Borrelia burgdorferi infection induced persistent IgM secretion controls bacteremia but not bacterial dissemination or tissue burden

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Abstract

Infection with *Borrelia burgdorferi (Bb)* causes Lyme disease in humans. In small rodents, the natural reservoir species of this spirochete, infections lead to only modest disease manifestations, despite causing persistence infection. While B cell responses are central for controlling bacterial tissue burden and disease manifestations, they lack classical aspects of T-dependent responses, such as sustained IgG affinity maturation and longevity, corresponding with a rapid collapse of germinal centers. Instead, the antibody response is characterized by strong and ongoing secretion of IgM, whose origins and impact on protective immunity to *B. burgdorferi* remains unknown. Here we demonstrate that *Bb* infection-induced IgM in mice was produced continuously, mainly by conventional B, not B-1 cells, in a T-independent manner. While IgM was passively protective and restricted early bacteremia, its production had no effects on bacterial dissemination into solid tissues, nor did it affect *Borrelia* tissue-burden. The latter was controlled by the induction of bactericidal IgG, as shown comparing infections in wild type mice with those of mice lacking exclusively secreted (s)IgM$^{-/-}$, all class-switched antibodies via deletion of *aicda* (AID$^{-/-}$), and all secreted antibodies (sIgM$^{-/-} \times$ AID$^{-/-}$). Consistent with the notion that *B. burgdorferi* infection drives production of IgM over more tissue-penetrable IgG, we demonstrated increased short- and long-term IgM antibody responses also to a co-administered, unrelated antigen. Thus, the continued production of IgM may explain the absence of *Bb* in the blood.

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Introduction

Borrelia burgdorferi sensu lato (Bb) is the causative agent of Lyme disease, a chronic, non-resolving, and often debilitating systemic emerging tick-transmitted infectious disease in humans, dogs and horses, among other species (1, 2). In mice, major natural reservoir hosts, *Bb* causes persistent infections with minimal clinical manifestations of disease, indicating that the bacteria have evolved complex strategies to evade mammalian immune responses. Indeed, our previous work showed that *Bb* rapidly enters draining LNs at the site of infection altering tissue structures of experimentally-infected mice (3, 4). While strong antibody production ensues and germinal centers initially form, a rapid collapse of germinal centers is noted around one month of infection, despite ongoing infection (3–5). This collapse in T-dependent germinal center responses correlated with the loss of high-affinity *Borrelia*-specific serum IgG antibodies (6). Together, these data demonstrate that *Bb* targets the inductive site of adaptive immunity and suggests that the antibody responses, while strongly induced and measurable, may be functionally compromised.

LNs are sites of early, strong and *Borrelia*-specific extrafollicular antibody production in infected mice (3). Nearly half of this response consisted of IgM (3). Beginning around three months after *Bb* infection, specific IgM-secreting plasma cells also begin to accumulate in the bone marrow (5). Bb-specific IgM has been found previously in the sera of 28 day Bb-infected mice (7). This is consistent with clinical studies, which have shown the presence of IgM responses to multiple *Bb* antigens even as far out as 10 years after the appearance of erythema migrans, an early manifestation of the disease in some humans, including individuals treated with antibiotics (8, 9). A remarkable observation, given the short half-life of IgM, considered to be under 24h.

It is unknown whether the ongoing, long-term production of *Bb*-specific IgM ultimately benefits the host or the pathogen. IgM has numerous functional characteristics that may benefit the host. IgM's multiple binding sites enable high-avidity binding, particularly to repeat antigens on bacterial or viral surfaces and to bacterial lipoproteins, which are abundant on the surface of *Bb* (10). Indeed, while class-switch recombination to IgG is considered a hallmark of protective immunity, studies with various pathogens have shown a necessary role for antigen-specific IgM in clearing infections and protecting the host from future challenge (11–13). IgM responses were shown also to be highly protective against infections with the blood-resident *B. hermsii*, the relapsing fever spirochete. In that infection the presence of IgM strongly reduced bacteremia, and therefore disease (14, 15). On the other hand, pentameric IgM, because of its large size, does not usually extravasate into connective tissues where *Bb* resides largely in the apparent absence of inflammation, thus perhaps precluding it from interactions once *Bb* has disseminated.

To define the function of IgM in immunity to Bb we studied its origins, protective capacity, and effects on the course of Bb infection. We demonstrate the strong and persistent production of passively protective *Bb*-specific IgM mainly by conventional B cells in a T-independent manner. IgM was shown non-redundant for controlling bacteremia, but not Bb dissemination and tissue burden, the latter was shown to require IgG. Immunization with a non-related antigen during *Bb* infection enhanced IgM responses compared to

responses in non-infected mice, suggesting that *Bb* may drive IgM responses to reduce antibody-mediated tissue clearance.

Material and Methods

Borrelia burgdorferi

A low-passage clonal strain of sensu stricto *Borrelia burgdorferi* (cN40) was grown in modified Barbour-Stoenner-Kelley II medium at 33°C and enumerated with a Petroff-Hauser bacterial counting chamber (Baxter Scientific, McGaw Park, IL) for inoculation of SCID mice. *Borrelia burgdorferi* lysate was obtained by growing N40 to log-phase (8–10 days) followed by pelleting by centrifugation, resuspended in cold PBS with $MgCl_2$ and centrifuged repeatedly at 17,500 g for 5 min at 4°C. Protein concentrations were determined by Bradford assay (Bio-Rad). Aliquots were stored at −20°C.

Mice

Eight- to twelve-week old female C57BL/6J, B6.Cg-*Igh^a^Thy1^a^Gpi1^a*/J (Igh^a^), B6.CB17-*Prkdc^scid^*/SzJ (SCID), B6.129P2-*Tcrb^tm1Mom^Tcrd^tm1Mom^*/J (ß/ T cell KO), B6.129S2-*Igh6^tm1Cgn^*/J (B cell KO) BALB/cByJ, B6.129S2-Cd40lg^tm1Imx^/J (CD40L^−/−^), CBySmn.CB17-*Prkdc^scid^*/J (BALB/c-SCID) female or male mice were purchased (The Jackson Laboratory). Late term pregnant Hsd:ICR (CD-1) mice (Harlan) and their 21-days old pups were used for passive protection assays. Breeders of mice that lack secreted IgM but not membrane bound IgM (sIgM^−/−^) originally generated by Jianzhu Chen (16), AID (AID^−/−^) (17), and mice knocked out for both sIgM and AID (sIgM × AID^−/−^) (18) on a C57BL/6 background, were kindly provided by Frances Lund (University of Alabama at Birmingham). All mice were kept under SPF housing conditions in ventilated microisolator cages. Mice were euthanized by over-exposure to carbon dioxide.

Ig allotype chimeras were generated as previously described (19, 20) to distinguish B-1 and B-2 cells and the IgM they secrete, using allotype and isotype specific reagents. In brief, newborn B6 pups (Igh-b) were treated with 0.1 mg of anti-IgM-b (AF6–78.25) i.p. to deplete all host B cells. On day 2 after birth, pups received 5×10^6 peritoneal cavity (PerC) cells from 8–12 weeks old congenic Igh-a donors as a source for B-1 cells. Pups were treated with 0.2 mg of AF6–78.25 i. p. biweekly for an additional six weeks. At that time *de novo* B-1 cell development from host bone marrow is strongly abrogated (21, 22). Mice were used two months after cessation of antibody treatment to allow full reconstitution of conventional B cells (Igh-b). The B-1 cell pool remains largely Igh-a (23, 24). Reverse chimeras were made also, in which newborn Igh-a mice were treated with anti-IgM-a (DS-1.1) and received cells from B6 (Igh-b) donors.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols involving animals were approved by the Animal Use and Care Committee at UC Davis.

Bactericidal assay

Bb was grown for 5 −7 days to mid-log phase at 33°C and adjusted to a density of 2.6×10^7 (Fig. 3B); 7.28×10^5 (Fig. 3C) and 8×10^5 (Fig. 3D) cells/ml, respectively. Sera pooled from day 31-infected C57BL/6 Igh-a or Igh-b expressing mice, sIgM$^{-/-}$ and SCID mice or graded doses purified IgG from indicated sources were diluted in modified Barbour-Stoenner-Kelley II (BSK) medium. To some cultures, as indicated, 1% serum from non-infected RAG−/− mice was added as a source of complement. Antibody/complement mixtures were added together with *Bb* to 0.5ml tubes containing BSK medium to generate a total volume of 0.2ml. As indicated, graded doses of antibodies were added to achieve concentrations of 0.0125, 0.025, 0.0375 and 0.05 mg IgG/ml, respectively. Tubes were briefly vortexed and then cultured for 48 hours at 33°C. Live *Bb* were counted using a hemocytometer and a darkfield microscope.

Infections and immunizations

Mice were infected with tissue-adapted *Bb* cN40 by transferring small pieces of ear tissue from SCID mice, infected for at least 14 days, into the right hind leg under the skin of recipients, as described previously (3). To generate *Bb*-infected ear tissue, B6 SCID mice were infected s.c. with 10^4 culture-grown *Bb* or 1×10^6 in BALB/c-SCID. Infected SCID mice were euthanized and ears were cleaned with 70% ethanol and diluted Nolvasan (Pfizer) before removal. Lateral tail vein blood collections were performed to obtain serum. For influenza immunization, mice were injected subcutaneously on the right side of the tail base with 500 hemagglutinating units (HAU) of A/PR8 (H1N1) in alum (Imject Alum, Thermo Fisher). Virus was propagated in embryonated hen eggs, and HAU determined as previously described (25).

Antibiotic treatment

Groups of mice infected with *Bb* were treated i.p. twice daily for 5 days followed by treatment once daily for 25 days with 16 mg/kg ceftriaxone in 100 μL PBS. Controls received PBS only.

Antibody purification

IgM was purified from sera of wildtype and β/δ T cell KO mice by column affinity chromatography, using immobilized anti-IgM mAb (clone 331, in-house generated with Thermo Scientific Pierce AminoLink Plus Immobilization Kit, pH 7.2 coupling buffer). IgM concentrations were determined by ELISA in comparison to a purified mouse IgM standard (Southern Biotech).

ELISA

Borrelia burgdorferi-specific ELISAs were performed by coating 96-well plates (Maxisorb, Thermo Fisher Scientific) with 1 μg/mL of N40 *Bb* lysate overnight at room temperature. Alternatively, coating was done with four immunodominant recombinant *Bb* proteins, decorin binding protein A (DbpA), outer surface protein C (OspC), arthritis related protein (Arp) and *Borrelia* membrane protein A (BmpA), as previously described (3). ELISAs were performed as previously described (3, 5). In brief, after blocking for 1 hour, diluted

sera were added to the plates and incubated for 2 hours. Antibody binding was revealed using biotin conjugated anti-IgM, anti-IgG, or anti-IgA (Southern Biotech) followed by an incubation with SA-HRP (Vector Laboratories) for 1 hour and then substrate for 20 min. Reactions were stopped with 1 N sulfuric acid and absorbance was read at 450 nm and reference wavelength of 595 nm using a SpectraMax M5 (Molecular Devices). Relative units antibody levels were calculated by comparison to a day-120 immune serum titrated on the same plates from mice infected with host-adapted spirochetes. To determine total concentrations of IgM, IgA or total Ig, ELISA was done as above but plates were coated with anti-IgM, -A or IgG (Southern Biotech) and binding was revealed with biotinylated antibodies to the matching Ig-isotype. Standards used were purified mouse IgM, -A or -G (Southern Biotech).

ELISPOT

To probe for *Bb*-specific antibody producing cells, 96-well plates (Multiscreen HA filtration; Millipore) were coated overnight as for ELISA plates. For influenza specific antibody producing cells, plates were coated with influenza A/PR8 (1000 HAU/mL). IgM-secreting cells were identified on plates coated with anti-IgM (clone 331, in-house generated by purification from hybridoma supernatants via protein G column affinity chromatography). After blocking with PBS/4% BSA for 1 h, our 5×10^5 cells in medium (RPMI 1640, 292 µg/mL L- glutamine, 100 µg/mL of penicillin and streptomycin, 10% heat inactivated FCS, and 0.03 M 2-ME) were placed in the starting well and 2-fold serially diluted. Cells were incubated at 37°C overnight then lysed with water. Antibody binding by B-1 and B-2 cells was revealed by adding biotin conjugated anti-IgM-a (DS-1.1) or anti-IgM-b (AF6–78.25), respectively, (both in house generated) in PBS/2% BSA for 2 hours, SA-HRP (Vector Laboratories) in PBS/2% BSA for 1 hour and revealing with 3-amino-9-ethylcarbazole (Sigma-Aldrich) for 10 min. Plates were dried and spots were counted in all wells with dilutions resulting in visible and countable spots using a stereomicroscope. Mean numbers ± SD were calculated from cell counts of all wells with countable spots.

Passive protection assay

To test for passive protective capacity of serum IgM from wildtype and ß/ T cell KO mice, purified serum IgM from day 60 infected mice was injected i. p. at various concentrations into 3-weeks old CD-1 pups prior to challenge with culture grown *Bb*. Injection of PBS or pre-immune sera served as negative controls. For all *Bb* challenge infections, s.c. inoculation of 10^4 N40 *Bb* between the shoulder blades was done 18 hours post antibody transfer. Mice were euthanized two weeks post infection and urinary bladder, heart base, and inoculation site were collected for qPCR and/or for culture in BSK II medium.

Flow cytometry

Single cell suspensions from inguinal LNs, spleens, peritoneal cavity, and bone marrow were stained as previously described (3). After Fc receptor blocking (10 µg anti-CD16/32, clone 2.4G2, in-house generated), the following antibody conjugations were used at predetermined optimal concentrations to determine frequencies of B cell subsets: CD19-Alexa700, CD8-Cy5PE (eBiosciences), CD24-FITC, CD23-FITC, CD43-PE, CD4-Cy5PE, F4/80-Cy5PE, CD5-biotin, IgM-allophycocyanin, IgM-a-allophycocyanin, IgM-b-allophycocyanin,

IgD-Cy7PE, IgD-a-Cy7PE, IgD-b-Cy7PE (all in-house generated), Streptavidin-Qdot605 (Invitrogen). Staining was performed on ice for 20 min. For dead cell discrimination, cells were stained with live/dead violet stain (Invitrogen by Life Technologies) on ice for 30 min. Data acquisition was performed on a 13-color FACSAria instrument (BD Biosciences) (26). Data were analyzed using FlowJo software.

qPCR for detection of *Bb* DNA

Quantitative real-time PCR was utilized to detect *Bb* flagellin (*flaB*) DNA in LNs, urinary bladder, injection sites and heart base, using primers and probe for *flaB* as described (27). Samples were amplified with 40 cycles of denaturation at 95°C for 15 sec, annealing at 50°C for 2 min and extension at 60°C for 1 min. Tissue samples were weighed and DNA was extracted from tissues with DNeasy kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Purified N40 DNA served as positive control and DNA from uninfected mice and water as negative controls. Data were calculated as the copy number of *flaB* DNA per mg of tissue based on DNA standard.

Western blotting

Whole cell *Bb* N40 proteins, loaded at 8μg/lane, were fractionated on 10–20% Tris-Glycine gel (Novex) before transfer to a nitrocellulose membrane (Pharmacia Biotech). Blots were blocked with 1% BSA in Tris-buffered-saline solution followed by incubation with immune sera from wild-type and sIgM$^{-/-}$ mice 31 days after infection or serum from uninfected wild-type mice (negative control), diluted at 1:100 for 2 hours. Blots were probed with goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma). Bands were revealed with a substrate solution containing 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium.

Statistical analysis

Statistical analysis was performed using the Student's t-test, and the Holm-Sidak method for multiple pairwise comparisons with help from Prism 8 software (GraphPad Software) as well as ANOVA for multi-group comparisons. A p-value of 0.05 was considered statistically significant.

Results

Continued production of Bb-specific IgM after infection

The presence of pathogen-specific serum IgM (with or without IgG) is often used as a diagnostic criterion of early infections. However, infection with *Bb* has been reported to induce the long-term elaboration of *Borrelia*-specific IgM in humans (8, 9) and mice (7). We previously showed that during acute infection, IgM antibodies binding to both, lysates of culture-grown *Bb* as well as to a pool of four immunodominant recombinant *Borrelia* proteins: Arthritis-resolving protein (Arp, Genbank AF050212), Outer surface protein C (OspC, AY275221), BmpA (AF288609.1) and Decorin-binding protein A (DbpA, AF069252.1) (3, 5), which we also used to assess anti-Borrelia antibodies here.

Borrelia-specific serum IgM increased during the first 30 days of infection of mice with host-adapted spirochetes and persisted for at least 24 weeks (Fig. 1A). 120-day infected mice lacking CD40L, a critical co-stimulator of T-dependent B cell activation, showed similar levels of anti-Borrelia IgM compared to controls, indicating that IgM production did not require cognate T cell help (Fig. 1B). 30-day treatment of mice with antibiotics (ceftriaxone) starting from 45 day after infection let to a significant drop in serum IgM levels against both, Arp and DbpA, compared to Bb-infected and sham-treated mice (Fig. 1C), similar to what we had observed previously for anti-Borrelia IgG responses (28). However, even 5 months after infection serum IgM levels were significantly higher than prior to infection in both the treated and non-treated groups ($p = 0.024$ and $p = 0.029$, respectively). Thus, persistent infection with *Bb* leads to long-term production of T-independent IgM such that IgM and IgG co-production occurred throughout infection (Fig. 1A). Despite extensive antibiotic treatment, Bb-IgM production was retained for months after infection to levels above those prior to infection.

IgM responses are generated mainly by follicular B cells

Given previous studies reporting that protective serum IgM responses to *B. hermsii* are derived from innate-like, T-independent CD5^{neg} B-1 cell responses (15), we considered whether the IgM response to *Bb* response was due to the activation of B-1 cells. To determine the contribution of B-1 cells to Bb-induced immunity, we used an antibody-mediated B cell depletion and adoptive transfer approach to generate chimeric mice in which B-1 and B-2 cells and the IgM they secrete can be distinguished by the Ig-allotype they express (Fig. 2A and Materials and Methods). In the chimeric mice the conventional B cells are derived exclusively from the bone marrow of the host mice (here Igh-b), while the B-1 cells are derived nearly exclusively from the transfer of congenic but allotype-disparate peritoneal cavity B-1 cells (here Igh-a). This approach allows identification of B lineage cells by criteria other than their potentially changing phenotype B cells. We have extensively validated this approach in the past (11, 19, 20, 24, 29). FACS-analysis confirmed that CD19+ IgMa+ IgDa+ cells were CD23- CD43+ CD5+ and CD5- B-1 cells (Fig. 2B), and IgDb^{hi} IgMb^{lo} B cells were host bone marrow-derived follicular B cells (Fig. 2C).

Bb infection of the chimeras resulted in a significant increase of both, B-1 and B-2 cells in the draining lymph nodes (LNs) at day 10 post infection, although the total number of B-1 cells remained very low (Fig. 2D). Only the CD5^{neg} but not CD5+ B-1 cells accumulated to higher levels in the LNs, while their numbers remained unchanged by the infection in peritoneal cavity and spleen (Fig. 2E). This was expected, as our recent studies have identified the CD5^{-} B-1 cells accumulating in draining LNs after infections (influenza and *Salmonella*) as activated CD5+ B-1 cells that have lost CD5 (30). Total (Fig. 2F), but not *Bb*-specific (Fig. 2G) IgM secretion by LN B-1 cells increased, when compared to non-infected mice, the vast majority of IgM was produced by host bone marrow-derived, thus mostly B-2 cells, in the LNs (Fig. 2F, G). Nonetheless, the latter may contain a very small amount (<1%) of CD5- B-1 cell-derived IgM, given that previous studies suggested that up to 10% of CD5- B-1 cells to be host-derived in these Ig-allotype chimeras (11, 19, 29). B-1 cells In the spleen of the chimeras, both B-1 and B-2 cell-derived total IgM increased significantly (Fig. 2H), possibly due to the activation of marginal zone B

cells (31). Studies in B6 mice showed no significant increases in the frequency of *Borrelia*-specific IgM-secreting cells in the spleens of day 10 infected B6 mice over a relatively high background, when probed with the cocktail of four recombinant proteins (Fig. 2I). The background presumably represents natural IgM secreting cells (3, 5). Collectively the data demonstrate that conventional LN B cells are major producers of the acute *Bb*-specific and T-independent IgM response. The data further indicate increased production of non-Bb specific IgM in the spleens in response to Bb infection.

IgM has passive protective capacity

Next, we studied the passive protective capacity of the IgM secreted in response to Bb infection. For this we adoptively transferred graded doses affinity chromatography-purified IgM from 60-day immune serum of either C57BL/6 or T cell-deficient (TCRβ/δ$^{-/-}$) mice into three-week old mouse pubs, which were challenged with 10^4 *Bb* spirochetes 18h later via syringe inoculation. One-week old pups were chosen because, due to their smaller size, they require less serum Ig for protection compared to adult mice. The level of protection was determined by establishing duplicate bacterial cultures of three organs (heart base, urinary bladder and LNs) from each recipient two-weeks after challenge. Mice were scored as positive if at least one culture showed the presence of spirochetes two weeks after culture onset. As little as 18μg purified immune IgM/mouse, derived from either wild type or T-deficient (TCRβ/δ$^{-/-}$) mice provided full immune protection, while 9μg IgM was partially protective (Table 1). Consistent with the T-independent nature of the IgM (Fig. 1B), there was no difference in the levels of protection with IgM purified from infected wild type or TCRβδ$^{-/-}$ mice. Furthermore, immune serum from TCRβδ$^{-/-}$ also provided full passive protection, while serum from non-infected B cell-deficient mice was non-protective, as expected (Table 1). Thus, infection-induced, T cell-independent IgM can provide passive protection from Bb challenge.

Bactericidal activity of immune sera is independent of sIgM

To explore potential mechanisms of sIgM-mediated immune protection we studied Bb infection in secreted (s)IgM-deficient mice (sIgM−/−). B cells of sIgM−/− mice express surface IgM, and can undergo class-switch recombination, but they cannot secrete IgM due to the deletion of the splice region for sIgM (16). Total serum IgG and IgA concentrations were comparable between C57BL/6 and sIgM−/− mice infected for 31 days with *Bb* (Suppl. Fig. 1A, C). As seen previously following influenza infection (11, 32), sIgM−/− mice mounted reduced *Bb*-specific IgG responses, when measured against a cocktail of 4 recombinant Bb proteins compared to wild type C57BL/6 mice (Fig. 3A, p< 0.0015 by two-way ANOVA). Since sIgM−/− mice express the Igh-a allele and C57BL/6 express Igh-b, we also measured Bb-specific responses in Igh-a expressing, C57BL/6 congenic wild type mice. Those mice showed similar serum IgG responses as C57BL/6 mice, but enhanced responses compared to sIgM−/− mice (Fig. 3A). Similarly, sIgM−/− mice had reduced Bb IgG compared to C57BL/6 mice on day 31 after infection when tested on bacterial lysates (Suppl. Fig. 1A). The strongest reductions were noted against DbpA (Suppl. Fig. 1B). As expected, IgM was absent in sIgM−/− mice (Suppl. Fig. 1D). Western-blot analysis did not reveal obvious differences in the pattern of bands from Borrelia lysates recognized by immune serum from either mouse strain (Suppl. Fig. 1E).

We then compared the *in vitro* borreliacidal activity of sera from day 31-infected C57BL/6 mice and sIgM$^{-/-}$ mice. Serum from infected SCID mice, lacking both sIgM and IgG, served as a negative control (Fig. 3B). Matching the volume of sera from control and sIgM$^{-/-}$, but not from infected SCID mice, when added to the cultures strongly, and in a dose-dependent manner facilitated killing of spirochetes *in vitro*, indicating the presence of antibodies with strong borreliacidal activity (Fig. 3B). The activity was lost when sera were heat-inactivated, presumably due to the loss of complement (not shown and see below). In multiple repeats of this assay, we found no consistent statistically significant differences in the bactericidal activity of sIgM–/– and control sera. This was surprising given the relatively large amount Borrelia-IgM in the sera of wild type mice and the reduction in IgG (Fig. 3A).

To determine whether enhanced bactericidal activity of purified IgG in the sera of sIgM–/– mice might offset the lack of sIgM and reduced amounts of IgG, we conducted similar in vitro studies comparing purified IgG from day 31 *Bb* infected C57BL/6 and sIgM–/– mice. Addition of 5μg IgG from either mouse strain resulted in strong borreliacidal activity when complement was provided to the cultures in form of serum from non-infected RAG1–/– mice. Control IgG from influenza infected mice had no significant effect (Fig. 3C). We repeated the assay also comparing bactericidal activity of graded doses purified IgG from *Bb*-infected C57BL/6-congenic mice expressing Igh-a. Again, maximal killing of Borrelia was achieved with about 5μg/ml immune-IgG, independent of the Ig-allotype (Fig. 3D). The activity required the presence of complement (Fig. 3D). Together the data suggested that despite the passive immune protective capacity of sIgM, the absence of sIgM does not significantly reduce the antibody-mediated *in vitro* borreliacidal activity of *Bb*-immune serum, or its purified IgG.

IgM does not significantly contribute to serum-mediated passive protection

Despite the potential of sIgM to provide immune protection in adaptive transfer experiments (Table 1), the *in vitro* studies did not support a strong contribution of sIgM in complement-mediated Bb killing. To determine whether the Bb-induced serum IgM contributed significantly to passive protection in the context of whole serum transfer, we compared the passive protective capacity of sera from C57BL/6 mice with that of sIgM-/–. Groups of three-week old mouse pubs (n= 5–7/group) received graded doses (50, 15, and 10 μg/ mouse) of serum adjusted to total Ig, as assessed by ELISA. A control group received PBS only. All groups of mice were challenged with 10^4 *Bb* spirochetes 18h later via syringe inoculation. Two weeks later, three tissues were taken, and DNA extracted for analysis of Bb copy number by PCR for Bb FlaB (Table 2). All mice that received PBS or sera from non-infected mice were positive for FlaB with high Bb copy numbers. In contrast, all but one mouse in each group were protected from Bb challenge when mice received 50 μg and 15 μg total Ig from either sIgM–/– or control mice (Table 2). The one infected mouse per group had greatly reduced copy numbers compared to the PBS or non-immune serum controls (Table 2). Partial protection was afforded by transfer of 10 μg/mouse immune serum and no significant differences were seen between C57BL/6 and sIgM–/– mice (Table 2). Thus, while IgM can provide passive protection, it did not seem to play a significant role in serum-mediated passive immune protection.

Secreted (s)IgM controls bacteremia but not bacterial tissue burden following Bb infection

To further determine the impact of sIgM on the control of *Bb* infection we compared Borrelia tissue burdens in the LNs, heart base and tibiotarsus joints in C57BL/6 and sIgM$^{-/-}$ mice by qPCR. Despite the significant and continued production of sIgM throughout the infection (Fig. 1A), sIgM did not have a noticeable impact of either Bb tissue load or dissemination kinetics (Fig. 4A). The one exception was a significant increase in Borrelia-burden at day 7 in the heart base of sIgM–/– compared to C57BL/6 control mice ($p < 0.028$; Fig, 4A, middle panel). The absence of sIgM also did not affect the overall very modest arthritis and carditis manifestations in mice on the B6 background at 121 days after infection (Suppl. Table 1).

Given the rapid dissemination of Bb into various organs, we compared the incidence rate of bacteremia in these mice. In contrast to the lack of effect on Borrelia tissue-loads, the presence of sIgM had a strong and significant impact on the control of bacteremia, particularly during the first two weeks of infection. While bacteremia was observed in at most 10% of infected B6 mice at any time point tested (Fig. 4B, C), over 70% of infected sIgM–/– mice had culturable bacteria in their blood on day 7 of infection, and bacteremia was observed still at day 21 (Fig. 4B, C). As the infection progressed the impact of sIgM became less pronounced, presumably due to the appearance of IgG (Fig. 3A). This is supported by analysis of total Ig-deficient SCID mice, in which all animals became bacteremic by day 10 and remained so throughout the study (Figure 4B, C). Thus, a major role of sIgM was identified in the control of Bb in the blood.

Next we compared the Borrelia tissue loads measured in C57BL/6 and sIgM–/– mice with that of *aicda (AID–/–)* mice (17), which lack the ability to generate any class-switched Ig but have supra-normal serum IgM concentrations, and with mice lacking all secreted Ig due to a lack of both, sIgM and *aicda* (sIgM × AID–/–) at day 121 after Bb infection. The data confirmed a requirement for IgG but not IgM in control of Borrelia tissue loads (Fig. 4D). The lack of class-switched antibodies alone in the AID–/– mice enhanced Borrelia-loads to levels similar to that of mice lacking all antibody secretion. And their levels were significantly higher than that of mice that lacked only sIgM or that had normal antibody levels (Fig. 4D). Similar results were obtained when we compared sIgM–/– and Ig-deficient (sIgM–/– × AID–/– mice) on day 30 of infection, the latter showing significantly increased Bb tissue burden (Suppl. Fig. 2A). Since sIgM–/– mice were engineered to express the Igh-a allotype we compared the sIgM–/– to control C57BL/6 mice carrying the Igha allotype. Similar to our previous studies, there was no significant difference in Bb tissue loads between sIgM$^{-/-}$ and wild type Igha-expressing mice, either on day 30 (Suppl. Fig. 2A), or on day 120 (Suppl. Fig. 2B) after Bb infection, suggesting that the Igh-allotype does not significantly affect the quality of the humoral response to Bb.

Together the data demonstrated that IgG but not IgM is critical for the long-term control of *Bb* tissue-burden or disease induction. Despite that, Borrelia tissue dissemination in mice appeared very little affected by the rate of bacteremia, suggesting Bb main mode of dissemination in mice occurs by means other than via the blood.

Bb infection enhances IgM responses

We had previously noted the relative abundance of IgM secretion in the lymph nodes of Bb infected mice, with Bb-specific antibody production contributing nearly half of antibody-secreting cells even beyond early timepoints of infection (3). Given that IgG but not sIgM controls *Bb* tissue loads, we aimed to determine whether *Borrelia*-infection steers immune responses towards exaggerated IgM production. For that we measured antibody-responses to immunization with an unrelated viral antigen in mice simultaneously infected with *Bb* (Fig. 5A). We chose influenza A/Puerto Rico/8/34 (A/PR8), as it results in strong IgG and only short-lived IgM responses (33). Both groups of mice also received A/PR8-specific transgenic CD4 T cells to provide strong T cell help. Remarkably, the frequencies of A/PR8-specific IgM secreting cells were strongly increased in the LNs of mice that were infected 10 days prior with *Bb* compared to mice that received A/PR8 immunization alone (Fig. 5B). In addition, 9 weeks after immunization significantly greater numbers of A/PR8-specific IgM-secreting plasma cells were found in the bone marrow of mice infected with *Bb* (Fig. 5C). Thus, infection with *Bb* drives B cell responses towards production of IgM, which reduces bacteremia and provides passive protection, but cannot affect *Bb* once disseminated into solid tissues.

Discussion

The complex enzootic life cycle of *Bb* requires the establishment of infected host reservoirs that enable the continued infection of newly hatched tick larvae and nymphs (34). Mice are one such reservoir species and the investigation into their immune responses can provide important clues about immune evasion strategies the bacteria have developed to establish persistence. Data presented here suggest that the regulation of B cell responses away from class-switched IgG and towards IgM production is one such mechanism. While immune IgM could provide passive immune protection, it did not affect Borrelia-clearance from solid host tissues, presumably due to its large size and the absence of continued tissue-inflammation during infection that otherwise could facilitate IgM extravasation. Interestingly, a heightened induction of IgM was observed not only to *Bb*, but also to a co-administered vaccine antigen, further strongly suggesting that it is not the quality of the *Borrelia* antigens, i.e. lipoproteins and carbohydrates, that drives the induction of IgM, but the inflammatory conditions induced by *Bb* (3, 35). Thus, we propose that the continued production of immune IgM is a manifestation of *Bb*-mediated B cell response subversion and represents an immune evasion strategy of *Bb*. It may promote Bb dissemination out of the blood and into the skin, where it can remain until attachment and bite of a tick will induce it to migrate towards the site of the tick-bite.

Strong humoral responses to many infections, both acute and chronic, often induce a serological response pattern characterized by the early and transient appearance of IgM that disappears within a couple weeks following initial infection, followed by an IgG response that is delayed by a few days, but then raises and remains present throughout infection (5). From this, critical serological diagnostic testing and interpretation have been developed that are widely used in the clinics, where the simultaneous measurement of IgM and IgG is used to diagnose acute (IgM alone or IgM plus IgG) from later (IgG only) stages of disease (36).

While such a pattern is observed widely with acute resolving infections, it is clear that such as "switch" from IgM to IgG does not occurs in response to all infections. For example, others had previously reported continued IgM production in response to infection of mice with the intracellular bacterium *Ehrlichia muris* (13). With regards to infection with *Bb*, our findings are consistent with findings in human patients, in which the continued presence of IgM in patients with positive confirmed diagnosis of Lyme disease was reported previously (8, 9, 37). Thus, classification of *Bb* infection based on the ratio of IgM : IgG into acute and chronic appears problematic.

Others showed previously that the multiple binding sites of the mostly pentameric IgM, compared to the monomeric IgG, as well as the flexibility of its molecular structure allow for better "coating" of bacterial surface antigens compared to IgG, and thus that IgM might have superior protective capacity (10). However, our in vitro studies did not support a superior bactericidal activity of sIgM at least for complement-mediated killing of *Bb*, given that the absence of sIgM from serum had no measurable effects on serum bactericidal activity. Interestingly, some previous studies suggested that the presence of sIgM can be detrimental for the host. During malaria infections non-specific IgM was shown to mask IgG binding sites on *Plasmodium falciparum* (38). During infection with *Schistosoma mansoni,* specific IgM was shown to bind the outer surface of this parasite and to block eosinophil-mediated killing (39), while IgG of identical specificity supported parasite killing. During *Salmonella* infection excess antibodies against an erroneous antigen was reported to block the protective capacity of other antigen specific antibodies (40). In that study, HIV infected individuals with non-typhoidal *Salmonella* infection had an excess of non-bactericidal lipopolysaccharide-specific IgG, which when bound to *Salmonella* blocked access for bactericidal outer membrane-specific IgG (40).

Of significance, previously studies showed a non-redundant role for IgM in the control of *B. hermsii* infection, a pathogen closely related to *Bb* (15). However, the pathogenesis of these two Borrelia-species is distinct, especially with regards to their tissue distribution. *B. hermsii* causes strong bacteremia and only sporadically infects tissues. In contrast, *Bb* predominantly resides in connective tissues of multiple organs with only episodic periods of bacteremia (41). Given its size, pentameric IgM may be most effective in binding to pathogens in the blood, as it seems to be able to extravasate into tissues only in the presence of inflammation and other forms of vascular leakage. Continued infection of *Bb*, in its persistent state, causes very little inflammation. Thus, *Bb* may evade the immune system by inducing strong IgM responses and residing in tissue where IgM is unable to reach.

It was surprising that the strong increase in the rate of bacteremia in mice lacking sIgM did not significantly affect bacterial burden in the various organs we tested, given that hematogenous dissemination of *Bb* has been previously indicated (reviewed in (42)). Instead, our data suggest that at least in mice, bacterial dissemination via blood may not contribute greatly to the rapid dissemination of *Bb* after infection. This is consistent with our previous findings that the dissemination to lymphoid tissues seem to follow lymph, rather than blood flow (3). However, it is conceivable that higher bacterial rates in blood of sIgM−/− mice may induce stronger levels of systemic inflammation and a heightened immune

response. However, we did not note any significant differences in clinical or pathological signs of disease between wild type and sIgM−/− mice (Suppl. Table 1).

While much of the *Bb* infection-induced IgM is generated independent of T cell help (5) and previous studies showed a role for B-1b cell-derived IgM in control of *B. hermsii* infection (15), we demonstrate here that the vast majority of the IgM response to *Bb* is produced by conventional B cells (Fig. 2). Only relatively few B-1 cells accumulated in the LNs, and their contribution to overall IgM production was low. The data agree with our findings that the induction of conventional B cell responses to an unrelated vaccine antigen (influenza) shifted towards increased IgM production during infection with *Bb*. This remarkable effect of the infection on promoting IgM responses rules out the nature of the *Bb*-antigens alone as the main driver of an IgM-dominated response. Instead, the data suggest that *Bb* might actively drive IgM over IgG responses.

IgM antibodies are evolutionarily conserved, participating in immune responses as far back as the jawed fish (43). Our findings that *Bb* might "exploit" this response to its advantage provides an opportunity not only to better understand *Bb* infections and to identify the unique serological fingerprints that aids their diagnosis, but also to explore the varied functions of this often-overlooked immunoglobulin isotype.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations:

Arp	arthritis-related protein
Bb	Borrelia burgdorferi
DbpA	Decorin-binding protein A
HA	hemagglutinin
LN	lymph node
OspC	Outer surface protein C
sIgM	secreted IgM

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Key Points

- *B. burgdorferi* induces B-2 cells to continuously secrete passively protective IgM

- IgM suppresses *B. burgdorferi* burden in the blood but not in tissues

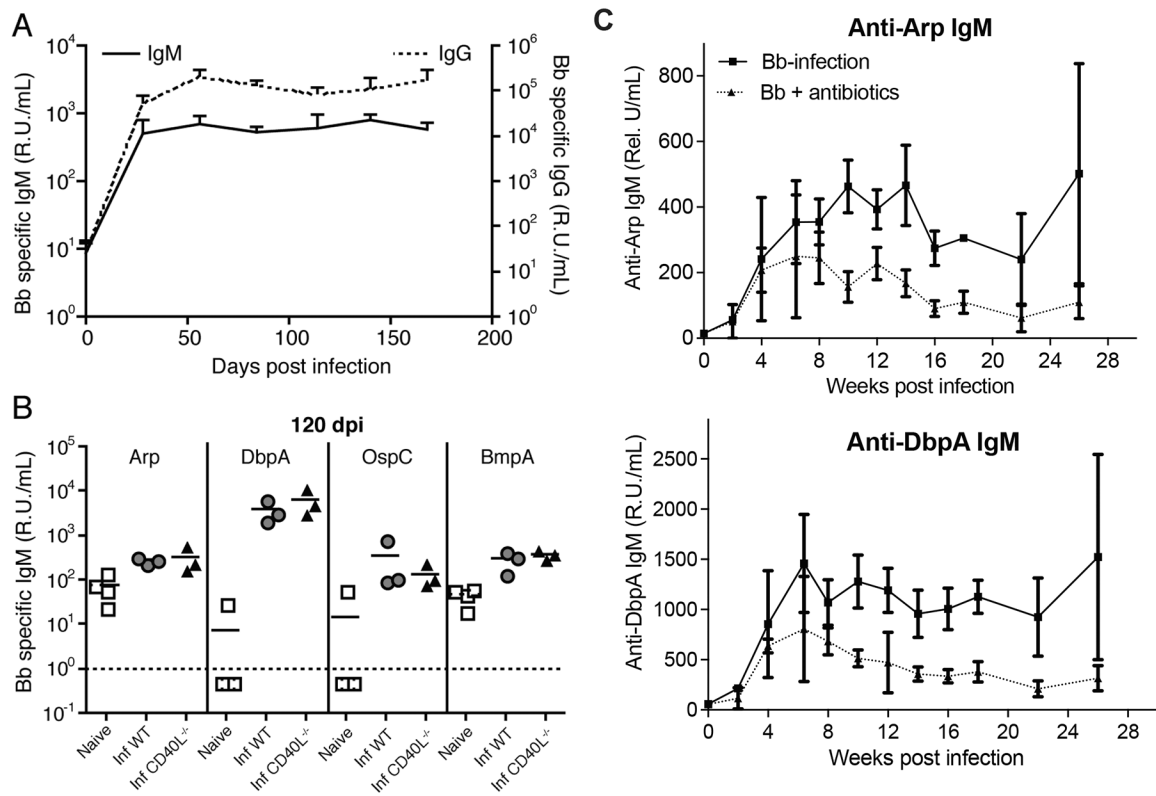- *B. burgdorferi* promotes enhanced IgM secretion after vaccination

Figure 1. Continuous production of T-independent IgM following *Bb* infection.
(**A**) Shown are relative units *Bb*-specific serum IgM (stippled line) and IgG (solid line) of mice at indicated times after infection with host-adapted spirochetes, as assessed by ELISA with a pool of recombinant *Borrelia*-proteins (DbpA, Arp, OspC, and BmpA). Each data point represents the mean of the group, + SD (n = 5). Data are from one experiment. Serum IgG titers are shown for comparison and have been reported previously (5). (**B**) Shown are relative units *Bb*-specific serum IgM to Borrelia proteins Arp, DbpA, OspC, BmpA in non-infected "naïve" mice, as well as B6 (WT) and congenic CD40L$^{-/-}$ mice day 120 post infection (inf) as assessed by ELISA. Symbols indicate results from individual mice and horizontal line indicates the mean for the group. (**C**) Relative units Arp- (top) and DbpA- (bottom) specific serum IgM of mice (n = 4) at indicated times after infection treated or not with ceftriaxone for 30 days starting on day 45.
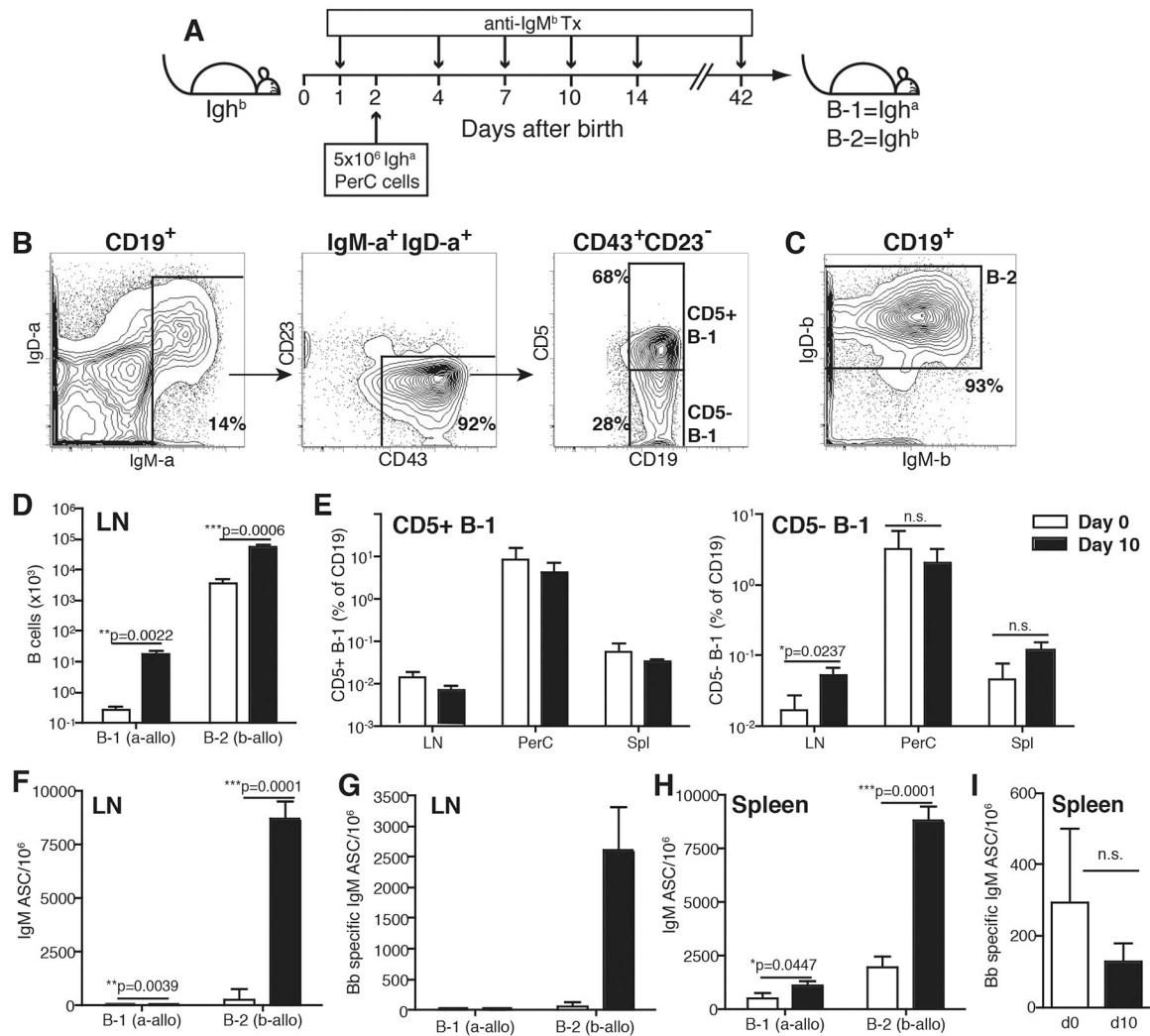
Figure 2. B-1 cells do not contribute significantly to IgM production in spleen or LNs after *Bb* infection.

(**A**) Experimental outline of neonatal Ig-allotype chimera generation. (**B**) Representative 5% FACS contour plots with outliers of CD19+ B cells from the peritoneal cavity of non-infected mice after gating of live single CD19+ lymphocytes. B-1 cells were identified as IgM-a+ IgD-a^lo (left panel) CD43+ and CD23− (middle). B-1 cells were further separated based on CD5 expression (right). (**C**) B-2 cells were identified as host-derived IgM-b^{+/−} IgD^hi cells. (**D**) Total numbers lymph node (LN) CD19+ B cells that were B-1 and B-2, as assessed by Ig-allotype. Bars indicate the mean number + SD before (open) and at 10 days after (filled) infection; (**E**) relative frequencies CD5+ and CD5- B-1 cell subsets in lymph node (LN), peritoneal cavity wash-out (PerC) and spleen (Spl). (**F**) Mean numbers + SD of total and (**G**) Bb-specific IgM-secreting B-1 (Igh-a) and B-2 (Igh-b) cells in LN; (**H**) Mean numbers + SD of total IgM-secreting B-1 (Igh-a) and B-2 (Igh-b) cells in spleen of chimeras. (**I**) Total *Bb*-specific IgM-secreting ASCs in the spleen of B6 mice prior to and at 10 days after Bb infection (n=4/group). Significant statistical values as indicated using unpaired, two-tailed Student's t-test. Results are from one of two (A-H, n = 3/group) and 4 (I; n=4/group) experiments that gave similar results.
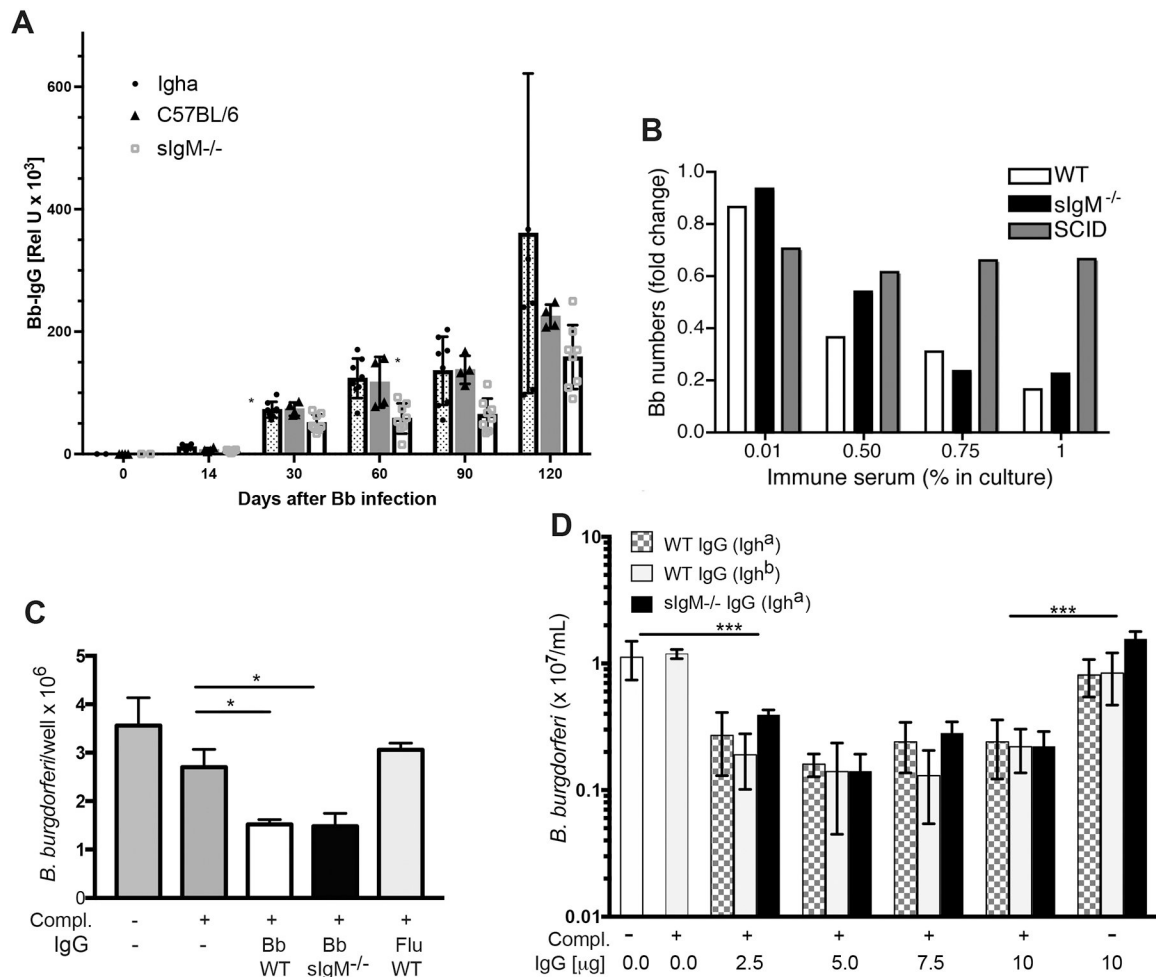
Figure 3. Similar bactericidal activity of immune sera from sIgM−/− and wildtype Bb-infected mice and their purified IgG

(**A**) Shown are mean serum Bb-specific IgG levels measured by ELISA at indicated times of Bb infection, expressed as relative units compared to a standard immune serum. Compared were sera from C57BL/6 congenic Igh-a mice and sIgM−/− mice (n = 8/group) and C57BL/6 mice (n=4). (**B**) Shown are mean fold-changes of *Bb* numbers in BSK cultures grown for 48h in the presence of indicated concentration serum from 31-days *Borrelia*-infected B6 (open bars), sIgM$^{-/-}$ (black) or SCID (grey) mice compared to cultures with medium alone. Shown is one of four experiments done using duplicate cultures that gave similar results. (**C**) Shown are mean ± SD *Bb* counts in cultures with BSK medium plus addition of 1% RAG−/− serum from non-infected mice as source of complement, where indicated, and 5μg purified IgG from sera of C57BL/6 or sIgM−/− mice infected for 31 days with *Bb*, or from C57BL/6 mice infected with influenza virus as a negative control. Results are pooled from 3 experiments done with cultures in triplicate. (**D**) Cultures were set-up as in (C) with indicated graded concentrations of purified IgG from C57BL/6 mice (checkered), B6 congenic but Igh-a expressing mice (light grey) or sIgM−/− mice (black) infected for 31 days with *Bb*. Cultures were done in quadruplicate and data represent one of 3 experiments done. Significance was tested for (A) by two-way ANOVA (p < 0.0015); for

(C) and (D) using unpaired, one-tailed Student's t-test comparing each culture to the BSK only control. *p < 0.05; ***p < 0.001.
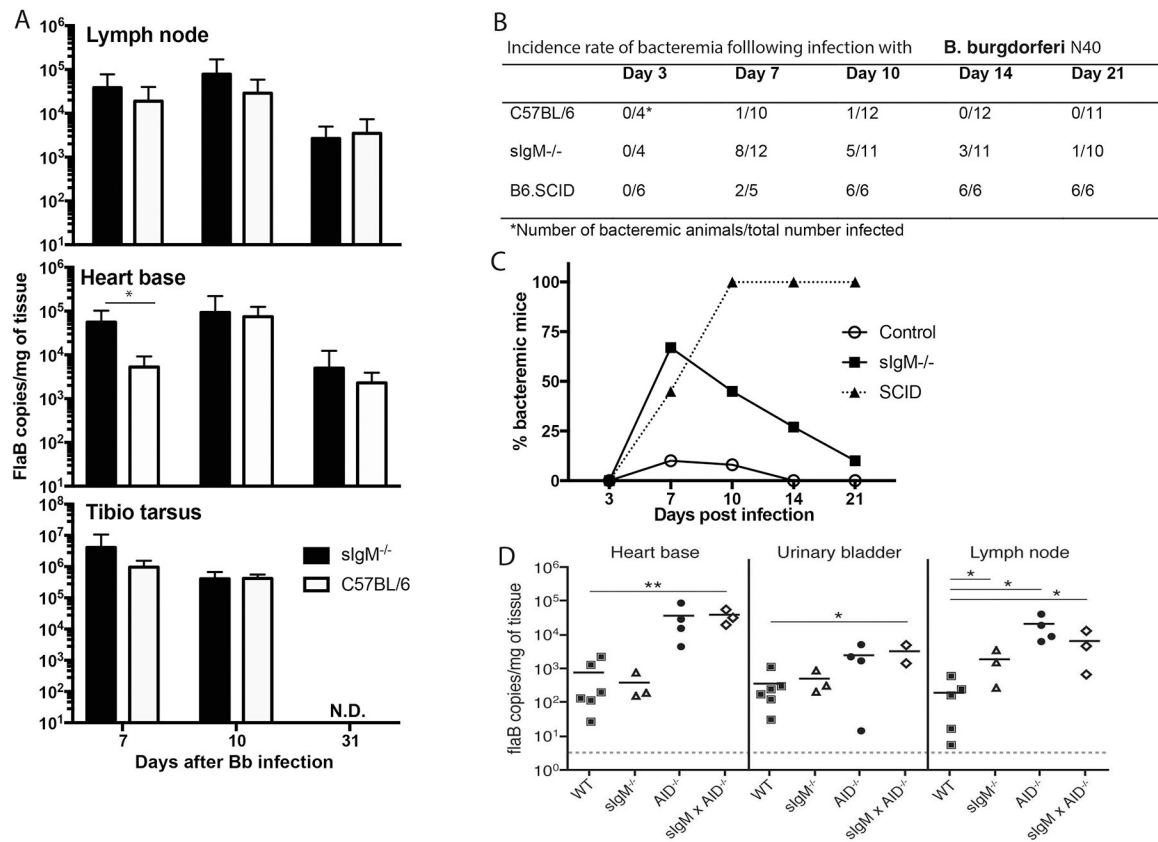
Figure 4. IgM controls *Bb* bacteremia but not tissue burden.

(A) Mean ± SD *Bb* tissue burden as assessed by qPCR, measuring flaB DNA copy number in indicated tissues of female C57BL/6 controls and sex-matched sIgM−/− mice at indicated days after infection. **(B)** Incidence of bacteremia in C57BL/6, sIgM−/− and B6.SCID mice at indicated time points after infection with host-adapted *Bb* N40 (n = 4–12/time point), as assessed by cultures of blood samples. **(C)** Data from (B) expressed as frequency bacteremic animals per group (culture positive/total infected). **(D)** *Bb* tissue burden of indicated tissues of day 121 *Bb*-infected female B6 (WT), sIgM−/−, AID−/− (mice lacking all class-switched antibodies) and sIgM−/− × AID−/− mice, (mice lacking all secreted antibodies). Each symbol represents one mouse, horizontal line indicates the mean for the group. Adjusted p-values were calculated using the Holm-Sidak method for pairwise multiple comparisons showing differences to WT controls. $*p < 0.05$; $**p < 0.001$. N.D., not determined.
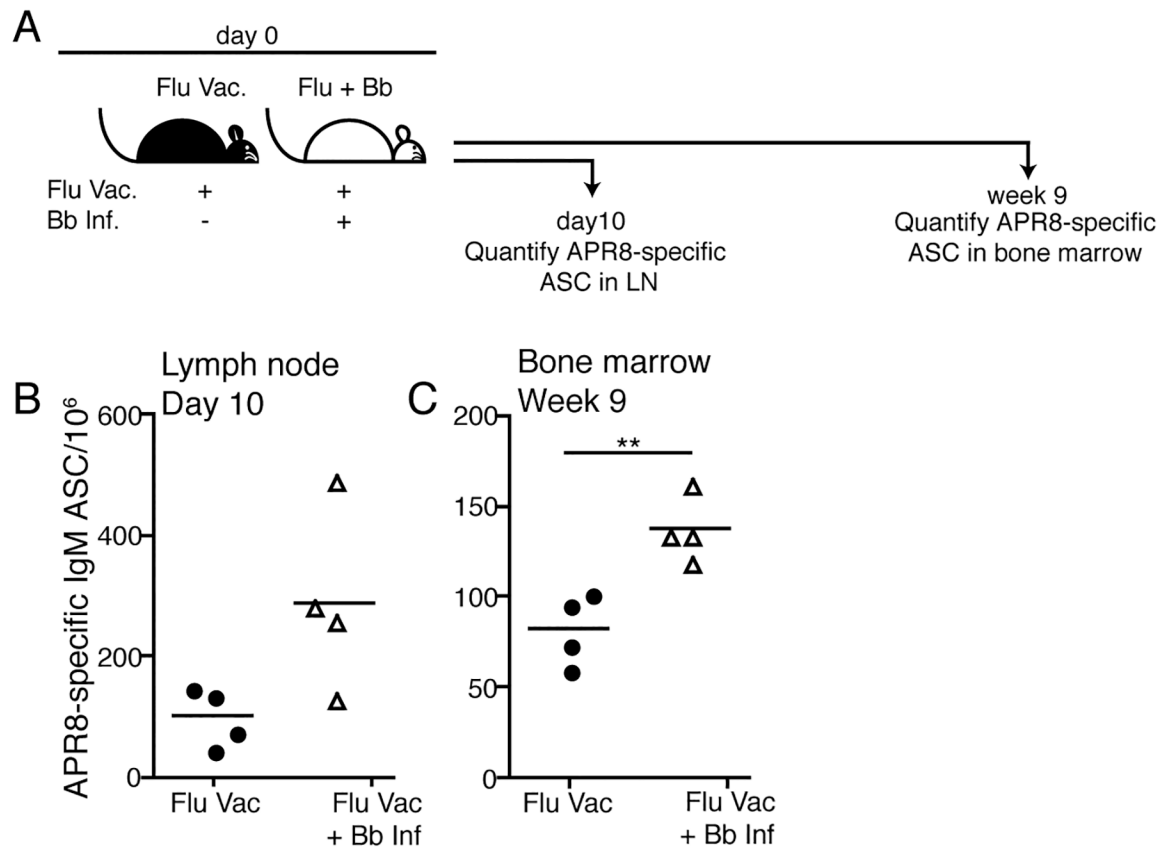
Figure 5. Infection with *Bb* increases IgM responses to an irrelevant antigen
(**A**) Experimental outline. Two groups of BALB/cByJ mice (n=4 per group) received 1 × 10^6 CD4 transgenic T cells specific for the hemagglutinin of influenza A/PR8 and were vaccinated s.c. with influenza A/PR8 in alum. One group was also infected with *Bb*. (**B**) At day 10 and (**C**) 9 weeks after infection, respectively, right inguinal LNs and bone marrow were analyzed by ELISPOT to quantify APR8-specific IgM ASC. Each symbol represents one mouse, horizontal line indicates the mean for the group. Data are representative of two independent experiments. Significant statistical values were determined using unpaired, one-tailed Student's t-test.

Table 1.

Protective capacity of purified IgM[a]

Serum Origin	Day after Infection	Passive Transfer	Dose	[a] Culture Positive/Total
B-deficient (μMT)	Day 0	Serum	50 μL	3/3
TCRβ/δ [−/−]	Day 60	Serum	50 μL	0/3
B6	Day 60	IgM	75 μg	0/4
			37 μg	0/4
			18 μg	0/4
			9 μg	1/4
βδ T cell KO	Day 60	IgM	75 μg	0/4
			37 μg	0/4
			18 μg	0/4
			9 μg	2/4

[a]Number of Bb-culture positive /total number tested. For each mouse multiple organs were cultured in duplicate for two weeks to grow *Bb*. Animals were regarded positive if at last one culture contained *Bb*.

Table 2.

The passive protective capacity of wildtype and sIgM$^{-/-}$ sera

		C57BL/6 Serum	sIgM$^{-/-}$ Serum	PBS (100 μL)
Inoculation site[a]	d0 sera	**5/5**[b] 19,309 (34,580)[c]	**5/5** 2,304 (677)	**7/7** 102,841 (241647)
	50 μg	**1/4** 0.2 (0.4)	**1/4** 0.2 (0.5)	
	15 μg	**1/7** 6 (16)	**1/7** 68 (181)	
	10 μg	**3/7** 315 (729)	**2/7** 43 (110)	
Urinary bladder	d0 sera	**4/5** 6,605 (14,258)	**2/5** 639 (908)	**6/7** 2,016 (2454)
	50 μg	**0/4** 0 (0)	**0/4** 0 (0)	
	15 μg	**0/7** 0 (0)	**0/7** 0 (0)	
	10 μg	**3/7** 67 (142)	**0/7** 0 (0)	
Heart base	d0 sera	**5/5** 42,419 (69,133)	**5/5** 8,258 (10,787)	**7/7** 102,841 (241647)
	50 μg	**1/4** 1 (2)	**1/4** 1 (2)	
	15 μg	**1/7** 73 (193)	**1/7** 89 (237)	
	10 μg	**4/7** 322 (407)	**2/7** 89 (154)	

[a]Groups of 3-week-old CD-1 pups (n=7) were treated with 100μl total serum from non-infected (d0 sera) wildtype or sIgM−/− mice, or with sera containing indicated amounts of total Ig from sera of day 31 infected mice as assessed by ELISA. All groups were challenged with *Bb* 18 hours post serum treatment and inoculation site, urinary bladder and heart base were collected from individual mice and assayed by qPCR for copy number of *Bb FlaB*.

[b]Number of pups positive by PCR/total number of pups in the group

[c]Mean copy number (SD) of *Bb* flagellin/mg of tissue of mice positive for *B. burgdorferi*