfection. There was body weight loss at a moderate level of an average of 8% in the group of MyD88^{-/-} mice during day 8 to 10 postchallenge (Fig. 6A). In contrast, the WT mice did not show any loss in body weight. Both MyD88^{-/-} and WT mice vaccinated with influenza VLPs were 100% protected (Fig. 6B). To better assess the protective efficacy, lung viral titers were determined at day 4 postchallenge ($n = 4$ out of 10 challenged mice). High viral titers over 10^7 PFU per ml were observed in naïve infected mice (Fig. 6C). Approximately 1,000-fold lower levels of virus titers, but still over 10^4 PFU per lung, were observed with MyD88^{-/-} mice than with naïve infected mice. However, no virus was detected in the immunized WT mice.

Considering reduced levels of serum antibodies in MyD88^{-/-} mice after vaccination with influenza A/PR8 VLPs, it is intriguing to determine the contribution of serum antibodies to the provision of protection. Immune sera from vaccinated MyD88-deficient, WT, or unvaccinated naïve mice were mixed with a lethal dose of A/PR8 virus and used to infect naïve mice as described in previous studies (28, 35). Groups of mice infected with A/PR8 virus mixed with immune sera from either MyD88 or WT mice did not show body weight loss and were 100% protected (Fig. 6D and E). The group of mice that was infected with a mixture of A/PR8 virus and naïve sera showed a significant weight loss and 20% survival. These results indicate that antibodies induced even at low levels in

FIG. 6. Protection of MyD88^{-/-} mice against challenge infection after intramuscular immunization with influenza VLPs. MyD88^{-/-} and wild-type mice ($n = 10$) were challenged with a lethal dose of live A/PR8 influenza virus ($6 \times LD_{50}$) 12 weeks after prime boost immunization. Mice were observed daily for 14 days, and body weight changes (A) and survival rates (B) were recorded. "Naïve" indicates unvaccinated C57B6 wild-type mice. The asterisk indicates a significance between MyD88^{-/-} and WT groups (*, $P < 0.05$, Student's 2-tailed t test). (C) Lung viral titers. At day 4 postchallenge, lungs were collected from sacrificed mice ($n = 5$), and viral titers determined by a plaque assay using MDCK cells. (D and E) Protective efficacy of immune sera from vaccinated MyD88^{-/-} mice. Immune sera collected from influenza A/PR8 VLP-vaccinated MyD88^{-/-} or WT mice at 2 weeks after boost immunization or unvaccinated naïve mice were incubated with a lethal dose of influenza A/PR8 virus ($3 \times LD_{50}$) at room temperature for 30 min. Groups of mice ($n = 4$) were intranasally infected with a lethal infectious dose mixed with immune sera (MyD88, WT) or naïve sera (Naïve). Body weight (D) and survival rate (E) were monitored for 14 days. +, one surviving mouse in the naïve serum group showed a significant delay in recovery.

MyD88^{-/-} mice contributed to conferring protection. These results suggest that influenza VLP vaccination of MyD88^{-/-} mice can induce protective immunity, but the protective efficacy was lower as evidenced by greater lung viral replication.

Protective immunity by mucosal vaccination is compromised in MyD88^{-/-} mice. MyD88-mediated innate immune signaling might be also important for inducing immune responses to mucosal vaccination. MyD88^{-/-} and WT mice were intranasally immunized with influenza VLPs ($3 \mu\text{g}$) at weeks 0 and 4. MyD88^{-/-} mice induced 6- to 8-fold lower levels of IgG antibody responses than WT mice after prime and boost intranasal immunization with influenza VLPs (Fig. 7A). Also, virus-specific IgM antibody levels were reduced 3- to 4-fold in MyD88^{-/-} mice compared to WT mice (Fig. 7B). In addition, virus-specific serum IgG antibodies were induced in MyD88^{-/-} mice at approximately 10-fold lower levels after intranasal immunization (Fig. 7A) than after intramuscular immunization of MyD88^{-/-} mice (Fig. 1). Particularly, MyD88^{-/-} mice showed levels of IgG2a/c and IgG2b isotype antibodies significantly lower than those of the corresponding isotypes in WT mice (Fig. 7C). The hemagglutination inhibition titers in MyD88^{-/-} mice intranasally immunized with influenza VLPs were observed to be at background levels, similar to those in unvaccinated mice (Fig. 7D). These results

indicate that the MyD88 innate signaling molecule is important for inducing isotype-switched antibodies after intranasal immunization with influenza VLPs, which is similar to results with intramuscular immunization.

To determine the differential effects on protective efficacy in MyD88^{-/-} mice, a stringent challenge condition was applied to intranasal vaccination. At 16 weeks after boost immunizations with influenza VLPs, MyD88 and WT mice were intranasally challenged with a lethal dose of A/PR8 influenza virus ($12 \times LD_{50}$), and body weight changes and survival rates were monitored (Fig. 8). Both naïve WT and MyD88^{-/-} mice showed severe body weight loss and died on day 7 or 8 postinfection. Vaccinated MyD88^{-/-} mice also showed similar severe body weight loss, and all died with a delay of 2 days compared to naïve MyD88^{-/-} mice. In contrast, WT mice showed 100% survival despite some body weight loss. To better understand the protective immune responses induced by intranasal immunization of MyD88^{-/-} mice with influenza VLP vaccines, an immune serum transfer experiment was performed and was followed by challenge with a low lethal dose of A/PR8 virus (Fig. 8C and D). Immune sera from WT mice conferred good protection without showing body weight loss, whereas immune sera from MyD88^{-/-} mice conferred survival, but significant body weight loss was observed. Naïve sera

FIG. 7. MyD88^{-/-} mice intranasally immunized with influenza VLPs show a defect in inducing antibody responses. MyD88-deficient mice or C57BL/6 immunocompetent wild-type mice ($n = 5$) were immunized intranasally at weeks 0 and 4 with 5 μ g/mouse of influenza VLPs. (A) Total IgG. A/PR8 virus-specific serum IgG antibody levels were determined at 2 weeks after prime and boost immunizations. (B) IgM. A/PR8 virus-specific serum IgM antibody levels were determined at 2 weeks after prime and boost immunizations. (C) Isotype antibodies. A/PR8 virus-specific isotype antibodies were determined with diluted sera (200 $\times$) and presented as OD values at 450 nm. (D) HAI titers. HAI titers of immune sera against influenza A/PR8 virus were determined by using 4 HA units of virus. Asterisks indicate significances between MyD88^{-/-} and WT groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$, Student's 2-tailed t test).

did not show any protective effect. Thus, a lower protective efficacy was observed with immune sera from intranasally immunized MyD88^{-/-} mice than with those from WT mice, but that protection was significantly higher than that observed with

naïve sera. These results are consistent with a previous study reporting that mice lacking MyD88 were defective in inducing neutralizing antibodies and extremely sensitive to intranasal infection with vesicular stomatitis virus (37). Therefore, the re-

FIG. 8. MyD88^{-/-} mice intranasally immunized with influenza VLPs were not protected against A/PR8 virus challenge. MyD88^{-/-} and wild-type mice ($n = 5$) intranasally immunized with influenza VLPs were challenged with a lethal dose of influenza A/PR8 virus ($12 \times LD_{50}$) 4 weeks after boost. (A) Weight changes (%). (B) Survival (%). (C and D) Protective efficacy of immune sera collected from intranasally vaccinated MyD88^{-/-} mice. Immune sera collected from influenza A/PR8 VLP intranasally immunized MyD88^{-/-} or WT mice after boost immunization or unvaccinated naïve mice were incubated with a lethal dose of influenza A/PR8 virus ($3 \times LD_{50}$) at room temperature for 30 min. Groups of mice ($n = 4$) were intranasally infected with a lethal infectious dose mixed with immune sera (MyD88, WT) or naïve sera (Naïve). Body weight (C) and survival rate (D) were monitored for 14 days. Naïve (WT), naïve unvaccinated wild-type mice; naïve (MyD88), naïve unvaccinated MyD88^{-/-} mice; MyD88^{-/-}, intranasally vaccinated MyD88^{-/-} mice; WT, intranasally vaccinated wild-type mice.

TABLE 1. Serum antibody levels in mice after influenza VLP vaccination^a

Mouse group	Average $\pm$ SD (ng/ml)
Naïve	602 $\pm$ 134
MyD88 ^{-/-}	49,728 $\pm$ 5,999
CD4 ^{-/-}	168,833 $\pm$ 17,927
WT	336,924 $\pm$ 22,908

^a Groups of mice ($n = 6$ per group) were intramuscularly immunized with 5 μ g of influenza VLP vaccine containing HA of the A/PR8 strain at weeks 0 and 4. At 4 weeks after boost vaccination, A/PR8 virus-specific antibody levels were determined. Naïve, unimmunized mice; MyD88^{-/-}, MyD88 knockout mice; CD4^{-/-}, CD4 knockout mice; WT, wild-type mice.

sults show that the protective immunity induced by VLP vaccination via an intranasal route is less effective in MyD88^{-/-} mice.

DISCUSSION

In the present study, we found that mice deficient in MyD88, a key signaling molecule for the innate immune system, induced significantly lower antibody responses after vaccination, indicating that MyD88-mediated signaling is important for inducing adaptive immune responses to VLP vaccination. Similar defects in inducing adaptive humoral immune responses were reported in previous studies using a MyD88^{-/-} mouse model with ovalbumin and LPS in incomplete or complete Freund's adjuvant (27, 31), inactivated influenza virus (20), *Salmonella* bacterial infection (26), live virus infections (9, 20), or vesicular stomatitis virus infection in a route-dependent manner (38).

Importantly, we found that MyD88^{-/-} mice were selectively defective in inducing isotype-switched antibodies after influenza VLP vaccination. The IgG2a/c isotype antibody, followed by IgG2b and IgG3 isotype antibodies, was most severely affected. It has long been considered that dendritic or B cells exposed to a pathogen or an immunogen activate CD4 Th1 or Th2 cells, subsequently mediating the production of antibodies through the cognate interaction between B cells and CD4 T cells. Alternatively, TLR signaling plays a critical role in IgG2a/c class switching independent of effector T cell responses in response to influenza viral infection (13). T cell-independent B cell responses were shown to induce protection or class-switched antibodies (3, 6, 22, 36). Our preliminary studies suggested that immunization of CD4 knockout mice with influenza VLPs induced substantial amounts of antibodies, which were slightly lower than those in wild-type mice but significantly higher than those in MyD88^{-/-} mice (Table 1). It was also demonstrated that influenza-specific IgG antibody responses induced in CD40- or major histocompatibility complex class II (MHC-II)-deficient mice were protected from challenge infection (21). Dendritic cells were shown to induce Ig class switching without CD40-dependent T cell help by expressing B cell-activating proteins, such as BAFF (B cell-activating factor of the TNF family) and APRIL (a proliferation inducing ligand) in the presence of IL-10 or transforming growth factor β (22). These innate immune mediators (BAFF and APRIL) are known to trigger Ig class-switching recombination in B cells by engaging the receptor transmembrane activator and calcium modulator and cyclophilin ligand inter-

TABLE 2. *In vitro* antibody production in MACS-purified B cells^a

<i>In vitro</i> exptl group	Average $\pm$ SD (ng/ml)
WT T cells	4.5 $\pm$ 0.5
Naïve B cells + WT T cells	10.8 $\pm$ 4.3
MyD88 (prime) + WT T cells	20.6 $\pm$ 8.4
WT (prime) + WT T cells	84.8 $\pm$ 20.3
MyD88 (boost) + WT T cells	95.2 $\pm$ 45.3
WT (boost) + WT T cells	310.0 $\pm$ 42.4

^a Lymphocytes were separated using the magnetic cell sorting system (MACS). After 7 days of *in vitro* cultures of B cells (2×10^6 cells/ml), virus-specific antibody levels were determined. WT T cells, CD4 T cells from vaccinated wild-type mice; naïve B cells, B220⁺ B cells separated from naïve mice; MyD88 (prime), B220⁺ B cells separated from prime-immunized MyD88^{-/-} mice; WT (prime), B220⁺ B cells separated from prime-immunized WT mice; MyD88 (boost), B220⁺ B cells separated from boost-immunized MyD88^{-/-} mice; WT (boost), B220⁺ B cells separated from boost-immunized WT mice.

actor (TACI) through MyD88-mediated NF- κ B activation (12). MyD88^{-/-} mice were reported to be impaired in the B cell-intrinsic TACI-dependent pathway for Ig diversification (12). However, it remains to be determined whether T cells are required for induction of virus-specific antibody responses observed in this study by vaccination of MyD88^{-/-} mice.

Using bacteriophage particles loaded with a CpG motif oligonucleotide, Jegerlehner et al. demonstrated that TLR9 signaling in B cells but not in non-B cells can directly stimulate B cells to undergo isotype switching to IgG2a (16). In addition, recent studies suggest that the generation of T-dependent antigen-specific antibody responses, such as with IgG2a, requires the intrinsic activation of B cells through MyD88-mediated signaling (9, 13, 27). The presence of intact CD4 T cell responses or the induction of CD4 T memory responses could not overcome the reduced or undetectable levels of IgG2a/c antibody production by MyD88-deficient B cells as demonstrated by B cell transfer studies of μ MT mice (9, 16, 27). Consistent with these studies, our preliminary studies using fractionated B cells and CD4⁺ T helper cells by the use of antibody-coated magnetic-beads suggested that MyD88^{-/-} B cells were not effective in differentiating into antibody-secreting cells even in the presence of wild-type CD4 T helper cells (Table 2). Therefore, B cell-intrinsic MyD88 signaling might have an important role in inducing virus-specific antibody responses and generating antibody secreting plasma cells after vaccination. The contribution of B-cell intrinsic MyD88 remains to be further addressed by adoptive transfer of MyD88^{-/-} B cells into B cell-deficient mice or by depleting CD4 T cells in MyD88^{-/-} mice. Taken together, the present results provide evidence that influenza VLP vaccines may stimulate TLR/MyD88 signaling pathways on B cells to induce humoral immune responses.

In contrast to events involved in the activation of B cells, the possible roles of the innate immune system in generating long-lived memory B cells and antibody secreting plasma cells are not well understood. Activation of naïve B cells occurs at the margins of the T cell-rich areas and B cell follicles, and the activated cells initiate a germinal center reaction resulting in the generation of high-affinity B cells (19, 24). Such positively selected B cells migrate to the plasma foci and differentiate into long-lived memory B cells or antibody-producing cells eventually trafficking to the bone marrow to become long-lived plasma cells (2, 4, 32). Memory B cells respond faster in dif-

ferentiating into antibody-producing plasma cells as shown by significant increases in serum antibody levels and antibody-secreting cell responses from WT mice after VLP vaccination. However, the capacity of bone marrow and spleen cells from the MyD88^{-/-} mice to produce antibodies was severely impaired after vaccination with influenza VLPs, indicating the critical role of MyD88 in generating recall B cell and antibody secreting plasma cell responses. Alternatively, MyD88 might have a critical role in the transition of germinal center B cells into long-lived, antibody-secreting plasma cells in bone marrow. Prior studies have identified a role for intrinsic MyD88 signaling in B cells for long-lived plasma cell accumulation bone marrow (9, 26). MyD88^{-/-} mice were defective in antibody-secreting cell responses in bone marrow but not in the generation of germinal center B cell responses to mouse polyomavirus infection (9). Also, MyD88^{-/-} mice developed significantly lower levels of CD138⁺ plasma cells, as well as a delay in generating plasma cells, upon bacterial infection than WT mice despite the apparently normal germinal center B cells in MyD88^{-/-} mice (26). Consistent with these previous studies, we also found that MyD88^{-/-} mice did not display a significant defect in generating germinal center B cells as shown by the flow cytometry analysis of phenotypic germinal center B cells in MyD88^{-/-} mice. In contrast to the apparently normal germinal center total B cells, MyD88^{-/-} splenic B cells were found to have a significant defect in differentiating into vaccine antigen-specific antibody-producing cells.

In contrast to vaccination with nonreplicating influenza VLPs, live-virus infection might be less dependent on MyD88 in inducing antibody responses. MyD88^{-/-} mice were previously shown to induce comparable (20) or higher (13) levels of IgG1 antibody responses after influenza virus infection, although lower but easily detectable levels of IgG2a/c antibodies were induced (13, 20). However, immunization of MyD88^{-/-} mice with influenza VLPs induced lower levels of IgG1 antibody responses and revealed defects in inducing other isotypes, such as IgG2a/c. This might be due to the fact that live virus infection utilizes redundant mechanisms for innate immune recognition, such as RIG-1 or melanoma differentiation-associated gene 5 (MDA5). RIG-1 and MDA5 are known to recognize double-strand RNA activating transcription factors NF- κ B and IFN regulatory factor 3, which leads to the production of type 1 IFN cytokines influencing antibody responses (13, 17, 37). Finally, this study provides evidence that MyD88 innate signaling is also important for inducing protective immunity after vaccination. MyD88^{-/-} mice intranasally or intramuscularly immunized with influenza VLPs showed less effective protection in controlling viral loads. Consistent with our study, MyD88^{-/-} mice were previously reported to be only partially protected after vaccination with inactivated viral vaccines (20). It was also demonstrated that MyD88^{-/-} mice were extremely sensitive to intranasal infection with vesicular stomatitis virus, correlating with the impaired induction of neutralizing antibodies probably by virtue of different cell populations (38).

In summary, isotype-switched IgG antibodies were observed at significantly lower levels with MyD88^{-/-} mice than with WT mice after intramuscular or intranasal vaccination with a non-replicating vaccine. Particularly, MyD88^{-/-} mice showed a defect in inducing effective boost immune responses and

IgG2a/c isotype antibody responses and in generating antibody-secreting plasma cells after vaccination. Challenge studies also showed that the protective efficacy of vaccination in MyD88^{-/-} mice was lower than that in WT mice. Therefore, these studies demonstrate an essential role for MyD88 in generating antibody-secreting plasma cells and in inducing Th1-type immune responses, as well as in inducing protective immunity.

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