Cutting Edge: Role of B Lymphocytes in Protective Immunity Against Salmonella typhimurium Infection

Hans-Willi Mittrücker, Bärbel Raupach, Anne Köhler and Stefan H. E. Kaufmann

*J Immunol* 2000; 164:1648-1652; doi: 10.4049/jimmunol.164.4.1648
http://www.jimmunol.org/content/164/4/1648

**References**
This article cites 40 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/164/4/1648.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Role of B Lymphocytes in Protective Immunity Against Salmonella typhimurium Infection

Hans-Willi Mittrücker,1 Bärbel Raupach, Anne Köhler, and Stefan H. E. Kaufmann

Infection of mice with Salmonella typhimurium gives rise to a disease similar to human typhoid fever caused by S. typhi. Since S. typhimurium is a facultative intracellular bacterium, the requirement of B cells in the immune response against S. typhimurium is a longstanding matter of debate. By infecting mice on a susceptible background and deficient in B cells (Igμ−/− mice) with different strains of S. typhimurium, we could for the first time formally clarify the role of B cells in the response against S. typhimurium. Compared with Igμ+/+ mice, LD50 values in Igμ−/− mice were reduced during primary, and particularly secondary, oral infection with virulent S. typhimurium. After systemic infection, Igμ−/− mice cleared attenuated aroA− S. typhimurium, but vaccine-induced protection against systemic infection with virulent S. typhimurium involved both B cell-dependent and -independent effector mechanisms. Thus, B cell-mediated immunity plays a distinct role in control of S. typhimurium in susceptible mice. The Journal of Immunology, 2000, 164: 1648–1652.

Salmonella enterica serotype Typhimurium (Salmonella typhimurium) is the causative agent of murine typhoid fever. After oral uptake, S. typhimurium trespasses the intestinal epithelium via M cells and enters the Peyer’s patches. From there, bacteria spread via mesenteric lymph nodes to spleen and liver, where they replicate. The initial phase of infection is characterized by the production of inflammatory cytokines and the activation of phagocytes (1). Laboratory mouse strains differ widely in their susceptibility to S. typhimurium infection and at least four gene loci control the level of innate susceptibility (2). One of these genes codes for Nramp1, a transmembrane protein of unknown function that is structurally related to metal ion channels (3). The expression of a functional Nramp1 molecule (Nramp1+) in phagocytes is a critical component during the early phases of the anti-Salmonella response, since mouse strains with a natural mutation of Nramp1 (Nramp1−) fail to adequately restrict initial multiplication of S. typhimurium (3).

Salmonella infection is eventually controlled by the acquisition of specific immunity. Both T and B lymphocytes are thought to participate in the clearance of bacteria and in protection against secondary infection but the exact function and the importance of B and T cell-mediated immunity for this process is still a matter of debate (1, 2). Data on the role of Abs are mainly derived from experiments in which serum from pre-exposed mice was transferred to naive animals which were subsequently infected with S. typhimurium or S. enteritidis. After transfer of salmonella-immune serum, some laboratories observed profound protection against challenge infection whereas others did not (4–13). Eisenstein et al. (14) demonstrated that serum transfer protected inherently resistant mice but not congenic mouse strains with defects in different susceptibility genes. This observation suggests that the degree of Ab-mediated protection depends on different susceptibility genes. This notion offers an explanation for the inconsistent results regarding Ab-mediated protection (2, 14).

The use of attenuated bacteria allows analysis of the immune response against S. typhimurium in susceptible mice, particularly from the Nramp1+ C57BL/6 background, in which most of the gene-deficient mice are currently available. Depending on the degree of bacterial attenuation, Nramp1+ mice control infection and are protected against reinfection with wild-type S. typhimurium (15–18). Experiments with gene-deficient mice demonstrated that control of primary infection with an attenuated S. typhimurium strain (aroA−) strictly depends on CD4+ T lymphocytes (19). Furthermore, in vivo depletion of CD4+ or CD8+ T cells reduces protection of immune mice against reinfection with wild-type S. typhimurium (15). Transfer of either serum or lymphocytes from protected mice to Nramp1+ recipients only marginally protects against oral infection with wild-type S. typhimurium, and simultaneous transfer of both serum and T lymphocytes is necessary for significant protection of recipients (6). The availability of Igμ−/− knockout mice has made it possible to determine the role of B cells in immunity against S. typhimurium. These mice lack all B lymphocytes. Because Ab secretion is an exclusive and major function of B lymphocytes, these animals are also devoid of Abs. Yet, B cells perform additional functions such as Ag presentation.

In the present study, we analyzed the role of B cells in protection against S. typhimurium using B cell-deficient Igμ−/− mouse mutants of susceptible background. Our experiments establish that protective immunity to S. typhimurium strongly, though not exclusively, depends on B cells.
Materials and Methods

Mouse strains in this study were C57BL/6 and Igμ−/− mice (20), backcrossed eight times onto the C57BL/6 background. Nramp1 genotype of individual Igμ−/− mice was analyzed by PCR (21).

S. typhimurium strains used were SL1344 (wild-type, rpsL, hisG) and SL7207 (aroA−) (22). Bacteria were grown overnight in Luria Bertani (LB) medium, washed twice in PBS, frozen, and stored at −80°C. Aliquots were thawed and bacterial titers were determined by plating serial dilutions. Bacteria were appropriately diluted and injected into the lateral tail vein of mice in 200 μl of PBS. For per os (p.o.) infection, S. typhimurium SL1344 was grown in LB medium containing 0.3 M NaCl without agitation overnight. Bacteria were washed twice in PBS, bacterial density was determined by absorption at 600 nm, and bacteria were appropriately diluted in PBS. Mice were starved overnight and bacteria were applied in a total volume of 200 μl of PBS by gastric intubation. Mice were vaccinated by i.v. injection of 5 × 10^5 S. typhimurium SL7207. Forty days after immunization, mice received two s.c. injections of 10 mg of ampicillin within 1 wk. Mice were challenged at least 50 days after primary infection as indicated. Alternatively, mice were vaccinated by i.v. injection of 5 × 10^4 S. typhimurium SL7207 and challenged 3 wk later by i.v. injection of wild-type salmonellae (1 × 10^4). Survival of mice was recorded daily and is given as percentage of live animals per time point. Bacterial burden was determined by plating serial dilutions of homogenized organs on LB plates. Statistical significance of results was determined with the statistic program included in the GraphPad Prism program (version 2.0; GraphPad, San Diego, CA). Survival curves were analyzed with the log rank test. LD50 values were determined with the method described by Reed and Muench (23).

Results

Targeted mutation in the Igμ-chain leads to a lack of mature B cells and complete absence of Igσ (20). Igμ−/− mouse mutants were generated from Sv129-derived embryonic stem cells and backcrossed onto the C57BL/6 background. Because C57BL/6 and Sv129 strains differ in their Nramp1 genotype, tail DNA of individual Igμ−/− mice was analyzed for Nramp1 by PCR and all mice analyzed were found to have the Nramp1 stimulated by i.v. injection of 5 × 10^4 SL7207 protected Igμ+/− mice from infection (Fig. 1A). Both strains succumbed to infection with 100 bacteria within 5–9 days.

Since typhoid fever is a food-borne disease, the natural way of infection is via oral uptake. In C57BL/6 mice, LD50 values for i.v. infection with S. typhimurium are <10 bacteria, but 10^4–10^5 bacteria for oral infection (unpublished result), indicating that efficient mechanisms restrict bacterial spreading from the intestine to the bloodstream. To analyze the role of Abs in this process, Igμ+/− and Igμ−/− mice were infected p.o. with different doses of S. typhimurium SL1344 and survival was recorded daily (Fig. 1B). Some of the Igμ+/− mice died after oral infection with 10^4 and 10^5 salmonellae, but none of these mice succumbed to an infection of 10^3 bacteria. In contrast, in the experiment depicted in Fig. 1B, all Igμ−/− mice died within 16 days, independent of the dose of infection. In all experiments, we observed at least a 10-fold lower LD50 value for Igμ−/− mice after primary oral infection with wild-type bacteria.

To analyze the function of Abs during systemic infection, Igμ−/− and Igμ+/− mice were infected with attenuated S. typhimurium SL7207. As a result of a block in the aromatic synthesis pathway (aroA−) and the low abundance of required metabolites in mammalian tissues, the growth of S. typhimurium SL7207 is restricted in infected animals (22). Mice were i.v. infected with 5 × 10^3 bacteria of S. typhimurium SL7207 and bacterial burden in liver and spleen was determined (Fig. 2). Both Igμ−/− and Igμ+/− mice controlled bacterial growth and at day 42 of infection, most of the mice had cleared the bacteria. Both Igμ+/− and Igμ−/− mice showed similar bacterial titers in livers at all time points analyzed. Spleens of Igμ−/− mice had slightly lower bacterial loads compared with spleens of wild-type mice.

In C57BL/6 mice, the efficacious immune response against attenuated S. typhimurium strains gives rise to protection against subsequent oral challenge with doses of wild-type S. typhimurium that are fatal for nonvaccinated animals (16). We therefore assessed whether vaccination with attenuated S. typhimurium SL7207 protected Igμ−/− mice against oral infection with virulent S. typhimurium SL1344. Vaccinated mice were infected p.o. with different doses of S. typhimurium SL1344 organisms including doses of up to 10,000-fold of the LD50 value for Igμ+/− mice.

---

2 Abbreviations used in this paper: LB, Luria Bertani; p.o., per os.
of three and two independent experiments, respectively. Ig\textsubscript{m} mice; Ig\textsubscript{m} Ig\textsubscript{m} (Fig. 3A) S. typhimurium were at least 1000-fold less well protected. Doses of S. typhimurium infection is significantly different (p < 0.05) but there is no significant difference between vaccinated Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsubscript{−/−} mice (p = 0.076). B. Groups consisted of 7 to 9 naive mice. Survival curves of vaccinated Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsubscript{−/−} mice are significantly different (p = 0.0042). Experiments in A and B are representative of three and two independent experiments, respectively. Ig\textsubscript{m} \textsuperscript{−/−} naive, ○; Ig\textsubscript{m} \textsuperscript{+/+} vaccinated, □; Ig\textsubscript{m} \textsuperscript{−/−} naive, ◆; and Ig\textsubscript{m} \textsuperscript{−/−} vaccinated, ▼. Vaccination with the aroA\textsuperscript{−} strain induced protection in Ig\textsubscript{m} \textsuperscript{+/+} mice and some of these mice survived even challenge infection with 2 × 10\textsuperscript{9} virulent bacteria. In contrast, Ig\textsubscript{m} \textsuperscript{−/−} mice were at least 1000-fold less well protected. A similar experimental set up was used to analyze the function of seven to nine mice. Survival curves of vaccinated Ig\textsubscript{m} and Ig\textsubscript{m} in the experiment shown in Fig. 3A, the mean survival time for naive Ig\textsubscript{m} \textsuperscript{+/+} mice was 6 and 12 days, respectively. However, neither in Ig\textsubscript{m} \textsuperscript{+/+} nor in Ig\textsubscript{m} \textsuperscript{−/−} mice full protection against a systemic challenge with wild-type S. typhimurium SL1344 was observed and only part of the mice survived infection. Accordingly, with this vaccination regimen statistically significant differences (p < 0.05) in the survival curves of vaccinated Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsuperscript{−/−} mice were not found when challenge was performed 7 wk after vaccination. We therefore modified the experimental protocol and challenged Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsuperscript{−/−} mice i.v. with wild-type S. typhimurium 3 wk after vaccination with attenuated S. typhimurium SL7207 (Fig. 3B). At this early time point of challenge infection, attenuated salmonellae are still present and antibacterial mechanisms should be fully operative. Injection of wild-type S. typhimurium at this time point therefore represents a less stringent challenge for the mice compared with the experiments described in Fig. 3A. In both Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsuperscript{−/−} mice, partial protection was observed and mean survival times in both vaccinated groups were prolonged. Importantly, with this vaccination/challenge scheme, significant differences between survival of Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsuperscript{−/−} mice were observed (p < 0.05). These experiments emphasize contribution of B cells to protection against systemic infection with S. typhimurium. Yet, in the absence of B cells and Abs partial protection existed, arguing that B cells and Abs are not absolutely required for control of systemic infection with virulent S. typhimurium.

### Discussion

In mice, the targeted mutation of the \( \mu \) gene causes a failure of B cell maturation that leads to a lack of peripheral B cells. The use of these mice allowed us to analyze the role of B cells in the immune response against S. typhimurium. Although, generation of Abs is a central function of B cells, they can perform alternative functions such as Ag presentation, initiation of T cell responses, and cytokine production (24–29). We therefore do not exclude Ab-independent B cell functions during S. typhimurium infection, although we assume that at least part of our results are best explained by the absence of Abs.

Our results formally prove that B cells are important for protection against oral infection with virulent S. typhimurium. In contrast, for controlling systemic infection with attenuated aroA\textsuperscript{−} S. typhimurium, B cells and Abs were dispensable. Vaccination of Ig\textsubscript{m} \textsuperscript{−/−} mice failed to induce protection against oral infection. After systemic infection, we observed partial protection in both vaccinated Ig\textsubscript{m} \textsuperscript{+/+} and Ig\textsubscript{m} \textsuperscript{−/−} mice, although control mice were better protected than mutant animals. These results do not only indicate that mechanisms other than B cells are important for control of systemic infection, but also that B cells participate in protection. Thus, our findings for the first time formally define a distinct role of B cells in protective immunity to S. typhimurium.

These data are reminiscent of and extend results from experiments with CBA/N mice. CBA/N mice, also named Xid mice (X-linked immunodeficiency), have a mutation in the gene for the protein tyrosine kinase Btk (Bruton’s tyrosine kinase) that is located on the X chromosome and show a deficiency in B cell maturation, leading to decreased levels of IgM and IgG3 (30). Compared with female littermates, male F\(_1\) (CBA/N × BALB/c) mice are more susceptible to i.p. infection with virulent salmonellae, but both male and female animals equally control systemic infection with attenuated aroA\textsuperscript{−} S. typhimurium (31, 32). However, data on the role of Abs derived from experiments with CBA/N mice have to be considered with care since Ab production is only partially inhibited in these mice. Furthermore, Btk is not only expressed in B cells but also in myeloid and erythroid cells, and recently it has been shown that mutation of the Btk gene affects peripheral expansion of myeloid cells and activation of mast cell (33, 34).
Given the marked difference between the lethal dose of oral vs systemic infection, efficient mechanisms must be operative which restrict spreading of *S. typhimurium* from the intestine to lymphatic organs and liver. Our results reveal that B cells are fundamental to this process. B cell-deficient mice were more susceptible to primary oral infection and were not protected against secondary oral infection. Different mechanisms can be assumed by which B cells could achieve this goal: In the gut, *S. typhimurium*-specific IgA can block bacterial adhesion to epithelial cells and agglutinate bacteria (35). It is also possible that opsonization with Abs leads to more effective uptake and destruction of bacteria by phagocytes in gut-associated lymphoid tissues. In addition, by the production of cytokines such as IFN-γ, B cells in the gut-associated lymphoid tissues can activate phagocytes and induce bactericidal mechanisms in these cells (28). As a net result fewer salmonellae reach spleen and liver in the presence of B cells.

Interestingly, IgM<sup>-/-</sup> mice were already more susceptible to primary oral infection with virulent *S. typhimurium*. Bacteria leave the gut within the first days of infection. At this time point, salmonella-specific Abs should not yet be available and Ab-independent B cell-mediated mechanisms could operate (28). An alternative explanation is the presence of Abs with cross-reactivity against salmonella Ags. Although mice were kept under specific pathogen-free conditions, animals had contact with Gram-negative bacteria of the resident gut flora and could develop cross-reactive Abs. There is also evidence for “natural Abs,” a fraction of Abs with specificities for a wide range of bacterial Ags that are at least in part generated by a specialized population of CD5<sup>+</sup> B cells (B1 cells) (36, 37).

Systemic infection results in rapid uptake of *S. typhimurium* by mononuclear phagocytes in liver and spleen and salmonella-specific Abs enhance this uptake (5). In liver and spleen, *S. typhimurium* survives within mononuclear phagocytes (38) where bacterium-specific Abs enhance this uptake (5). In liver and spleen, *S. typhimurium* survives within mononuclear phagocytes (38) where bacteria of the resident gut flora and could develop cross-reactive Abs. There is also evidence for “natural Abs,” a fraction of Abs with specificities for a wide range of bacterial Ags that are at least in part generated by a specialized population of CD5<sup>+</sup> B cells (36, 37).

The importance of macrophage activation is emphasized by the high susceptibility to *S. typhimurium* of mice deficient in IFN-γ receptor or of mice in which IFN-γ or TNF-α are neutralized with specific Abs (15, 19, 39, 40). Both IFN-γ and TNF-α are crucial for macrophage activation. T cells are involved in this process either by secreting macrophage-activating cytokines like IFN-γ or by direct T cell-macrophage interactions. Accordingly, mice deficient in T cells, and especially in CD4<sup>+</sup> T cells, suffer from chronic infection with attenuated aroA<sup>-</sup> *S. typhimurium* strains (19, 41). An important function of CD4<sup>+</sup> T cells is to provide help for the activation and differentiation of B cells (42). In contrast to mice deficient in CD4<sup>+</sup> T cells (19), IgM<sup>-/-</sup> mice were able to control and eliminate the same aroA<sup>-</sup> strain of *S. typhimurium*, indicating that the main function of CD4<sup>+</sup> T cells in this infection model is not to provide help for B cells but to activate macrophages.

Our finding that IgM<sup>-/-</sup> mice can control systemic infection with attenuated *S. typhimurium* argues against an essential role for B cells and Abs in clearing systemic infection. This notion is further supported by our result that IgM<sup>-/-</sup> mice vaccinated with attenuated *S. typhimurium* were partially protected against challenge infection with virulent *S. typhimurium*. Probably, primary infection induced salmonella-specific T cells, activation of macrophages, and other effector mechanisms capable of controlling subsequent infection with virulent *S. typhimurium* in the absence of Abs. However, control is incomplete and B cells participate in vaccine-induced protection.

In summary, our results prove that B cells are necessary for efficient protection of hypersusceptible mice against primary and secondary oral infection with *S. typhimurium*, indicating that B cells have an essential role in host defense. During systemic infection, B cell-independent mechanisms gain increasing importance in these mice. Although there is no absolute requirement for B cells and Abs in protection to systemic infection, B cell-mediated immunity clearly improves protection in salmonella-susceptible mice.

### Acknowledgments

We thank Drs. B. A. D. Stocker and K. Rajewsky for providing *S. typhimurium* strains or IgM<sup>-/-</sup> deficient mice, respectively, and we acknowledge support by the “Fonds der Chemischen Industrie.”

### References


