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TLR2 and TLR4 signaling shapes specific antibody responses to *Salmonella typhi* antigens

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TLR directly induce innate immune responses by sensing a variety of microbial components and are critical for the fine-tuning of subsequent adaptive immune responses. However, their impact and mechanism of action on antibody responses against bacterial antigens are not yet fully understood. *Salmonella enterica* serovar Typhi (*S. typhi*) porins have been characterized as inducers of long-lasting specific antibody responses in mice. In this report, we show that immunization of TLR4-deficient (TLR4$^{-/-}$), myeloid differentiating gene 88-deficient and Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient mice with *S. typhi* porins led to significantly reduced B-cell responses. TLR2$^{-/-}$ mice, as well, showed reduced IgG titers with a more pronounced impairment in the production of IgG3 anti-porins antibodies. Adoptive transfer of TLR2$^{-/-}$- or TLR4$^{-/-}$-B cells into B-cell-deficient mice revealed a direct effect of TLR4 on B cells for the primary IgM response, whereas stimulation of B cells *via* TLR2 was important for IgG production. Furthermore, *S. typhi* porins were found to efficiently elicit maturation of CD11c^{+} conventional DC. Taken together, *S. typhi* porins represent not only a suitable B-cell antigen for vaccination, but exhibit potent TLR-dependent stimulatory functions on B cells and DC, which help to further enhance and shape the antibody response.

Key words: Antibody response · porins · *S. typhi* · TLR

Introduction

Recognition of PAMP by TLR is critical for the induction of the innate immune response [1]. TLR can stimulate adaptive immune responses indirectly through the activation of innate immune cells that produce inflammatory cytokines and chemokines, and upregulate co-stimulatory molecules. Furthermore, TLR ligands participate in shaping adaptive immune responses by triggering their respective receptors on B and/or T cells [2–6]. The direct participation of TLR in B-cell activation was described by Leadbetter *et al.* [7], who showed that an effective activation of Rheumatoid factor^{+} B cells requires a synergistic engagement of the BCR and a member of the TLR family. Subsequent studies revealed that TLR9 and TLR7 engagement contributes to the activation of autoreactive B cells [8, 9].

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It has been proposed that TLR provide a third signal together with antigen recognition through the BCR and cognate T cell help to achieve the maximal activation of B cells and class switch recombination [10–12]. Likewise, during antiviral immune responses, TLR7 and TLR9 modulate the antibody response by regulating class switch recombination [13, 14]. These findings provided insight into the importance of particular TLR signals on antibody responses against viral antigens. During bacterial infections, particular TLR such as TLR4 and TLR2 are triggered [15]; however, their participation in the antibody response against bacterial antigens and their mechanism of action have remained largely unexplored.

Current protective vaccines against pathogens induce long-lasting neutralizing antibody responses [16]. Recent studies have shown that efficient B-cell immunity using vaccination with model antigens can only be achieved when the antigen is applied in conjunction with appropriate TLR stimulation [17]. However, this view has been challenged in a study by Nemazee and colleagues [18], who showed that antigen-induced antibody responses can be generated in the absence of TLR signaling. A major drawback in these previous studies is the use of typical model antigens such as trinitrophenol-hemocyanin, keyhole limpet hemocyanin or ovalbumin, which may not behave like vaccine preparations or pathogen-derived antigens. In the current study, we have analyzed the importance of TLR signaling in the antibody responses against a *Salmonella enterica* serovar Typhi (*S. typhi*) porins' preparation. Porins are the most abundant proteins found in the outer membrane of Gram-negative bacteria; they assemble in trimers and achieve concentrations of up to 10^5 molecules *per* cell [19]. Porins purified from WT *S. typhi* (hereafter porins' preparation) consist of a mixture of two proteins: outer membrane protein C (OmpC) and F (OmpF), which assemble as homotrimers [20–22]. These antigens are highly immunogenic, both in mice and humans [20, 23, 24]. Immunization of mice with a preparation of *S. typhi* porins elicits a lifelong antibody response and protection against *S. typhi* challenge [22]. Furthermore, it has been shown that sera of patients in the acute and convalescent phases of typhoid fever contain IgM and IgG antibodies that mainly recognize porins [25, 26]. Healthy volunteers vaccinated with porins produced high titers of anti-porins antibodies without showing adverse effects. Therefore, these proteins have been proposed as vaccine candidates against typhoid fever [21].

We report here that TLR4-deficient ($TLR4^{-/-}$), myeloid differentiating gene 88-deficient ($MyD88^{-/-}$) and Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient ($TRIF^{-/-}$) mice immunized with *S. typhi* porins showed a pronounced impairment in the generation of a specific antibody response against *S. typhi* porins. Likewise, $TLR2^{-/-}$ mice displayed impaired production of IgG anti-porins antibodies with a particularly pronounced alteration in the IgG3 subclass. Moreover, adoptive transfer experiments revealed that TLR4 expression on B cells impacts on the IgM response, whereas TLR2-mediated signaling in B cells was important for the generation of IgG. Furthermore, *S. typhi* porins elicited maturation of both B cells and DC. Overall, these results indicate that the recognition of the porins' preparation by TLR impacts on the quantity and quality of the specific antibody response. Moreover, these findings bear important implications for the rational design of vaccines against bacterial pathogens such as *S. typhi*, and help in the understanding of TLR-mediated adjuvant effects.

Results

Impaired antibody response against *S. typhi* porins in the absence of TLR signaling

Immunization with *S. typhi* porins induces an antibody response even in the absence of adjuvant co-administration [21, 22]. Porins from several Gram-negative bacteria bind to TLR2 [27–29], leading to upregulation of co-stimulatory molecules and MHC class II (MHC II) antigens on macrophages, B cells and DC [30–32], cytokine release by macrophages and upregulation of surface immunoglobulin molecules on B cells [33, 34]. To assess the contribution of TLR signaling to the antibody response against *S. typhi* porins, we first analyzed the induction of anti-porins IgM and IgG in mice deficient of the main adaptor proteins of the TLR signaling pathway. Two downstream adaptor proteins have been shown to control all TLR signals: MyD88 transduces signals from all TLR except TLR3, whereas TRIF transduces signals from TLR3 and partly from TLR4 [35–37]. B6, $MyD88^{-/-}$ and $TRIF^{-/-}$ mice were immunized with 10 μg of *S. typhi* porins, and antibody titers were determined during a period of 30 days. Both $MyD88^{-/-}$ and $TRIF^{-/-}$ mice showed impaired IgM and IgG responses, even after booster immunization on day 15 (Fig. 1A and B). Analysis of the different IgG subclasses on day 30 post immunization revealed that $MyD88^{-/-}$ mice displayed significantly reduced IgG1, IgG2a/c, IgG2b and IgG3 titers (Fig. 1C). Likewise, in $TRIF^{-/-}$ mice, IgG2a/c, IgG2b and IgG3 subclasses were reduced; however, IgG3 titers were only mildly affected (Fig. 1C). These results indicate that TLR signaling enhances the antibody response against *S. typhi* porins and that both $MyD88^{-/-}$ and $TRIF^{-/-}$ signals contribute to the observed adjuvant effect.

TLR2 and TLR4 shape *S. typhi* porins-specific B-cell immunity

As mentioned in the previous part of the *Results* section, porins from different Gram-negative bacteria are TLR2 ligands [27, 28]. Moreover, the presence of undetectable trace amounts of LPS in the *S. typhi* porins' preparation could provide stimulation *via* TLR4, which might explain why $TRIF^{-/-}$ mice displayed reduced antibody responses. In order to study the contribution of TLR2 and TLR4 signaling in the antibody response against *S. typhi* porins, B6, $TLR2^{-/-}$ and $TLR4^{-/-}$ mice were immunized with the *S. typhi* porins' preparation. $TLR2^{-/-}$ mice did not show impairment in anti-porins IgM, but roughly four times reduced anti-porins IgG titers (Fig. 2A and B). $TLR4^{-/-}$ mice displayed a more pronounced decrease in both IgM and IgG titers (Fig. 2A and B). Analysis of IgG subclasses on day 30 post immunization revealed a small reduction in IgG1 and IgG2b titers, but

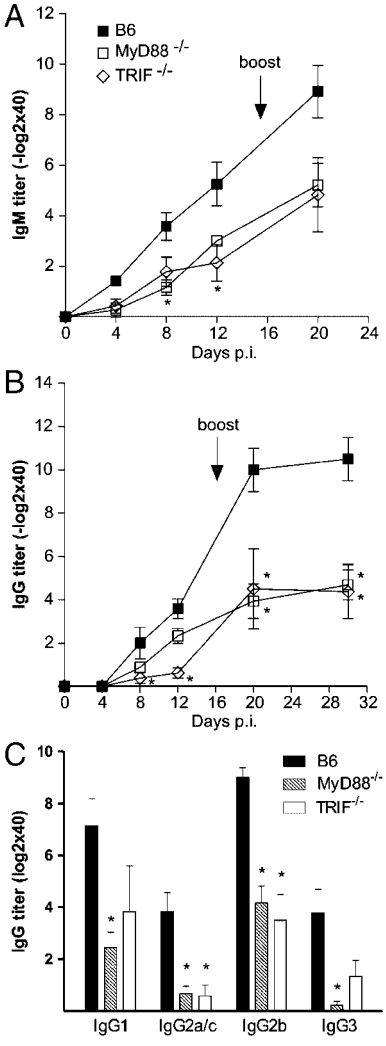

Figure 1. Antibody response against *S. typhi* porins is impaired in MyD88$^{-/-}$ and TRIF$^{-/-}$ mice. MyD88$^{-/-}$, TRIF$^{-/-}$ and B6 mice were immunized with 10 μg of *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time point. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of six mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*p<0.05).

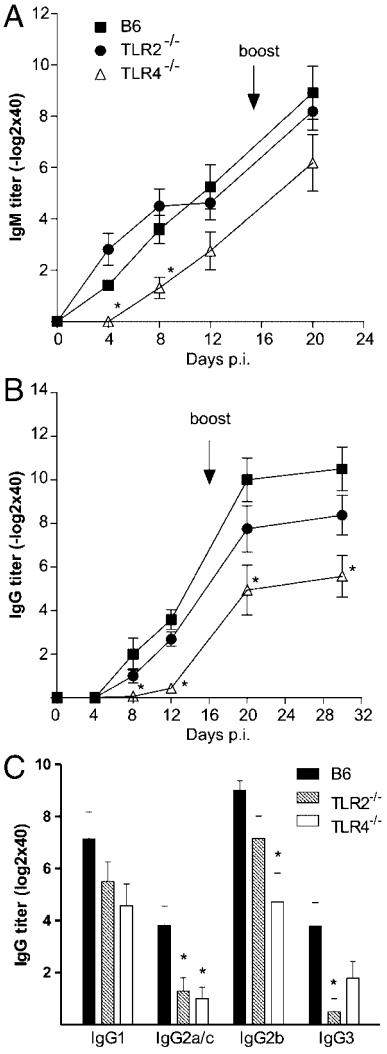

Figure 2. Antibody response against *S. typhi* porins is reduced in TLR4$^{-/-}$ and TLR2$^{-/-}$ mice. TLR4$^{-/-}$, TLR2$^{-/-}$ and B6 mice were immunized with 10 μg *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of eight mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*p<0.05).

a pronounced alteration in IgG2a/c and IgG3 titers in TLR2$^{-/-}$ mice (Fig. 2C). TLR4$^{-/-}$ mice showed a generally reduced production of IgG subclasses, with the decrease in titers of IgG2a/c and IgG2b being most dramatic (Fig. 2C). Thus, these data suggest that both TLR4 and TLR2 enhance the production of anti-porins antibodies and shape IgG subclass expression.

Type I IFN do not impact on the antibody response against *S. typhi* porins

Studies on the antibody response against viral antigens have shown that type I IFN induced through TLR receptor signals impact on the production of the different IgG subclasses [13, 38]. Because TLR4-mediated signals transmitted *via* the adaptor molecule TRIF lead to induction of IFN-β [36], we analyzed in the next experiments, the participation of type I IFN in the anti-porins antibody response. B6 and type I IFN receptor-deficient (IFNAR$^{-/-}$) mice were immunized with 10 μg of the *S. typhi* porins' preparation as described in the *Materials and methods* section. Both anti-porins IgM and IgG titers were unaltered in IFNAR$^{-/-}$ mice as compared with B6 mice (Fig. 3A and B). Moreover, the analysis of IgG subclasses on day 30, post immunization, revealed no differences between IFNAR$^{-/-}$ and B6 mice (Fig. 3C). Therefore, we conclude that type I IFN signals are neither necessary to elicit or enhance IgM or IgG antibody

responses against *S. typhi* porins, nor do they participate in the IgG class switch.

Activation of B cells and DC *via* TLR2 or TLR4 ligands

TLR2 and TLR4 ligands could enhance the *S. typhi* porins-specific antibody response by directly activating B cells, and thereby augment the BCR-mediated stimulation. To address this question, B cells from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were isolated and stimulated with 1 μg/mL of *S. typhi* porins. TLR2$^{-/-}$, TLR4$^{-/-}$ and TRIF$^{-/-}$ B cells upregulated expression of the co-stimulatory molecules CD86 and of MHC II (Fig. 4A and B). In contrast, MyD88$^{-/-}$ B cells were not activated (Fig. 4A and B), suggesting that a stimulation of B cells through TLR4 or TLR2 induced by the *S. typhi* porins' preparation was sufficient for the activation of B cells, but that B cells were not activated in the absence of both signals.

DC are not only important for the activation of CD4 T cells that will later provide cognate T help to the B cells, but can also supply signals directly to B cells [39]. To assess the stimulation of DC by the *S. typhi* porins' preparation, primary splenic DC (DC) were stimulated with 1 μg/mL of *S. typhi* porins. Monitoring of CD86 (Fig. 5A), CD40 (Fig. 5C), CD80 and MHC II expression (data not shown) revealed that TLR4$^{-/-}$ DC were stimulated by porins. It is noteworthy that both B cells and DC from TLR2$^{-/-}$ mice upregulated the activation marker CD86 (Fig. 5A and B), whereas TLR2$^{-/-}$ B cells failed to respond with CD40 upregulation after porins stimulation (Fig. 5D). To test for the presence of other bacterial TLR2 ligands, such as lipoproteins and peptidoglycans in the *S. typhi* porins' preparation, the different B-cell and DC preparations were stimulated with proteinase K-digested porins. Although B6 and TLR2$^{-/-}$ DC and B cells were still activated by this preparation, TLR4$^{-/-}$ DC and B cells did not respond, indicating the absence of non-protein TLR2 ligands in the porins' preparation, and suggesting that *S. typhi* porins or other proteins in the preparation bind to TLR2. Stimulating TLR4$^{-/-}$ DC and B cells with a preparation from *ΔOmpCΔOmpF* (*i.e.* porins-deficient) *S. typhi* did not induce the activation of the cells, providing further support that the porins' preparation did not contain flagellin or another TLR2 binding protein (Fig. 5). However, this porins-deficient preparation contains other proteins that are normally not present in the *S. typhi* porins' preparation plus detectable levels of LPS (data not shown), further indicating that such "contaminating" molecules are not responsible for TLR2 activation. These results could be confirmed using *in vitro* stimulation of HEK293 cells stably transfected with TLR2 and TLR2/6 (our unpublished data). Because the observed differences in the *S. typhi* porins-mediated activation of B cells and DC could be due to traces of LPS that were not detectable in our *Limulus* amoebocyte lysate assay, B cells and DC were stimulated with 100 and 2 ng/mL LPS (the latter being a concentration below the limit of detection of our *Limulus* assay). B6, TLR2$^{-/-}$ and to a lesser extent, MyD88$^{-/-}$ and TRIF$^{-/-}$ B cells were activated at a high concentration of LPS, but did not respond when 2 ng/mL were

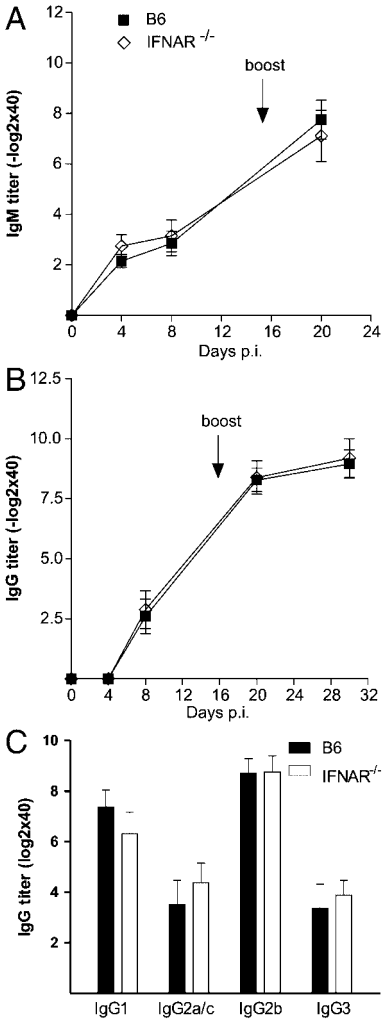

Figure 3. Antibody response against *S. typhi* porins is independent of type I IFN. IFNAR$^{-/-}$ and B6 mice were immunized with 10 μg porins at day 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM of six mice *per* group. Statistical analysis was performed using Student *t*-test.

provided (Fig. 5B and D). In contrast, even this low concentration of LPS was able to induce upregulation of CD86, and CD40 in DC from B6 and TLR2$^{-/-}$ mice (Fig. 5A and C). As expected, TLR4$^{-/-}$ B cells and DC remained unstimulated both at high and low LPS concentrations (Fig. 5). Overall, these results suggest that DC activation through TLR signals could contribute to the antibody response against *S. typhi* porins. Furthermore, it appears that TLR2 and TLR4 signals provided by the *S. typhi* porins' preparation differentially affect the activation of DC and B cells.

TLR2 and TLR4 signals on B cells contribute to antibody responses against *S.typhi* porins

TLR2 and TLR4 signals could act directly on B cells, or through the activation of other APC such as DC. To determine the contribution

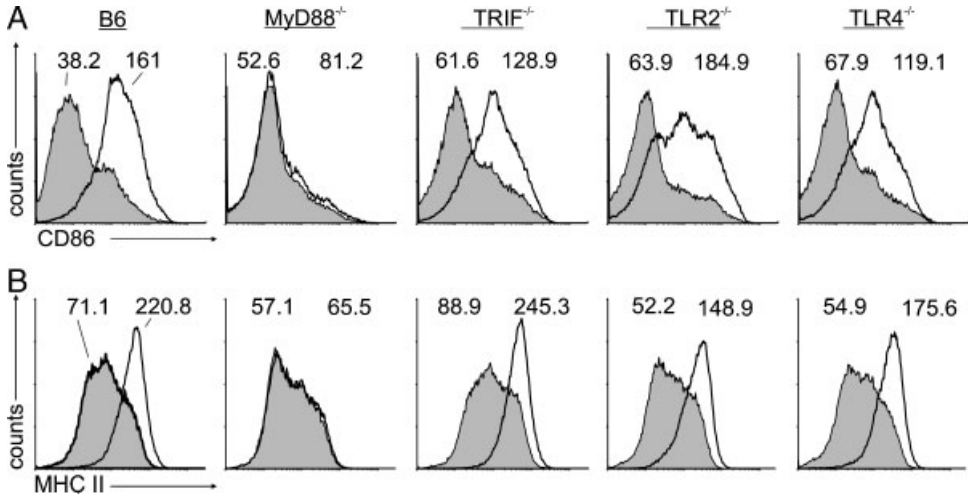

Figure 4. *S. typhi* porins induce B-cell activation. B cells from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with 1 μg/mL *S. typhi* porins. After 24 h, the expression of (A) CD86 and (B) MHC II on CD19^{+} B cells was analyzed by flow cytometry. Results are from one of three independent experiments with filled histograms representing B cells incubated in medium alone, and open histograms representing B cells incubated with porins. Values represent the MFI of the respective cell population.

of TLR2 and TLR4 signaling in B cells to the anti-porins response, splenic B cells from B6, MyD88$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice were adoptively transferred into μ chain membrane exon deficient mice (μMT), which lack B cells. Reconstituted mice were immunized on days 0 and 15 with 10 μg of the *S. typhi* porins' preparation and antibody titers were analyzed at the indicated days. In the absence of TLR4 on B cells, anti-porins IgM titers were reduced (Fig. 6A). However, booster immunization elicited an IgG response comparable with B6 B-cell recipient mice (Fig. 6B and C), indicating that TLR4 expression on B cells is most important during the induction of the primary IgM antibody response. It is noteworthy, that mice receiving TLR2$^{-/-}$ B cells showed a normal IgM response but a reduced IgG production (Fig. 6), suggesting that TLR2 signals on B cells are critical for anti-porins IgG production. Mice receiving MyD88$^{-/-}$ B cells exhibited both impaired IgM and IgG response. (Fig. 6). Overall, these experiments confirm the importance of TLR2 and TLR4 signals on B cells for the anti-porins antibody production, and their differential contribution in shaping the adaptive immune response.

Discussion

TLR ligands are used in several vaccine preparations as adjuvants to enhance specific immune responses; however, the contribution of TLR signaling to induction and maintenance of antibody responses is still controversial. For example, it has been proposed that TLR are required to elicit an antibody response [17]; on the other hand, mice with a total deficiency in TLR signaling were able to produce specific antibody responses when immunized with antigens in TLR ligand-free adjuvants [18]. Recently, it has been also proposed that intrinsic TLR signals in B cells enhance the specific antibody responses, but are not required for their induction [40]. Most of these studies have used model antigens that only partially reflect the situation in a response against bacterial antigens. Here, we show that TLR4- and TLR2-mediated signals are important for the enhancement of anti-porins antibody responses. Moreover, both TLR4- and TLR2-dependent signals contributed differentially in shaping the IgG antibody response against *S. typhi* porins. We decided to analyze the participation of TLR in the antibody response against bacterial antigens using immunization of *S. typhi* porins as a model because these proteins induce life-lasting bactericidal antibody responses [22]. In the present study, the porins-specific IgM and IgG antibody responses in MyD88$^{-/-}$ and TRIF$^{-/-}$, as well as TLR4$^{-/-}$ mice were reduced, whereas in TLR2$^{-/-}$ mice, a reduction in anti-porins IgG was observed. These results suggest that signaling through TLR participates in the enhancement of the anti-porins antibody response. The general reduction in the production of IgG subclasses in TLR4$^{-/-}$ and TRIF$^{-/-}$ mice, the pronounced reduction in IgG2a/c and IgG3 titers in TLR2$^{-/-}$ mice and, finally, the pronounced reduction in IgG2a/c and IgG3 in MyD88$^{-/-}$ mice indicated furthermore an important role of these molecules in shaping the IgG subclass expression. These results corroborate previous findings showing that MyD88 signaling is required for the production of IgG2a [14, 41]. Moreover, the similarities in the IgG subtype production between MyD88$^{-/-}$ and TLR2$^{-/-}$ also suggest that signaling through TLR2 and MyD88 impacts on the isotype class switch to IgG3. This could be mediated by the effect of TLR signals on the production of IFN-γ, because it has been shown that IFN-γ participates in the induction of IgG3 [42]; however, this issue remains to be addressed in future studies.

TLR signals could participate at different stages in the induction of antibody responses: through direct signals in the B cells enhancing their activation, proliferation and antibody production [12, 43, 44] or by the activation of DC to further trigger the adaptive immune response [45].

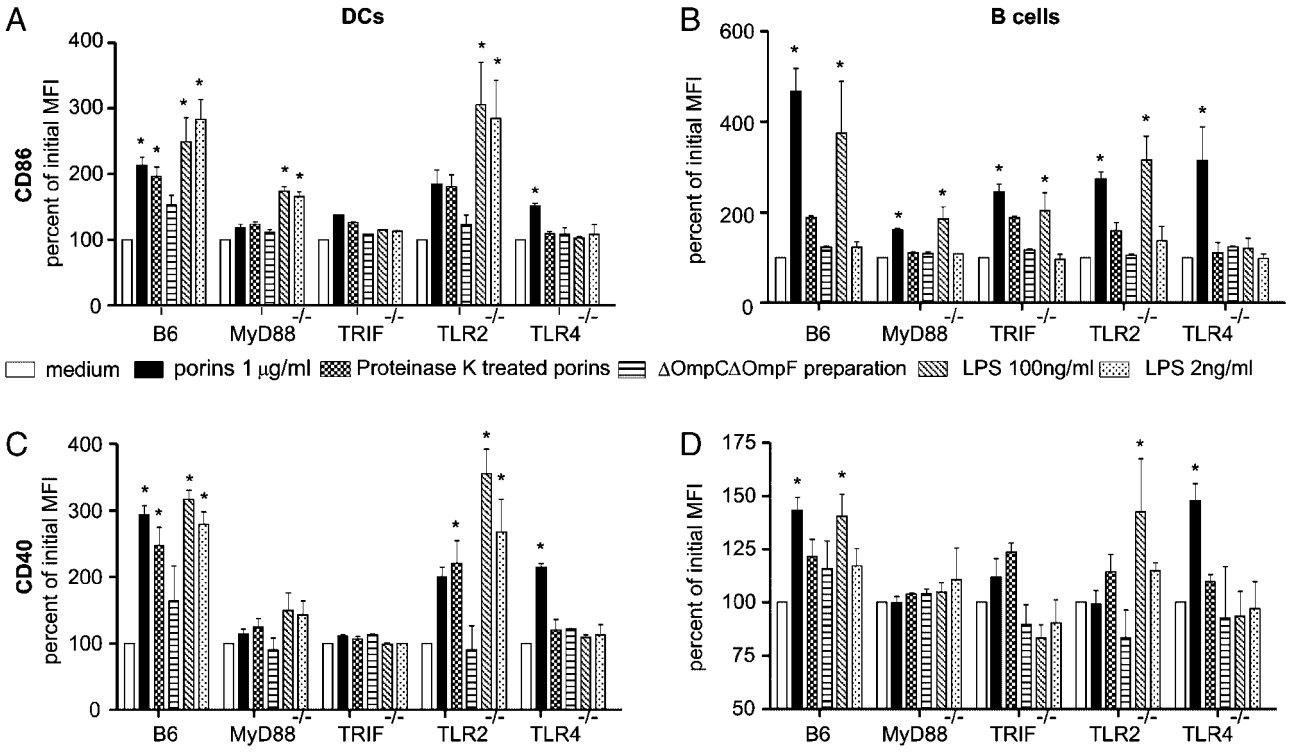

Figure 5. Activation of B cells and DC by *S. typhi* porins. B cells and DC from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were stimulated with 1 μg/mL *S. typhi* porins, 1 μg/mL proteinase K-digested *S. typhi* porins, *ΔOmpCΔOmpF S. typhi* preparation, 100 ng/mL LPS, 2 ng/mL LPS or medium alone. After 24 h, the expression of CD86 (A, B) and CD40 (C, D) on CD11c^{+} DC (A, C), and CD19^{+} B cells (B, D) was analyzed by flow cytometry. Results are expressed as percentage of initial MFI and represent the mean ± SEM of four independent experiments. Statistical analysis was performed using one-way ANOVA. Significant differences between the MFI of the cells treated with the different stimuli and the MFI from the medium-treated cells of the corresponding genotype are indicated (*p<0.05).

The presented *in vitro* and *in vivo* evidence shows that the *S. typhi* porins' preparation directly activates B cells *via* TLR2 and TLR4. It has been recently proposed that intrinsic TLR signals on B cells amplify the antibody response through MyD88, but are not required for initial triggering [40]. The present study supports this notion, and furthermore indicates that TLR2 and TLR4 signals act differentially during B-cell activation.

B cells can activate the T-dependent antibody responses by directly presenting antigen to Th cells [46]. However, other APC, in particular DC, contribute importantly to the antibody response by activating Th cells or by direct interaction with B cells [39]. DC from MyD88$^{-/-}$ and TRIF$^{-/-}$ mice showed no activation after *S. typhi* porins stimulation, whereas DC from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were activated. A possible difference between B cells and DC could be their activation through different TLR ligands, in particular to traces of LPS, which is supported by the fact that TLR4$^{-/-}$ mice showed a deficient antibody response. Although the low concentration of LPS probably present in the porins' preparation was not able to activate B cells, it was enough to activate DC. Importantly, the concerted stimulation of specific B cells through TLR2 and BCR by the porins' preparation, acting as a PAMP and an antigen (Pamptigen) [47], is probably sufficient in TLR4$^{-/-}$ mice to induce anti-porins IgM antibodies. Of note is that very small amounts of LPS, undetectable by conventional methods, can still participate in immune responses *in vivo*, which should be considered for the analysis of the immune response to isolated bacterial components.

Taken together, we propose that TLR4-mediated, B-cell intrinsic signals enhance the *S. typhi* porins-specific IgM production, whereas TLR2-mediated, B-cell intrinsic signals participate in the enhancement and shaping of the IgG response. These findings will help to guide further improvements in the development of vaccines against important human pathogens.

Materials and methods

Mice and bacteria

C57BL/6 (B6) mice were obtained from Charles River Laboratories (Sulzfeld, Germany). MyD88$^{-/-}$ [35], TRIF$^{-/-}$ [48], TLR4$^{-/-}$ [49], TLR2$^{-/-}$ [50] and μMT [51] mice on the B6 background were bred in the Institut für Labortierkunde (University of Zurich, Switzerland). IFNAR$^{-/-}$ [52] on the B6 background were kindly provided by Martin Bachmann, Cytos AG, Schlieren, Switzerland, and bred in the Research Department Kantonal Hospital St Gallen. All mice were maintained in individually ventilated cages and were used between 6 and 9 weeks of age. All animal experiments were performed in

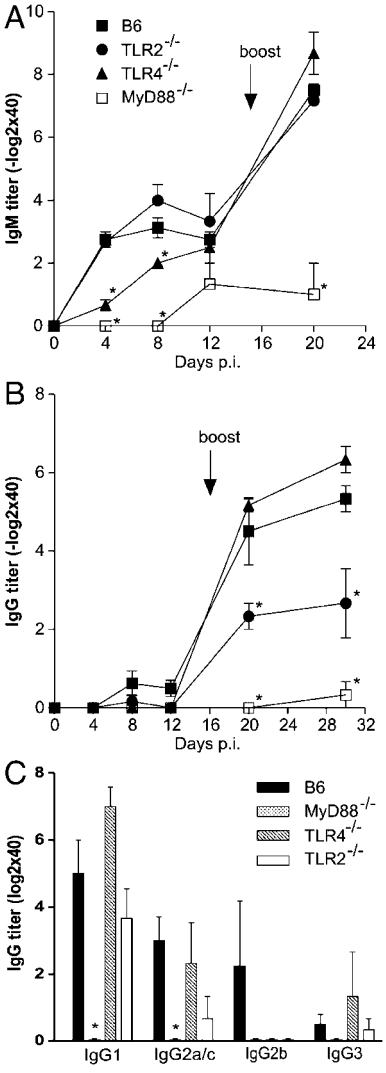

Figure 6. TLR2 and TLR4 signals on B cells contribute differentially to the antibody response. 5×10^7 B cells from B6, MyD88$^{-/-}$, TLR4$^{-/-}$ or TLR2$^{-/-}$ mice were adoptive transferred to μMT mice. Mice were immunized with 10 μg *S. typhi* porins on days 0 and 15. (A) Anti-porins IgM and (B) anti-porins IgG antibody titers were measured by ELISA at the indicated time points. (C) Anti-porins IgG subclasses IgG1, IgG2a/c, IgG2b and IgG3 were determined on day 30 post immunization. Results are expressed as the mean ± SEM, of at least three mice *per* group. Statistical analysis was performed using one-way ANOVA. Significant differences between the respective gene-deficient mice and the WT control at the same time point are indicated (*$p<0.05$).

accordance with the Swiss Federal legislation on animal protection. For porins' preparation the *S. typhi* strain from ATCC (No. 9993) and the isogenic *S. typhi* mutant strain VALE39*ΔompF* Km^R*ΔompC* Cm^R [22] were used.

Preparation of porins and immunization protocol

The porins (*i.e.* OmpC and OmpF) were purified from *S. typhi* or its isogenic mutant as previously described [21]. LPS content was determined by means of the *Limulus* amoebocyte lysate assay (LAL; Endosafe® KTA, Charles River Endosafe Laboratories, Charleston, SC, USA), detection limit 0.2 ng LPS/μg protein. Groups of mice were immunized i.p. on day 0 and boosted on day 15 with 10 μg of the *S. typhi* porins' preparation. Blood samples were collected at various times post immunization as indicated. Individual serum samples were stored at −20°C until analysis.

Determination of antibody titers by ELISA

High-binding 96-well polystyrene plates (Corning®, New York, NY, USA) were coated with 10 μg/mL of porins in 0.1 M carbonate–bicarbonate buffer, pH 9.5. Plates were incubated for 1 h at 37° and then overnight at 4°. Before use, plates were washed three times in PBS (pH 7.2) containing 0.05% Tween-20 (PBS-T) (Sigma-Aldrich). Non-specific binding was blocked with 5% non-fat dry milk diluted in PBS (PBS-M) for 2 h at 37°. After washing, sera were diluted 1:40 in PBS-M and twofold serial dilutions were added to the wells. Plates were incubated for 2 h at 37°, followed by four washes with PBS-T. The optimal dilution, 1:1000, of peroxidase-conjugated rabbit anti-mouse IgM, IgG, IgG1, IgG2b antibodies (Zymed, San Francisco, CA, USA), IgG2a/c (Acris GmbH, Germany; mice from the B6 express the subclass IgG2c that cross react with antibodies against the IgG2a subclass and therefore we refer to them as IgG2a/c [53, 54]), IgG3 (Rockland, Gilbertsville, PA, USA) in PBS-M was added, followed by 1 h of incubation at 37°C and four additional washes with PBS-T. Ortho-phenylenediamine (0.5 mg/mL; Sigma) in 0.1 M citrate buffer, pH 5.6, containing 0.08% H_2O_2 was used as the enzyme substrate. The reaction was stopped with 1.25 M H_2SO_4 and the optical densities were read at 492 nm using an automatic ELISA plate reader (Tecan). Antibody titers are given as $-\log_2$ dilution $\times$ 40. Positive titers were defined as 3 SD above the mean values of the negative controls.

B lymphocyte and DC isolation

Single-cell splenocyte suspensions were obtained from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice by digestion with collagenase (Sigma-Aldrich). The B-lymphocyte fraction was enriched using mouse CD45R (B220) microbeads (Miltenyi Biotec) and the CD11c$^+$ DC fraction using mouse CD11c (N418) microbeads (Miltenyi Biotec) as recommended by the manufacturer. For adoptive transfer experiments, 5×10^7 B6, MyD88$^{-/-}$, TLR2$^{-/-}$ or TLR4$^{-/-}$ B cells (>96% purity as assessed by CD19 expression) were injected intravenously to μMT mice; reconstituted mice were immunized with porins 3 h later.

B lymphocyte and DC stimulation

B lymphocytes or DC from B6, MyD88$^{-/-}$, TRIF$^{-/-}$, TLR2$^{-/-}$ and TLR4$^{-/-}$ mice (2×10^6 cells/well) were seeded in RPMI 1640

(Gibco) 5% FCS (Gibco) and stimulated with 1 μg/mL of *S. typhi* porins' preparation, *S. typhi* porins' preparation previously digested with proteinase K (Roche), *ΔOmpCΔOmpF S. typhi* preparation, 2 or 100 ng/mL protein-free *S. typhi* LPS (provided by Dr. John S. Gunn, Ohio State University, Columbus, OH, USA) or medium alone. After 24 h, cells were harvested, stained with mAbs against CD19 PE (Immunotools), CD11c PE, CD40 APC, CD80 FITC, I-A/I-E APC and CD86 APC (BD Biosciences Pharmingen). Differential regulation of CD86 and CD40 was best suitable as read-out for DC *versus* B-cell activation because CD80 was only weakly expressed on B cells and MHC II was highly expressed on freshly isolated splenic DC and could not be further upregulated with the stimuli used here. 7-amino-actinomycin D (7AAD) (Sigma-Aldrich) was added at a concentration of 2.5 μg/mL 20 min prior to the analysis to exclude non-viable cells. Cells were analyzed with a FACS Calibur flow cytometer using the CellQuest software (BD Biosciences).

Statistical analysis

Statistical analyses were performed using Student *t*-test, or one-way ANOVA with Prism 5.0 (GraphPad Software). A p-value of <0.05 was considered significant.

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Abbreviations: **IFNAR$^{-/-}$:** type I IFN receptor-deficient · **MHC II:** MHC class II · **μMT:** μ chain membrane exon deficient mice · **MyD88$^{-/-}$:** myeloid differentiating gene 88-deficient · **Omp:** outer membrane protein · **S. typhi:** *Salmonella enterica* serovar typhi · **TLR4$^{-/-}$, TLR2$^{-/-}$:** TLR4-, TLR2-deficient · **TRIF$^{-/-}$:** Toll/IL-R domain-containing adaptor-inducing IFN-β-deficient

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