MyD88 Signaling Is Not Essential for Induction of Antigen-Specific B Cell Responses but Is Indispensable for Protection against *Streptococcus pneumoniae* Infection following Oral Vaccination with Attenuated *Salmonella* Expressing PspA Antigen

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MyD88 Signaling Is Not Essential for Induction of Antigen-Specific B Cell Responses but Is Indispensable for Protection against Streptococcus pneumoniae Infection following Oral Vaccination with Attenuated Salmonella Expressing PspA Antigen

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TLRs directly induce innate host defense responses, but the mechanisms of TLR-mediated adaptive immunity remain subject to debate. In this study, we clarified a role of TLR-mediated innate immunity for induction of adaptive immunity by oral vaccination with a live recombinant attenuated Salmonella enterica serovar Typhimurium vaccine (RASV) strain expressing Streptococcus pneumoniae surface protein A (PspA) Ag. Of note, oral or intranasal vaccination with RASV expressing PspA resulted in identical or even significantly higher levels of PspA-specific IgG and IgA responses in the systemic and mucosal compartments of MyD88−/− mice of either BALB/c or C57BL/6 background when compared with those of wild-type mice. Although PspA-specific CD4+ T cell proliferation in the MyD88−/− mice was minimal, depletion of CD4+ T cells abolished PspA-specific IgG and IgA responses in the MyD88−/− mice of BALB/c background. Of the greatest interest, MyD88−/− mice that possessed high levels of PspA-specific IgG and IgA responses but minimal levels of CD4+ T cell responses died earlier than nonvaccinated and vaccinated wild-type mice following i.v. or intranasal challenge with virulent S. pneumoniae. Taken together, these results suggest that innate immunity activated by MyD88 signals might not be necessary for Ag-specific Ab induction in both systemic and mucosal sites but is critical for protection following oral vaccination with attenuated Salmonella expressing PspA. The Journal of Immunology, 2008, 181: 6447–6455.

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3 Abbreviations used in this paper: TRIF, Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF); TRIM, Toll/IL-1 receptor domain-containing adaptor protein (TRAP), and TRIF-related adaptor molecule (TRAM) (1, 3). These adaptor molecules trigger the activation of various transcriptional factors and then induce inflammatory and antimicrobial responses, which not only lead to stimulation of innate immunity but also help to initiate the development of Ag-specific adaptive immunity (4).

Recent studies have indicated that TLRs sense the microbial infection and engage multiple mechanisms that control the initiation of adaptive T and B cell responses (4). However, the essential role of innate immunity for control of adaptive immunity remains controversial. One previous study demonstrated that activation of TLRs is essential to generate T cell-dependent Ag-specific B cell responses in addition to CD4+ T cell help (5). More recently, innate immune sensing of peptidoglycan by a nucleotide-binding oligomerization domain (Nod)-1 was reported to be essential for onset of Ag-specific T and B cell responses (6). In contrast, several typical adjuvants elicited robust T cell-dependent Ag-specific B cell responses in the absence of both MyD88 and TRIF signals, indicating that TLR signal is not a key factor for induction of B cell activation (7). To resolve these controversial issues, we investigated the role of TLR-mediated innate immunity for induction of adaptive immunity by mucosal (oral and intranasal [i.n.]) vaccination with a live attenuated Salmonella vaccine strain that potentially has several TLR agonists such as lipoprotein, LPS, and flagellin.

Mucosal surfaces that serve as boundaries with the exterior environment are covered with special epithelial layers that act as barriers against exogenous challenges by pathogens and soluble Ags (8, 9). The mucosal immune system, which is functionally independent of the systemic immune apparatus, has developed its own highly organized immunological tissues (8, 9). These tissues maintain homeostasis in the vast mucosa by mounting specialized antiinflammatory immune defenses such as the production of...
secretory IgA (SIgA) Ab and the induction of tolerance against innocuous soluble substances as well as commensal bacteria. Furthermore, due to the migration of IgA Ab-secreting cells (ASCs), local mucosal immunization leads to Ag-specific IgA production at distant mucosal sites (10).

Modified virulent genes in bacteria have potential for mucosal vaccines and Ag carrier vehicles (11, 12). Mucosally administered attenuated Salmonella typhimurium expressing recombinant Ag from other pathogens elicits primarily a Th1-type dominant immune response to both recombinant and Salmonella Ags (13, 14). We previously reported that oral administration of recombinant attenuated S. enteric serovar Typhimurium vaccine (RASV) strains expressing pneumococcal surface protein A (PspA) Ag resulted in high levels of PspA-specific IgG responses and efficient protection against challenge with virulent Streptococcus pneumoniae (15). Because Salmonella organisms express a variety of TLR agonists both on their surface and internally (e.g., lipoprotein, LPS, and flagellin), all known as strong adjuvants for enhancement of Ag-specific T and B cell responses (16–18), we questioned whether the TLR agonists in bacterial cell components are involved in inducing complementary and synergistic effects that modulate adaptive immunity following oral immunization with RASV strain expressing PspA Ag. In the present study, we found that a T cell-dependent Ag-specific B cell response was normally induced in MyD88−/− mice while Ag-specific CD4+ T cell responses were minimal. Of interest, MyD88−/− mice did not have efficient protection against S. pneumoniae infection following oral vaccination with attenuated Salmonella expressing PspA Ag. Thus, we conclude that TLR-mediated MyD88 signaling is not crucial for induction of Ag-specific adaptive immunity but is indispensable for protection against bacterial infection.

Materials and Methods

Mice

Wild-type (WT) BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. Polymeric Ig receptor (pIgR)−/− mice of BALB/c background, MyD88−/− and MyD88−/−/Trif−/− mice of both BALB/c or C57BL/6 background were kindly provided by Drs. Masanobu Nanno (Yukut Central Institute for Microbiological Research, Tokyo, Japan) and Shizuo Akira (Research Institute for Microbial Diseases, Osaka University, Osaka, Japan), respectively. To generate the Peyer’s patch-null mice, pregnant BALB/c mice were injected i.p. with 600 μg of anti-IL-7Rα mAb on gestational day 14 (19). All mice used in experiments were between 6 and 12 wk of age. Mice were maintained under pathogen-free conditions in the experimental facility at the International Vaccine Institute (Seoul, Korea), where they received sterilized food and water ad libitum. The experiments were approved by the Institutional Animal Care and Use Committee of the International Vaccine Institute.

Bacterial strains

S. enterico serovar Typhimurium (9241 ΔpabA1516 ΔpabB323 ΔaasA16 ΔaroABD3 ΔaraA198::araCPBADlacIATGTT containing pY3A3802) expressing rPspA-RX1 (15, 20) or WT U.K.-1 strain was grown at 37°C in Luria-Bertani (LB) broth or LB agar. Recombinant S. typhimurium BRD 847 strain, a double aroA aroD mutant that expresses the nonotopoic, immunogenic 50-kDa ToxC fragment of tetanus toxin from plasmid pTETni15 under the control of the androgenically inducible nrb promoter (3Salmonella-ToxC), was cultured in LB broth or LB agar containing ampicillin (21). Bacterial suspensions for mouse immunization were prepared in PBS from LB broth. For the protection assay, S. pneumoniae virulent capsular type 3 strain WU2 was cultured on tryptosec acid agar containing 5% sheep blood (BD Biosciences) or Todd-Hewitt broth plus 0.5% yeast extract (THY medium, BD Biosciences) (22). WU2 strain was grown in 100 ml of THY medium until late log phase, adjusted with 3% glycerol, and frozen in 1-ml aliquots containing ~10^6 CFU/ml. For inoculation, a fresh aliquot was thawed and appropriately diluted (~1000-fold) for injection. The actual number of CFU injected was confirmed by plating on the 5% sheep blood (BD Biosciences).

Immunization

Mice were immunized intragastrically with a single 100-μl aliquot of bacterial suspension (equivalent to 10^7 CFU) via an intubation needle (Fuchiformi). For i.n. vaccination, mice were immunized under anesthesia by pipette with bacterial suspension (1 × 10^7 CFU in a 20-μl PBS aliquot). All immunizations were performed at 2-wk intervals.

ELISA and ELISPOT

Serum and fecal extracts were obtained, and Ag-specific Ab titers were determined by ELISA as described elsewhere (23). Endpoint titers were expressed as the reciprocal log of the last dilution giving an OD at 450 nm of 0.1 greater than background. To assess the number of ASCs, mononuclear cells were obtained from the spleen, lung, and small and large intestines as previously described (23). Ag-specific ASCs were measured by an ELISPOT assay according to an established protocol (24) and were counted with the aid of a stereomicroscope (SZ2-ILST, Olympus).

Ag-specific CD4+ T cell proliferation

Mesenteric lymph node and spleen were isolated from WT and MyD88−/− mice of BALB/c background 7 days after the final oral dose of RASV-PspA, and CD4+ T cells from each tissue sample were isolated after staining with anti-CD4 microbeads (clone L3T4; Miltenyi Biotec) by MACS (Miltenyi Biotec). Dendritic cells (DCs) from spleen of naive BALB/c mice were sorted by the FACSARia system (BD Biosciences) and used as professional APCs. For CD4+ T cell proliferation, different numbers of CD4+ T cells from each tissue and DCs (1 × 10^6) were cocultured in the absence and presence of PspA Ag (1 μg/ml) for 72 h. Incorporation of [3H]thymidine after a 16-h culture period was determined. For neutralizing experiments, anti-CD4 mAb was injected three times per week throughout the immunization period.

S. pneumoniae challenge

Groups of mice were challenged i.v. (2 × 10^6 or 2 × 10^7 CFU in 200 μl of PBS) or i.n. (5 × 10^6 CFU in 50 μl of PBS) with virulent S. pneumoniae WU2 strain. Survival was monitored after challenge.

Neutralizing Abs

Hydromas producing anti-CD4 (GK1.5) Ab were purchased from the American Type Culture Collection and mAb was obtained from ascites. Mice were injected i.p. with 150 μg of depleting anti-CD4 or isotype control rat IgG (Sigma-Aldrich) Abs 1 day before vaccination and every 3 or 4 days thereafter. Almost 95% of CD4+ T cells were depleted in the spleen after treatment of anti-CD4 neutralizing Ab.

Adoptive transfer

To reconstitute BALB/c WT mice with sera, CD4+ T cells, or B cells obtained from RASV-immunized MyD88−/− mice of BALB/c background, MyD88−/− were orally administered 10^6 CFU of RASV twice at 2-wk intervals. The CD4+ or B220- cells were isolated from the spleen and mesenteric lymph node of the immunized MyD88−/− mice using microbead-conjugated mAbs (Miltenyi Biotec) according to the manufacturer’s instructions. Groups of BALB/c WT mice were i.v. transferred with 500 μl of sera or 2 × 10^7 cells of CD4+ T cells or B220+ cells, and challenged the next day with the virulent S. pneumoniae WU2 strain.

Data and statistical analysis

The Kaplan-Meier method was used to determine the statistical significance of differences in survival time. We performed the log-rank test (Mantel-Cox) using SPSS 12.0K for Windows. To compare the differences between two experimental groups, we used the Student t test. To compare multiple groups, we conducted one-way ANOVA followed by the Tukey honestly significant difference (HSD) post hoc test. Each experiment was repeated at least three times using 5–10 mice per group. p values of <0.05, <0.01, and <0.001 were assumed to be statistically significant.

Results

Oral immunization of RASV expressing PspA Ag elicited high levels of Ag-specific IgG and SIgA responses in serum and intestine

We first investigated the immunogenicity of RASV strain expressing PspA Ag in systemic (i.e., serum) and mucosal (i.e., fecal extract) compartments after priming and boosting via the oral route. At day 14 after the first vaccination (priming), high levels of
PspA-specific IgG Ab were detected in serum of WT BALB/c mice (Fig. 1a). The levels of PspA-specific IgG and IgA Abs in serum and IgA Abs in fecal extract were much higher 14 days after boosting than after priming (Fig. 1a). To determine whether IgA Abs in fecal extracts are in secretory form, we used pIgR−/− mice lacking the IgA secretion pathway (25). These mice showed complete loss of IgA responses in fecal extracts but had levels of IgG and IgA Abs comparable to those in WT mice (Fig. 1b). To assess the protective efficacy of oral vaccination with RASV strains expressing PspA Ag, groups of WT and pIgR−/− mice with or without vaccination were challenged intratracheally (i.t.) with a lethal dose of virulent S. pneumoniae WU2 strain and their survival was monitored (26). As expected, nonvaccinated WT mice died within 2 days following i.t. challenge with S. pneumoniae (Fig. 1c). Interestingly, all vaccinated WT BALB/c mice survived, whereas vaccinated pIgR−/− mice of BALB/c background died within 5 days after i.t. challenge (Fig. 1c). This result strongly suggests that oral vaccination with RASV expressing PspA Ag elicits effective protection against a lethal dose of S. pneumoniae and further that S IgA is essential for protection.

Both Peyer’s patches (PP) and lamina propria in the small intestine are main entry sites for bacterial invasion (27, 28). To identify Ab inductive sites by oral RASV-PspA vaccination, PP-null mice were produced. The levels of PspA-specific IgA responses both in serum and fecal extracts were significantly impaired in PP-null mice after oral doses of RASV-PspA (Fig. 1d). In contrast, PP-null and WT mice of BALB/c background had identical levels of PspA-specific IgG in serum (Fig. 1d). These data suggest that PP play an indispensable role for induction of PspA-specific IgA in both systemic and mucosal compartments following oral administration of RASV-PspA.

Ag-specific B cell response was elicited in MyD88−/− mice after oral vaccination of a bacteria-based vaccine strain expressing exogenous protein Ag

We used MyD88−/− mice to look for a role of innate immunity in development of B cell response following oral vaccination with RASV expressing PspA. Since we found no defect in function of colonization by oral administration with RASV expressing PspA in the MyD88−/− mice (data not shown), we further addressed inductive levels of T cell-dependent Ag-specific Ab
in both serum and fecal extracts after administration of oral RASV expressing PspA. Both WT and MyD88−/− mice of BALB/c background were orally immunized three times 2 wk apart with RASV expressing PspA. Unexpectedly, we found brisk levels of PspA-specific IgG and IgA in serum and intestinal IgA in fecal extract were measured at day 14 after priming and day 7 after final boosting. PspA-specific ASCs were determined from spleen (SP), small intestines (SI), large intestines (LI), and lung 7 days after the last immunization. c, BALB/c background WT and MyD88−/− mice were immunized orally with recombinant *S. typhimurium* expressing tetanus toxoid. Tetanus toxoid Ag-specific IgG and IgA in serum and intestinal IgA in fecal extract were measured at day 14 after priming and day 7 after boosting two times. Titers of tetanus toxoid-specific IgG subclasses in serum were measured at day 14 after boosting. *p < 0.05; **p < 0.01; ***p < 0.001 vs WT mouse group.

We next queried whether normal induction of B cell responses occurred in MyD88−/− mice given other attenuated *Salmonella* strains. For this experiment, we used an attenuated *Salmonella* vaccine strain expressing the C fragment of the tetanus toxin (*rSalmonella*-Tox C) (14, 29). Interestingly, identical levels of tetanus toxoid-specific IgG and IgA responses were found in serum and fecal extracts in WT and MyD88−/− mice
Following oral vaccination with rSalmonella-Tox C strain (Fig. 2c). Consistent with results obtained with the RASV expressing PspA (Fig. 2, a and b), dominant Th2-type responses (high IgG1) were found in the MyD88−/− mice after oral administration with rSalmonella-Tox C strain (Fig. 2c). We also checked for C57BL/6 background in MyD88−/− mice at same time because Ag-specific Ab induction might be related to mouse genetic background (Fig. 3a). Oral administration of RASV expressing PspA resulted in predominant levels of Ag-specific IgG and IgA both in serum and fecal extract of C57BL/6 background MyD88−/− mice (Fig. 3a). Additionally, identical levels of PspA-specific IgG and IgA both in the serum and fecal extract of C57BL/6 background MyD88−/−TRIF−/− mice were elicited following oral vaccination with RASV expressing PspA (Fig. 3b). Of note, high levels of both IgG1 (Th2 type) but reduced levels of IgG2c (Th1 type) Abs were determined in the serum of C57BL/6 background MyD88−/− mice when compared with those of C57BL/c WT mice (Fig. 3). Overall, MyD88- and TRIF-dependent innate immunity does not involve development of T cell-dependent Ag-specific B cell responses.

A critical role of CD4+ T cells in MyD88−/− mice for induction of Ag-specific Ab

Several previous studies found impaired proliferation of MyD88 signal-deficient CD4+ T cells both in vivo and in vitro (5, 30). Thus, we next examined Ag-specific proliferation of CD4+ T cells in WT and MyD88−/− mice following oral administration with RASV expressing PspA Ag. CD4+ T cells were isolated from vaccinated WT and MyD88−/− mice and cocultured with DCs from spleens of naive mice with or without PspA Ag in vitro. Of note, PspA-specific CD4+ T cell proliferation was significantly decreased in MyD88−/− mice compared with those in WT mice, but minimal levels of proliferation were maintained in MyD88+/− mice (Table 1). To further confirm a role of CD4+ T cells for induction of B cell responses, CD4+ T cells were depleted by neutralizing anti-CD4 mAb during the vaccination period (Fig. 4a). In WT mice, PspA-specific IgG and IgA levels were dramatically decreased while intact levels of IgA response remained in fecal extract after depletion of CD4+ T cells. Interestingly, no detectable levels of PspA-specific IgG and IgA responses were found in serum and fecal extracts after depletion of CD4+ T cells in the MyD88−/− mice (Fig. 4a).

Since previous studies suggested an important role of CD4+ T cells for protection against colonization of S. pneumoniae (31, 32), we next looked for a direct role of CD4+ T cells for protection against S. pneumoniae infection. First, CD4+ T cells from RASV-vaccinated mice were isolated and reconstituted in naive BALB/c mice. Then, recipient mice were challenged i.v. with a lethal dose of virulent S. pneumoniae WU2 strain and their survival was monitored (Fig. 4b). Recipient mice reconstituted with CD4+ T cells of RASV-vaccinated BALB/c mice died at an early time point following i.v. challenge whereas RASV-vaccinated mice had 100% survival. Taken together, even though Ag-specific CD4+ T cell proliferation was impaired in the MyD88−/− mice compared with WT mice, it

Table I. Proliferation of CD4+ T cells following oral administration of RASV-PspA Ag

<table>
<thead>
<tr>
<th>Mice</th>
<th>Tissue</th>
<th>CD4+ T Cells Alone (+PspA)</th>
<th>Ratio of DCs to CD4+ T Cells (+PspA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1:20</td>
</tr>
<tr>
<td>BALB/c</td>
<td>Spleen</td>
<td>3,797.7 ± 640.6</td>
<td>17,882.6 ± 3,008.5**</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>Spleen</td>
<td>677.1 ± 43.5</td>
<td>2,045.8 ± 637.4*</td>
</tr>
<tr>
<td>BALB/c</td>
<td>MLN</td>
<td>1,572.1 ± 527.6</td>
<td>3,975.9 ± 1,115.6*</td>
</tr>
<tr>
<td>MyD88−/−</td>
<td>MLN</td>
<td>914.5 ± 294.4</td>
<td>1,412.2 ± 431.5</td>
</tr>
</tbody>
</table>

*Different numbers of CD4+ T cells from each tissue of BALB/c or MyD88−/− mice of BALB/c background and splenic DCs from naive BALB/c mice as APCs (1 × 10^5) were co-cultured in presence of PspA Ag (1 mg/ml) for 5 days. *, p < 0.05; **, p < 0.01; and ***, p < 0.001 vs CD4+ T cell alone group. MLN indicates mesenteric lymph node.
seems likely that minimal CD4⁺ T cell function might be involved in enhancing Ag-specific B cell responses in the MyD88⁻/⁻ mice. Furthermore, CD4⁺ T cells are indispensable for B cell activation but might not directly manipulate protection against S. pneumoniae infection.

MyD88 signal plays an indispensable role for protection against bacterial infection in the absence or presence of Ag-specific B cell responses

Our next question was whether enhanced levels of acquired immunity in the systemic and mucosal tissues of MyD88⁻/⁻ mice are sufficient and efficient for protection against S. pneumoniae infection. To clarify this issue, orally vaccinated WT and MyD88⁻/⁻ mice were challenged with virulent S. pneumoniae via i.v. (sepsis model) or i.t. (lung pneumonia model) routes. As expected, PBS-vaccinated BALB/c mice died within 4 days following challenge with S. pneumoniae (Fig. 5a). Importantly, MyD88⁻/⁻ mice that possessed high levels of PspA-specific IgG and IgA Abs but minimal CD4⁺ T cell responses died 4–6 days following i.v. or i.n. challenge with virulent S. pneumoniae while vaccinated WT BALB/c mice had a 100% survival rate (Fig. 5a). These results suggest that innate immunity activated by MyD88 signals might not be necessary for Ag-specific Ab induction but is critical for protection against S. pneumoniae infection.
S. pneumoniae infection following oral vaccination with attenuated Salmonella expressing PspA.

To directly assess the neutralizing effects of PspA-specific Abs that induce the MyD88-deficient condition, sera or CD4+ T cells or B220+ cells from RASV-vaccinated MyD88−/− mice of BALB/c background were isolated and reconstituted in naive BALB/c mice. Then, recipient BALB/c mice were i.v. challenged with a lethal dose of S. pneumonia 1 day after reconstitution (Fig. 5b). Of note, the recipient mice reconstituted with sera of RASV-vaccinated MyD88−/− mice had 100% survival (Fig. 5b) and no loss of body weight (data not shown). Additionally, recipient BALB/c mice reconstituted with B220+ cells of RASV-vaccinated MyD88−/− mice had ~50% survival whereas mice reconstituted with CD4+ T cells died at early time points following challenge with S. pneumonia. These results clearly demonstrate that PspA-specific Abs induced by MyD88−/− B cells possess normal neutralizing function even though RASV-vaccinated mice died earlier than nonvaccinated BALB/c mice.

**Discussion**

To clarify the role of innate immunity for induction of Ag-specific adaptive immunity following mucosal vaccination with bacteria-based vaccine possessing several TLR ligands, we adopted attenuated Salmonella vaccine strains expressing T cell-dependent Ag (e.g., PspA or tetanus toxoid). We found that Ag-specific B cell responses, including systemic IgG and intestinal SlgA Abs, were induced after oral administration through PP-dependent, CD4+ T cell-dependent, and MyD88/TRIF signaling-independent pathways. Of interest, innate immunity mediated by MyD88 signal was not essential for production of PspA-specific Abs, but was indispensable for survival against S. pneumoniae infection.

The necessity of innate immunity is controversial, specifically the role of TLR signals as an inducer to provoke Ag-specific adaptive immunity in which T and B cells become armed against bacteria/virus infection (33). It is thought that to induce an effective immune response, microorganisms must stimulate complex sets of pattern-recognition receptors, which exist both within and outside the cells. It has been suggested that both TLRs on B cells and DCs are required for optimal Ab responses to T cell-dependent Ag (5). However, a recent study indicated that TLRs are not essential to link between innate and adaptive immunity following vaccination with a protein Ag with different kinds of adjuvants (7). Gavin et al. observed mainly IgG2b and IgG2c responses in MyD88−/− TRIF−/− mice given Ag with alum or CFA (7). Our results support those findings. We found that TLR-mediated innate immunity is dispensable for induction of Ag-specific acquired immunity induced by bacteria-based vaccine (Fig. 2). Previous studies demonstrated that MyD88−/− mice have a profound defect in the activation of Ag-specific Th1 but not Th2 immune responses (34–36). Although mucosal vaccination with RASV strain enhanced Th1-dominant responses in innate immunity intact mice, MyD88−/− mice had a Th2-dominant condition (IgG1 > IgG2a or IgG2c) following oral vaccination with RASV expressing PspA. In this regard, DCs from WT mice induced allogenic T cells to secrete IFN-γ, but DCs from MyD88−/− mice skewed T cells to secrete IL-4 instead of IFN-γ in response to LPS (37). When MyD88−/− mice were immunized with keyhole limpet hemocyanin (KLH)-CFA via the systemic route, predominant levels of IL-4 but not IFN-γ were produced by CD4+ T cells isolated from draining lymph node in the presence of APCs (37). Possibly Th2-dominant responses support stronger humoral immune responses in MyD88-deficient mice.

It has been postulated that there are alternative pathways through which the innate immune response influences an adaptive immune response: TLR-dependent or TLR-independent (38–40). Two different families of pattern-recognition receptors are expressed in epithelial cells; Nod proteins are located intracellularly, whereas many TLRs are typically expressed on the cell surface. Recent findings indicate that once Salmonella is taken up in the cytoplasmic area, intracellular innate immune recognition systems other than surface molecules contribute to the onset of Ag-specific T and B cell responses (38, 41). The relevance of Nod1 and Nod2 for bacterial infections was clearly demonstrated for microbial infections such as Helicobacter pylori and Listeria monocytogenes (41). Ifap, caspase recruitment domain (CARD)-containing nucleic acid localization domain leucine-rich repeat protein, is responsible for S. typhimurium-induced caspase-1 inflammasome activation (42). Additionally, caspase-1 inflammation activation can also be triggered by cytosolic microbial and host DNA in an ASC (apoptosis-associated specklike protein containing a CARD)- or NALP (also known as cryopyrin)-3-dependent manner (43). Since caspase-1 is responsible for the secretion of proinflammatory cytokines such as IL-1β, IL-18, and IL-33, which are known to be involved in generating Th2-dominant immune responses (44), the Th2 responses in Myd88−/− mice after RASV vaccination can be ascribable to the activation of caspase-1 inflammasome by attenuated Salmonella vaccine strain. Thus, it might be necessary to clarify the role of intracellular signals by NOD-like receptors including NOD, Ifap, and NALP-3 signals for induction of Ag-specific acquired immunity following oral administration with attenuated Salmonella vaccine strain, although so far there is no evidence for this hypothesis.

Bacteria such as Shigella or Salmonella or Yersinia can invade M cells on PP and the villous epithelium and DCs on the lamina propria of the small intestine and subsequently result in the acquired phase of Ag-specific immune responses (28, 45, 46). A recent study that used different S. typhimurium strains that targeted the two mechanisms of bacterial entry, M cells in PP (InvA+) and DCs in lamina propria of the small intestine (invA−), showed high numbers of M cell-penetrating invasive S. typhimurium in PP, leading to fecal IgA induction (47). In contrast, both strains of bacteria (InvA+ or invA−) were equally capable of reaching the mesenteric lymph node and the spleen and inducing IgG responses (47). A recent study also showed that PP but not isolated lymphoid follicle in the small intestine is required for Ag-specific intestinal SlgA induction of oral-attenuated Salmonella expressing tetanus toxoid (29). Our present results support the finding that PP is essential for induction of PspA-specific IgA but not for IgG responses in the serum and fecal extracts following oral administration of RASV expressing PspA Ag (Fig. 1d). Furthermore, PP-null MyD88−/− mice did not mount intestinal IgA responses but had intact levels of systemic IgG responses after oral administration of RASV expressing PspA (data not shown). Overall, these data suggest that M cell entry into PP is crucial for development of Ag-specific IgA Ab but not for IgG response following oral vaccination with attenuated bacteria-based vaccine candidates, as shown by the independent manner of MyD88-mediated innate immunity.

Of special interest was our finding that MyD88−/− mice vaccinated with RASV expressing PspA, which possessed brisk levels of neutralizing PspA-specific IgG and IgA Abs and minimum activation of PspA-specific CD4+ T cells, died early and at a time point similar to that of nonvaccinated WT mice following lethal
challenge with S. pneumoniae (Fig. 5). These results provide convincing evidence that the MyD88 signal is more essential for induction of effective protection against S. pneumoniae infection than the enhanced PsPα-specific B cell response. In this regard, MyD88−/− mice are more likely to have bacteremia, gangrenous mucosal necrosis, severe colitis, and death following infection with microbiota than are MyD88-intact mice (48–50). The terminal point of signal through the TLR pathway can activate the level of NF-κB, which causes production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-6 (1, 2). Our data support previous findings that IL-6 but not TNF-α in the serum of MyD88−/− mice is completely impaired following oral vaccination with RASV expressing PsPα (data not shown). On the other hand, another study suggests a crucial role of MyD88 in killing of Gram-negative bacteria (e.g., Escherichia coli and attenuated S. typhimurium) due to diminished NADPH oxidase activity in phagocytic cells, such as macrophages in MyD88−/− mice (51). Taken together, our results imply that although an adaptive response occurs, dysfunction of proinflammatory cytokines and/or phagocytosis mediated by MyD88-dependent signaling might be one factor that explains insufficient clearance of pathogens such as S. pneumoniae in MyD88−/− mice.

Khan et al. reported that MyD88−/− mice are markedly defective in their ability to induce multiple proinflammatory cytokine- and chemokine-specific mRNAs in the spleen after i.p. immunization with heat-killed S. pneumoniae capsular type 14 (36). However, prior i.p. immunization of MyD88−/− mice with heat-killed S. pneumoniae type 14 resulted in efficient protection although naive MyD88−/− mice were more susceptible than were naive WT mice against otherwise lethal challenge with live S. pneumoniae type 14 (36). The challenge dose of live S. pneumoniae in the study by Khan et al. was moderated (no naive WT mice were killed) compared with that used in our present study (100% of naive WT mice were killed within 4 days after challenge). Taken together, whereas low amounts of live S. pneumoniae can be protected by MyD88-independent Abs, MyD88 signal might be indispensable for protection against infection by large quantities of live S. pneumoniae.

In other work, we demonstrated that LPS-specific adaptive immunity is normally induced in MyD88−/− mice following oral administration of RASV strain (H.-J. Ko, J.-Y. Yang, D.-H. Shim, S.-M. Park, Y.-H. Park, S. Akira, R. Curtiss III, and M.-N. Kweon, submitted for publication). However, orally vaccinated MyD88−/− mice with high levels of neutralizing Abs in both systemic and mucosal compartments died earlier than nonvaccinated WT mice when challenged orally with virulent Salmonella. Thus, we can conclude that, regardless of T cell-dependent or -independent Ags, MyD88 signal is dispensable for induction of adaptive immune responses but essential for protection against bacterial infection that mainly invaded via the mucosal surface.

Vaccination is the most effective means of preventing infectious diseases. The information learned in this study will greatly facilitate the rational design of novel bacteria-based vaccines against a multitude of infections common in developing countries.

Disclosures
The authors have no financial conflicts of interest.

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