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IFN-γ–Producing CD4+ T Cells Promote Generation of Protective Germinal Center–Derived IgM+ B Cell Memory against *Salmonella* Typhi

Christian Perez-Shibayama,*,†,1 Cristina Gil-Cruz,*,†,1 Rodolfo Pastelin-Palacios,‡ Luisa Cervantes-Barragan,*,§,¶ Emiliano Hisaki,† Qian Chai,* Lucas Onder,* Elke Scandella,* Tommy Regen,‖ Ari Waisman,‖ Armando Isibasi,† Constantino Lopez-Macias,† and Burkhard Ludewig*

Abs play a significant role in protection against the intracellular bacterium *Salmonella* Typhi. In this article, we investigated how long-term protective IgM responses can be elicited by a *S.* Typhi outer-membrane protein C– and F–based subunit vaccine (porins). We found that repeated Ag exposure promoted a CD4+ T cell–dependent germinal center reaction that generated mutated IgM-producing B cells and was accompanied by a strong expansion of IFN-γ–secreting T follicular helper cells. Genetic ablation of individual cytokine receptors revealed that both IFN-γ and IL-17 are required for optimal germinal center reactions and production of porin-specific memory IgM+ B cells. However, more profound reduction of porin-specific IgM B cell responses in the absence of IFN-γR signaling indicated that this cytokine plays a dominant role. Importantly, mutated IgM mAbs against porins exhibited bactericidal capacity and efficiently augmented *S.* Typhi clearance. In conclusion, repeated vaccination with *S.* Typhi porins programs type I T follicular helper cell responses that contribute to the diversification of B cell memory and promote the generation of protective IgM Abs. *The Journal of Immunology*, 2014, 192: 000–000.

Salmonella enterica infections remain an important health problem. Recent global estimates for enteric fever report >27 million annual cases and 200,000 deaths (1, 2). The prevalence of *Salmonella*-related illness is particularly high in developing countries; however, because the number of travelers crossing international borders exceeds one billion people per year (3), the incidence of *S.* Typhi infection causing typhoid fever continues to increase in developed countries. Moreover, nontyphoidal *Salmonella* (NTS) serovars such as *S.* Typhimurium cause bacteremia in young children and immunologically compromised adults (4). Because *S.* Typhi and NTS serovars frequently persist in their hosts and thereby contribute to the transmission to naive individuals (5), these pathogens are considered important targets for vaccination-controlled diseases (6–8). Hence it is important to develop and to characterize subunit vaccines that focus protective immune responses on the critical components of the pathogen.

Salmonella and other Gram-negative bacteria express outer-membrane proteins (Omps) that are highly immunogenic and elicit innate and adaptive immune responses in mice (9–14). In humans, IgG and IgM Abs against pore-forming Omps (porins) can be found in individuals recovering from typhoid fever (15, 16). Moreover, Abs directed against porins exert protective functions in HIV patients suffering from NTS infection (4). *S.* Typhi OmpC and OmpF can be purified and produced at large scale for application in humans (17). Such highly purified *S.* Typhi porins induce long-lasting bactericidal Ab responses in mice (18) and exhibit intrinsic adjuvant activity (14, 19). Intriguingly, *S.* Typhi porins have been applied to humans showing that this subunit vaccine is safe and immunogenic and that the B cell response in humans generates IgG and IgM Abs (17), suggesting that both isotypes could contribute to protection against this intracellular pathogen.

Recent studies have established that a protective effect of anti-*Salmonella* Abs in humans is due to their ability to facilitate complement-mediated lysis (18, 20). Notably, Abs against porins (4, 20) or against LPS (21) are associated with protective humoral immunity. For example, NTS porins elicit an immediate IgM response from B1b B cells that reduces bacterial titers in mice (22). Likewise, B1b B cells recognize porins and generate unmutated IgM Abs that can suffice to control bacteremia (23). These studies

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Abbreviations used in this article: ASC, Ab-secreting cell; B6, C57BL/6; DC, dendritic cell; GC, germinal center; IMSS, Mexican Social Security Institute; MZ, marginal zone; NTS, nontyphoidal *Salmonella*; PNA, peanut agglutinin; Tfh, T follicular helper.

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indicate that B cells expressing unmutated IgM specific for *Salmonella* Ags are present in naive animals and suggest that the protective capacity of such B cells may be enhanced through selection processes that improve their performance.

B cell responses are optimized in germinal centers (GCs), which are follicular structures within secondary and tertiary lymphoid organs (24). The major functions of the GC reaction are Ab diversification through class-switch recombination and the generation of affinity-matured B cells through the process of somatic hypermutation (25). Moreover, GCs facilitate the generation of B cell memory and produce large numbers of short- and long-lived plasma cells that provide high Ab titers in serum (26). Interestingly, IgM^+ memory B cells appear to be of high importance for secondary GC reactions, whereas IgG memory B cells more rapidly promote plasma cell generation (27, 28). However, it has remained unexplored whether and how long-lived, affinity-matured IgM^+ memory B cells emerging from GCs contribute to the heterogeneity of B cell responses against porins derived from the persisting pathogen *S.* Typhi.

Defense against persisting pathogens requires the concerted action of the different arms of the immune system (29). For example, maintenance of protective B cell responses against viruses hiding in distinct sanctuaries depends on the continued support from $CD4^+$ T cells (30, 31). The GC reaction that generates such B cell responses is regulated by a specific $CD4^+$ T cell type known as T follicular helper (Tfh) cells, which can differentiate into various subtypes (e.g., Th1-like, Th2-like, Th17-like) (25, 32). Tfh cell differentiation is initiated through contact with Ag-expressing dendritic cells (DCs) (33), leading to the rapid induction of the Tfh signature transcription factor BCL-6 (34). Hence the immediate Tfh differentiation program impinges on the subsequent T–B cell interaction including GC reaction and B cell memory formation. One prediction from this scenario for B cell responses against *Salmonella* serovars suggests a dominant role of IFN-γ in these processes because anti-*Salmonella* T cell responses in mice are dominated by IFN-γ (35, 36), and humans and mice with deficiencies in the IFN-γ/IL-12 axis exhibit pronounced susceptibility to infection with these intracellular bacteria (37, 38). Indeed, we found that the GC reaction generating IgM^+ memory B cells against *S.* Typhi porins was almost exclusively dependent on IFN-γ–producing Th cells and that the generation of mutated IgM^+ memory B cells was accompanied by a strong expansion of BCL-6–expressing Th1-like Tfh cells. Moreover, repeated Ag exposure was required to maintain the GC reaction and to fully elicit Th cell–dependent B cell memory diversification. In conclusion, this study reveals that the signals driving Tfh cell differentiation are imprinted in particular pathogen substructures and suggests that type I Tfh cells support the generation of mutated IgM^+ memory B cells and secure the long-term production of bactericidal IgM Abs against *S.* Typhi porins.

Materials and Methods

Ethics statement

Experiments in Switzerland were performed in accordance with federal and cantonal guidelines (Tierschutzgesetz) under permission numbers SG08/79, SG09/83, and SG09/87 following review and approval by the Cantonal Veterinary Office (St. Gallen, Switzerland). Experiments in Mexico were performed in accordance with national guidelines (Norma oficial Mexicana, NOM-062-ZOO 1999) in the Unidad de Investigación Médica en Inmunoquímica, Mexican Social Security Institute [IMSS], Mexico, following review and approval by the IMSS National Scientific Research Committee (project CNIC-2006-785-076). Human samples were obtained from adult subjects who had provided written informed consent under a protocol approved by the IMSS National Scientific Research Committee (composed by Ethics, Scientific and Biosafety committees, project CNIC 2010-785-002).

Mice and bacteria

C57BL/6 (B6) mice were purchased from Charles River Laboratories (Sulzfeld, Germany) or Harlan (Mexico City, Mexico). B6.129X1-H2-$Ab1^{tm1Koni}$/J (MHC class II–deficient; $Iab^{-/-}$) and B6.129S7-$Ifngr^{tm1Agt}$/J (IFN-γR–deficient; $Ifngr^{-/-}$) mice were maintained locally and at the Institute for Laboratory Animal Sciences at the University of Zürich. $Il17ra^{-/-}$ mice were described previously (39). All mice were on the B6 genetic background, were maintained in individually ventilated cages, and were used between 6 and 9 wk of age. Virulent *S.* Typhi strain ATCC 9993,9,12,Vi,d was used for infection, complement-mediated bactericidal assays, and Ab and complement fixation analysis. *Escherichia coli* K12 strain was used for complement and Ab fixation analysis.

Human sera

Blood samples from the volunteers of the clinical trial (17) were obtained 10 y after vaccination: 5 volunteers from the porins vaccinated group and 2 volunteers from the placebo group. As control group, 20 healthy male volunteers from the Mexico City area were selected after a medical examination consisting of a complete clinical history, physical examination, and clinical laboratory tests. Volunteers suffering from any disease and those who had been previously vaccinated against typhoid or treated with immune modulators were excluded from the study. Further details on the study subjects can be found in Supplemental Table I.

Ab detection

For measurement of anti-porin Ab titers, high-binding 96-well polystyrene plates (Cornings, New York, NY) were coated with 1 μg *S.* Typhi porins per well. The assay was performed as described previously (14). Ab titers are given as −log2 dilution ×40. Positive titers were defined as 3 SD above the mean values of the negative controls. ELISPOT assays were performed following the manufacturer's instructions (Mabtech AB). Plates with 1 μg *S.* Typhi porins per well were incubated for 24 h at 37°C with 10^5 peritoneal, spleen, or bone marrow cells obtained from porin-immunized or naive mice. Plates were counted using an ELISPOT-Reader and analyzed with the software ELISPOT 3.1SR (AID). Individual samples were tested in duplicate. Values are expressed as mean number of specific Ab-forming cells (experimental sample − naive control).

Purification of porins and immunization protocol

Porins were purified from *S.* Typhi ATCC 9993 as previously described (14, 17). LPS content was determined using the limulus amebocyte lysate assay (Endosafe KTA; Charles River Endosafe Laboratories); all batches were negative for limulus amebocyte lysate assay (detection limit, 0.2 ng LPS/mg protein). Moreover, Western blot analysis using anti-LPS polyclonal sera confirmed that LPS was not detectable by these means (data not shown). Biotinylated porins were prepared using the EZ-Link NHS-biotin reagents (Thermo Scientific) following the manufacturer's instructions. Proteinase K–digested porins were prepared as described previously (14). Mice were immunized i.p. on day 0 and boosted on day 15 with 10 μg *S.* Typhi porins. Sera were collected at various time points after immunization and stored at −20°C until analysis.

Flow cytometric analysis and sorting

Serum samples from porin-immunized mice or human volunteers were heat inactivated at 56°C for 30 min. For IgM-binding analysis, heat-inactivated sera were incubated with *S.* Typhi or *E. coli* K12 for 30 min at 4°C. PE-Cy7–labeled anti-mouse IgM or anti-human allophycocyanin-labeled IgM (Biolegend) were used for detection in flow cytometry. For C3b binding analysis, heat-inactivated sera (30 min at 56°C) were incubated with *S.* Typhi or *E. coli* for 30 min at 4°C followed by incubation for 20 min at 37°C with sera from naive B6 mice as a source of complement. FITC-labeled anti-mouse C3 Abs (MP Biomedicals) were used for detection. Samples were analyzed by flow cytometry using a FACSCanto (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star). Marginal zone (MZ) B cells were first enriched by negative selection using biotinylated Abs against follicular B cells (anti-CD23), plasma cells (anti-CD138), and T cells (anti-CD3; all from eBioscience) and anti-biotin coupled MACS beads (Miltenyi Biotec). $B220^+CD21^+CD23^{low/neg}$ MZ B cells were sorted using a FACS Aria III flow cytometer (Becton Dickinson). Purity of postsort MZ B cells was 98–99%. GC B cells were enriched by positive selection using biotin-labeled peanut agglutinin (PNA; Vector Laboratories) and anti-biotin–coupled MACS beads. FACS-sorted $B220^+GL7^+FAS^+$ GC B cells showed a purity of 95–98%.

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Detection of $CD4^+$ T cell responses

Libraries containing 15-mer peptides with a 5-residue overlap were designed from *S.* Typhi OmpC and OmpF sequences (40). Peptides were synthesized by JPT Peptide Technologies GmbH (Berlin Germany). For peptide-specific cytokine production, 10^6 splenocytes were restimulated with each peptide in the presence of brefeldin A (5 μg/ml) for 5 h at 37°C. Cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml; both purchased from Sigma) as positive control or left untreated as a negative control. For intracellular staining, restimulated cells were surface stained and fixed with Cytofix/Cytoperm (BD Biosciences) for 20 min. Fixed cells were incubated at 4°C for 40 min with permeabilization buffer (2% FCS/0.5% saponin/PBS) containing anti–IFN-γ mAb (BD Biosciences). Samples were analyzed by flow cytometry using a FACSCanto flow cytometer (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star).

Cytokine determination

Bone marrow–derived DCs were stimulated either with porins (0.1 μg or 1 μg) or with a mixture of peptides $OmpF_{201-215}$ and $OmpC_{241-255}$, and cocultured with MACS-sorted splenic CD4 T cells from naive or porin-immunized mice. Cells were stimulated with phorbol myristate acetate (50 ng/ml) and ionomycin (500 ng/ml; both purchased from Sigma) as positive control or left untreated as a negative control. At the indicated time points, supernatants were collected and IFN-γ, IL-17A, and IL-21 concentration was determined using the respective ELISA assay (eBioscience) following the manufacturer's instructions. IL-6, TNF, IL-4, IL-2, and IL-23 were determined using cytometric bead array (BD biosciences).

$CD4^+$ T cell depletion

Mice were immunized i.p. on day 0 and boosted on day 15 with 10 μg *S.* Typhi porins. $CD4^+$ T cells were depleted either before boosting (at day 13) or after boosting (day 20) by using 0.5 mg/mouse of the monoclonal anti-CD4 depleting Ab (clone YTS191) every third day. Depletion of $CD4^+$ T cells was assessed by flow cytometry and was usually >98%.

Immunofluorescence analysis

For immunofluorescence analysis, spleens were fixed in PBS/4% PFA. Twenty-micrometer sections were cut using a Vibratome (Leica VT 1200S). Fixation and staining was performed as described previously (41) using Abs against B220 and CD4 (eBioscience) and PNA (Vector). Images were acquired using Zeiss LSM710 microscope and processed using ZEN software (Zeiss) and Adobe Photoshop (Adobe Systems).

Generation of anti–S. Typhi porin mAbs

B6 mice were immunized i.p. on day 0 and boosted on day 15 with 10 μg *S.* Typhi porins. Splenocytes were obtained at day 40 and were fused with P3x63Ag8.653 myeloma cells. Supernatant of wells containing growing cells were screened for specific Abs against *S.* Typhi porins by ELISA. After expansion of porin-specific hybridomas, a limiting dilution step was performed. Monoclonal hybridomas were expanded and used for Ab purification and BCR sequencing. Purification of IgM was done using HiTrap IgM purification HP (GE Life Sciences) according to the manufacturer's instructions. Mutations in Ig variable regions were determined following RNA isolation from 10^7 hybridoma cells using TRIzol (Invitrogen), cDNA transcription using a high-capacity cDNA reverse transcription kit (Applied Biosystem), and amplification with degenerate PCR as previously described (42). Degenerate primers were designed to amplify nine of the variable gene families and Cμ. Sequences were determined on an ABI 3130 Prism sequencer (Applied Biosystems). Variable genes were identified using the IgBLAST software from the National Center for Biotechnology Information.

Complement-mediated bactericidal assay

In vitro bactericidal activity of IgM mAbs was determined using monoclonal IgM from anti-porin clones 32-6B and 11-6B and the 21-C7 IgM clone specific for the murine hepatitis virus (isotype control). Two-fold serial dilutions of the Abs starting at 0.2 mg/ml were added to wells containing 200 ± 50 CFUs *S.* Typhi, and guinea pig serum as complement source (9% v/v). Guinea pig sera were collected from healthy animals. Sera were pooled and maintained at −80°C until used; the same batch of sera was used to perform all experiments. Controls included murine anti–*S.* Typhi serum as positive control and bacteria plus guinea pig serum as negative control. Plates were incubated for 18 h at 37°C. Bactericidal titers are provided as −log2 dilution ×20 and represent the highest dilution at which 50% bactericidal activity was observed.

In vivo opsonization assay

B6 mice received i.v. with 10^6 CFU *S.* Typhi preincubated for 30 min at 4°C with monoclonal IgM (0.5 mg/ml) from anti-porin hybridomas 32-6B or 11-6B, or the 21-C7 isotype control in a final volume of 200 μl. Heat-inactivated anti–*S.* Typhi serum was used as positive control. Bacterial loads in spleens and livers were determined 24 h after i.v. application.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 5.0 using two-tailed Student *t* test. Longitudinal comparison between different groups was done with one-way ANOVA with Tukey's posttest or by Kruskal–Wallis test with Dunn's posttest. Statistical significance was defined as $p < 0.05$.

Results

S. Typhi porins induce long-lasting Ab responses in humans

Humans infected with *S.* Typhi develop substantial IgM and IgG Ab titers against porins (15, 16), and preparations of *S.* Typhi porins containing OmpC and OmpF induce robust Ab responses in human volunteers within 2 wk after s.c. vaccination (17). To assess the longevity of anti-porin Ab responses in humans, we obtained serum samples from volunteers who had participated in a *S.* Typhi porin vaccination study in 2002 (17). Even after more than a decade, *S.* Typhi porin–vaccinated individuals exhibited still significantly higher IgG (Fig. 1A) and IgM (Fig. 1B) titers compared with the placebo group. Because the number of individuals in the placebo group who could be retrieved was rather low ($n = 2$), we determined anti–*S.* Typhi porin Ab titers in individuals with a negative clinical history for typhoid fever and without vaccination against typhoid fever. Importantly, these control serum samples showed anti-porin titers that were comparable with the placebo group and were significantly lower compared with the *S.* Typhi porin serum group (Fig. 1A and 1B). These data indicate that the individuals vaccinated with *S.* Typhi porins in 2002 had maintained specific Ab titers against the immunogen. To confirm these findings and to assess recognition of the Ag in its natural context, we determined serum IgM binding to *S.* Typhi and *E. coli* by flow cytometry. We found that IgM from *S.* Typhi porin–vaccinated

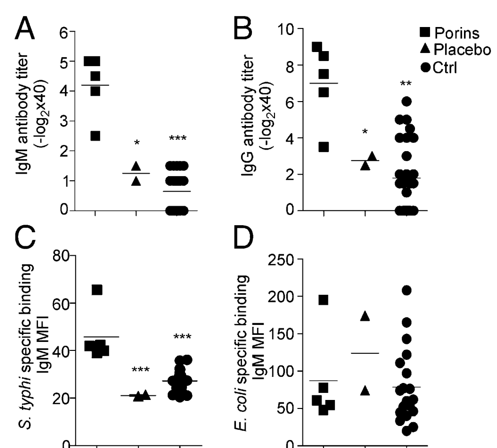

FIGURE 1. *S.* Typhi porins induce long-lasting IgM Ab responses in humans. Blood samples of *S.* Typhi porin–immunized individuals ($n = 5$) or placebo recipients ($n = 2$) were obtained 10 y after vaccination. Sera obtained from healthy volunteers ($n = 20$, control [Ctrl]) served as further controls. *S.* Typhi porin–specific IgM (**A**) and IgG (**B**) Ab titers were determined by ELISA. Binding of serum IgM to *S.* Typhi (**C**) and *E. coli* (**D**) was determined by flow cytometry. Values of each individual are shown; horizontal bars represent mean values. Statistical analysis was performed using Kruskal–Wallis test with Dunn's posttest (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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individuals specifically recognized the surface of *S.* Typhi (Fig. 1C), but not of *E. coli* (Fig. 1D). Thus, individuals exposed to *S.* Typhi porins not only develop long-lasting IgG Ab titers, but also maintain substantial IgM Ab responses that facilitate specific recognition of the pathogen.

Sustained CD4$^+$ T cell help secures long-term persistence of S. *Typhi porin–specific IgM*

To elucidate the mechanisms that facilitate generation of IgM B cell memory against *S.* Typhi porins in mice, we examined first whether repeated Ag exposure is required to sustain the IgM response. Single application of 10 μg porins (prime) elicited strong IgG responses that lasted for >100 d and was not further augmented by a boost at day 15 (Fig. 2A). Notably, IgM serum Abs waned after day 8 in the prime regimen, whereas prime/boost application elicited a persisting IgM serum response (Fig. 2A). Ag exposure in prime/boost regimen also precipitated recruitment of IgM Ab-secreting cells (ASCs) to the bone marrow (Fig. 2B), which could represent long-lived plasma cells that can contribute to the maintenance of high serum titers (43). Alternatively, IgM B cell memory and high serum Ab titers can be maintained by a CD4$^+$ T cell–dependent GC reaction (25, 26). To dissect the contribution of CD4$^+$ T cells in the anti-porin IgM responses, MHC class II–deficient mice ($Iab^{-/-}$), which lack CD4$^+$ T cells but maintain a normal CD8 T cell compartment (44), were immunized in a prime/boost regimen and B cell responses against porins were evaluated. Indeed, not only IgG (Fig. 2C), but also high IgM serum titers (Fig. 2D), and persistence of splenic IgM ASCs (Fig. 2E) were strictly CD4$^+$ T cell dependent. Moreover, assessment of IgM binding to the surface of *S.* Typhi by flow cytometry (Fig. 2F) revealed that CD4$^+$ T cells significantly augmented IgM binding (Fig. 2G) and the ability to fix complement on the surface of *S.* Typhi (Fig. 2H). These data suggest that prime/boost vaccination with *S.* Typhi porins fostered not only sustained high-level IgM production, but also improved the effector function of these Abs.

To further substantiate that CD4$^+$ T cells are critical for the maintenance of anti-porin IgM, we depleted CD4$^+$ T cells either before (day 13) or after reexposure to the Ag (day 20; Supplemental Fig. 1A and 1B). We found that preboost depletion almost completely abolished the CD4$^+$ T cell–dependent elevation of serum IgM (Supplemental Fig. 1C) and the increase of splenic IgM ASCs (Supplemental Fig. 1D and 1E). Interestingly, postboost depletion reduced not only the levels of serum IgM (Supplemental Fig. 1F) and numbers of splenic IgM ASCs, but also almost completely blocked recruitment of IgM ASCs to the bone marrow (Supplemental Fig. 1G and 1H). Preboost CD4$^+$ T cell depletion did not have a profound effect on IgG serum titers or ASC accumulation in the spleen (Supplemental Fig. 1I–K). Taken together, CD4$^+$ T cells are critical for the sustenance of *S.* Typhi porin–specific IgM B cell responses in a prime/boost vaccination scheme.

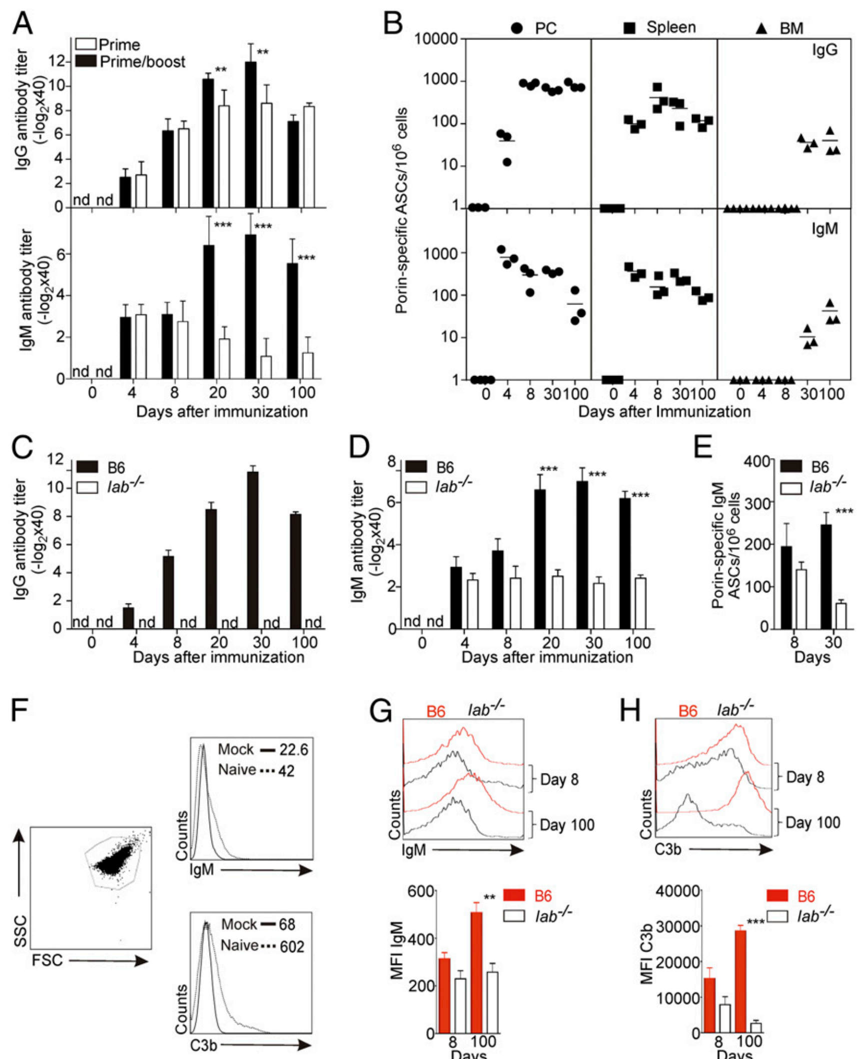

FIGURE 2. CD4$^+$ T cell–dependent maintenance of murine IgM responses against *S.* Typhi porins. (**A**) B6 mice were immunized i.p. once on day 0 (prime) or twice on days 0 and 15 (prime/boost) with 10 μg *S.* Typhi porins. IgG (*upper panel*) and IgM (*lower panel*) Ab titers as determined by ELISA at the indicated time points (mean ± SEM, n = 6–11 mice per time point). (**B**) Porin-specific IgG or IgM ASCs were enumerated by ELISPOT at the indicated time points in the prime/boost regimen. Data from one representative of three independent experiments with three mice per group; symbols represent values from individual mice; horizontal bars represent mean values. (**C**–**H**) B6 or $Iab^{-/-}$ mice were immunized in the prime/boost immunization protocol, and IgG (C) and IgM (D) Ab titers were measured by ELISA at the indicated time points. (E) Porin-specific IgM ASCs in spleens of B6 and $Iab^{-/-}$ mice on days 8 and 30 (mean ± SEM, representative data from one of three independent experiments, four mice per group). (F) Determination of serum IgM and C3b binding to *S.* Typhi by flow cytometry. Values indicate mean fluorescence intensity (MFI) from bacteria incubated with secondary reagent only (mock) or serum from Ag naive mice (naive). (G) Binding of IgM from B6 or $Iab^{-/-}$ mice at the indicated days. *Upper panel* shows representative histograms; *lower panel* shows mean MFI ± SEM. (H) C3b deposition on *S.* Typhi preincubated with serum from B6 or $Iab^{-/-}$ mice. *Upper panel* shows representative histograms; *lower panel* shows mean MFI ± SEM ($n \geq 5$ mice/group, pooled from two independent experiments). Statistical analysis was performed using Student t test (** p < 0.01, *** p < 0.001). nd, not detectable.

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CD4⁺ T cells maintain S. *Typhi porin–specific GC B cell responses*

CD4⁺ T cells support B cell differentiation at different levels (25). Because splenic MZ B cells can participate in CD4⁺ T cell–dependent (45) and –independent (46) Ab responses, we assessed first whether the absence of CD4⁺ T cells would affect the splenic $B220^+CD21^+CD23^{low/neg}$ MZ B cell population. As shown in Supplemental Fig. 2A, expansion and contraction of splenic MZ B cells after prime/boost immunization with *S.* Typhi porins was not altered in MHC class II–deficient animals. Moreover, the frequency of porin-specific IgM ASCs in sorted splenic MZ B cells (Supplemental Fig. 2B) and their total number per spleen (Supplemental Fig. 2C) were not affected by the absence of CD4⁺ T cells, indicating that MZ B cells did not contribute to long-term maintenance of IgM Ab responses after repeated vaccination with *S.* Typhi porins.

Next, we analyzed spleen sections of *S.* Typhi porin–immunized mice at different time points for evidence of GC formation. As shown in Fig. 3A, PNA-binding GCs in B cell areas were present already on day 8 after immunization. PNA⁺ GCs were still detectable on day 100 (Fig. 3A). Quantification of GC B cells using a combination of anti-B220, anti-FAS, and anti-GL7 Abs (Fig. 3B) revealed a substantial expansion of GC B cells after primary and secondary immunization until day 30 and a maintenance of high GC B cell numbers in spleen until day 100 (Fig. 3C). Importantly, immunization with proteinase K–digested porins failed to elicit a GC B cell response (Supplemental Fig. 2D), indicating that the presence of the native protein was required for the induction of the GC reaction. To determine the number of porin-specific GC B cells, we stained B cells with biotin-labeled porins, revealing that ~2% of IgM⁺ GC B cells bound the Ag on day 30 after immunization (Fig. 3D). Specificity of this staining was controlled by preincubation with an excess of unlabeled porins, followed by labeling with biotinylated porins (Fig. 3D, Ctrl). Enumeration of porin-specific cells revealed that ~10,000 cells, that is, 0.02% of total splenic B cells, were Ag-specific IgM⁺ GC B cells at the maximal expansion on day 30 (Fig. 3E). Moreover, we found that repeated immunization enhanced expansion of GC B cells (Fig. 3F and 3G) and porin-specific IgM⁺ GC B cells (Fig. 3H) on day 30 by ~3-fold. Moreover, preboost depletion revealed that the increase in GC B cells (Fig. 3F and 3G) and porin-specific IgM⁺ GC B cells (Fig. 3H) induced by the booster immunization was almost completely dependent on the presence of CD4⁺ cells. Likewise, repeated immunization significantly increased the frequency of porin-specific IgM ASCs in sorted splenic GC B cells (Supplemental Fig. 2E) and their total number per spleen (Supplemental Fig. 2F). Taken

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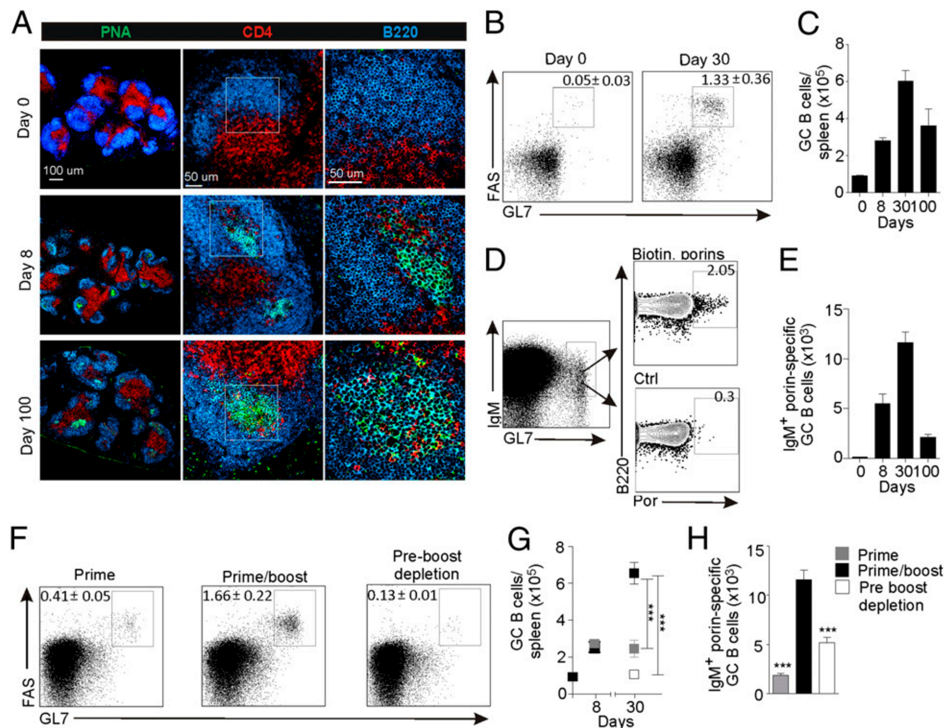

FIGURE 3. Induction of a sustained *S.* Typhi porin–specific GC reaction after vaccination in the prime/boost scheme. (**A**) Immunofluorescence in situ analysis of GCs at different time points using the indicated staining. Representative spleen sections from one of three mice analyzed per time point. (**B**) Detection of $B220^+FAS^+GL7^+$ GC B cells by flow cytometry at the indicated time points after vaccination. Plots show representative staining from one of four mice per time point; values indicate mean percentage ± SEM of GC B cells in spleen ($n = 8$ mice, pooled data from two independent experiments). (**C**) Number of splenic GC B cells at different time points after immunization (mean ± SEM, $n = 8$ mice, pooled data from two independent experiments). (**D**) Representative dot plots showing enumeration of *S.* Typhi porin–specific IgM⁺ GC B cells by flow cytometry using incubation with biotinylated porins. Control stains included preincubation with an excess of unlabeled porins before staining with biotinylated porins. Values indicate percentage of porin-specific cells in IgM⁺ GC B cells with values from control stains subtracted. (**E**) Total numbers of porin-specific IgM⁺ GC B cells per spleen at the indicated time points (mean ± SEM, $n = 9$ mice, pooled from three independent experiments). (**F**) Assessment of splenic $B220^+FAS^+GL7^+$ GC B cell expansion on day 30 in mice receiving either the *S.* Typhi porin prime, prime/boost vaccination, or prime/boost vaccination with Ab-mediated CD4⁺ T cell depletion on day 13. Plots show representative staining from one of four mice per time point; values indicate mean percentage ± SEM of GC B cells in spleen ($n = 8$ mice, pooled data from two independent experiments). (**G**) Number of splenic GC B cells at different time points after immunization in prime, prime/boost, and prime/boost/CD4⁺ T cell depletion conditions (mean ± SEM, $n = 8$ mice, pooled data from two independent experiments). (**H**) Total numbers of porin-specific IgM⁺ GC B cells per spleen at day 30 after immunization (mean ± SEM, $n = 9$ mice, pooled from three independent experiments). Statistical analyses in (G) and (H) were performed using one-way ANOVA with Tukey's post analysis (***$p < 0.001$).

together, these data show that repeated exposure to *S.* Typhi porins induced a CD4$^+$ T cell–dependent GC reaction that facilitated the maintenance of IgM$^+$ B cell memory and supported long-term production of IgM.

IFN-γ–dependent maintenance of S. *Typhi porin–specific B cell memory*

IFN-γ is a crucial protective cytokine during *Salmonella* infections of mice and humans (38). Moreover, T cells from *S.* Typhi porin–vaccinated individuals produce IFN-γ after in vitro restimulation (17). Likewise, CD4$^+$ T cells from porin-immunized mice secreted substantial amounts of IFN-γ after in vitro restimulation with the whole protein (Supplemental Fig. 3A). To make porin-specific CD4$^+$ T cells amenable to more detailed analysis, we tested libraries containing overlapping *S.* Typhi OmpC and OmpF peptides for their ability to induce IFN-γ secretion in T cells derived from porin-vaccinated mice. We found five peptides that detected CD4$^+$ IFN-γ–producing cells after ex vivo restimulation (Supplemental Fig. 3B and 3C). For the subsequent experiments, the combination of the two immunodominant $OmpF_{201–215}$ and $OmpC_{241–255}$ peptides was used to further characterize porin-specific CD4$^+$ T cell responses. Using this approach, we found that the prime/boost regimen significantly enhanced the expansion of porin-specific CD4$^+$ T cells (Fig. 4A and 4B). Moreover, ∼25% of porin-specific CD4$^+$ T cells from mice that were immunized once with porins exhibited properties of Tfh cells; that is, they coexpressed CXCR5 and PD1 (Fig. 4A and 4C). Importantly, repeated vaccination with porins significantly increased the expansion of CD4$^+$ CXCR5$^+$PD1$^+$ T cells that were specific for *S.* Typhi porin peptides (Fig. 4A and 4C). To confirm that porin-specific CD4$^+$ T cells expressing CXCR5 and PD1 were indeed Tfh cells, we determined expression of the transcription factor BCL-6 by intracellular staining. As shown in Fig. 4D, only those porin-specific CD4$^+$ T cells that exhibited the Tfh cell signature were BCL-6$^+$, under conditions of both single and repeated Ag application. This finding suggests that vaccination with *S.* Typhi porins immediately programs CD4$^+$ to become Th1-type Tfh cells and that prolonged Ag exposure drives more cells into the pathway of Tfh differentiation.

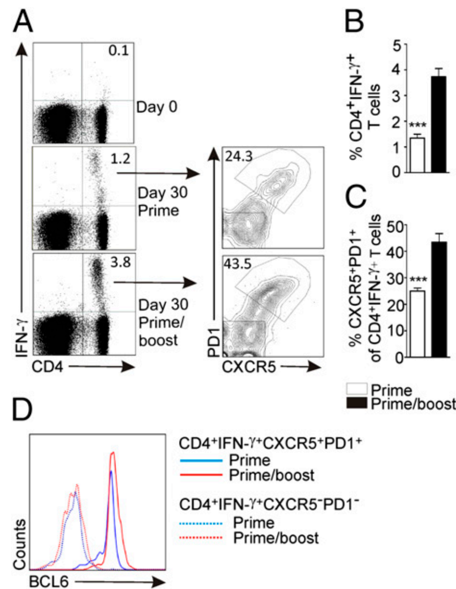

FIGURE 4. Tfh cell responses induced by *S.* Typhi porins. B6 mice were i.p. vaccinated in the prime or prime/boost regimens, and splenic CD4$^+$ T cell responses were analyzed on day 30. (**A**) Representative dot plots showing intracellular IFN-γ staining in CD4$^+$ T cells (*left panels*) and corresponding analysis of Tfh cell signature marker (CXCR5 and PD1) expression on IFN-γ–producing cells shown in the right plots. Values in dot plots indicate percent of IFN-γ$^+$ cells of CD4$^+$ T cells; values in contour plots indicate Tfh signature-positive cells of IFN-γ–producing CD4$^+$ T cells. (**B**) Percentage of *S.* Typhi porin–specific, IFN-γ–secreting CD4$^+$ porins T cells and (**C**) percentage of Tfh signature–positive, IFN-γ–producing CD4$^+$ T cells (mean ± SEM, $n = 9$ mice, pooled from three independent experiments). (**D**) FACS analysis of BCL-6 expression in the indicated porin-specific CD4$^+$ T cell populations on day 30 either in the prime (blue lines) or prime/boost (red lines) schemes; one representative plot of three is shown for each condition. Statistical analyses were performed using Student *t* test (***$p < 0.001$).

Tfh cells exhibit a high plasticity shown by the fact that they can produce different combinations of cytokines that support distinct B cells responses (32). Ex vivo restimulation assays of purified splenic CD4$^+$ T cells from porin-immunized mice with OmpC/OmpF peptide-pulsed DCs revealed that indeed IFN-γ was the dominant cytokine produced in these cultures. Moreover, porin-specific CD4$^+$ T cells responded as well with moderate IL-17A production, but did not produce IL-21, IL-2, IL-4, IL-13, or IL-23 (Supplemental Fig. 3D). To assess the extent to which IFN-γ or IL-17A contribute to the porin-specific B cell memory, we immunized mice deficient for the IFN-γR or the IL-17RA with porins in the prime/boost scheme. Genetic ablation of the IFN-γR resulted in stronger impairment of long-term serum IgM and IgG production (Fig. 5A) and generation of splenic IgM and IgG ASCs (Fig. 5B). Moreover, the IFN-γ pathway mediated strong expansion of GC B cells (Fig. 5C and 5D) and efficient production of porin-specific IgM$^+$ GC B cells (Fig. 5E). Importantly, IL-17RA signaling as well contributed to the induction and maintenance of anti-porin IgM and IgG responses (Fig. 5A and 5B), whereas IL-21R deficiency did not affect the B cell response against this Ag (data not shown). Taken together, these data indicate that IFN-γ is the dominant cytokine for the generation of T cell–dependent B cell responses against *S.* Typhi porins.

Mutated anti-porin IgM exerts effector functions against S. *Typhi*

To assess whether the CD4$^+$ cell–dependent GC reaction generated mutated IgM, we fused splenic B cells with myeloma cells on day 40 of the prime/boost scheme. Interestingly, >75% of the hybridomas (40/49) produced IgM. Those two IgM-producing hybridoma clones that showed highest binding in the *S.* Typhi porin ELISA (Supplemental Fig. 3E) were chosen for further analysis. Sequencing of the BCR region revealed that both clones have accumulated several mutations in the framework and complementary-determining regions (Supplemental Fig. 3F). Moreover, purified IgM Abs from both clones bound to the surface of *S.* Typhi (Fig. 6A) and led to complement fixation on the bacteria (Fig. 6B). Notably, clone 11-6B, which showed higher complement fixation in vitro (Fig. 6B), also exhibited higher bactericidal activity in vitro (Fig. 6C). Next, we assessed whether porin-specific IgM mAbs enhance bacterial clearance in vivo. To this end, 10^6 CFUs virulent *S.* Typhi was preopsonized with anti-porin IgM mAbs or with highly bactericidal anti-porin serum and subsequently applied to naive B6 mice. Assessment of bacterial content in spleens (Fig. 6D) and livers (Fig. 6E) after 24 h revealed that opsonization with the 11-6B monoclonal IgM Ab facilitated efficient removal of the bacteria from infected tissues. Thus, mutated IgM Abs that exert highly efficient complement fixation on the surface of *S.* Typhi and mediate bacterial lysis in vitro can provide rapid in vivo clearance of the pathogen.

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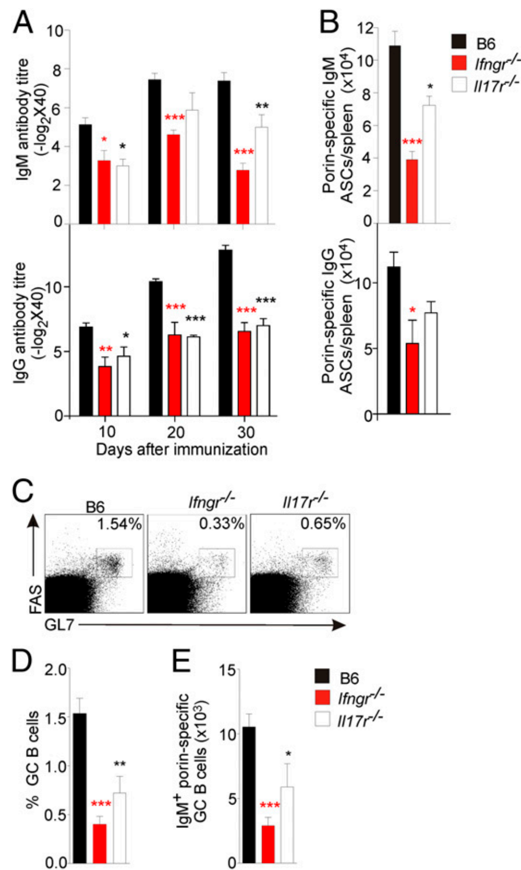

FIGURE 5. Contribution of different cytokines to the maintenance of the *S.* Typhi porin–specific IgM B cell response. Anti-porin IgM and IgG serum responses at the indicated time points (**A**) and porin-specific IgM and IgG ASCs per spleen at day 30 (**B**) were measured in the indicated mouse strains vaccinated in the prime/boost scheme (mean ± SEM, $n = 6$ mice per group and time point). (**C**–**E**) GC B cell responses were determined on day 30 in the prime/boost regimen. Representative dot plots with values showing percentage of GC B cells in spleens (C) and summary of GC B cell expansion (D) in the indicated mouse strains (mean percentage ± SEM, $n = 8$ mice, pooled data from two independent experiments). (E) Total numbers of porin-specific IgM$^+$ GC B cells per spleen in the indicated mouse strain (mean ± SEM, $n = 8$ mice, pooled data from two independent experiments). Statistical analyses were performed using one-way ANOVA with Tukey's post analysis (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). nd, not detectable.

Discussion

Memory B cells generating neutralizing Abs against specific pathogens in humans can persist for decades, as shown in survivors of the 1918 H1N1 influenza virus pandemic (47). Interestingly, human IgM$^+$ memory B cells can produce neutralizing Abs against existing and extinct influenza virus species, thus providing efficient cross-protection (48). Notably, the major proportion of CD27$^+$ memory B cells in human blood expresses IgM (49), and BCL-6 mutation analysis suggests that these cells are derived from a T cell–dependent GC reaction (50). Together with these previous studies, the data presented in this article suggest that vaccination with *S.* Typhi porins may also elicit such persisting IgM B cell memory responses in humans. Unfortunately, consecutive PBL samples from the *S.* Typhi porin vaccination study participants had not been acquired, thus prohibiting detailed cellular and molecular analysis of specific anti-porin B cell responses in humans at this time point. Certainly, future clinical trials with *S.* Typhi subunit vaccines or attenuated strains should consider acquisition and

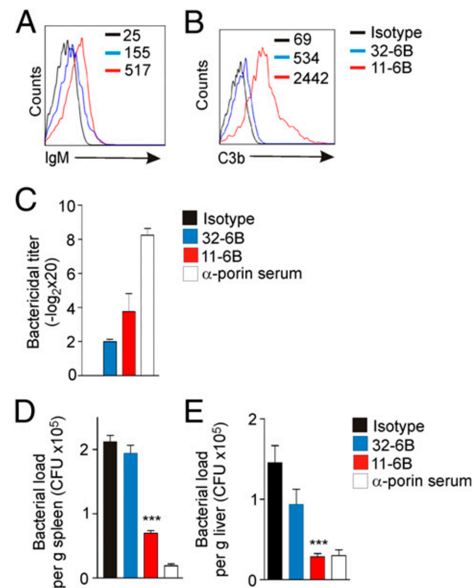

FIGURE 6. Mutated IgM mAbs against *S.* Typhi porins exhibit bactericidal capacity in vivo and in vitro. *S.* Typhi was incubated with IgM antiporins 32-6B and 11-6B mAbs or isotype control mAb recognizing an irrelevant Ag. (**A**) IgM and (**B**) C3b deposition on the bacteria was determined by flow cytometry. Values indicate mean fluorescence intensity for the indicated Ab; one representative of two independent experiments. (**C**) In vitro bactericidal capacity of anti-porin mAbs against *S.* Typhi was compared with isotype control and anti-porin hyperimmune serum. Bactericidal titers represent the lowest dilution at which 50% killing of bacteria was observed (mean ± SD, samples were tested in duplicates in two independent experiments). (**D** and **E**) *S.* Typhi that was opsonized with 32-6B, 11-6B, isotype, or heat-inactivated anti-porin serum and B6 mice were i.v. infected with 10^6 CFUs. Bacterial loads in spleen (D) and liver (E) were determined 24 h postinfection (mean ± SEM values of pooled data from two independent experiments, $n = 8$ mice). Statistical analyses were performed using one-way ANOVA with Tukey's post analysis (***$p < 0.001$).

storage of such samples for analysis of this interesting aspect of human B cell biology.

The major mechanisms that warrant the maintenance of high levels of protective Abs in serum include: 1) long-term survival and activity of Ab-producing plasma cells in specific bone marrow niches (51); 2) unspecific, mainly TLR-mediated stimulation of memory B cells to differentiate into short-lived plasma cells (52); and 3) a persisting, CD4$^+$ T cell–dependent GC reaction that depends on Ag deposition on follicular DCs (53). Our data suggest that the third scenario best describes generation of B cell memory during *S.* Typhi porin immunization of mice: porins rapidly elicit a GC reaction that produces initially mainly IgG memory and plasma cells in a strictly CD4$^+$ T cell–dependent fashion. Notably, the initial IgM response was CD4$^+$ T cell independent and promoted by extrafollicular MZ B cells. Because porins are hydrophobic and very stable proteins, it is likely that these proteins can be trapped by follicular DCs in GCs as Ab–Ag complexes and are displayed to B cells for a prolonged period once specific Abs had been generated. Thus, repeated Ag exposure, that is, the second immunization on day 15, probably further fostered the GC reaction through increased Ag deposition, leading to a >5-fold elevation in porin-specific IgM$^+$ GC B cells and a roughly 3-fold increase in porin-specific, IgM-secreting B cells. It has been suggested that such B cell memory diversification is determined by the nature of the Ag with particulate Ag driving both IgG and IgM memory from persisting GCs, whereas soluble protein Ags do

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not induce a persisting GC reaction, and hence generate mainly IgG memory (27). The results from the present *S.* Typhi porin immunization study suggest a remarkably simple regulation of B cell memory diversity in response to soluble porin proteins, namely, that repeated Ag application with provision of Ag for an ongoing GC reaction is a dominant factor in this process. Indeed, repeated application of soluble subunit vaccines would ideally mimic persisting low-level infections that are known to efficiently broaden B cell memory responses in humans (54). Importantly, an increase in B cell memory diversity further supports resistance of the host against infection through the generation of Abs against potential escape mutants (55). Thus, it is likely that constant Ag supply by persisting *Salmonella* drives increasing heterogeneity of B cells to better cope with the infection and to preempt appearance of new variants.

Studies on primary immunodeficiencies in humans have revealed an almost exclusive role of the Th1 T cell pathway for the defense against intracellular bacteria such as *Salmonella enterica* serovars (38). These findings were extended in murine studies that showed that lack of the Th1 T cell master transcription factor T-bet enhanced susceptibility to *Salmonella* infection (56). Moreover, development of optimal Th1 responses against *S.* Typhimurium with maximal expansion of IFN-γ–secreting $CD4^+$ T cells and high IgG titers in serum requires exposure to the pathogen of at least 14 d (57). Interestingly, in this setting of antibiotic treatment–mediated abrogation of *Salmonella* growth in vivo, IgM serum responses against whole bacteria were not influenced by the variation in Ag exposure (57), indicating that application of the well-defined *Salmonella* porin subunit vaccine permits optimization of all protective immune mechanisms with high IgG responses and boosting of Th1 $CD4^+$ T cells and GC IgM B cells. Moreover, our data indicate that repeated exposure to *S.* Typhi porins elicited a focusing of the $CD4^+$ T cell population on a Th1-biased Tfh cell response. The absence of IL-21 in the supernatants of $CD4^+$ splenocytes with porin peptide-pulsed DCs and the lack of significantly altered anti-porin Ab responses in *Il21r*-deficient animals suggest that this cytokine does not play a role in the IgM GC B cell response. This finding is in line with a previous study showing that IL-21 is not required for the protective Ab response against *Salmonella* infection (58). However, IL-21 may provide critical support for IgM to IgG Ig switch during infection with other Gram-negative bacteria such as *Ehrlichia muris* (59), suggesting that these bacteria imprint a different cytokine pattern during infection.

It has been shown that IL-17–producing Tfh cells are crucial for the production of high-affinity T cell–dependent IgA in Peyer's patches (60). Moreover, both IFN-γ and IL-17 are important for the generation and maintenance of GCs during autoimmune reactions (61). Our findings revealed that *S.* Typhi porins induce not only IFN-γ, but also an IL-17A response in $CD4^+$ T cells, and that both *Ifngr*$^-$ *and Il17ra* deficiency impacts on GC and anti-porin Ab generation. Thus, it is possible that IFN-γ and IL-17 exhibit synergistic and/or overlapping effects in the GC reaction. Clearly, further investigation is needed to clarify potential interactions between porin-specific Th1- and Th17-like Tfh cells on B cell memory formation.

In conclusion, our study shows that vaccination with the subunit vaccine *S.* Typhi porins efficiently recapitulates the Th1 T cell differentiation pathway that is typical for *Salmonella* infection. Moreover, it is likely that porin-specific type I Tfh cells support the generation of mutated IgM^+ memory B cells that contribute to the long-term production of bactericidal IgM Abs. Future clinical studies using porins as a subunit vaccine against typhoid fever should thus aim to optimize the induction of bactericidal memory IgM.

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Disclosures

The authors have no financial conflicts of interest.

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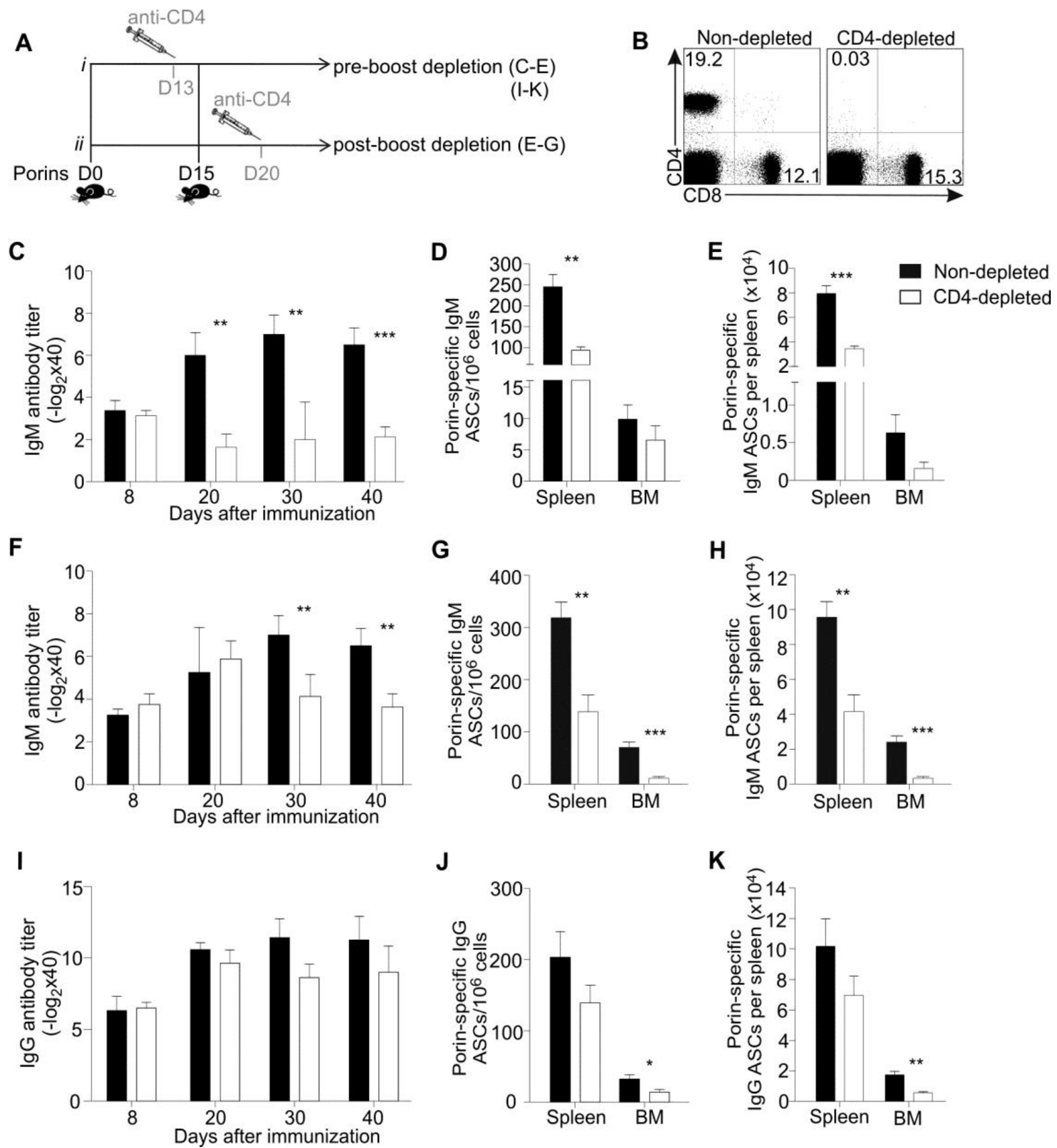
Supplementary information

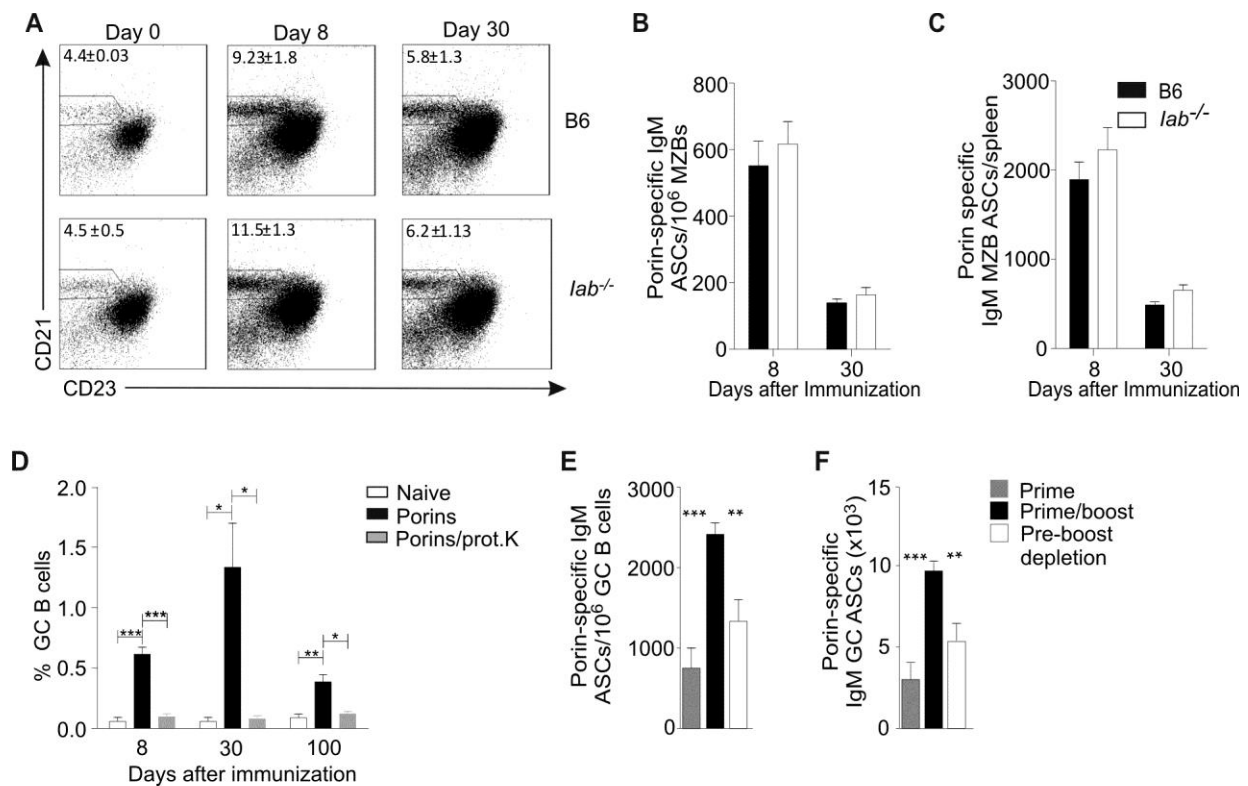
Supplementary Table 1. Demographic details on vaccinated and control subjects.

Patient group	Treatment [a]	N	Gender	Mean age ±SEM (years)
Porins	10 µg *S*. Typhi porins s.c.	5	Male	51.20±12.9
Placebo	Isotonic salt solution	2	Male	46±9.8
Control	None	20	Male	42±11.3

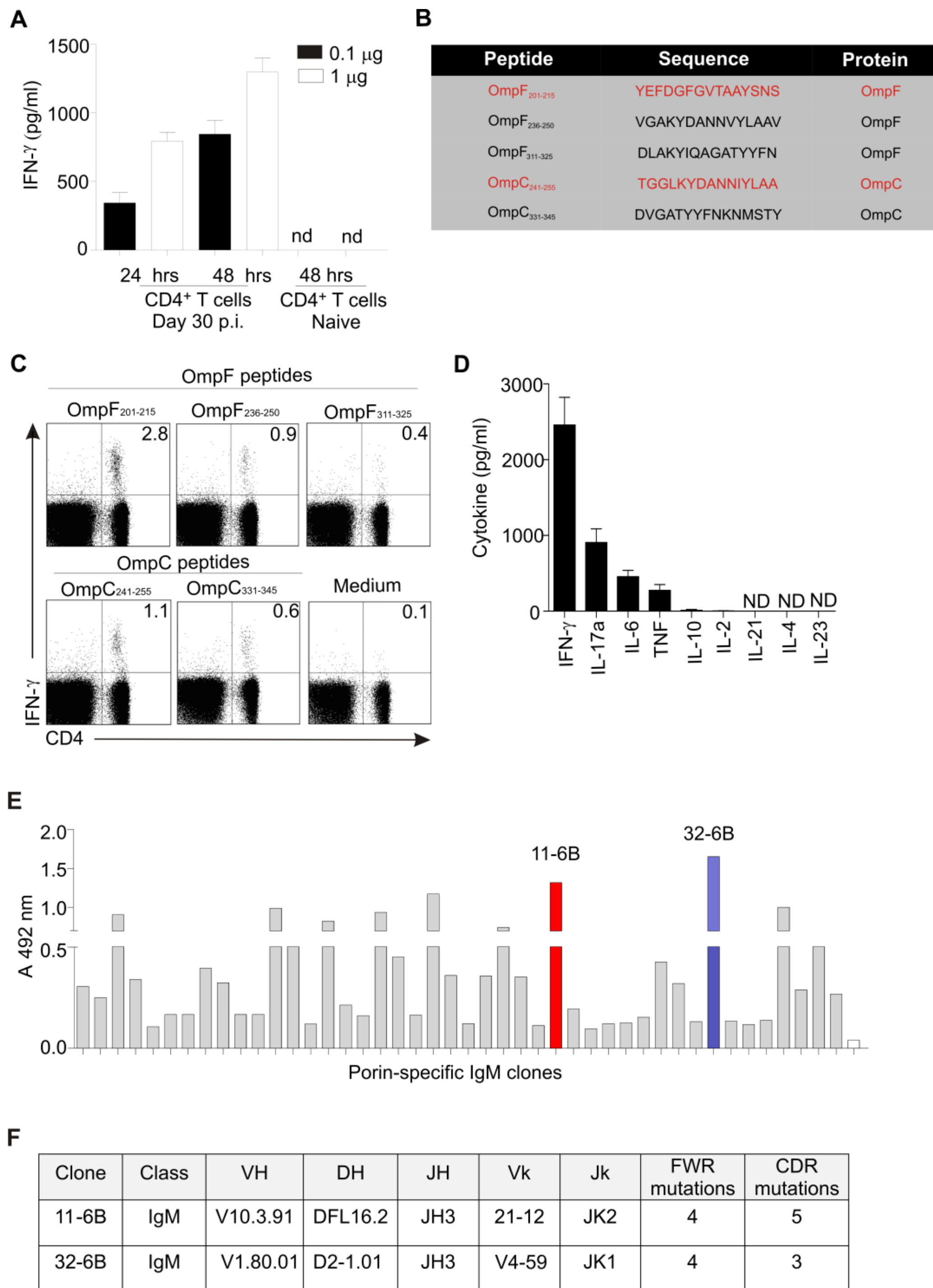
[a] As described in Salazar-Gonzalez et al. 2004. *Immunol. Lett.* 93: 115-122.



Supplementary Figure 1. $CD4^+$ T cells enhance and sustain anti-porin IgM antibody reponses. (A) Schematic representation of $CD4^+$ T cell depletion strategies: application of 0.5 mg i.p. every third day starting either starting either (i) on day 13 (results in C-E and I-K) or (ii) day 20 (results in F-H) of the prime/boost porin vaccination. (B) Representative dot plots from blood sample showing frequencies of $CD4^+$ and $CD8^+$ T cells before and 3 days after administration of $CD4^+$ T cell-depleting antibodies. (C,F) Serum IgM or (I) IgG antibody titers were measured by ELISA at the indicated time points after immunization. Frequency (D,G or J) and total numbers (E,H or K) of splenic and bone marrow porin-specific, IgM or IgG-producing ASCs were determined 20 days after starting the $CD4^+$ T cell depletion by ELISPOT, i.e. day 33 for pre-boost and day 40 for post-boost depletion. Data from one independent experiment out of three with n = 3 mice per group. Statistical analysis was performed using Student's *t* test (*, $p< 0.05$; **, $p< 0.01$, ***, $p<0.001$).



Supplementary Figure 2. Marginal zone B cells contribute to the T cell-independent IgM antibody response induced by *S.* Typhi porins. (A) Frequencies of $B220^{+}CD21^{+}CD23^{low/neg}$ marginal zone (MZ) B cells in B6 and $CD4^{+}$ T cell-deficient *Iab*$^{-/-}$ mice in spleens were determined at the indicated time points by flow cytometry (mean ± SEM, n=4 mice per group from one representative of three experiments). Frequencies (B) and total numbers (C) of porin-specific, IgM-secreting ASCs were determined by ELISPOT using FACS-sorted MZ B cells from the indicated time points after vaccination (mean ± SEM, n=6 mice with pooled data from two independent experiments). (D) Percentage of $B220^{+}FAS^{+}GL7^{+}$ GC B cells in B6 mice immunized with porins or proteinase K-digested porins (Porins/prot.K) as determined by flow cytometry at the indicated time points after prime/boost vaccination (mean ± SEM, n≥4 mice per group and time point, pooled from two independent experiments). Frequencies (E) and total numbers (F) of porin-binding IgM^{+} GC B cells as determined by flow cytometry on day 30 after vaccination either in prime, prime/boost or prime/boost regimen with pre-boost antibody depletion of $CD4^{+}$ T cells (mean ± SEM, values pooled data from 6 mice from two independent experiments). Statistical analyses in D-F were performed using one way ANOVA with Tukey's post analysis (*, p<0.05; **, p<0.01; ***, *p*<0.001).



Peptide	Sequence	Protein
$OmpF_{201-215}$	YEFDGFGVTAAYSNS	OmpF
$OmpF_{236-250}$	VGAKYDANNVYLAAV	OmpF
$OmpF_{311-325}$	DLAKYIQAGATYYFN	OmpF
$OmpC_{241-255}$	TGGLKYDANNIYLAA	OmpC
$OmpC_{331-345}$	DVGATYYFNKNMSTY	OmpC

Clone	Class	VH	DH	JH	Vk	Jk	FWR mutations	CDR mutations
11-6B	IgM	V10.3.91	DFL16.2	JH3	21-12	JK2	4	5
32-6B	IgM	V1.80.01	D2-1.01	JH3	V4-59	JK1	4	3

Supplementary Figure 3. Assessment of anti-porin CD4^{+} T cell response, peptide library screen and generation of anti-*S.* Typhi porins B cell hybridomas (A) Determination of IFN-γ production by sorted splenic CD4^{+} T cells from porin-vaccinated or naïve B6 mice co-cultured with dendritic cells pulsed with 0.1 µg or 1 µg of porins. Supernatants were collected after 24 h and 48 h and the concentration of IFN-γ was measured by ELISA (mean ± SEM, n = 4 mice). (B and C) Fifteen-mer peptides with 5 amino acid overlap were designed from OmpF or OmpC sequences. 71 peptides derived from OmpF and the 74 peptides derived from OmpC were tested for their capacity to induce IFN-γ production in CD4^{+} T cells derived from splenocytes at day 30 post immunization.

Representative FACS plots showing intracellular cytokine staining following restimulation with the indicated peptides. Values in upper right quadrant indicate percentage of IFN-γ^+ $CD4^+$ T cells. Sequences details of those peptides that induced specific IFN-γ responses, immunodominant peptides used for further analysis are highlighted in red. (D) Determination of cytokine production by sorted splenic $CD4^+$ T cells co-cultured with dendritic cells pulsed with $OmpF_{201-215}$ and $OmpC_{241-255}$ peptides. Supernatants were collected after 24 h and the concentration of IL-21, IFN-γ and IL-17A, IL-6, TNF, IL-10, IL-2, IL-4 or IL-23 was measured by ELISA or CBA (mean ± SEM, n = 6 mice). (E and F) B cell hybridomes generation.B6 mice were immunized i.p. on day 0 and boosted on day 15 with 10 µg of the *S. typhi* porins. Splenocytes were obtained at day 40 after first immunization and were fused with P3x63Ag8.653 myeloma cells. (E) Porin-binding of the 40 IgM-producing hybridomas as determined by ELISA. Monoclonal antibodies 11-6B (red) and 32-6B (blue) that exhibited the highest binding efficacy were used for further experiments. (F) Sequence analysis of the recombined IgM locus of 11-6B and 32-6B hybridomas.