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IgM Production by Bone Marrow Plasmablasts Contributes to Long-Term Protection against Intracellular Bacterial Infection

Rachael Racine,* Maura McLaughlin,† Derek D. Jones,* Susan T. Wittmer,‡ Katherine C. MacNamara,* David L. Woodland,‡ and Gary M. Winslow*‡

IgM responses are well known to occur early postinfection and tend to be short-lived, which has suggested that this Ig does not significantly contribute to long-term immunity. In this study, we demonstrate that chronic infection with the intracellular bacterium *Ehrlichia muris* elicits a protective, long-term IgM response. Moreover, we identified a population of CD138highIgMhigh B cells responsible for Ag-specific IgM production in the bone marrow. The IgM-secreting cells, which exhibited characteristics of both plasmablasts and plasma cells, contributed to protection against fatal ehrlichial challenge. Mice deficient in activation-induced cytidine deaminase, which produce only IgM, were protected against fatal ehrlichial challenge. The IgM-secreting cells that we have identified were maintained in the bone marrow in the absence of chronic infection, as antibiotic-treated mice remained protected against challenge infection. Our studies identify a cell population that is responsible for the IgM production in the bone marrow, and they highlight a novel role for IgM in the maintenance of long-term immunity during intracellular bacterial infection. The Journal of Immunology, 2011, 186: 1011–1021.

Long-lasting humoral immunity is typically associated with the presence of high-affinity isotype-switched Abs (1). Although IgM is well known to act as the first line of defense during microbial infections, production of this Ig typically wanes during the development of IgG responses. Accordingly, IgM is not usually considered to play an important role in long-term immunity, even though this Ig is highly effective at host defense. IgM has a pentameric structure, which affords a higher valency than do the structures of other Igs, thus allowing for this Ig to bind Ags with high avidity (2, 3). IgM is also more efficient in agglutination, a process that facilitates the removal of foreign pathogens or Ags (4). IgM has been shown to regulate B cell development (5), to facilitate the clearance of apoptotic cells (6), and to modulate both inflammatory responses (7) and autoimmune diseases (8). Moreover, long-term IgM responses have been elicited in several infection and immunization models (9–14), and IgM-mediated protection has been reported against infection with the extracellular pathogens *Borrelia hermsii* (15) and *Streptococcus pneumoniae* (16). Although there are reports of IgM-mediated immunity to intracellular bacteria during acute infection (17, 18), the role of this Ig in the generation and maintenance of long-term immunity has not been studied.

Both plasmablasts and plasma cells are Ab-secreting cells. Plasmablasts primarily secrete IgM, but they also have the capacity to produce low-affinity isotype-switched Abs. Following their activation, plasmablasts usually undergo apoptosis within 3–4 d (19), but their lifespan can be extended by pathogen-associated inflammation or persistent Ag (11, 20, 21). Plasmablasts are found in the red pulp of the spleen, adjacent to the T cell zone (22, 23), and in the medullary cord of the lymph nodes (LNs) (24). Plasma cells are responsible for the production of isotype-switched Abs required for immunological memory. This population can be detected in secondary lymphoid organs, but long-lived plasma cells are more often found within specialized niches within the bone marrow (BM), accounting for ~0.5–1.0% of total mononuclear cells (25–28). Although plasmablasts and plasma cells have been studied extensively, IgM-secreting cells have not been characterized phenotypically in the BM.

Our studies of long-term IgM production during bacterial infection have used a model of protective immunity against fatal ehrlichial infection. The ehrlichiae are tick-borne, rickettsial pathogens, which can cause serious flu-like symptoms in both animals and humans (29). Prior infection with *Ehrlichia muris* generates B cell-dependent immunity against an ehrlichia known as *Ixodes ovatus* ehrlichia (IOE) (30). IOE, unlike *E. muris*, causes fatal infection in immunocompetent mice (31). *E. muris* infection is associated with the early expansion of splenic CD11c-expressing plasmablasts that are responsible for the production of CD4 T cell-independent Ag-specific IgM (32). The plasmablast response is associated with impaired germinal center formation, with a localized suppression of the splenic IgG production (33). Because Ag-specific IgM is maintained indefinitely in *E. muris*-immunized mice, we addressed a protective role for IgM in long-term immunity. In the current study, we characterized a population of IgM-secreting cells in the BM that contributes to the maintenance of long-term, Ag-specific IgM. Moreover, we demonstrated
that the IgM produced by this population is sufficient for protection against fatal challenge infection. Our findings thus highlight a novel role for IgM-secreting cells in long-term immunity during intracellular bacterial infection.

Materials and Methods

Mice

The C57BL/6 mice used in these studies were obtained from The Jackson Laboratory (Bar, Harbor, ME) or were bred in the Animal Care Facilities at the Wadsworth Center (Albany, NY) or the Trudeau Institute (Saranac Lake, NY) under microisolator conditions, in accordance with each institution’s guidelines for animal welfare. μs-deficient (B6;129S4-Igh-μm(S/J)) and activation-induced cytidine deaminase (Aicda)-deficient mouse strains were provided by Dr. J. Chen (Massachusetts Institute of Technology, Boston MA) and Dr. T. Honjo (Kyoto University, Kyoto, Japan), respectively. The μS/Aicda doubly deficient mice were provided by Dr. Troy Randall (University of Rochester, Rochester, NY).

Bacterial Infections

Details regarding the bacterial strains and infection protocols have been described previously (30). Mice were infected, via the peritoneum, with 5 × 10^8 copies of E. muris or 1 × 10^9 copies of IOE.

Quantification of bacteria

Bacterial copy number was determined by probe-based PCR, using primers and probes for the E. muris and IOE dsb genes, as described previously (34). The PCR products were analyzed with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City CA). The copy number of the E. muris dsb gene was determined using known quantities of dsb amplicons as standards. The limit of detection of the assay was found to be one copy of the dsb gene per 10 ng mouse genomic DNA. We have made the simplifying assumption that bacterial copy number and numbers of viable bacteria were equivalent in our experimental model.

Abs and flow cytometry

The following Abs used for flow cytometry were purchased from eBioscience (San Diego, CA): CD49d-FTTC (clone R1-2), CD44-FTTC (clone Pgp-1), IgM-FTTC (clone HI/41), IgD-FTTC (clone 11-26c), CD45R-PerCP-Cy5.5 (clone B220), CD93-allophycocyanin (clone AA4.1). The following Abs were purchased from BD Biosciences (Franklin Lakes, NJ): IA^a^-PE (clone AF6-120), IgM-PE (clone R6-620), CD138-PE (clone 281-2), CD184/CXCR4-PE (clone 2B11), CD19-PerCP-Cy5.5 (clone ID3), and CD138-alkaline phosphocyanin (clone AA4.1). The following Abs used for flow cytometry were purchased from eBio-Abs and flow cytometry were purchased from eBio-Sci-ence: CD4, CD8, CD11b, CD43, CD49b, Ly-6G (Gr-1), and Ter119 (obtained from Stem Cell Technologies, Vancouver, BC). The cells were incubated with Fc blocking solution, as described above, and were then stained with Abs directed against IgM and CD138; the cells were then separated using a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ).

Flow cytometric sorting of BM B cells

BM cells were obtained from six to eight age-matched mock-infected and E. muris-infected mice on day 100 postinfection. Single-cell suspensions were prepared, and the cells were resuspended at 1 × 10^6 cells/ml in calcium- and magnesium-free PBS containing 2% FBS. Erythrocytes were not lysed by the B cells were enriched by magnetic depletion, using mAbs to the following mouse cell surface Ags: CD4, CD8, CD11b, CD43, CD49b, Ly-6G (Gr-1), and Ter19 (obtained from Stem Cell Technologies, Vancouver, BC). The cells were incubated with Fc blocking solution, as described above, and were then stained with Abs directed against IgM and CD138; the cells were then separated using a FACSArray cell sorter (BD Biosciences, Franklin Lakes, NJ).

ELISA

Ab titers were determined by ELISA using purified recombinant E. muris outer membrane protein (OMP)-19 (35). Goat anti-mouse Abs conjugated to alkaline phosphatase were used to determine the serum titers of IgM, IgG, and the subclasses of IgG (SouthernBiotech, Birmingham, AL).

ELISPOT analyses

Detection of OMP-specific IgM-producing and IgG-producing cells was conducted using an ELISPOT assay. BM and splenocytes were obtained as

FIGURE 1. Ag-specific IgM-secreting cells reside in the spleen and BM. A. Serum OMP-19 IgM and IgG titers were determined on the indicated days after E. muris infection. B. The frequency and number of OMP-19 IgM- (top panels) and IgG-secreting cells (bottom panels) were assessed in the spleen, LN, BM, and PECs on day 150 postinfection by ELISPOT analysis. C. The frequency and number of Ag-specific IgG-secreting cells was assessed in the spleen and BM on day 366 postinfection. Spots produced by cells isolated from mock-infected mice, which were detected at frequencies of <0.001%, were enumerated and subtracted from the spots measured in the infected mice. The data are representative of two experiments in which four mice were analyzed at each time point.
described above. LN cells were obtained by mechanical disruption in HBSS containing 2% FBS. Erythrocytes were removed from the spleen and BM by brief hypotonic lysis. Peritoneal exudate cells (PECs) were obtained by lavage, and the cells were passed through a 70-μm pore size nylon strainer. Nitrocellulose plates (Multiscreen-HA; Millipore, Bedford, MA) were coated overnight at 4°C with a polyclonal IgM capture Ab (Southern-Biotech) or purified recombinant *E. muris* OMP-19 (10 μg/ml) (36). The assay plates were then incubated in blocking solution (IMDM supplemented with 10% FBS) for 2–3 h at 37°C. The cells were cultured in IMDM, supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 50 μM 2-ME, and 10% FBS, and were then seeded in 96-well plates at a concentration of 1 × 10^5 cells/ml, in duplicate, in a volume of 100 μl. BM populations isolated by flow cytometric sorting were plated at a concentration of 1 × 10^5 cells/ml, in duplicate, in a volume of 100 μl. The cells were further diluted in the microtiter plate using 2-fold doubling dilutions. After an 18-h incubation at 37°C in 5% CO₂, bound IgM or IgG was detected by using goat anti-mouse IgM or IgG conjugated to alkaline phosphatase (SouthernBiotech); 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (Sigma-Aldrich, St. Louis, MO) was used as the substrate. Spots were enumerated using a CTL ImmunoSpot S5 core analyzer, and the data were analyzed using CTL ImmunoSpot software (Cellular Technology). The frequency of Ag-specific Ig-secreting cells was determined by dividing the number of spots per well by the number of cells plated. Spots produced by cells from mock-infected control mice (which were <0.001%) were subtracted from the number of spots obtained from the infected mice.

**Doxycycline treatment**

C57BL/6 mice were infected with *E. muris*, and beginning on day 28 postinfection, half of the mice in the mock-control and infected groups were administered weekly i.p. injections of doxycycline (400 μg/mouse; Sigma-Aldrich). A total of three injections were performed during 2 wk. During the treatment period, doxycycline was also provided in drinking water at a concentration of 200 μg/ml.

**Statistical analyses**

Statistical analyses were performed with an unpaired Student *t* test (GraphPad Prism; GraphPad Software, La Jolla CA).

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**FIGURE 2.** *E. muris* infection is associated with an expansion of CD138<sup>high</sup>IgM<sup>high</sup> cells in the BM. A, BM cells from age-matched mock-infected and day 100 *E. muris*-infected mice were analyzed for cell surface expression of IgM and CD138. The gated regions used for the phenotype analysis are indicated (R1, R2, and R3). B, The cell frequency (top panel) and number (bottom panel) of each population was determined. C and D, The cell populations indicated in A from both mock-infected (top panels) and infected mice (bottom panels) were analyzed for expression of the indicated cell markers. The data are representative of two experiments in which four mice were used per group. A Student *t* test was used to determine statistical significance of the indicated (bracketed) differences. *p* < 0.05; **p** < 0.01.
Results

Both the spleen and bone marrow are sources of long-term IgM

We previously demonstrated that *E. muris* infection elicits a robust IgM plasmablast response (32) that is associated with impaired GC formation and suppressed IgG responses in the spleen (33). In those studies, however, we did not address whether IgM contributes to immunity following challenge infection. In this study, to determine the longevity of the humoral response during chronic *E. muris* infection, we first monitored the kinetics of the OMP-19 IgM and IgG production, beginning on day 30 postinfection. Ag-specific IgM was detected, and by day 150 postinfection, reciprocal serum titers were as high as 2400 and remained elevated for as long as 366 d (Fig. 1A). OMP-19 IgM titers were also detected in the sera from days 30 to 366 postinfection, at titers consistently $>300$, indicating that chronic infection elicited long-term IgM production. Therefore, we asked whether B cells residing in the peritoneal cavity, BM, LNs, or spleen contribute to long-term IgM and IgG production during *E. muris* infection. The highest frequencies and numbers of IgM- and IgG-producing cells were found in the spleen and BM on day 150 postinfection (Fig. 1B). OMP-19 IgM-secreting cells were not detected in the PECs, and only low cell frequencies were observed in the LNs (Fig. 1B). On day 366 after *E. muris* infection, both IgM-secreting and IgG-secreting cells were detected in the spleen and BM, but the frequency and number of cells were reduced relative to the values seen on day 150 postinfection (Fig. 1C). Although IgG-secreting cells are well known to reside in the BM (37), our results indicate that BM Ab-secreting cells can contribute to the long-term production of Ag-specific IgM.

Identification of an Ag-specific IgM-secreting population in the BM

The development of IgG-secreting plasma cells in the BM has been extensively investigated. Although IgM-secreting cells in the BM have been detected in mice (38–40) and humans (41, 42), the cells responsible for IgM production have not been phenotypically characterized. To identify the IgM-secreting cells in the BM, we analyzed *E. muris*-infected mice on day 100 postinfection for cell surface expression of IgM and CD138 (Syndecan-1) (43). We identified three B cell populations that expressed CD138:

- CD138$^\text{high}$IgM$^\text{high}$ cells, CD138$^\text{+}$IgM$^\text{+}$ cells, and CD138$^\text{+}$IgM$^\text{−}$ cells (Fig. 2A). We observed that the frequency and number of CD138$^\text{high}$IgM$^\text{high}$ cells were 3-fold higher than the frequency and number of cells in uninfected mice (Fig. 2A, 2B). In contrast, the frequencies of CD138$^\text{+}$IgM$^\text{+}$ and CD138$^\text{+}$IgM$^\text{−}$ cells were lower in the infected mice than in the uninfected mice.

Even though CD138 is a good cell surface marker for Ab-secreting cells, the marker can also be expressed on developing B lymphocytes in the BM (44). Therefore, we also analyzed the three CD138-expressing populations, in both uninfected and infected mice, to characterize the expression of other cell surface markers associated with the differentiation of Ab-secreting cells. Plasmablasts express a number of B lymphocyte markers, including, CD19, B220, IgM, IgD, and MHC class II, although at lower levels than do mature B cells. During the final stages of plasma cell maturation, Ab-secreting cells lose surface expression of CD19, B220, IgM, IgD, and MHC class II. In addition to CD138, both plasmablasts and plasma cells express a number of other cell surface receptors, including CD93, CXCR4, CD44, and CD49d (45–49). Therefore, we addressed whether these B lymphocyte Ags were expressed on the three populations of CD138-expressing cells identified in Fig. 2A. Both CD19 and B220 were detected on cells from all three CD138-expressing populations; however, cell surface expression of B220 was much lower on the CD138$^\text{high}$IgM$^\text{high}$ cells than on the CD138$^\text{+}$IgM$^\text{+}$ and CD138$^\text{+}$IgM$^\text{−}$ cells (Fig. 2C). Intermediate to high cell surface expression of IgD was only detected on some of the CD138$^\text{+}$IgM$^\text{+}$ cells (R2 in Fig. 2C), whereas MHC class II was most highly expressed on the CD138$^\text{high}$IgM$^\text{high}$ cells. The CD138$^\text{+}$IgM$^\text{high}$ cells also exhibited high surface expression of CXCR4, CD49d, CD44, and CD93, all of which are cell surface receptors, including CD93, CXCR4, CD44, and CD49d (45–49). Therefore, we addressed whether these B lymphocyte Ags were expressed on the three populations of CD138-expressing cells identified in Fig. 2A. Both CD19 and B220 were detected on cells from all three CD138-expressing populations; however, cell surface expression of B220 was much lower on the CD138$^\text{high}$IgM$^\text{high}$ cells than on the CD138$^\text{+}$IgM$^\text{+}$ and CD138$^\text{+}$IgM$^\text{−}$ cells (Fig. 2C). Intermediate to high cell surface expression of IgD was only detected on some of the CD138$^\text{+}$IgM$^\text{+}$ cells (R2 in Fig. 2C), whereas MHC class II was most highly expressed on the CD138$^\text{high}$IgM$^\text{high}$ cells. The CD138$^\text{+}$IgM$^\text{high}$ cells also exhibited high surface expression of CXCR4, CD49d, CD44, and CD93, all of which are cell surface receptors, typically found on Ab-secreting cells (45) (Fig. 2D, Table I). Although CXCR4 was detected on a portion of the CD138$^\text{+}$IgM$^\text{−}$ cells, there was no apparent upregulation of the other cell surface markers on the CD138$^\text{+}$IgM$^\text{+}$ or CD138$^\text{+}$IgM$^\text{−}$ cells (Fig. 2D). These data demonstrate that the CD138$^\text{high}$IgM$^\text{high}$ population increased in frequency and number during ehrlichial infection, and this population exhibited phenotypic characteristics of both plasmablasts and plasma cells.

To determine whether the CD138-expressing cells produced OMP-19–specific IgM in the BM, we next isolated each of the

Table 1. Expression of cell surface markers on BM B cells

<table>
<thead>
<tr>
<th>Marker</th>
<th>Status</th>
<th>CD138$^\text{high}$IgM$^\text{high}$ (R1)</th>
<th>CD138$^\text{+}$IgM$^\text{+}$ (R2)</th>
<th>CD138$^\text{+}$IgM$^\text{−}$ (R3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>Mock</td>
<td>45.2 ± 15.3</td>
<td>31.6 ± 2.2</td>
<td>27.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>33.5 ± 1.2</td>
<td>32.4 ± 4.5</td>
<td>28.0 ± 0.9</td>
</tr>
<tr>
<td>B220</td>
<td>Mock</td>
<td>51.3 ± 12.9</td>
<td>83.7 ± 4.1</td>
<td>53.0 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>33.3 ± 6.1*</td>
<td>82.9 ± 5.0</td>
<td>53.3 ± 2.4</td>
</tr>
<tr>
<td>IgD</td>
<td>Mock</td>
<td>42.7 ± 15</td>
<td>151.7 ± 9.5</td>
<td>7.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>64.7 ± 12.1</td>
<td>100.7 ± 24.0*</td>
<td>8.4 ± 0.9</td>
</tr>
<tr>
<td>IA$^b$</td>
<td>Mock</td>
<td>133.8 ± 17.0</td>
<td>25.5 ± 4.1</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>108.3 ± 31.9</td>
<td>36.3 ± 5.0*</td>
<td>9.0 ± 2.0</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Mock</td>
<td>84.8 ± 16.5</td>
<td>34.7 ± 11.5</td>
<td>7.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>99.2 ± 23.4</td>
<td>36.6 ± 7.5</td>
<td>7.3 ± 0.0</td>
</tr>
<tr>
<td>CD49d</td>
<td>Mock</td>
<td>44.4 ± 4.1</td>
<td>12.7 ± 4.6**</td>
<td>11.6 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>68.2 ± 5.9*</td>
<td>20.8 ± 5.8**</td>
<td>12.8 ± 1.7</td>
</tr>
<tr>
<td>CD44</td>
<td>Mock</td>
<td>668.3 ± 92.2</td>
<td>165.7 ± 7.8</td>
<td>244.7 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>829.7 ± 25.5*</td>
<td>166.0 ± 13.5</td>
<td>232.0 ± 13.1</td>
</tr>
<tr>
<td>CD93</td>
<td>Mock</td>
<td>222.8 ± 49.2</td>
<td>122.3 ± 15.5</td>
<td>242.3 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>Infected</td>
<td>595.3 ± 16.8**</td>
<td>122.0 ± 9.0</td>
<td>249.0 ± 2.3</td>
</tr>
</tbody>
</table>

Data indicate the mean fluorescence intensities of the major cell populations analyzed in Fig. 2C, 2D. The flow cytometry regions are from Fig. 2A. The means and SD are shown. Statistically significant differences between the infected and mock controls are indicated in boldface type.

$^p < 0.05$; $^{**} p < 0.01$. 

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three populations described in Fig. 2 (Supplemental Figs. 1, 2) and determined the frequency and number of Ag-specific IgM and IgG responses by ELISPOT analysis. Up to 5% of the BM CD138<sup>high</sup> IgM<sup>high</sup> cells secreted OMP-19 IgM following ehrlichial infection, accounting for ~4000 cells in the BM on day 100 postinfection (Fig. 3A). OMP-19 IgM was also produced by the CD138<sup>high</sup> IgM<sup>−</sup> population, but the frequencies and numbers of these cells were decreased by at least 10-fold relative to the CD138<sup>high</sup>IgM<sup>high</sup> cells. Furthermore, the CD138<sup>high</sup>IgM<sup>high</sup> cells secreted only IgM; IgG was not produced by this population (Fig. 3B). OMP-19 specific IgG was only secreted by the CD138<sup>high</sup>IgM<sup>−</sup> population, and the frequency of such B cells was <0.03% (Fig. 3A). These results demonstrate that the CD138<sup>high</sup>IgM<sup>high</sup> cells are responsible for most Ag-specific IgM production in the BM during chronic ehrlichial infection.

IgM is sufficient for protection against fatal ehrlichial challenge infection

Previous studies have demonstrated that prior E. muris infection protected mice against an otherwise fatal ehrlichial challenge (30, 50). Although adaptive IgG responses are a hallmark of immunological memory, we addressed whether the IgM that we detected during chronic infection contributed to their protection. To address roles for IgM and IgG, we used three different Ig-deficient mouse strains that lacked either IgM, IgG, or all Abs. Secretory IgM (μS)-deficient mice carry a targeted deletion in the part of the Ig μ H chain that removes both the translation termination and polyadenylation site; thus, in mice of this strain, IgM is expressed on the B cell surface, but it is not secreted (51). Mice deficient for activation-induced cytidine deaminase (Aicda), an enzyme that is required for Ig class-switching, produce IgM, but not IgG (52). μS/Aicda doubly deficient mice were also used in these studies; they have no secretory Abs, but contain a quasination B cell repertoire (53).

We first confirmed that only the expected Abs were produced in the gene-targeted strains by analyzing the serum Igs in day 28 E. muris-infected mice. Aicda-deficient and μS-deficient mice lacked the production of IgG and IgM, respectively, although, OMP-19 Abs titers, in both gene-targeted strains, were higher than in C57BL/6 mice (Fig. 4A,4B) as reported previously (51). Because the μS-deficient mice were on the B6.129 background, we used IgG2a to detect production of Abs in this strain, whereas IgG2c was used for the C57BL/6 control mice. Ag-specific IgG2a/c was 10-fold higher in the infected μS-deficient mice than in the wild-type mice; 3- to 4-fold higher titers of IgG2b and IgG3 were also detected (Fig. 4C). Neither OMP-19–specific IgM or IgG was observed in the sera of μS/Aicda-deficient mice, as expected (data not shown).

### FIGURE 3. The CD138<sup>high</sup> IgM<sup>high</sup> cells produce most OMP-19 IgM in the BM.

A. The CD138<sup>high</sup>IgM<sup>high</sup>, CD138<sup>+</sup>IgM+, and CD138<sup>+</sup>IgM<sup>−</sup> populations were separated by flow cytometric sorting from both mock-infected (open histograms) and day 100 E. muris-infected C57BL/6 mice (filled histograms). The frequency and total number of OMP-19 IgM (top row) and IgG (bottom row) were determined by ELISPOT analysis.

B. The frequency and number of total IgM- and IgG-secreting cells produced by the CD138<sup>high</sup>IgM<sup>high</sup> population were determined by ELISPOT analysis. These data are representative of two independent experiments, where six to eight mice were used per group. A Student t test was used to determine statistical significance of the indicated (bracketed) differences. *p < 0.05; **p < 0.01.
We infected the three gene-targeted strains and C57BL/6 mice with *E. muris* to determine whether IgM and IgG were required for survival. Both the μS-deficient and Aicda-deficient strains survived infection. However, 80% of the μS/Aicda-deficient mice became moribund, thus confirming findings from previous studies by our laboratory and others that Abs are essential for immunity to *E. muris* (Fig. 4D) (30, 50). Infection was similar in the μS-deficient and C57BL/6 mice, but bacterial numbers were reduced by 2- to 7-fold in the spleen and PECs, respectively, in Aicda-deficient mice on day 10 postinfection (Fig. 4E). Although bacteria were no longer detected in the spleens of the three gene-targeted strains by day 30 postinfection, an ∼2-fold decrease in bacterial burden in the PECs, relative to wild-type mice, was observed in both the Aicda-deficient and μS-deficient mice (Fig. 4F). Thus, protection against *E. muris* infection was observed in the presence of either IgM or IgG.

We next addressed whether IgM or IgG alone was sufficient for protection against fatal IOE infection. The μS-deficient and Aicda-deficient mice that survived *E. muris* infection were challenged with IOE on day 30 after *E. muris* infection. Both immunized gene-targeted strains, as well as C57BL/6 mice, survived otherwise fatal IOE challenge infection (Fig. 4G). Although other cells and factors, including CD4 T cells, likely contribute to immunity, these studies demonstrate that either IgM or IgG is sufficient for the protective humoral response against an otherwise fatal challenge infection. Long-term IgG-mediated immunity is not unexpected, but the ability of IgM to provide protection during intracellular bacterial infection is a novel observation and is of potential importance for immunity to other intracellular pathogens. Although we cannot rule out the potential contribution of IgA in mediating protection, it is unlikely that this Ig contributes to immunity, as *E. muris* is not a mucosal pathogen.

Our studies demonstrated a protective role for IgM on day 30 postinfection; however, we had not investigated the duration of IgM-mediated immunity. Even though our results demonstrated that Ag-specific IgM could consistently be detected in the sera of *E. muris* infection elicits long-term, IgM-mediated immunity. We monitored serum OMP-19 IgM titers in Aicda-deficient mice between days 30 and 135 postinfection. On day 250 after *E. muris* infection, OMP-19 IgM and IgG sera titers were determined in Aicda-deficient and μS-deficient strains, as well as in C57BL/6 mice. The indicated strains were challenged with a fatal dose of IOE on day 250 after *E. muris* infection. Three mice were used per group. A Student *t* test was used to determine statistical significance of the indicated (bracketed) differences. *p < 0.05; **p < 0.01.

**FIGURE 4.** IgM or IgG is sufficient for immunity against fatal *ehrlichia* infection. A and B, *Aicda*-deficient and μS-deficient mice were infected with *E. muris*, and serum titers of OMP-19 IgM and IgG were assessed on day 30 postinfection by ELISA. C, Serum OMP-19 IgG1, IgG2a/c, IgG2b, and IgG3 titers were determined by ELISA, in both μS-deficient mice and C57BL/6 mice, on day 30 postinfection. D, The indicated strains were challenged with *E. muris*, and morbidity was monitored. E and F, *E. muris* infection was quantitated in the spleen and PECs on day 10 and day 30 postinfection. G, On day 30 after *E. muris* infection, *Aicda*-deficient, μS-deficient, and C57BL/6 mice were challenged with IOE (2 × LD₅₀) and morbidity was monitored. These data are representative of two experiments in which four mice were used per group. A Student *t* test was used to determine statistical significance of the indicated (bracketed) differences. *p < 0.05; **p < 0.01.

**FIGURE 5.** *E. muris* infection elicits long-term, IgM-mediated immunity. A, Serum OMP-19 IgM titers in *Aicda*-deficient mice were monitored between days 30 and 135 postinfection. B and C, On day 250 after *E. muris* infection, OMP-19 IgM and IgG sera titers were determined in *Aicda*-deficient and μS-deficient strains, as well as in C57BL/6 mice. D, The indicated strains were challenged with a fatal dose of IOE on day 250 after *E. muris* infection. Three mice were used per group. A Student *t* test was used to determine statistical significance of the indicated (bracketed) differences. *p < 0.05; **p < 0.01.
mice, were enumerated and subtracted from the untreated and treated mock-infected untreated mice, as well as by cells from doxycycline-treated panels.

The number of OMP-19 IgM (serum titers were determined by ELISA.

Detection of Ag-specific IgM-secreting cells in the spleen and BM during chronic infection. Mock-infected and PECs was analyzed by quantitative PCR. Detection of Ag-specific IgM and IgG responses were assessed.

FIGURE 6. Detection of Ag-specific IgM-secreting cells in the spleen and BM during chronic infection. Mock-infected and E. muris-infected C57BL/6 mice were administered doxycycline (doxy) on days 28, 35, and 42 after E. muris infection. On day 77 postinfection, the OMP-19 IgM and IgG responses were assessed. A, Bacterial infection in the spleen, BM, and PECs was analyzed by quantitative PCR. B, OMP-19 IgM and IgG serum titers were determined by ELISA. C and D, The frequency and number of OMP-19 IgM (top panels) and IgG-secreting cells (bottom panels) were determined by ELISPOT. Spots produced by cells from mock-infected untreated mice, as well as by cells from doxycycline-treated mice, were enumerated and subtracted from the untreated and treated E. muris-infected C57BL/6 mice, we next addressed whether OMP-19 IgM was present in Aicda-deficient mice throughout chronic infection. Indeed, between days 45 and 135 after E. muris infection, OMP-19 IgM was detected in the sera at reciprocal titers >1000 (Fig. 5A). Moreover, day 135 E. muris-infected, Aicda-deficient mice survived fatal IOE challenge (data not shown). In a second experiment, both Aicda-deficient and μS-deficient mice were challenged on day 250 after E. muris infection. Compared to the titers in infected C57BL/6 mice, Ag-specific IgM and IgG titers were 10-fold higher in the Aicda-deficient and μS-deficient strains (Fig. 5B, 5C). After challenge with IOE, 75% of mice from both gene-targeted strains survived fatal IOE challenge infection (Fig. 5D). Thus, either IgM or IgG is sufficient for long-term immunity against chronic E. muris infection and against fatal IOE challenge.

Persistent infection is not required for long-term IgM responses

Because E. muris establishes a low-level, chronic infection (54, 55), we next addressed whether persistent infection was required for the maintenance of the long-term Ag-specific IgM responses. Although our laboratory has previously demonstrated that infection can persist in PECs for as long as 60 d postinfection (54), using a PCR assay, we were unable to detect bacteria in the PECs, spleen, LNs, or BM on either day 150 or day 366 postinfection (data not shown). Nonetheless, it is possible that low numbers of E. muris were present but were undetectable by PCR assay.

Because chronic infection can promote long-term Ab production (37), we next addressed whether persistent infection was required for the maintenance of the IgM response. Because the ehrlichiae are sensitive to doxycycline, we treated E. muris-infected mice beginning at day 30 postinfection; the antibiotic was administered every 7 d for 2 wk, and the mice were maintained on the antibiotic in drinking water for that period. Doxycycline treatment was effective: following antibiotic treatment, bacteria were undetectable by PCR assay in the PECs on day 77 after E. muris infection (Fig. 6A). This finding is consistent with published reports on the efficacy of doxycycline treatment during chronic ehrlichial infection (55). We also addressed whether antibiotic treatment prevented splenomegaly, given that the latter criterion has been used as a measure of chronic infection (56). Following doxycycline treatment, spleen cellularity was similar to that in mock-infected control mice, supporting the conclusion that infection had either been eliminated or greatly reduced in the treated mice (data not shown).

We addressed whether Ag-specific IgM or IgG responses were maintained in the absence of a detectable chronic infection using ELISA and ELISPOT analyses. On day 77 postinfection, the OMP-19 IgM and IgG serum titers were 10-fold higher in the infected C57BL/6 mice, respectively. E. muris (EM)-infected untreated mice, doxycycline-treated infected mice, and doxycycline-treated uninfected mice were challenged with a fatal dose of IOE and morbidity was monitored. The data are representative of two experiments in which four mice were used per group. A Student t test was used to determine statistical significance of the indicated (bracketed) differences.

*p < 0.05; **p < 0.01.
postinfection, but these differences were not significant (Fig. 6D). Thus, chronic infection was required for the production of splenic IgM and IgG, as well as for the production of Ag-specific IgG in the BM. However, IgM production in the BM was maintained, even in the apparent absence of chronic infection.

To determine whether chronic infection was required for protection against fatal IOE challenge infection, we treated *E. muris*-infected mice and mock-infected C57BL/6 mice with doxycycline and challenged them with a fatal dose of IOE on day 77 after *E. muris* infection. Regardless of antibiotic treatment, mice previously infected with *E. muris* survived infection and, as expected, both the antibiotic-treated and untreated, unimmunized mice were susceptible to fatal IOE infection on about day 10 postinfection (Fig. 6E). These data demonstrate that antibiotic treatment does not abolish protection against fatal IOE challenge, suggesting that Ab-mediated protection is maintained in the absence of chronic infection.

Because the CD138$^{\text{high}}$IgM$^{\text{high}}$ cells were responsible for most Ag-specific IgM production in the BM, we also addressed whether this population was present in the BM and spleen of *E. muris*-infected C57BL/6 mice that had been treated with doxycycline. Relative to mock-infected mice, a 3- to 5-fold increase in the

Discussion

Although IgM-mediated immunity is usually considered to be short-lived and to be effective only during the early stages of infection, our studies reveal a novel role for IgM in the maintenance of long-term immunity during a chronic intracellular bacterial infection. Moreover, we identified the population of cells in the BM that are responsible for the long-term production of IgM. Our findings indicate that IgM may be of greater utility during chronic bacterial and viral infections than has previously been appreciated (57). Other investigators have also provided evidence for long-term IgM responses, although such reports are relatively rare. For example, immunization with 4-hydroxy-3-nitrophenyl acetyl-

![FIGURE 7](chart.png)

**FIGURE 7.** Chronic infection is not required for the expansion of the CD138$^{\text{high}}$IgM$^{\text{high}}$ population in the BM. Mock- and *E. muris*-infected C57BL/6 mice were administered doxycycline on days 28, 35, and 42 after *E. muris* infection. A and C, BM cells and splenocytes from mock-infected and day 77 *E. muris*-infected mice that were left untreated (top panels) or were administered doxycycline (bottom panels) were analyzed for the expression of CD138 and IgM. B and D, The frequency and number of CD138$^{\text{high}}$ and IgM$^{\text{high}}$ cells were determined. A Student *t* test was used to determine statistical significance of the indicated (bracketed) differences. *p* < 0.05; **p** < 0.01.
Ficoll (11), capsular polysaccharides (12, 58), or Salmonella enterica serovar typhi porins (13, 59) has been shown to generate long-term IgM responses. Long-term IgM responses have been shown to be effective during infections by other pathogens, including Borrelia hermsii (15, 60), Streptococcus pneumoniae (16), and Plasmodium chabaudi chabaudi (9). Taken together, these findings indicate that IgM is not only of utility early following infections.

IgM-secreting cells have been detected in the BM of both mice and humans (38, 41, 42), but our study provides both phenotypic and functional characterizations of BM cells responsible for the production of Ag-specific IgM. Although our study is, to our knowledge, the first that characterizes such IgM-producing cells, immunization with OVA elicited an expansion of CD138<sup>high</sup> IgM<sup>high</sup> cells in the BM of Aicda-deficient mice (53). The CD138<sup>high</sup>IgM<sup>high</sup> cells that we have identified are relatively rare, accounting for 0.1–0.7% of BM mononuclear cells, and they exhibit a novel phenotype with characteristics of both plasmablasts and plasma cells. The high cell surface expression of IgM, MHC class II, and CD19 on the CD138<sup>high</sup>IgM<sup>high</sup> cells suggest that they are short-lived plasmablasts. In contrast, the high expression of CD138 and the downregulation of B220 expression are characteristic of plasma cells, given that the loss of general B lymphocyte cell surface markers is associated with increased maturity and a longer lifespan (43, 61). Long-lived plasma cells are typically terminally differentiated, nondividing cells that secrete IgG in the absence of either chronic infection or persistent Ag (37, 61). However, other studies have suggested that long-lived IgM-secreting plasma cells can persist in the BM of mice either infected with Plasmodium chabaudi chabaudi (62) or immunized with serotype 3 capsular polysaccharide from Streptococcus pneumoniae (63). We observed long-term IgM production and detected the CD138<sup>high</sup>IgM<sup>high</sup> population in the BM after extended, and apparently effective, antibiotic treatment; those characteristics suggest that the population that we have identified can be maintained in the BM independent of infection. However, we cannot rule out the possibility that persistent Ag or low-level inflammation was responsible for maintenance of the Ab-secreting cells. Indeed, available evidence suggests that plasma cells compete for survival in the BM and that survival niches are maintained by inflammation associated with chronic infection (27, 64, 65).

Another explanation for the maintenance of the CD138<sup>high</sup> IgM<sup>high</sup>-producing cells is that they originate from IgM<sup>+</sup> memory B cells. In a recent study, Ag rechallenge caused activation-induced cytidine deaminase-expressing IgM<sup>+</sup> memory B cells to differentiate into IgM-secreting cells (66). However, in our study, IgM production was maintained in Aicda-deficient mice, suggesting that the population we have identified is not derived from IgM<sup>+</sup> memory B cells. Alternatively, persistent Ag or non-specific inflammatory signals may contribute to the maintenance of the CD138<sup>high</sup>IgM<sup>high</sup> population in the BM (67). Persistent Ag may also drive the differentiation of naive B cells into IgM-producing cells that migrate to and establish residence in the BM (68). Another possibility is that the BM functions as a secondary lymphoid organ-like structure (69), thereby facilitating the local generation of Ag-specific IgM-producing cells (39, 70).

The identification of Ag-specific IgM-secreting cells during intracellular bacterial infection has obvious implications for vaccine design. Previous studies that have examined vaccine efficacy have focused primarily on isotype-switched Abs. However, many childhood vaccines elicit low but persistent IgM responses, which can afford protection against infection (71–74). Sustained IgM production has also been reported in a phase I clinical trial of a Francisella tularensis vaccine (75). Vaccination with the envelope glycoprotein gp120 from the HIV elicited Ag-specific IgM responses for as long as 24 wk postimmunization (76). Thus, long-term IgM immunity could be important in a range of bacterial and viral infections, and the Ag-specific IgM plasma blasts identified in our present studies could be important in such responses.

IgM may also be useful in passive immunotherapy. IgG has been the primary focus of mAb treatments; however, improved techniques to overcome the size and heavy glycosylation of IgM has allowed this Ig to be exploited therapeutically (77). Intravenous polyclonal IgM-enriched Ig therapy has yielded promising results in reducing mortality rates in neonatal and pediatric patients with severe sepsis caused by Gram-negative bacteria (78, 79). Moreover, a monoclonal IgM therapy has been developed to treat the most common hospital-acquired infection, by Pseudomonas aeruginosa (80, 81). Monoclonal IgM has also been effective in preventing both disease and mortality in mice infected with Cryptococcus neoformans (82). Thus, we predict that IgM will find increasing utility in the treatment and prevention of infectious diseases.

In summary, we have identified a population of Ag-specific IgM-secreting cells in the BM that contributes to long-term IgM-mediated immunity against an intracellular bacterial infection. Our findings suggest that greater efforts should be made to exploit IgM for the treatment of bacterial and viral infections.

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Disclosures
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