Immunity
Article

Signaling via the MyD88 Adaptor Protein in B Cells Suppresses Protective Immunity during *Salmonella typhimurium* Infection

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SUMMARY

The myeloid differentiation primary response gene 88 (*Myd88*) is critical for protection against pathogens. However, we demonstrate here that MyD88 expression in B cells inhibits resistance of mice to *Salmonella typhimurium* infection. Selective deficiency of *Myd88* in B cells improved control of bacterial replication and prolonged survival of the infected mice. The B cell-mediated suppressive pathway was even more striking after secondary challenge. Upon vaccination, mice lacking *Myd88* in B cells became completely resistant against this otherwise lethal infection, whereas control mice were only partially protected. Analysis of immune defenses revealed that MyD88 signaling in B cells suppressed three crucial arms of protective immunity: neutrophils, natural killer cells, and inflammatory T cells. We further show that interleukin-10 is an essential mediator of these inhibitory functions of B cells. Collectively, our data identify a role for MyD88 and B cells in regulation of cellular mechanisms of protective immunity during infection.

INTRODUCTION

B lymphocytes can produce antibodies, present antigen to T cells, and secrete cytokines. The production of antibodies is usually induced upon triggering of the B cell receptor (BCR) by antigen and provision of costimulatory signals from T helper lymphocytes. Accordingly, defects in BCR signaling or in T cell help can result in antibody deficiencies and increased susceptibility to infections (Conley et al., 2009). B cells can also be activated directly by microbial products through Toll-like receptors (TLRs) and the adaptor protein encoded by myeloid differentiation primary response gene 88 (*Myd88*). However, the role of intrinsic MyD88 signaling in B cells during bacterial infections is not yet well defined.

TLR agonists can increase antibody responses, suggesting a role for TLRs in humoral immunity (Krieg, 2007). Indeed, *Myd88*$^{-/-}$ mice fail to generate bone marrow (BM) plasma cells and to maintain long-term humoral immunity after infection with polyoma virus (Guay et al., 2007). Similarly, neutralizing antibody titers rapidly decline in *Myd88*$^{-/-}$ mice infected with vesicular stomatitis virus, as compared to wild-type mice (Zhou et al., 2007). These effects may result from a lack of TLR signaling in B cells. B cells are known to express TLRs, and TLR agonists can induce their proliferation and secretion of antibodies in vitro (Gururajan et al., 2007; Krieg et al., 1995). Furthermore, B cell-deficient mice reconstituted with *Myd88*-deficient B cells make weaker antibody responses than mice with wild-type B cells when lipopolysaccharide (LPS) is used as adjuvant (Meyer-Bahlburg et al., 2007). *Myd88*-deficient B cells also produce fewer specific antibodies than wild-type B cells in mixed BM chimera infected with a herpes virus (Gargano et al., 2008). However, other studies found that mice with *Myd88*-deficient B cells can mount normal primary antibody responses after immunization with certain adjuvants, suggesting that this pathway differently contributes to antibody responses depending on the type of immune challenge (Gavin et al., 2006; Lampropoulou et al., 2008).

TLRs can also stimulate antibody-independent functions of B cells, including cytokine production. B cells can secrete interleukin (IL)-10, and thereby suppress autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), experimental ulcerative colitis, and collagen-induced arthritis (Fillatreau et al., 2002; Mauri et al., 2003; Mizoguchi et al., 2002). Similarly, human B cells can produce IL-10 and inhibit autoimmune pathology (Duddy et al., 2007; Duddy et al., 2004; Goetz et al., 2007). Remarkably, TLRs can initiate the production of IL-10

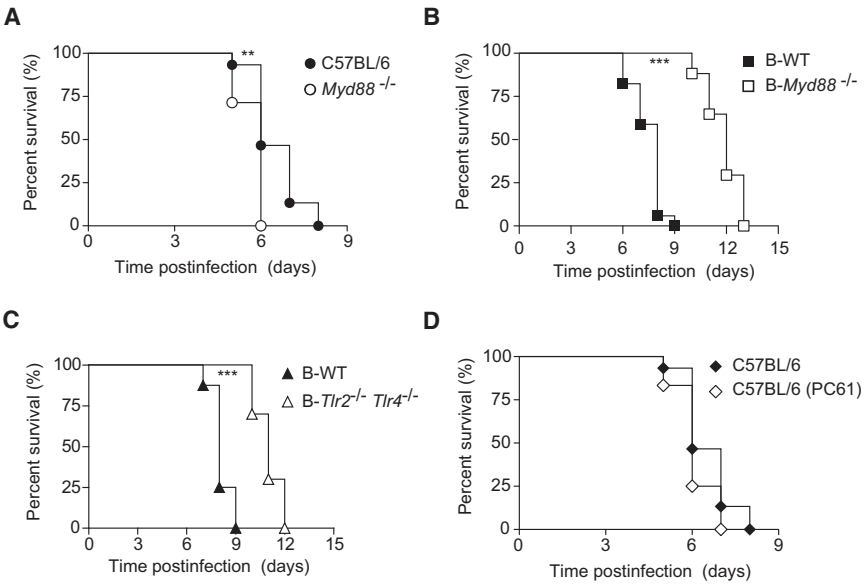

Figure 1. Ablation of *Myd88* in B Cells Improves Survival of Mice Infected with Virulent *Salmonella*

Mice were infected intravenously (i.v.) with 100 CFU virulent *Salmonella typhimurium* (SL1344).

(A) Survival of C57BL/6 (n = 15) and *Myd88*$^{-/-}$ (n = 14) mice after infection.

(B) Survival of B-WT (n = 17) and B-*Myd88*$^{-/-}$ (n = 17) mice after infection.

(C) Survival of B-WT (n = 8) and B-*Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ (n = 10) mice after infection

(D) Survival of C57BL/6 (n = 15) and C57BL/6 mice treated i.v. with 200 μg anti-CD25 antibody 3 days prior to infection (n = 12).

See also Figure S1.

by naive B cells and are essential for the regulatory functions of B cells in EAE (Lampropoulou et al., 2008). Thus, mice with B cell-specific deficiency in both *Tlr2* and *Tlr4* or in *Myd88* develop a chronic form of EAE, similar to mice with *Il10*-deficient B cells, whereas mice with wild-type B cells recover after a short episode of paralysis (Fillatreau et al., 2002; Lampropoulou et al., 2008).

The function of MyD88 signaling in B cells during bacterial infection is poorly understood. This signaling may lead to increased presentation of antigen to T cells and/or to augmented production of protective antibodies, so that deletion of *Myd88* in B cells may impair resistance to infections. Alternatively, the recently identified role of MyD88 in the suppressive functions of B cells in EAE (Lampropoulou et al., 2008) suggests that ablation of *Myd88* specifically in B cells could unleash cell-mediated immune responses and improve protection against pathogens. Here, we addressed the specific role of MyD88 signaling in B cells in mice during *Salmonella typhimurium* infection. *Salmonella* are facultative intracellular Gram-negative bacteria that can cause a wide range of diseases. In mice *S. typhimurium* induces a disease with similarities to typhoid fever, which in humans causes 600,000 deaths annually (Mittrücker and Kaufmann, 2000; Pang et al., 1998). We found that selective deficiency of *Myd88* in B cells strongly improved resistance of mice against *S. typhimurium* infection. Thus, one of the major consequences of MyD88 signaling in B cells is the counter-regulation of protective immunity during infection by such intracellular bacteria.

RESULTS

Ablation of *Myd88* in B Cells Improves Survival of *Salmonella*-Infected Mice

The role of MyD88 in B cells was assessed in chimera mice in which only B cells lack *Myd88* (B-*Myd88*$^{-/-}$). To produce B-*Myd88*$^{-/-}$ mice, B cell-deficient JHT mice were lethally irradiated and reconstituted with a mixture of BM cells from JHT (80%) and *Myd88*$^{-/-}$ mice (20%). In such chimera, all B cells originate from the *Myd88*$^{-/-}$ BM, whereas other hematopoietic cell types are mostly (80%) of wild-type phenotype. The advantage of this experimental system is the absence of leakiness; that is, no wild-type B cells can be found in B-*Myd88*$^{-/-}$ chimera. Control mice (B-WT) were generated by reconstituting irradiated JHT mice with BM cells from C57BL/6 (80%) and *Myd88*$^{-/-}$ (20%) mice. In B-WT chimera all hematopoietic cells, including B cells, are mostly (80%) of wild-type phenotype. Thus, B-WT mice can be used to determine the function of MyD88 signaling in B cells, taking into account the 20% *Myd88*$^{-/-}$ hematopoietic cells other than B cells that are present in B-*Myd88*$^{-/-}$ mice. Characterization of these chimera revealed that their immune systems were comparable, except for a mild decrease in frequency of follicular B cells and increases in frequencies of marginal zone and CD1d^{hi}CD5^{+} B cells in B-*Myd88*$^{-/-}$ mice compared to B-WT mice (Figure S1A available online). Activation status of B cells, CD11c^{+} dendritic cells (DCs), CD11b myeloid cells, and T cells were similar in the two types of mice (Figures S1B and S1C).

To address the role of MyD88 in host protection, we first infected *Myd88*$^{-/-}$ and C57BL/6 mice via the intravenous (i.v.) route with virulent *S. typhimurium* (strain SL1344) and monitored their survival. Mice with complete *Myd88* deficiency succumbed to *S. typhimurium* infection earlier than C57BL/6 mice (Figure 1A). We then specifically examined the role of MyD88 signaling in B cells by infecting B-*Myd88*$^{-/-}$ and B-WT mice in the same way. Remarkably, lack of *Myd88* expression by B cells resulted in prolonged survival of the infected mice (Figure 1B). B-WT mice started to die on day 6 postinfection (p.i.) and all had succumbed by day 9 with a median survival time of 8 days (Figure 1B). In contrast, B-*Myd88*$^{-/-}$ mice started to die at day 10 and survived until day 13 with a median survival time of 12 days (Figure 1B). Thus, *Myd88* deficiency in B cells extended survival time by 4 days. These results demonstrate the dual role of MyD88 in host defense: expression of MyD88 in cells other than B cells promotes the survival of infected mice, whereas its expression in B cells counterbalances this protective effect. Similarly, mice lacking both *Tlr2* and *Tlr4* in B cells (B-*Tlr2*$^{-/-}$*Tlr4*$^{-/-}$) displayed prolonged survival upon infection with *Salmonella* compared to B-WT mice (Figure 1C), suggesting that the B cell-mediated suppression results from direct

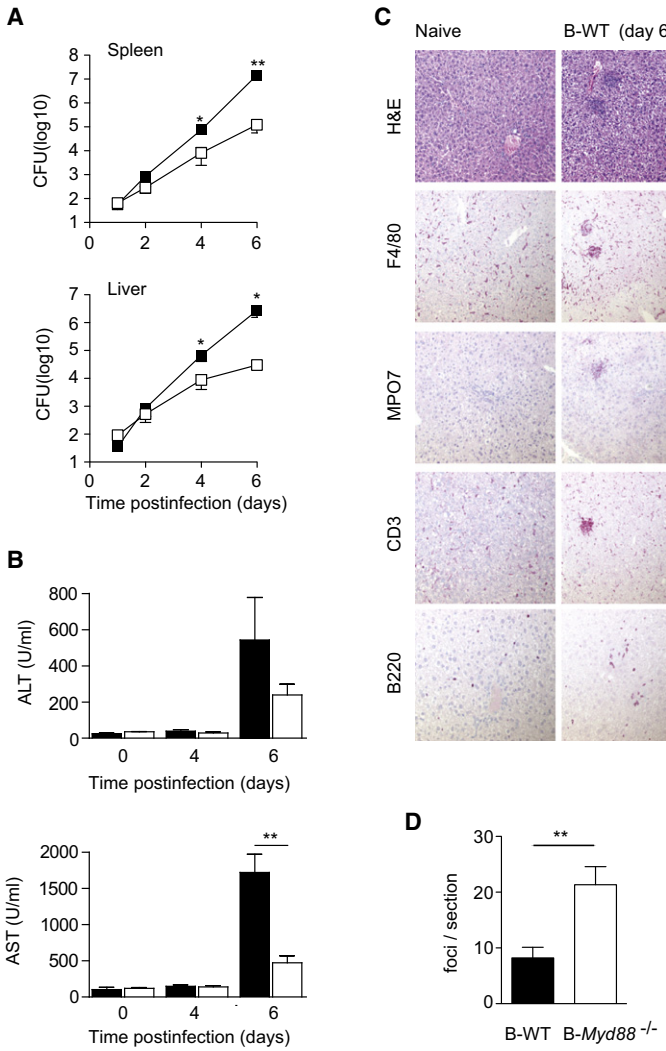

Figure 2. *Myd88* Deficiency in B Cells Improves Control of *Salmonella* Infection

(A) Bacterial loads in spleen (top) and liver (bottom) of B-WT (black square) and B-*Myd88*$^{-/-}$ (white square) mice after infection with SL1344 (n = 8–10 mice per group per time point).

(B) Serum titers of alanine aminotransferase (ALT; top) and aspartate aminotransferase (AST; bottom) in infected B-WT (black bars) and B-*Myd88*$^{-/-}$ mice (white bars).

(C) Liver histochemistry of total infiltrating cells (hematoxylin and eosin [H&E]), macrophages (F4/80), neutrophils (MPO7; myeloperoxidase 7), T cells (CD3), and B cells (B220) at day 6 p.i. A 100× magnification of the original image is shown.

(D) Number of the inflammatory foci per liver section at day 6 p.i. (B-WT, n = 5; B-*Myd88*$^{-/-}$, n = 6).

(A–D) Results shown are pooled from two independent experiments. Means ± SEM. *p < 0.05; **p < 0.01; ***p < 0.0001.

microbial sensing by the B cells. To address whether natural regulatory CD4$^+$CD25$^+$ T (Treg) cells also limit host protection to *S. typhimurium*, we treated C57BL/6 mice with aCD25 antibody 3 days before infection, given that such treatment was shown to impair the suppressive functions of Treg cells (Stephens and Anderton, 2006). Indeed, we observed complete disappearance of CD4$^+$CD25$^+$ T cells from peripheral blood within 3 days (data not shown), but this had no effect on the survival of infected mice (Figure 1D). These data highlight the unique role of B cells in the regulation of host protection during systemic *S. typhimurium* infection.

Although the functions of MyD88 in promoting host defense to pathogens have been extensively investigated, the specific effects resulting from MyD88-signaling in B cells during infection are poorly defined. Thus, we analyzed how the lack of *Myd88* in B cells could improve protection. The longer survival of B-*Myd88*$^{-/-}$ mice correlated with lower bacterial loads in spleen and liver as compared to B-WT mice (Figure 2A). At day 4 p.i., B-WT mice carried a 10-fold greater bacterial burden in spleen and liver than B-*Myd88*$^{-/-}$ mice, and this difference reached 100-fold by day 6, implying that expression of MyD88 in B cells impaired control of the infection (Figure 2A). This increased bacterial load correlated with higher liver tissue damage in B-WT mice than in B-*Myd88*$^{-/-}$ mice, as revealed by serum amounts of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) at day 6 p.i. (Figure 2B).

We then addressed whether MyD88 signaling in B cells suppressed the immune response to bacteria by performing histological analyses in infected livers. At day 6 p.i., livers contained inflammatory foci consisting of F4/80$^+$ macrophages, MPO7$^+$ neutrophils, and CD3$^+$ T cells (Figure 2C). Low numbers of B cells were found in the periphery of these foci and in adjacent portal tracts (Figure 2C). Remarkably, livers from B-*Myd88*$^{-/-}$ mice had ~2-fold more inflammatory foci than livers from B-WT mice, indicating that MyD88 signaling in B cells suppressed local immune protection in the liver (Figures 2C and 2D).

Taken together, these results indicate that MyD88 signaling in B cells impairs control of bacterial replication and resistance of mice to the infection upon intravenous challenge with *S. typhimurium*. This may involve an inhibitory effect of MyD88-signaling in B cells on the protective immune response.

MyD88 Signaling in B Cells Inhibits Innate Immunity to *Salmonella*

The higher bacterial loads in B-WT mice at days 4 and 6 p.i. suggested that MyD88 expression in B cells inhibited innate mechanisms of immune protection. We thus analyzed innate responses in spleens at early time points (days 1 and 2 p.i.), when the different groups of mice still carried similar numbers of virulent *S. typhimurium* (SL1344). In addition, we analyzed

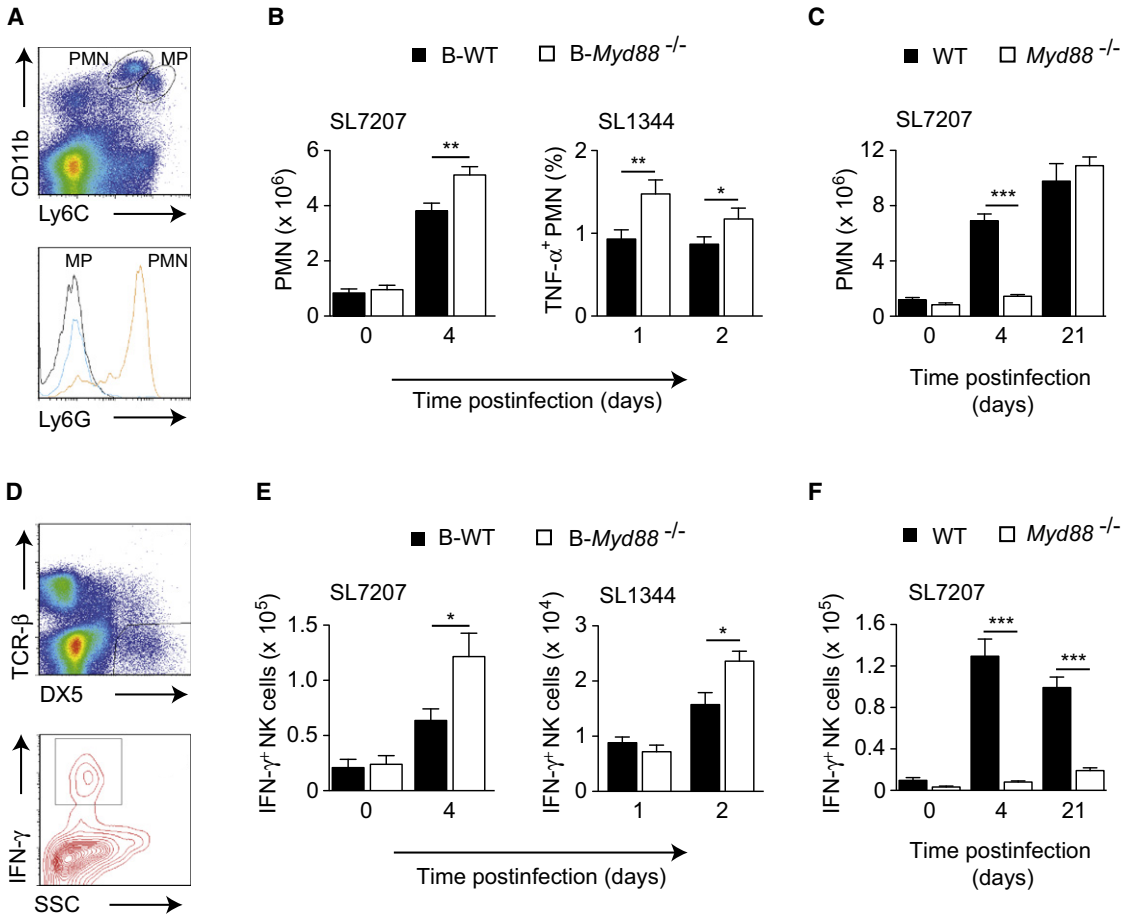

Figure 3. Suppression of Neutrophil and NK Cell Responses by MyD88 Expression in B Cells

B-WT, B-*Myd88*$^{-/-}$, C57BL/6, and *Myd88*$^{-/-}$ mice were infected either with 10^6 attenuated (SL7207) or with 100 virulent (SL1344) *Salmonella*.

(A) The top panel shows expression of CD11b and Ly6C by live spleen cells from a B-WT mouse at day 2 p.i. with SL7207. The gates used to identify neutrophils (PMN; CD11b^{hi}Ly6C^{+}) and mononuclear phagocytes (MP; CD11b^{+}Ly6C^{hi}) in infected spleens are indicated. The bottom panel shows levels of expression of Ly6G by live PMN (orange line), MP (blue line), and CD11b^{-}Ly6C^{-} cells (black line).

(B) The left panel shows the total numbers of PMN per spleen in B-WT and B-*Myd88*$^{-/-}$ mice infected with SL7207. The right panel shows the frequency of splenic TNF-α-producing PMN in these chimera mice infected with SL1344. For this analysis, splenocytes were restimulated for 6 hr with heat-killed *Salmonella* and stained for CD11b, Ly6C, Ly6G, and intracellular TNF-α.

(C) Numbers of PMN per spleen in C57BL/6 and *Myd88*$^{-/-}$ mice infected with SL7207.

(D) The top panel shows expression of DX5 and TCRβ by live spleen cells from a B-WT mouse at day 2 p.i. with SL7207. NK cells were identified as DX5^{+}TCR-β^{-} cells. The bottom panel shows production of IFN-γ by NK cells after restimulation of splenocytes with heat-killed *Salmonella* for 6h.

(E) Absolute numbers of IFN-γ-producing NK cells per spleen in B-WT and B-*Myd88*$^{-/-}$ mice infected with SL7207 (left panel) or virulent SL1344 (right panel) *Salmonella*.

(F) Absolute numbers of splenic IFN-γ-producing NK cells per spleen in C57BL/6 and *Myd88*$^{-/-}$ mice infected with SL7207.

(A–F) Data shown are pooled from two independent experiments (12 mice per group per time point). Mice were analyzed individually. Graphs show mean ± SEM. *p < 0.05. **; p < 0.01; ***p < 0.0001.

See also Figure S2.

innate responses in mice infected with an attenuated *S. typhimurium* strain (SL7207).

Upon infection with *S. typhimurium*, neutrophils, mononuclear phagocytes, and DCs are rapidly recruited to sites of bacterial replication where they contribute to containment of the infection (Tam et al., 2008). We first monitored the responses mediated by CD11b^{hi}Ly6C^{+}Ly6G^{hi} neutrophils and CD11b^{+}Ly6C^{hi}Ly6G^{-} mononuclear phagocytes (Figure 3A) (Kang et al., 2008). Accumulation of neutrophils was substantially higher in spleens of B-*Myd88*$^{-/-}$ mice than in B-WT mice after infection with attenuated *Salmonella*, whereas the two groups of mice carried similar numbers of bacteria (Figure 3B; Figure S2A and Table S1). Notably, we also detected a heightened accumulation of neutrophils in livers of infected B-*Myd88*$^{-/-}$ mice compared to B-WT mice (Figure S2B). *B-Myd88*$^{-/-}$ mice also developed an enhanced neutrophil response after infection with virulent *Salmonella*, as shown by a higher frequency of TNF-α-producing neutrophils compared to B-WT mice (Figure 3B), whereas in this case the numbers of neutrophils in the spleens were comparable in the two types of mice (Figure S2C). We deduce from these

observations that B cells activated via MyD88-dependent pathways suppress neutrophil-mediated protection. Notably, MyD88 signaling in B cells did not alter the numbers of either mononuclear phagocytes or DCs ($CD11c^{hi}$ cells) (Figures S2D and S2E). In contrast, responses of neutrophils, mononuclear phagocytes, and DCs were severely impaired in *Myd88*$^{-/-}$ mice at day 4 p.i. (Figure 3C; Figures S2D and S2E). Thus, MyD88 controlled the accumulation of DCs and mononuclear phagocytes in infected spleens without a contribution from B cells, whereas the response of neutrophils was counterregulated by MyD88 expression in B cells.

During *Salmonella* infection, NK cells are the first source of the essential macrophage-activating cytokine IFN-γ (Hess et al., 1996). Remarkably, B-*Myd88*$^{-/-}$ mice had substantially more IFN-γ-producing NK cells than B-WT mice at day 4 p.i. with attenuated *Salmonella*, whereas absolute numbers of NK cells were similar (Figures 3D and 3E; Table S1; data not shown). Likewise, stronger NK cell responses were observed in B-*Myd88*$^{-/-}$ mice than in B-WT mice after infection with virulent *Salmonella* (Figure 3E). Thus, MyD88 signaling in B cells results in inhibition of the NK cell response. In *Myd88*$^{-/-}$ mice, the NK cell response was severely impaired at days 4 and 21 p.i. (Figure 3F). Collectively, our observations reveal an inhibitory circuit linking MyD88 expression in B cells with innate immune defenses mediated by neutrophils and NK cells.

MyD88 Signaling in B Cells Suppresses Inflammatory T Cell Responses

Inflammatory $CD4^+$ T cell responses play a crucial role in protection from *Salmonella* infection (Hess et al., 1996). Thus, we assessed T cell immunity in B-WT, B-*Myd88*$^{-/-}$, C57BL/6, and *Myd88*$^{-/-}$ mice after i.v. infection with attenuated *S. typhimurium*.

At day 10 p.i., *Salmonella*-specific IFN-γ- and TNF-α-producing $CD4^+$ T cells were detected in comparable numbers in B-WT and B-*Myd88*$^{-/-}$ mice (Figures 4A and 4B). In B-WT mice, these responses were maintained to a similar degree until day 21. In contrast, in B-*Myd88*$^{-/-}$ mice the numbers of IFN-γ- and TNF-α-expressing $CD4^+$ T cells doubled between days 10 and 21 p.i. (Figures 4A and 4B; Table S1). Thus, MyD88 signaling in B cells resulted in selective inhibition of the expansion of the inflammatory $CD4^+$ T cell population. Similarly, spleens of infected B-*Myd88*$^{-/-}$ mice accumulated significantly more IFN-γ-producing $CD8^+$ T cells compared to B-WT mice (Figure 4C; Table S1). As expected, *Myd88*$^{-/-}$ mice had fewer IFN-γ- and TNF-α-producing $CD4^+$ T cells as well as fewer IFN-γ-producing $CD8^+$ T cells than control C57BL/6 mice at day 21 p.i. (Figures 4A–4C). Of note, we did not detect any IL-17-expressing $CD4^+$ T cells, implying a minor role of this cytokine during *Salmonella* infection (data not shown), in agreement with others (Schulz et al., 2008). In conclusion, these data imply that the balance between MyD88 signaling in B cells and in other cells determines the intensity of protective inflammatory T cell responses during the course of *Salmonella* infection in wild-type mice.

MyD88 Signaling in B Cells Accelerates the Development of Humoral Immunity

Because the proposed function of intrinsic MyD88 signaling in B cells is the amplification of antibody responses, we compared humoral immunity in B-*Myd88*$^{-/-}$ and B-WT mice after infection with attenuated *Salmonella*.

Rapid differentiation of activated B cells into short-lived plasma cells is a hallmark of humoral immunity. We found that accumulation of splenic $CD138^+$ plasma cells peaked at day 4 p.i. in B-WT mice and then declined to preinfection numbers by day 62 (Figure 4D). In contrast, B-*Myd88*$^{-/-}$ mice developed a delayed response, which was less intense and never reached the amount observed at day 4 in B-WT mice (Figure 4D).

We then assessed germinal center (GC) development, a T cell-dependent process of B cell activation. Histological analysis revealed GCs in both B-WT and B-*Myd88*$^{-/-}$ mice at day 10 p.i., although they were apparently smaller in the B-*Myd88*$^{-/-}$ mice (Figure 4E). Supporting this observation was the fact that spleens from infected B-*Myd88*$^{-/-}$ mice had lower frequencies of $GL7^+Fas^+$ GC B cells compared to B-WT mice (Figure 4F).

GC B cells give rise to long-lived BM plasma cells, which can maintain specific antibody responses for several years (Radbruch et al., 2006). Accumulation of plasma cells in BM became evident at day 62 p.i. (Figure 4G). At this time point, B-*Myd88*$^{-/-}$ mice had markedly fewer BM plasma cells than B-WT mice (Figure 4G). In line with this finding, BM cells from B-*Myd88*$^{-/-}$-infected mice produced fewer antibodies than those from B-WT mice after in vitro culture (data not shown). Thus, MyD88 signaling in B cells is important for the establishment of the BM pool of antibody-secreting plasma cells. It is yet unclear whether this defect is compensated by heightened accumulation of plasma cells in different tissues.

Subsequently, we compared antibody responses in B-*Myd88*$^{-/-}$ mice and B-WT mice. We first analyzed natural antibody titers in naive mice. Consistent with published data, relative titers of both total and *Salmonella*-specific natural IgM were reduced in naive B-*Myd88*$^{-/-}$ mice compared to B-WT mice (Figure S3A) (Barr et al., 2009). After *S. typhimurium* infection, specific IgM and IgG responses were initially lower in B-*Myd88*$^{-/-}$ than in B-WT mice, but these defects were no longer apparent at 4 weeks p.i. (Figure 4H). We then quantified the various IgG isotypes produced upon infection. B-*Myd88*$^{-/-}$ mice showed trends for increased IgG1, and decreased IgG3 titers compared to B-WT mice, but these differences were not statistically significant (Figure S3B). The two types of mice made similar IgG2b and IgG2c responses (Figure S3B). Our data indicate that MyD88 signaling in B cells can facilitate the formation of plasma cells and germinal center B cells and accelerate the production of specific antibodies (Table S1). However, most of the observed differences were only transient and modest.

B Cell-Derived IL-10 Inhibits Immunity to *Salmonella*

We have previously found that in EAE, the suppressive function of B cells on T cell-mediated immunity depends on both MyD88 and IL-10 (Fillatreau et al., 2002; Lampropoulou et al., 2008). We therefore asked whether B cells counterregulate cellular mechanisms of protective immunity via IL-10 during *Salmonella* infection.

Salmonella can directly induce IL-10 production by B cells in vitro via a mechanism involving TLR2 and/or TLR4 (but not TLR9) and MyD88 (Figure S4A). Notably, DCs produced little IL-10 upon coculture with *Salmonella*, indicating the uniqueness

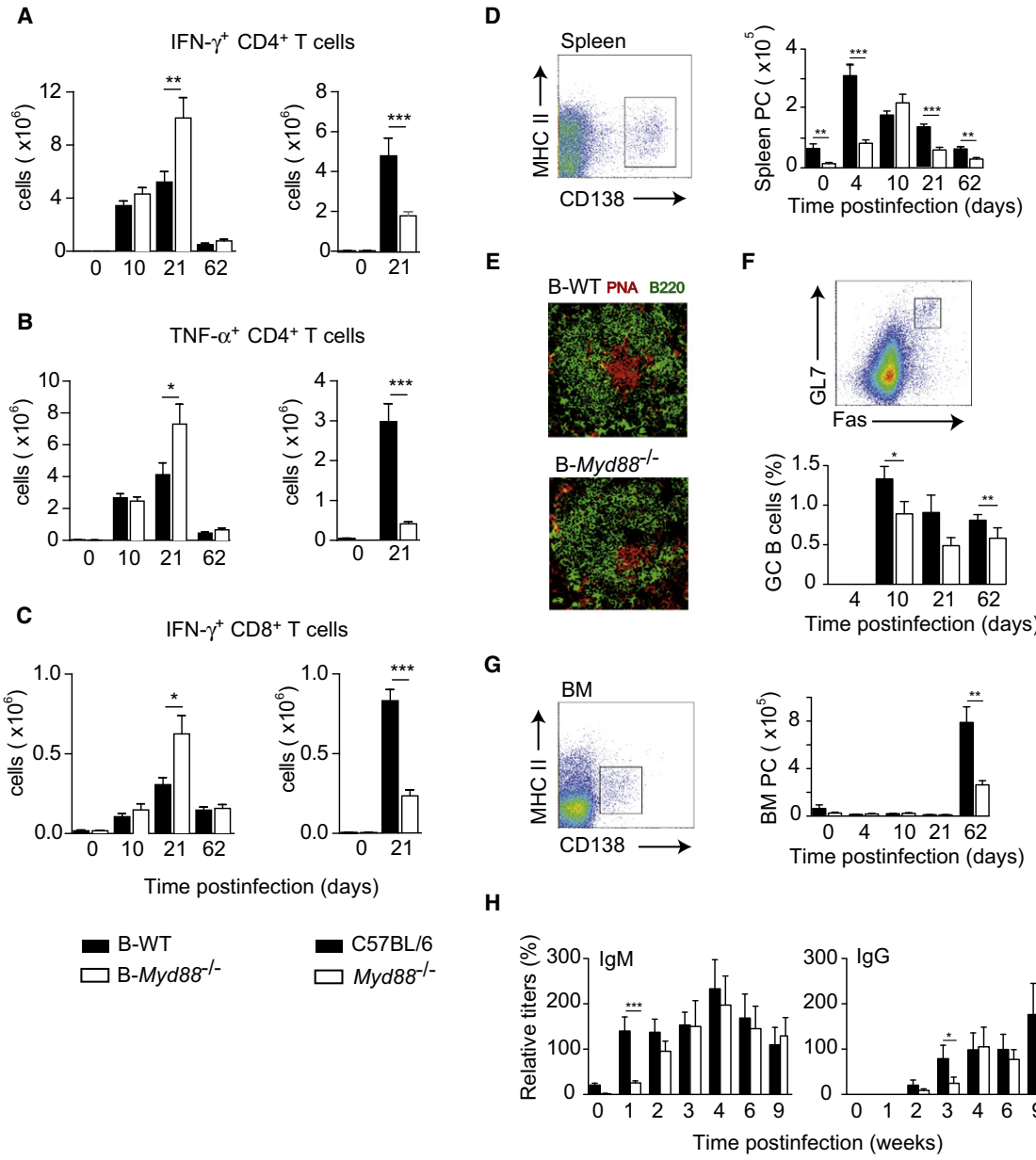

Figure 4. MyD88 Signaling in B Cells Suppresses Inflammatory T Cell Responses and Accelerates Humoral Immunity to *Salmonella*

B-WT, B-*Myd88*$^{-/-}$, C57BL/6, and *Myd88*$^{-/-}$ mice were infected i.v. with 10^6 CFU *Salmonella* (SL7207). At indicated time points, splenocytes were isolated, restimulated for 6 hr with heat-killed *Salmonella*, and stained for CD4, CD8, and intracellular cytokines.

(A and B) show total numbers of IFN-γ- and TNF-α-expressing CD4$^+$ T cells per spleen in B-WT and B-*Myd88*$^{-/-}$ mice (left panel) and in C57BL/6 and *Myd88*$^{-/-}$ mice (right panel).

(C) Total numbers of IFN-γ-expressing CD8$^+$ T cells per spleen in B-WT and B-*Myd88*$^{-/-}$ mice (left panel) and in C57BL/6 and *Myd88*$^{-/-}$ mice (right panel).

(D) Shown on the left are splenic plasma cells (PCs) identified by flow cytometry as CD138$^+$MHC-II^{lo}B220$^+$ cells (gated on live B220$^+$ cells; plot from B-WT at day 4 p.i.). Shown on the right is quantification of PC in spleens of B-WT (black bars) and B-*Myd88*$^{-/-}$ (white bars) mice at indicated time points after infection.

(E) Staining of splenic germinal centers (GCs) by immunofluorescence with PNA (peanut agglutinin; red) and B220 (green) in mice at day 10 p.i.

(F) As shown in the top panel, splenic GC B cells were identified by flow cytometry as GL7$^+$Fas$^+$B220$^+$ (gated on live B220$^+$ cells; B-WT mouse at day 10 post-infection). The bottom panel shows the frequency of GC cells among B cells in spleen of B-WT (black bars) and B-*Myd88*$^{-/-}$ (white bars) mice at indicated time points after infection.

(G) As shown on the left, BM cells were stained as in (A) to identify CD138$^+$MHC-II^{low}B220$^+$ PC (gated on live B220$^+$ cells, B-WT mouse at day 62 p.i.). Shown on the right are numbers of PCs in BM (two femurs and one tibia) in B-WT (black bars) and B-*Myd88*$^{-/-}$ (white bars) mice at indicated time points after infection.

(H) Serum titers of *Salmonella*-reactive IgM (left) and IgG (right) antibodies were determined at weekly intervals in B-WT (black bars) and B-*Myd88*$^{-/-}$ (white bars) mice by ELISA. Pooled data from three independent experiments, with 15 mice per group per time point, are shown.

(A–C) Data are from three independent experiments for B-WT and B-*Myd88*$^{-/-}$ mice (15 mice per group per time point) and two independent experiments for C57BL/6 and *Myd88*$^{-/-}$ mice (ten mice per group per time point).

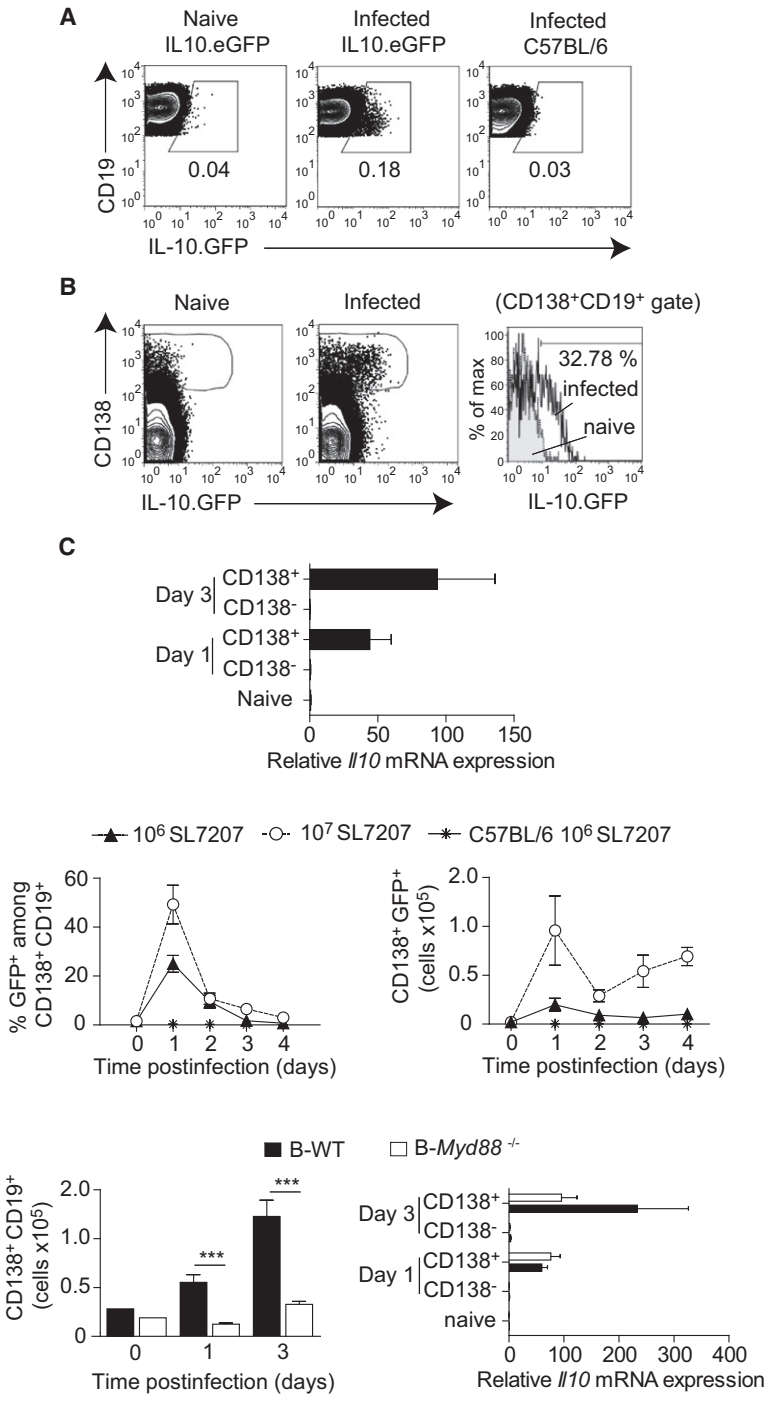

Figure 5. IL-10 Expression in B Cells during *Salmonella* Infection

(A and B) IL-10.eGFP and control C57BL/6 mice were infected i.v. with 10^6 *Salmonella* (SL7207). On day 1 p.i., live splenocytes were stained ex vivo for CD19 and CD138 without prior stimulation. Data are representative of three independent experiments.

(A) Expression of eGFP by CD19-gated live splenocytes from naive and infected IL-10.eGFP mice and infected C57BL/6 mice. Numbers indicate percentages of eGFP-expressing cells among $CD19^+$ B cells.

(B) Dot plots gated on $CD19^+$ cells show eGFP expression by $CD138^+$ cells. Histogram shows expression of eGFP by $CD138^+CD19^+$ cells from naive and infected IL-10.eGFP mice.

(C) Relative *Il10* mRNA expression by $CD138^+CD19^+$ and $CD138^-CD19^+$ cells sorted from splenocytes of C57BL/6 mice on days 1 and 3 p.i. with 10^6 attenuated *Salmonella*. *Il10* mRNA expression amounts were calculated relative to B cells from naive mice. Pooled data from two independent experiments are shown. Cells were sorted from a pool of six and mice mice on days 1 and 3, respectively, for each experiment. Graphs show the mean ± SEM.

(D) Frequency of $eGFP^+$ among $CD138^+CD19^+$ cells (left) and absolute numbers of $GFP^+CD138^+CD19^+$ cells (right) in spleen at indicated time-points after infection of IL-10.eGFP or C57BL/6 mice with 10^6 or 10^7 attenuated *Salmonella* (SL7207). Shown are pooled data from two independent experiments (six mice per group per time point). Graphs show the mean ± SEM.

(E) Numbers of $CD138^+CD19^+$ cells in spleen from B-WT and B-*Myd88*$^{-/-}$ chimera mice at the indicated time-points after infection with 10^6 attenuated *Salmonella*.

(F) Relative *Il10* mRNA expression by $CD138^+CD19^+$ and $CD138^-CD19^+$ cells sorted from splenocytes of B-WT and B-*Myd88*$^{-/-}$ mice on days 1 and 3 p.i. with 10^6 attenuated *Salmonella*. *Il10* mRNA expression was calculated relative to B cells from naive B-WT mice. Shown is pooled data from two independent experiments. Cells were sorted from a pool of six and 4 mice on days 1 and 3, respectively, for each experiment. Graphs show mean ± SEM. ***p < 0.0001.

See also Figure S4.

of this B cell response (data not shown). To examine the development of IL-10-producing B cells during the course of *Salmonella* infection in vivo, we used an IL-10.eGFP reporter mouse strain (called B-Green mouse), in which the eGFP coding sequence was gene-targeted to the *Il10* locus just downstream of the translation stop (Figure S4B). Remarkably, a population of $eGFP^+$ B cells could be detected in B-Green mice as early as day 1 p.i. (Figure 5A). These cells characteristically expressed the marker CD138, which is classically associated with plasmablast and plasma cell differentiation but can also be upregulated on other types of activated B cells (Figure 5B). In contrast, we could not detect induction of eGFP expression in $CD19^-$ cells or in $CD19^+CD138^-$ B cells at this early time point p.i. (Figures S4C and S4D). In order to verify that *Salmonella* infection results in rapid development of *Il10*-expressing $CD138^+$ B cells, we isolated $CD138^+$ and $CD138^-$ B cells from C57BL/6 mice at days 1 and 3 p.i. (Figure S4E) and quantified their *Il10* mRNA content. As expected, *Il10*

(D–G) Data shown are pooled from two independent experiments, with 10–16 mice per group per time point.
Mice were analyzed individually. Graphs show the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.0001.
See also Figure S3.

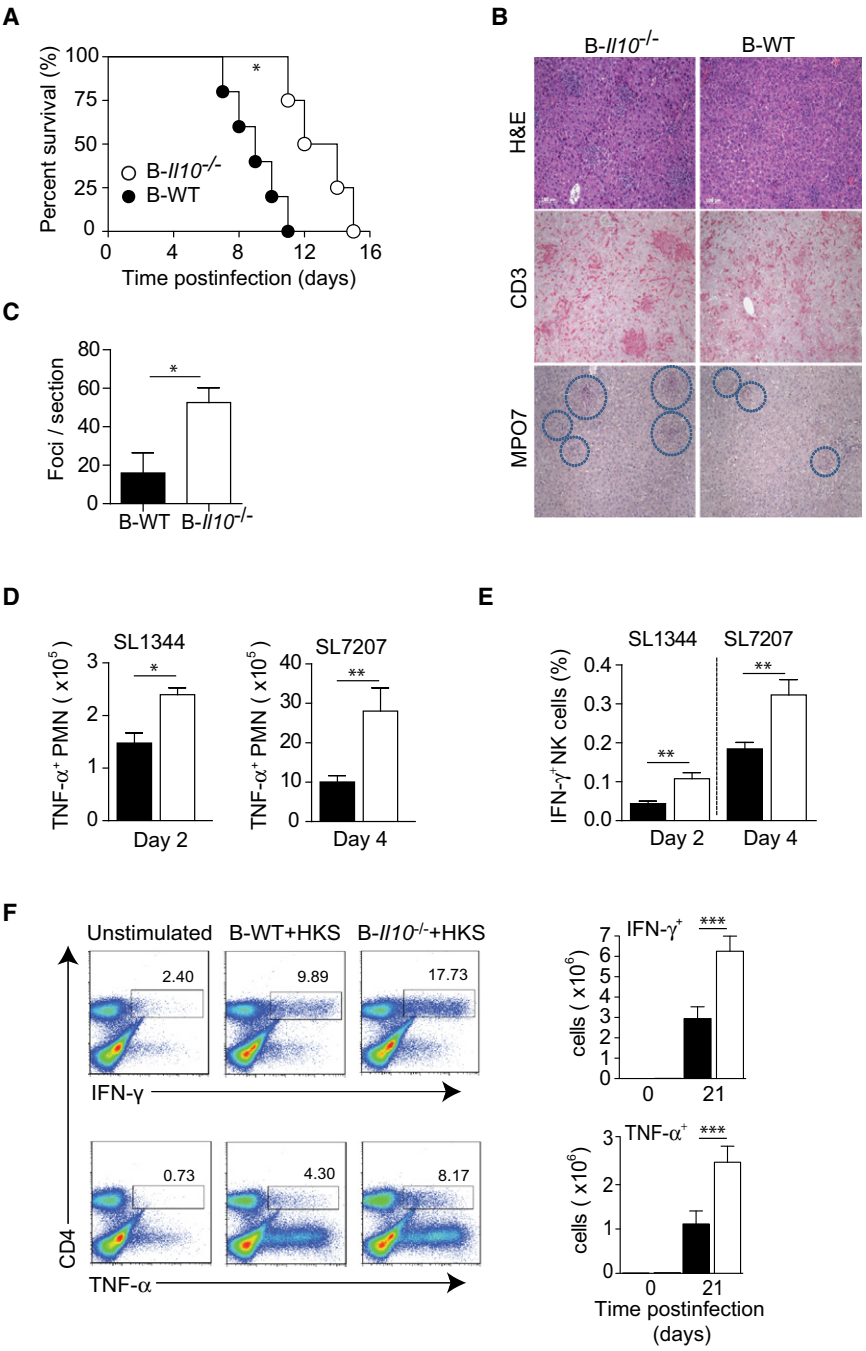

Figure 6. B Cells Suppress Immunity to *Salmonella* Infection via IL-10

(A) Survival of B-*Il10*$^{-/-}$ (n = 8) and control chimera with wild-type B cells (n = 9) after i.v. infection with 100 CFU virulent *Salmonella* (SL1344).

(B) Liver histochemistry of total infiltrating cells (H&E; hematoxylin and eosin), T cells (CD3) and neutrophils (MPO7; myeloperoxidase 7) at day 6 p.i. A 100× magnification of the original image is shown.

(C) Number of the inflammatory foci per liver section at day 6 p.i. (B-WT, n = 4; B-*Il10*$^{-/-}$, n = 7). Representative aggregates of MPO7^{+} neutrophils are encircled.

(D) Total numbers of TNF-α-producing PMN per spleen in B-WT and B-*Il10*$^{-/-}$ mice infected with SL1344 (left panel) or SL7207 (right panel) at indicated time-points p.i. For this analysis, splenocytes were restimulated for 6 hr with heat-killed *Salmonella* and stained for CD11b, Ly6C, and intracellular TNF-α.

(E) Frequency of IFN-γ-producing NK cells per spleen in B-WT and B-*Il10*$^{-/-}$ mice infected with SL1344 or SL7207. For this analysis, splenocytes were restimulated for 6 hr with heat-killed *Salmonella* and stained for TCR-β, DX5, and intracellular IFN-γ.

(F) B-*Il10*$^{-/-}$ and control chimera mice were infected i.v. with 10^6 *Salmonella* (SL7207). At indicated time points, splenocytes were isolated, restimulated for 6 hr with heat-killed *Salmonella* (HKS), and stained for CD4 and intracellular IFN-γ and TNF-α. Mice were analyzed individually. Left panels show an example of flow cytometry results for a BW-T and a B-*Il10*$^{-/-}$ mouse analyzed at day 21 p.i. The "unstimulated" plots correspond to unstimulated cells from the B-*Il10*$^{-/-}$ mouse. Numbers indicate percentages of cytokine-producing cells among CD4^{+} T cells. Right panels show absolute numbers of IFN-γ^{+} (top), and TNF-α^{+} (bottom) CD4^{+} T cells per spleen. Graphs show the mean ± SEM. *p < 0.05 and **p < 0.01.

mRNA was strongly upregulated in CD138^{+} cells (but not in CD138^{-} cells) both at days 1 and 3 p.i. (Figure 5C). We then used the B-Green reporter strain to characterize the dynamic of this B cell response during the course of *Salmonella* infection. Notably, the frequency of GFP-expressing cells among CD138^{+} B cells was maximum at day 1 p.i., when it was proportional to the number of bacteria administered and decreased thereafter (Figure 5D). Approximately 50% of splenic CD19^{+}CD138^{+} cells expressed eGFP at 24 hr p.i. with 10^7 colony forming unit (CFU) *Salmonella* (Figure 5D). Remarkably, this early response was markedly diminished in B-*Myd88*$^{-/-}$ mice compared to B-WT mice. In particular, lack of *Myd88* in B cells resulted in dramatically impaired development of CD138^{+}CD19^{+} B cells (Figure 5E). From these experiments, we conclude that *Salmonella* infection results in vivo in the rapid accumulation of IL-10-expressing CD19^{+}CD138^{+} B cells in spleen via a MyD88-dependent pathway.

To assess the role of IL-10 expression by B cells in vivo, we generated B-*Il10*$^{-/-}$ mice by reconstituting irradiated JHT mice with a mixture of BM cells from JHT mice (80%) and *Il10*-deficient mice (20%). Control chimera was obtained by reconstituting irradiated JHT mice with BM cells from C57BL/6 mice (80%) and *Il10*$^{-/-}$ mice (20%). Remarkably, B-*Il10*$^{-/-}$ mice showed improved survival after infection with virulent *Salmonella*, indicating that IL-10 production by B cells inhibited protection (Figure 6A). The similar improvement of survival seen in infected B-*Il10*$^{-/-}$ and B-*Myd88*$^{-/-}$ mice, together with the impaired development of IL-10-expressing B cells in B-*Myd88*$^{-/-}$ mice,

suggested to us that IL-10 could be an important mediator of the suppression resulting from intrinsic MyD88 signaling in B cells during *Salmonella* infection. To examine this notion further, we characterized the innate and adaptive immune responses of B-*Il10*$^{-/-}$ mice infected with *Salmonella*. Notably, lack of IL-10 in B cells was associated with heightened infiltration of immune cells in infected livers, so that B-*Il10*$^{-/-}$ mice had ~2-fold more inflammatory foci than B-WT mice at day 6 p.i. (Figures 6B and 6C), as previously seen for B-*Myd88*$^{-/-}$ mice (Figure 2C). Similarly, B-*Il10*$^{-/-}$ mice displayed enhanced neutrophil and NK cell responses after infection with either attenuated or virulent *Salmonella* (Figures 6D and 6E). We also found, as previously described during EAE (Fillatreau et al., 2002), that IL-10 production by B cells strongly suppressed the specific CD4$^+$ T cell response to *Salmonella*: B-*Il10*$^{-/-}$ mice had more IFN-γ-producing CD4$^+$ T cells and more TNF-α-producing CD4$^+$ T cells than control mice at day 21 p.i. (Figure 6F). Thus, the regulatory functions of B cells during *Salmonella* infection involve both MyD88 and IL-10, as previously observed in EAE (Fillatreau et al., 2002; Lampropoulou et al., 2008). Considering that *Salmonella* triggers the development of IL-10-producing B cells in a MyD88-dependent manner, these results suggest that IL-10 mediates at least part of the suppressive functions resulting from MyD88 signaling in B cells. However, MyD88-activated B cells may also exert suppressive functions via additional mechanisms.

Vaccination Fully Protects Mice with *Myd88*-Deficient B Cells from Lethal Infection

TLRs and MyD88 can promote long-lasting antibody responses and T cell memory, suggesting a critical role for this pathway in vaccine efficacy (Krieg, 2007; Pasare and Medzhitov, 2004). However, we found that MyD88 expression in B cells can lead to inhibition of protective cellular immunity toward *Salmonella* infection. Thus, we examined whether MyD88 signaling in B cells affects the protective efficacy of a live-attenuated *Salmonella* vaccine.

B-WT and B-*Myd88*$^{-/-}$ mice were vaccinated with attenuated *Salmonella* and then challenged with virulent *Salmonella* 90 days later, a time point when the attenuated bacteria had been completely cleared (Figure S2A). MyD88 signaling in B cells profoundly affected the protection afforded by the vaccine. Vaccinated B-*Myd88*$^{-/-}$ mice were completely protected against virulent *S. typhimurium*, demonstrating a 100% survival after 2 months (Figure 7A). In contrast, protection was only partial in B-WT mice, which started to succumb at day 7 with a 50% survival rate after 60 days (Figure 7A). Thus, the efficacy of the live-attenuated vaccine is impaired as a result of MyD88 expression in B cells.

To identify the protective mechanisms regulated by MyD88-signaling in B cells during this rechallenge experiment, we infected vaccinated mice with attenuated *Salmonella* (SL7207) and analyzed innate and adaptive immune responses 5 days later, when bacterial loads were similar in spleens and livers of B-WT and B-*Myd88*$^{-/-}$ mice (Figure S5A). Because the protection afforded by the attenuated *Salmonella* vaccine involves antibody production (Mastroeni et al., 1993; Mittrücker et al., 2000), we measured amounts of *Salmonella*-reactive antibodies in serum of vaccinated mice before and after rechallenge. Serum antibody titers were comparable at day 90 in vaccinated B-WT and B-*Myd88*$^{-/-}$ mice, and they were not significantly modulated at day 5 after reinfection (Figure S5B). We then examined whether MyD88 signaling in B cells influenced memory T cell responses occurring in spleen and BM (Tokoyoda et al., 2009). *Salmonella*-reactive IFN-γ-, TNF-α-, and IL-10-producing CD4$^+$ and CD8$^+$ T cells were present in equal numbers in spleen and BM of B-WT and B-*Myd88*$^{-/-}$ mice at day 90 postvaccination (Figures 7B and 7C; Figure S6C). Anamnestic T cell responses were also similar at day 5 after rechallenge in B-WT and B-*Myd88*$^{-/-}$ mice, as shown by analyzes of production of IFN-γ, TNF-α, or IL-10 by CD4$^+$ and CD8$^+$ T cells from spleen and BM (Figures 7B and 7C; Figure S6C). T cell responses were not detected at day 5 p.i. in naive mice (data not shown). Thus, MyD88 signaling in B cells does not appear to modulate the formation or the reactivation of memory T cells at the time points analyzed. This is consistent with our observations in naive chimera mice, in which differences in T cell responses were only observed between B-WT and B-*Myd88*$^{-/-}$ mice at day 21 p.i. (Figures 4A–4C).

In contrast, vaccinated B-*Myd88*$^{-/-}$ mice displayed substantially higher innate responses than vaccinated B-WT mice upon rechallenge (Figures 7D and 7E). Vaccinated B-*Myd88*$^{-/-}$ mice harbored more neutrophils in spleen and liver than B-WT mice at day 5 after rechallenge, and markedly more TNF-α-producing neutrophils in spleen, whereas these parameters were similar in the two types of mice on day 90 (Figure 7D). Similarly, B-*Myd88*$^{-/-}$ mice accumulated more NK cells in livers than B-WT mice (Figure 7E). Thus, as seen for naive mice, MyD88-signaling in B cells exerts an early regulatory effect on innate immune responses mediated by neutrophils and NK cells during rechallenge of vaccinated mice.

We then examined whether this B cell-mediated suppression represented a major contribution of the B cell compartment during rechallenge of vaccinated mice. To this end, we performed vaccination and rechallenge experiments with wild-type C57BL/6 mice treated after vaccination, 1 week prior to rechallenge, with a B cell-depleting aCD20 antibody. Remarkably, this treatment improved the survival of vaccinated mice (Figure 7F), confirming the important role of B cell-mediated regulation during *Salmonella* infection in wild-type mice. Thus, a major function of the B cell compartment during reinfection of vaccinated mice is to regulate the protective innate response against *Salmonella*.

DISCUSSION

The signaling adaptor protein MyD88 is critical for effective host defense against infections (von Bernuth et al., 2008). However, we demonstrate in this study that MyD88 expression in B cells markedly inhibits protective immunity during systemic *Salmonella* infection. Deficiency of *Myd88* in B cells improved the control of the infection and sustained the survival of the infected mice. Remarkably, vaccination afforded complete protection against infection in mice with a B cell-specific *Myd88* deficiency, but only partial protection in control mice. Our data demonstrate that one of the major consequences of MyD88 signaling in B cells during infection with an intracellular bacterium is the suppression of innate immunity. Furthermore, using a B cell-depleting

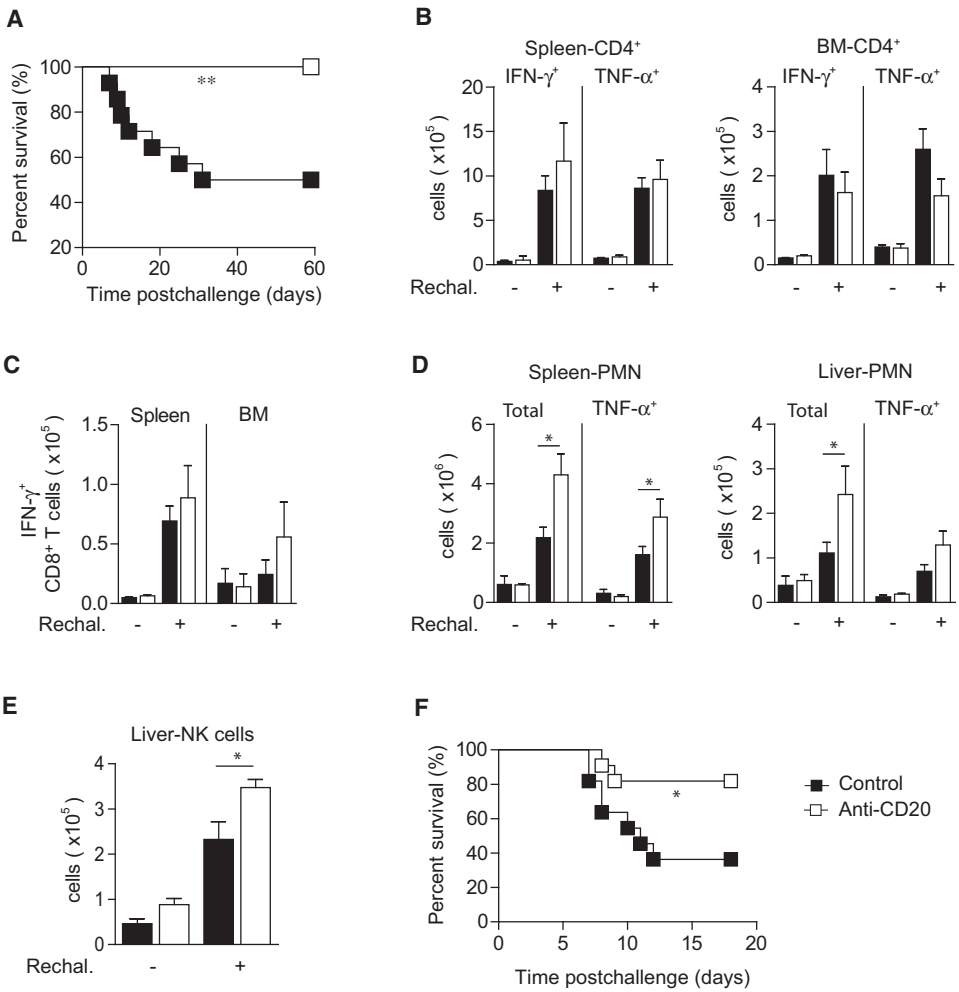

Figure 7. Vaccination Fully Protects Mice with *Myd88*-Deficient B Cells from Virulent *Salmonella* Infection

(A) Survival of vaccinated B-WT (black squares; n = 22) and B-*Myd88*$^{-/-}$ (white squares, n = 19) mice following i.v. challenge with virulent *Salmonella* SL1344. Mice were vaccinated i.v. with 10^6 attenuated *Salmonella* (SL7207) and challenged 90 days later with 100 CFU SL1344.
Results are pooled from two independent experiments; **p < 0.01.

(B–E) B-WT (black bars) and B-*Myd88*$^{-/-}$ (white bars) mice were vaccinated i.v. with 10^6 *Salmonella* (SL7207). After 90 days, mice were either re-challenged with 10^6 SL7207 i.v. (+) and analyzed 5 days later, or analyzed without rechallenge (–). Splenocytes, BM cells, and liver leukocytes were isolated from individual mice, restimulated for 6 hr with heat-killed *Salmonella*, and stained for T cell, PMN, and NK cell markers together with intracellular cytokines. Pooled data from three independent experiments (B-WT mice, n = 7; B-*Myd88*$^{-/-}$, n = 8) are shown. Graphs show the mean ± SEM; *p < 0.05.

(B) Total numbers of IFN-γ- and TNF-α-producing CD4$^+$ T cells in spleen (left) and BM (two femurs and two tibias) (right) in non-rechallenged (–), and rechallenged (+) mice.

(C) Total numbers of IFN-γ-producing CD8$^+$ T cells in spleen and BM, as described in (B).

(D) Total numbers of PMN and TNFα-producing PMN in spleen (left panel) and liver (right panel). PMN were defined as in Figure 3.

(E) Total numbers of NK cells in liver. NK cells were defined as in Figure 3.

(F) Survival of C57BL/6 mice vaccinated with 10^6 attenuated *Salmonella* (SL7207) and rechallenged 90 days later with 100 CFU virulent *Salmonella* (SL1344). A group of vaccinated mice received a single dose of B cell-depleting CD20 antibody 1 week prior to rechallenge. Shown are pooled data from two independent experiments (untreated group, n = 11; treated group, n = 11); *p < 0.05.

See also Figure S5.

antibody, we could show that B cells limit the resistance of vaccinated wild-type mice to rechallenge.

We show that *Salmonella* infection results in the rapid differentiation of IL-10-expressing B cells in spleens of infected mice. This response was already detected at 24 hr p.i. and persisted for at least 3 days. At these early time points *Il10* expression was restricted to B cells expressing the plasmablast and plasma cell marker CD138, as revealed using IL-10.eGFP reporter B-Green mice, and real-time PCR on cells isolated from infected wild-type mice. Intrinsic MyD88-signaling in B cells was essential for the optimal development of IL-10-producing CD19$^+$CD138$^+$ B cells, being absolutely required for the expansion of the CD19$^+$CD138$^+$ B cell subset during the early course of the infection. Altogether, these data position IL-10-producing B cells at the interface between the adaptive and the innate immune systems. Indeed, the rapidity of this response, as well as its

dependence on the signaling adaptor MyD88 are features characteristic of innate immunity. Furthermore, we observed that IL-10 produced by B cells regulates innate pathways involving neutrophils and NK cells. In vaccinated mice, such modulation of the innate immune system was associated with a marked inhibition of host resistance to reinfection. Thus, our observations suggest an unexpected regulatory role of IL-10-producing $CD138^{+}CD19^{+}$ B cells on innate immunity. Are these cells antibody-secreting plasmablasts or plasma cells? It will be important to further characterize these cells and to delineate their relationship with marginal zone B cells that express high amounts of TLRs, that rapidly secrete IL-10 upon TLR triggering, and that are among the first cells to encounter antigens from the blood.

Neutrophils provide an important first line of defense against *Salmonella* (Conlan, 1997). Soon after infection, they accumulate in spleen and liver, where they lyse infected cells, and prevent the spread of the bacteria (Conlan, 1996). Depletion of neutrophils results in dramatic expansion of *Salmonella* and dissemination of the infection to brain, lungs, and kidneys (Conlan, 1997). Remarkably, we found that neutrophils accumulated more effectively in spleens and livers of infected B-*Myd88*$^{-/-}$ and B-*Il10*$^{-/-}$ mice than of B-WT mice. MyD88 signaling in B cells also resulted in inhibition of this innate response upon rechallenge of vaccinated mice. Similarly, B cell-deficient mice mounted increased neutrophil responses after infection with *Francisella tularensis*, *Chlamydophila abortus*, *Mycobacterium tuberculosis*, or *Leishmania donovani*, which can all signal via TLRs and MyD88 (Bosio and Elkins, 2001; Buendía et al., 2002; Buendía et al., 2009; Maglione et al., 2007; Smelt et al., 2000). Although the signals driving B cell-mediated inhibitions of neutrophils were not defined in these studies, our observations together with these data suggest a general role for MyD88 signaling in B cells and for IL-10 in the regulation of neutrophil responses. Previous studies showed that IL-10 can regulate neutrophil responses during infections (Dai et al., 1997).

Control of *Salmonella* infection critically depends on IFN-γ (Hess et al., 1996; Spörri et al., 2006). At early stages of infection, NK cells are the major source of this cytokine. NK cell activation was severely impaired in *Myd88*-deficient mice, possibly due to the lack of intrinsic MyD88 signaling in NK cells (Spörri et al., 2006). Unexpectedly, we found that B-*Myd88*$^{-/-}$ and B-*Il10*$^{-/-}$ mice developed stronger NK cell responses than B-WT mice after *Salmonella* infection. Such regulation was also observed upon rechallenge of vaccinated B-WT and B-*Myd88*$^{-/-}$ mice. Similarly, mice lacking B cells showed increased NK cell activity after LCMV infection (Bründler et al., 1996). Such increased NK cell responses could result from heightened DC activation given that DCs can provide key signals for NK cell responses (Hochweller et al., 2008; Kang et al., 2008; Lucas et al., 2007). After *Listeria monocytogenes* infection, DCs recruit NK cells into foci where bacteria are trapped and activate them locally to produce IFN-γ via IL-12 and IL-18 (Kang et al., 2008). During *Salmonella* infection, the early production of IFN-γ also depends on IL-12 and IL-18 (Mastroeni et al., 1999; Mastroeni et al., 1996). Notably, B cells activated via MyD88 can directly suppress via IL-10 the provision of these NK cell-activating factors by DCs (Lampropoulou et al., 2008; Moulin et al., 2000). It is therefore plausible that an altered interaction between B cells and DCs underlies the increased NK cell responses in B-*Myd88*$^{-/-}$ mice. Others have recently shown that Treg cells can also suppress NK cell activity (Feuerer et al., 2009). However, it is unlikely that during *Salmonella* infection B cells could regulate NK cells via Treg cells given that Treg cell inactivation had no effect on survival of the infected mice.

We found here that B-*Myd88*$^{-/-}$ mice mounted stronger Th1 cell responses to *Salmonella* than B-WT mice. Intriguingly, B cells can also regulate T cell responses to self-antigens upon activation via MyD88, suggesting that MyD88 signaling in B cells operates within a general circuit of immune regulation (Lampropoulou et al., 2008; Skupsky et al., 2007; Zambidis et al., 1997). Our data indicate that this inhibitory function of B cells involves IL-10 given that *Salmonella* stimulates the accumulation of IL-10-producing B cells in a MyD88-dependent manner, and mice lacking IL-10-producing B cells also display enhanced inflammatory T cell responses during this bacterial infection, as previously observed in EAE (Fillatreau et al., 2002). It is likely that DCs are a major target of this suppression because IL-10 produced by TLR-activated B cells can inhibit the capacity of DCs to drive Th1 cell differentiation in vitro and in vivo (Lampropoulou et al., 2008; Sun et al., 2005). In addition, adoptive transfer experiments have shown that LPS-activated B cells can suppress T cell immunity via a mechanism independent of IL-10 but requiring expression of MHC-II and CD86 by the B cells (El-Amine et al., 2000; Litzinger et al., 2005). The role of this alternative mechanism remains to be tested during *Salmonella* infection.

The suppressive effects resulting from MyD88-signaling in B cells can be viewed as paradoxical considering the important roles of MyD88 and B cells for host defense. However, we recently proposed a model explaining how they could confer advantageous properties to the immune system by granting improved robustness of microbial sensing, and accelerated kinetic of induction of immunity, while preventing excessive tissue inflammation (Lampropoulou et al., 2010). This is possible because, as shown by our data, MyD88 signaling in B cells is part of a negative feed-forward loop controlling the responses triggered by MyD88 signaling in other cells (Lampropoulou et al., 2010; Lampropoulou et al., 2008; Sun et al., 2005). Notably, such negative feed-forward loops are one of the principal mechanisms for increasing robustness of biological systems toward external variation (Kitano, 2004). In the case of infection, mutations reducing the binding affinity of pathogen-associated molecular patterns (PAMPs) for the stimulatory pattern recognition receptors (PRRs) could facilitate pathogen escape. However, if PRRs can transmit both stimulatory and inhibitory signals, reduction in the affinity of the PAMP for the PRR would be compensated by a proportional diminution of the inhibitory signal, so that an effective immune response could still develop, as expected from a robust system. In addition, experiments and mathematical modeling have demonstrated that inhibitory feed-forward loops allow accelerated biological responses compared to simple stimulatory loops (Alon, 2007). The importance of rapid immune responses for the adequate control of pathogens is unambiguously demonstrated by the improved protection conferred by immunological memory and vaccines. Thus, it is not surprising to find that such loops, which are widely used in other biological and engineering systems, also contribute

to immune regulation (Alon, 2003). According to such model, suppression should be higher when the system is exposed to stronger stimulatory signals. Our finding that the amount of IL-10-producing B cells in spleens of infected mice was proportional to the number of bacteria administered is consistent with this notion.

In conclusion, we have identified an unexpected function of MyD88-signaling in B cells—the limitation of cell-mediated immune responses during bacterial infection—and confirmed its amplifying role in humoral immunity. Notably, we found that MyD88 signaling in B cells results in downmodulation of three crucial arms of cellular immunity: neutrophils, NK cells, and inflammatory T cells. It will be important to determine whether similar negative feed-forward loops, which can sustain the dynamics and the robustness of immunity, also operate in other systems of pathogen recognition. Unraveling the complexity of such multicellular networks is likely to facilitate the design of novel immune intervention strategies against pathogens.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, *Myd88*$^{-/-}$, *Il10*$^{-/-}$, *Tlr9*$^{-/-}$, *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$, and JHT (Gu et al., 1993) mice were bred under specific pathogen-free conditions. Chimera were made as described (Fillatreau and Gray, 2003). Experiments were performed in accordance with French, German, Portuguese, and Russian authorities.

Generation of B-Green IL-10^{iRESeGFP} Gene-Targeted Mouse

The long homology arm of the targeting construct comprised the third to fifth exons of the *Il10* gene (Figure S4B). An IRES-eGFP construct followed by loxP-flanked neomycin (neo^{R}) selection cassette was introduced just downstream of the translational stop. As a short homology arm we used the upstream 3′ untranslated region of IL-10's fifth exon along with the endogenous polyadenylation sequence. A herpes simplex thymidine-kinase expression cassette was cloned to the 3′ flank of the targeting construct as a counter-selectable marker for transgene-integration. E14.1 ESCs were electroporated with the linearized targeting construct and positively and negatively selected with G418 and gancyclovir, respectively. G418 and gancyclovir double-resistant ESC clones were screened for correct integration by Southern blotting and PCR. Finally, targeted clones were injected into C57BL/6 blastocysts. Chimeric males were bred to wild-type C57BL/6 mice, and offspring screened by PCR for the mutated allele. Deletion of the neomycin-resistant gene using Cre-mediated recombination of flanking loxP sites in the targeted locus was achieved by crossing with a Cre deletor transgenic line (Lewandoski et al., 1997).

Bacterial Infections

Salmonella typhimurium strains (virulent SL1344; attenuated aroA^{-} SL7207) were used as described (Mittrücker et al., 2000). Mice were infected i.v. with 1×10^6 colony-forming units (CFUs) SL7207 or 100 CFU SL1344. In vaccination and rechallenge experiments mice were first vaccinated with 1×10^6 CFU SL7207 and 90 days later were rechallenged i.v. with either 100 CFU SL1344 or 1×10^6 CFU SL7207, as indicated. In B cell depletion experiments, vaccinated C57BL/6 mice received a single i.v. dose of 250 μg anti-CD20 antibody (clone 18B12) 1 week prior to rechallenge with 100 CFU SL1344. The 18B12 IgG2a anti-CD20 was a kind gift of R. Dunn and M. Kehry at Biogen Idec (Hamel et al., 2008). Survival was recorded daily and presented as percentage of live animals. We determined bacterial burden by plating dilutions of homogenized organs on MacConkey agar plates. Heat-killed *Salmonella typhimurium* (HKST) was SL1344 inactivated by incubation at 70°C for 1 hr.

Histology

For immunostaining, 2- to 3-μm-thick sections of formalin-fixed, paraffin-embedded tissue were cut, deparaffinized, and subjected to a heat-induced epitope retrieval step. Slides were rinsed in cool running water and washed in Tris-buffered saline (pH 7.4) before incubation with primary antibodies against CD3 (N1580, Dako, Glostrup, Denmark, dilution 1:10), B220 (eBioscience, San Diego, CA, USA, 1:200), myeloperoxidase (A0398, Dako, 1:10000), and F4/80 (eBioscience, 1:50) for 30 min. For detection, biotinylated donkey anti-rat or donkey anti-rabbit (Dianova, Hamburg, Germany) secondary antibodies were used, and subsequently, the streptavidin-AP kit (K5005, Dako) was used. Alkaline phosphatase was revealed by Fast Red as chromogen. We performed negative controls by omitting the primary antibody.

Flow Cytometry

Cells were stained as follows: After blocking of Fc receptors (2.4G2), surface stainings were done with mAbs against B220 (RA3.6B2), CD4 (GK1.5), CD8α (53-6.7), TCRβ (H57-597), CD49b (DX-5), MHC-II (M5/114), CD138 (281-2), Fas (CD95), Ly-77 (GL7), CD11b (M1/70), CD11c (N418/HL3), CD80 (16-10A1), CD86 (GL1), CD1d (1B1), CD5 (53-7.3), CD21 (7G6), CD23 (B3B4), CD44 (IM7), CD62L (MEL-14), Ly6C (AL-21), and Ly6G (1A8). Antibodies were from BD PharMingen (San Diego, CA), eBioscience (San Diego, CA), or Miltenyi Biotec (Bergisch Gladbach, Germany) or produced in our facility. Dead cells were excluded with propidium iodide.

For intracellular stainings, splenocytes or BM cells were seeded at 3×10^6 cells per well in flat-bottomed 48-well plates with 1 ml RPMI 1640 (with 10% FCS, L-glutamine, penicillin, streptomycin, and 2-ME) and restimulated with HKST (1×10^8 bacteria/well). After 1h Golgistop (BD PharMingen) was added in recommended amounts. The cultures were left for another 4 hr 30 min, then surface and intracellularly stained with the Cytofix/Cytoperm kit (BD Biosciences) as recommended. Antibodies were anti-IL-10 (JES5-16E3), anti-IFN-γ (XMG1.2), and anti-TNF-α (MP6-XT22); all from BD PharMingen.

Data were acquired on FACSCalibur (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.). For FACS sorting, CD138^{+} and CD138^{-} CD19^{+} cells were sorted from live splenocytes gated on non-CD11b/CD11c/CD4 cell with a FACSAria II (BD Biosciences).

ELISA

To measure *Salmonella*-specific Abs, diluted sera were incubated on 96-well plates coated with 5×10^6 HKST/well. For measuring total Abs, plates were coated with anti-Ig(H^{+}L) (Southern Biotechnology Associates, Birmingham, AL, USA). Detection was done with alkaline phosphatase-conjugated anti-IgM and anti-IgGs Abs (Southern Biotechnology Associates).

Splenic B cells were obtained with anti-CD43 microbeads (Miltenyi Biotec), and activated at 5×10^5 cells per well in 96-well flat-bottom plates as described (Lampropoulou et al., 2008) with different amounts of HKST. IL-10 was measured after 3 days by cell-based ELISA, as described (Lampropoulou et al., 2008).

mRNA Expression Analysis

RNA was isolated from sorted cells with Trizol (AMS Biotechnology, Europe Ltd.), treated with DNase (Ambion Inc.), and reverse-transcribed with the Reverse Transcription System (Promega). Quantitative RT-PCR was performed on an MX3005P QPCR System (Stratagene), with LighCycler FastStart DNA Master SYBR Green I (Roche). Transcripts were quantified with β-actin as an internal standard. Forward primers (FPs) and reverse primers (RPs) were as follows (MWG Biotech): IL-10 FP: 5′-AGC CGG GAA GAC AAT AAC TG-3′, IL-10 RP: 5′-CAT TTC CGA TAA GGC TTG G-3′; β-actin FP: 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′, β-actin RP: 5′-TAA AAC GCA GCT CAG TAA CAG TCC-3′.

Alanine and Aspartate Aminotransferase Measurements

Serum samples were obtained on the indicated days, stored at −20°C, and analyzed on an automatic Roche modular analyzer (Grenzach-Wyhlen, Germany).

Statistical Analysis

Data were analyzed with an unpaired two-tailed Student's t test. Survival curves were compared with the log rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at doi:10.1016/j.immuni.2010.10.016.

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