The Omentum Is a Site of Protective IgM Production during Intracellular Bacterial Infection

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Infection of mice with the bacterium *Ehrlichia muris* elicits a protective T cell-independent (TI) IgM response mediated primarily by a population of CD11c-expressing plasmablasts in the spleen. Although splenic marginal zone (MZ) B cells are considered to be important for TI responses to blood-borne pathogens, MZ B cells were not responsible for generating plasmablasts in response to *Ehrlichia muris*. Moreover, antigen-specific serum IgM was decreased only modestly in splenectomized mice and in mice that lacked spleen, lymph nodes, and Peyer's patches (SLP mice). Both splenectomized and SLP mice were protected against lethal ehrlichial challenge infection. Moreover, we found a high frequency of *Ehrlichia*-specific plasmablasts in the omentum of both conventional and SLP mice. Omental plasmablasts elicited during *Ehrlichia* infection lacked expression of CD138 but expressed CD11c in a manner similar to that of their splenic counterparts. Selective ablation of CD11c-expressing B cells nearly eliminated the omental *Ehrlichia*-specific plasmablasts and reduced antigen-specific serum IgM, identifying the omental B cells as a source of IgM production in the SLP mice. Generation of the omental plasmablasts was route dependent, as they were detected following peritoneal infection but not following intravenous infection. Our data identify the omentum as an important auxiliary site of IgM production during intracellular bacterial infection.

The adaptive humoral immune response is characterized by the production of high-affinity, isotype-switched antibodies. The development of these antibodies is dependent on T cell help and typically takes place within germinal centers. However, germinal centers take nearly a week to develop following antigen exposure, potentially leaving the host at risk early after infection (1). Low-affinity, polyreactive, natural IgM, which is produced prior to pathogen exposure, serves as a first line of defense against many pathogens (2, 3). Natural IgM is secreted by two subsets of B cells: marginal zone (MZ) B cells and B-1 cells (1, 2, 4). A second line of adaptive humoral immunity is provided by T cell-independent (TI) antibodies, which are also produced by MZ and B-1 B cells, but only after antigen exposure. MZ B cells are a sessile population of cells that reside within the spleen. By virtue of their localization near the splenic arterial blood supply, these cells are optimally positioned to rapidly interact with blood-borne antigens and cells (5, 6). B-1 B cells, although present in the spleen at low frequencies, are enriched within the peritoneal and pleural cavities (7). B-1 cells also play an important role in host defense, as they are activated in response to both bacterial and viral infections (6, 8, 9). Thus, upon antigenic challenge, both MZ and B-1 B cell subsets in the spleen provide an important source of antibody.

The importance of a spleen as a site of TI immune responses has long been recognized; for example, asplenic humans are at elevated risk for developing bacterial infections (10, 11, 12), and asplenic mice exhibit diminished antibody responses to streptococcal polysaccharides (13). However, the requirement for a spleen is not absolute, as some studies have described antibody responses to TI antigens in asplenic patients, suggesting that alternate or induced lymphoid tissues may be able to elicit compensatory antibody responses (5, 14). One such tertiary tissue is the omentum, a fatty lymphoid organ located in the peritoneal cavity of mice and humans that connects the spleen, stomach, pancreas,

and colon (15). Immune cells enter and exit the peritoneal cavity via the omentum, a trafficking process that is critically dependent on the chemokine CXCL13 (16, 17). Omental tissue contains lymphocyte-rich foci known as milky spots, which are enriched for B-1 cells and macrophages (18, 19). Milky spots can organize T cell-dependent (TD) antibody responses to peritoneal antigens, as well as efficiently collect antigens and cells within the peritoneal cavity (20, 21). However, a role for the omentum as a site for the generation of TI immune responses has only begun to be addressed.

Our previous studies described the generation of TI IgM by a population of CD11c-expressing spleen plasmablasts induced by infection with the intracellular bacterium *Ehrlichia muris* (22). These cells produce the majority of the IgM in the spleen; the IgM is antigen-specific, polyreactive (23), and sufficient to mediate host protection during both acute and chronic ehrlichial infection (22, 24). In the present study, we addressed the role of conventional secondary lymphoid organs for the production of protective IgM during infection. We show that mice lacking all conven-

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tional secondary lymphoid tissues are nonetheless able to mount an early IgM response against infection. The IgM response occurs at least in part in the omentum, which acts to maintain IgM production during exposure to peritoneal antigens. These studies underscore the function of the omentum in host defense to pathogens and reveal compensatory roles for lymphoid tissues that allow for efficient humoral responses to both regional and systemic antigenic exposure.

MATERIALS AND METHODS

Mice. Sex-matched C57BL/6, CD19^{Cre} [B6.129P2(C)-Cd19^{tm1(cre)Cgn}/J], CD11c-DTR (diphtheria toxin receptor) [B6.FVB-Tg(Igax-DTR/EGFP)57Lan/J], IgH^a (B6.Cg-Igh^aThy1^aGpi1^a/J), and lymphotoxin α -deficient (LT α ^{-/-}; C57BL/6.129Lta^{tm1Dch}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred under microisolator conditions at the Wadsworth Center Animal Care Facility or at the Trudeau Institute (Saranac Lake, NY), in accordance with institutional guidelines for animal welfare. Splenectomized C57BL/6 and sham-splenectomized mice were purchased from Taconic Farms (Germantown, NY). Notch2^{flox} (B6.129S-Notch2^{tm3Grid}/J) mice were a gift from T. Gridley (The Jackson Laboratory) (25). Spleen, lymph node (LN), Peyer's patch-deficient (SLP) chimeric mice were generated as previously described (21) and were housed at the Trudeau Institute. As institutional guidelines do not allow the use of death as an endpoint in animal studies, mice were humanely sacrificed when judged to be moribund.

Infections. Mice were infected intraperitoneally (i.p.) or intravenously (i.v.) with 5×10^4 copies of *E. muris* or i.p. with 2 times the 50% lethal dose (LD₅₀) of *Ixodes ovatus* ehrlichia (IOE), as previously described (26).

Genotyping. Mouse genomic DNA was extracted from tail tissue by using hot sodium hydroxide, as previously described (27). PCR was performed using the following oligonucleotide primers: Notch2^{flox} (forward, 5'-TAGGAAGCAGCTCAGCTCACAG-3'; reverse, 5'-ATAACGCTAAACGTGCACTGGAG-3' [these yielded 201- and 161-bp products from the mutated and wild-type alleles, respectively]); CD19^{Cre} transgene (forward, 5'-GCGGTCTGGCAGTAAAACTATC-3'; reverse, 5'-GTGAAA CAGCATTGCTGTCACTT-3' [100-bp product]); CD19^{Cre} wild type (forward, 5'-CCTCTCCCTGTCTCCTTCCT-3'; reverse, 5'-TGGTCTG AGACATTGACAATCA-3' [477-bp product]); CD11c-DTR transgene (forward, 5'-AAGTTCATCTGCACCACCG-3'; reverse, 5'-TCCTTGAA GAAGATGGTGCG-3' [173-bp product]). The following PCR cycling conditions were utilized: for Notch2^{flox}, 94°C for 3 min, 35 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s, followed by 72°C for 2 min; for CD19^{Cre}, 94°C for 3 min, 35 cycles of 94°C for 30 s, 62°C for 60 s, and 72°C for 60 s, followed by 72°C for 2 min; for CD11c-DTR, 95°C for 30 s, 35 cycles of 95°C for 30 s, 60°C for 60 s, and 72°C for 60 s, followed by 72°C for 5 min.

Flow cytometry and antibodies. Spleens, lymph nodes, and omenta were mechanically disrupted using razor blades. The omenta were further dissociated by treatment with 1 mg/ml collagenase D (Roche, Indianapolis, IN) for 1 h at 37°C. The cells were disaggregated using a 70- μ m-pore-size cell strainer (BD Falcon), and erythrocytes were removed by hypotonic lysis, using ammonium chloride. Cells were treated with anti-CD16/32 (2.4G2) prior to incubation with the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-IgM (clone II/41) and PerCP-Cy5.5-conjugated B220 (RA3-6B2) (eBioscience, San Diego, CA) and phycoerythrin (PE)-conjugated CD138 (281-2), PE-conjugated CD11b (M1/70), PE-conjugated-CD5 (53.7.3), and allophycocyanin-conjugated CD11c (HL3) (BD Biosciences, Franklin Lakes, NJ). The cells were stained at 4°C for 20 min, washed, and analyzed without fixation. Unstained cells were used to establish the flow cytometer voltage settings, and single-color positive controls were used to adjust compensation. Data were acquired on a FACSCalibur flow cytometer with Cell Quest software (Becton Dickinson, Mountain View, CA) and were analyzed with FlowJo software (Tree Star, Inc.).

Enzyme-linked immunosorbent spot assay (ELISPOT)/enzyme-linked immunosorbent assay (ELISA) analyses. OMP-19-specific antibody-secreting cells and serum antibodies were detected as previously described (22, 23).

Statistical analyses. Statistical analyses were performed using a two-tailed Mann-Whitney test.

RESULTS

Marginal zone B cells are not required for the generation of IgM during bacterial infection. Our previous studies demonstrated an important protective role for a population of antigen-specific IgM-secreting plasmablasts in the spleen during ehrlichial infection (22). These B220^{low}CD11c^{low}-expressing cells are elicited at high frequency, generate nearly all of the T cell-independent (TI) IgM in the spleen, and exhibit characteristics of marginal zone (MZ) B cells (i.e., they exhibit an IgM⁺IgD^{low}CD9⁺ surface phenotype and are located in the extrafollicular region of the spleen [22]). Based on these characteristics, we hypothesized that the B220^{low}CD11c^{low}-expressing spleen plasmablasts were derived from MZ B cells during ehrlichial infection. As Notch2 signaling is critical for MZ B cell development, to address this hypothesis, we generated Notch2^{flox/flox}; CD19^{Cre/+} (B-Notch2^{-/-}) mice to eliminate Notch2 expression in CD19-expressing cells. Notch2^{-/-} mice lack MZ B cells but contain normal numbers of follicular and B-1 B cells (28). As expected, MZ B cells were effectively depleted in the B-Notch2^{-/-} mice, as confirmed by a loss of splenic CD21^{high}CD23^{low} B cells ($8.61\% \pm 2.17\%$ for B-Notch2^{+/+} compared to $0.46\% \pm 0.21\%$ for B-Notch2^{-/-}, $P < 0.001$; data not shown). To test a requirement for Notch2 signaling in the generation of the CD11c-expressing plasmablasts, we infected B-Notch2^{+/+}, B-Notch2^{+/+}, and B-Notch2^{-/-} mice with *E. muris* and monitored the spleen plasmablast population on day 9 postinfection (p.i.). B220^{low}CD11c^{low} spleen plasmablasts were elicited at similar frequencies in B-Notch2^{+/+}, B-Notch2^{+/+}, and B-Notch2^{-/-} mice, and the total number of plasmablasts was unaffected (Fig. 1A and B). Antigen-specific serum IgM titers were also comparable, regardless of Notch2 expression (Fig. 1C). These data indicate that splenic CD11c⁺ plasmablasts can be generated in the absence of MZ B cells and suggested that other B cell lineages, perhaps located in different tissues, could contribute to the IgM response.

Secondary lymphoid organs are dispensable for the generation of IgM. The finding that MZ B cells were not necessary for IgM production during ehrlichial infection prompted us to identify a nonsplenic source(s) of IgM-producing cells. To this end, splenectomized and sham-splenectomized mice were infected, and IgM and IgG production was monitored thereafter. Splenectomy resulted in only a minor reduction in antigen-specific serum IgM titers on day 14 p.i., and this difference was resolved by day 28 p.i., indicating that IgM could be elicited in the absence of a spleen (Fig. 2A). The ehrlichial outer membrane protein 19 (OMP-19)-specific IgG serum antibody response was also unaffected, consistent with our prior observation that antigen-specific IgG is generated primarily in the lymph nodes, rather than in the spleen, during acute infection (Fig. 2B) (29). Splenectomized mice immunized with *E. muris* were also protected against lethal *Ixodes ovatus* ehrlichia (IOE) challenge (Fig. 2C).

To determine whether conventional lymphoid organs were required for generating IgM responses during ehrlichial infection,

FIG 1 Marginal zone B cells are not required for IgM production during *Ehrlichia muris* infection. B-Notch2^{+/+}, B-Notch2^{+/-}, and B-Notch2^{-/-} mice were generated by intercrossing Notch2^{fllox/+}; CD19^{Cre/+} mice. (A) The indicated mice were infected i.p. with *E. muris*, and spleen cells were analyzed for surface expression of B220 and CD11c on day 9 postinfection. (B) The frequency and number of B220^{low}CD11c^{low} plasmablasts in the spleen were determined. (C) Sera from infected mice were tested for IgM reactivity to OMP-19 by ELISA. The data represent antibody titers detected in individual mice; horizontal lines indicate the mean. The data are representative of at least five experiments that used 2 to 10 animals per group; ns, not significant. The statistical analyses were performed using a two-tailed Mann-Whitney test throughout.

we next infected lymphotoxin α -deficient (LT α ^{-/-}) mice, which do not develop lymph nodes or Peyer's patches and fail to form an MZ or germinal centers in the spleen (30). We found that LT α ^{-/-} mice generated an IgM response comparable to that of C57BL/6 mice (Fig. 2D), although antigen-specific serum IgG titers were decreased in the LT α ^{-/-} strain (Fig. 2E). Immunized LT α ^{-/-} mice were also protected against lethal bacterial challenge (Fig. 2F).

Because alternate lymphoid tissues, aside from the spleen and lymph nodes, can contribute to a productive immune response, we next generated mice lacking spleen, lymph nodes, and Peyer's patches (SLP mice) by reconstituting splenectomized, lethally irradiated LT α ^{-/-} mice with C57BL/6 bone marrow (BM). Since LT α ^{-/-} mice do not develop lymph nodes or Peyer's patches, even after reconstitution with LT α -sufficient cells, the resulting chimeric mice entirely lack spleen, lymph nodes, and Peyer's patches (21, 31, 32). Infected SLP mice generated a serum IgM response comparable to that of wild-type mice, indicating that conventional secondary lymphoid tissues were not required (Fig. 2G). In contrast, antigen-specific IgG was not detected, consistent with our observation that the majority of antigen-specific IgG is produced in the lymph nodes during ehrlichial infection (Fig. 2H) (29). SLP mice were also fully protected against lethal ehrlichial challenge (Fig. 2I), indicating that IgM alone was effective. These data confirm our previous findings that IgM is sufficient to mediate protective immunity against lethal bacterial challenge (24) and indicate that conventional secondary lymphoid organs are dispensable for the generation of protective IgM during ehrlichial infection.

The omentum is a reservoir for antigen-specific IgM-secreting cells. The generation of protective IgM in the absence of a spleen, lymph nodes, and Peyer's patches suggested the presence of a discrete population of IgM-secreting cells. Therefore, we next investigated whether the omentum, a secondary lymphoid organ within the peritoneal cavity, was a source of IgM production during infection (21). We first infected splenectomized and sham-splenectomized mice, via the peritoneal cavity, and analyzed omenta and lymph nodes for antigen-specific IgM production by ELISPOT analysis. We found similar frequencies of ehrlichial OMP-19-specific IgM-secreting cells in both tissues in splenectomized and sham-splenectomized mice, although the number of antigen-specific cells was higher in the omenta of splenectomized mice (Fig. 3A). In comparison, we found 100-fold fewer antigen-specific IgM-secreting cells in the lymph nodes of both splenectomized and sham-splenectomized mice, indicating that the omentum was a much larger source of IgM-secreting B cells in both groups of mice.

Because CD11c expression uniquely identifies IgM-secreting B cells in the spleen during infection, we next analyzed B cells in the omentum for expression of this integrin. We identified a population of CD11c-expressing B cells in the omenta of both splenectomized and sham-splenectomized mice; however, these cells lacked surface expression of CD138, a marker typically associated with antibody-secreting cells, possibly due to collagenase-mediated cleavage from the cell surface during sample preparation (Fig. 3B and C) (33, 34). The CD11c-positive cells exhibited high surface expression of both CD11b and CD5, relative to spleen or CD11c-

FIG 2 Secondary lymphoid organs are dispensable for IgM-mediated protective immunity. (A and B) OMP-19-specific serum IgM and IgG titers were determined for splenectomized mice, or sham-splenectomized control mice, on the indicated days postinfection. (C) Splenectomized mice and sham controls were immunized with *E. muris*, via the peritoneal cavity. Thirty days later, immunized and unimmunized mice were challenged with a lethal dose of *Ixodes ovatus* ehrlichia (IOE; $2 \times LD_{50}$), and morbidity was monitored. (D and E) Antigen-specific serum IgM and IgG titers were determined for infected $LT\alpha^{-/-}$ and C57BL/6 mice. (F) $LT\alpha^{-/-}$ mice were immunized with *E. muris* i.p., and immunized and unimmunized mice were challenged with a lethal dose of IOE. (G and H) OMP-19-specific serum IgM and IgG titers were determined for SLP $^{-/-}$ and C57BL/6 mice. (I) *E. muris*-immunized or unimmunized C57BL/6 mice and immunized SLP-deficient mice were challenged with a lethal dose of IOE. The data are representative of at least two experiments that used 4 or 5 mice per group. *, $P < 0.05$; ns, not significant.

negative omental B cells, indicating that the omental response was generated from B-1 lineage cells (Fig. 3D).

To address whether the CD11c-expressing omental B cells secreted IgM, we generated mixed BM chimeric mice using CD11c-DTR donor BM (35). C57BL/6 mice were lethally irradiated and reconstituted with a 1:1 ratio of wild-type (IgM^a) and allotype congenic CD11c-DTR (IgM^b) BM. In the cells containing the CD11c-DTR transgene, the CD11c promoter drives the expression of the DTR, and administration of diphtheria toxin (DT) promotes the selective ablation of transgenic CD11c-positive cells. The use of BM chimeric mice limited possible effects due to DT-mediated ablation of non-B CD11c-expressing cells, such as dendritic cells. The chimeric mice were infected with *E. muris* and administered DT on days 5 and 7 p.i., and IgM production was

analyzed in the omenta on day 9 postinfection. CD11c-expressing B cells were detected in the omenta, and the frequency of this population decreased 2-fold upon DT treatment, indicating that a significant portion of the B cell population was depleted (Fig. 4A). This was likely due to specific depletion of the B cells derived from the CD11c-DTR bone marrow, as similar frequencies and numbers of IgM^a- and IgM^b-secreting B cells were detected in the omenta of non-DT-treated chimeric mice (Fig. 4B). Following DT treatment, the frequency and number of IgM^b-producing cells in the omentum was markedly reduced, indicating that the CD11c-positive omental B cells were responsible for IgM production. IgM^a-producing cells and serum IgM^a were, in contrast, increased following DT treatment, probably because these DT-resistant cells underwent expansion following the elimination of DT-susceptible

FIG 3 The omentum is a source of antigen-specific IgM during infection. (A) ELISPOT analysis was used to determine the frequency and number of OMP-19-specific IgM-secreting cells in the omentum and lymph nodes of splenectomized and sham-splenectomized mice, 9 days after intraperitoneal infection with *E. muris*. (B) B220⁺-positive omental B cells were analyzed for expression of CD11c and CD138. (C) The frequency and number of B220⁺CD11c⁺ cells in the omentum were determined. The data are representative of two experiments that used 5 mice per group. *, $P < 0.05$; ns, not significant. (D) CD11c-positive and -negative B220⁺ omental B cells (gated) were monitored for cell surface expression of CD11b and CD5. Staining of splenic B cells is shown for comparison (gray histograms). Representative data are shown.

ble B cells. DT administration also caused reduction of antigen-specific serum IgM^b (Fig. 4C), likely due to DT-mediated depletion of IgM^b-producing cells in both the omentum and spleen. The data together reveal CD11c-expressing B cells within the omentum to be a significant source of antigen-specific IgM production, and one that could fully compensate in the absence of a splenic B cell response.

B cells in the omentum are elicited in response to peritoneal antigen administration. Although we identified the omentum as a source of antigen-specific IgM in response to TI infection, our infection model utilized intraperitoneal inoculation of mice, an injection route that has been shown to target antigens to the omentum (21, 36, 37). To address the importance of the route of inoculation on the omental B cell response we have described, we infected splenectomized and sham-splenectomized mice intraperitoneally and intravenously (via the peritoneal cavity and bloodstream, respectively) and analyzed the immune response in the omentum and lymph nodes. Intraperitoneal infection generated a population of antigen-specific IgM-secreting cells in the omentum in both splenectomized and sham-splenectomized mice (Fig. 5A). Although similar cell frequencies were detected in both groups of mice, the number of IgM-secreting cells was in-

creased in splenectomized mice, perhaps because lymphocyte trafficking is modified in the absence of a spleen. In contrast, intravenous infection failed to elicit any IgM response in the omentum, demonstrating that the response to the peritoneal antigen was route dependent. In an inverse fashion, intraperitoneal infection did not elicit a large lymph node IgM response at this time, but intravenous infection resulted in a large expansion of IgM-secreting cells in the lymph nodes, and the response was of greater magnitude in splenectomized mice (Fig. 5A). The failure to elicit an IgM response in the omentum following intravenous infection was likely due to a much lower bacterial colonization in the omentum following this route of infection (Fig. 5B). These data also suggest that the omental B cell response was generated *in situ*. Both intraperitoneal and intravenous infection of splenectomized mice resulted in the production of antigen-specific serum IgM, although antibody concentrations were reduced in intravenously infected splenectomized mice, relative to those of sham-splenectomized controls (Fig. 5C). These data indicate that, in the absence of both a splenic and omental response in intravenously inoculated mice, IgM immunity is highly compromised. As reported previously, LN B cells make a relatively minor contribution to the IgM response during early ehrlichial infection (29). Our

FIG 4 CD11c-expressing B cells are responsible for nearly all of the antigen-specific IgM during infection. Recipient mice were lethally irradiated and reconstituted 1:1 with CD11c-DTR bone marrow and IgH^a bone marrow. The CD11c-DTR chimeric mice were infected with *E. muris* i.p. and were untreated or were administered DT on days 5 and 7 p.i. (A) B220⁺ cells from the omenta of DT-treated and untreated chimeric mice were analyzed for surface expression of CD11c and CD138 on day 9 postinfection. (B) ELISPOT analysis was used to determine the frequency and number of antigen-specific IgM^a- and IgM^b-secreting cells in the omentum on day 9 p.i. (C) OMP-19-specific serum titers of IgM^a and IgM^b were determined with or without DT administration. The data are representative of three experiments using 5 animals per group. *, $P < 0.05$; ns, not significant.

data indicate that the omentum is a site of IgM production and that this response is route dependent. These studies underscore the function of the omentum in antibody-mediated host defense to pathogens.

DISCUSSION

The omentum is recognized as a site of lymphocyte entry and exit from the peritoneal cavity, as well as a lymphoid tissue capable of initiating T cell-dependent (TD) and -independent (TI) immune responses. The spleen, the largest secondary lymphoid organ, serves to filter blood-borne antigens and coordinate immune re-

sponses. Its role in TD and TI immunity is thought to be invaluable, as it houses resident MZ, follicular, and B-1 cells and is a major site for extrafollicular IgM production during ehrlichial infection. Nevertheless, our studies demonstrate that the omentum, in the complete absence of conventional secondary lymphoid tissues, is sufficient to generate protective IgM in response to bacterial infection in mice. Although we cannot rule out the possibility that inducible lymphoid tissues also contribute to IgM production, this possibility appears less likely, given that *E. muris* is a blood-borne pathogen and is not known to colonize mucosal tissues that can support inducible lymphoid responses. Thus, the

FIG 5 Omentum B cells are elicited in response to peritoneal antigen. Splenectomized mice and sham controls were infected with *E. muris* intraperitoneally or intravenously. (A) On day 9 postinfection, ELISPOT analysis was used to determine the frequency and number of OMP-19-specific IgM-secreting cells in the omentum and lymph nodes. (B) Mice were infected intraperitoneally or intravenously, and on day 9 postinfection, quantitative PCR was used to determine *E. muris* copy number in both the omentum and pooled LNs (brachial and axillary). (C) Serum IgM titers were determined for mice infected intraperitoneally and intravenously. The data are representative of two experiments using 5 or 6 mice for each group. *, $P < 0.05$; ns, not significant.

omentum may contribute more to humoral immunity during bacterial infections than has been heretofore recognized.

Our findings contrast with a previous study, in which immunization of asplenic mice with TI bacterial polysaccharides, either intraperitoneally or intravenously, failed to elicit an IgM response (13). The absence of TI antibody production was attributed to a lack of B-1a cells, as congenitally asplenic mice are also deficient in this B cell subset (13). In our studies, splenectomy did not affect the generation of antigen-specific IgM, suggesting that B-1a cells do not play a role in IgM production during ehrlichial infection. Moreover, our data also indicate that MZ B cells, which are found only in the spleen in mice, are dispensable for the generation of IgM during infection. Thus, due to the high frequency of B-1 B cells present in the peritoneal cavity, it is likely that the CD11c plasmablast response elicited during ehrlichial infection is derived

from B-1b or resident or recruited follicular B cells. Although our studies did not directly address whether the ommental antibody response is TI, this is likely the case, as the ommental IgM-secreting B cells we identified in this study were phenotypically similar to the TI splenic plasmablasts we characterized previously, in that both populations expressed CD11c (22). Nevertheless, the relationship between the ommental and splenic B cell populations is unclear, and it is possible that the ommental B cells we identified are precursors of the spleen B cells.

Although our findings highlight an important role for omental B cell responses during intraperitoneal infection, we did not observe a significant B cell response, or infection, in the omentum following blood-borne ehrlichial inoculation. These data suggest that the omental response may be of limited relevance during natural ehrlichial infections. These findings were unexpected, as

studies in mouse models have found that the ehrlichiae infect most tissues, independent of the route of inoculation (38, 39), observations that we interpreted to be a consequence of tissue infiltration by infected monocytes. The current studies indicate, in contrast, that blood-borne ehrlichiae neither gain access to the omentum nor elicit a B-1 cell response, unless delivered via the intraperitoneal route. Notwithstanding, our findings are of wider relevance for our knowledge of B cell responses that occur independently of secondary lymphoid tissues but are also relevant to host immunity to infectious agents that access the peritoneal cavity, via natural or hospital-acquired infections (39–42).

Rangel-Moreno et al. previously demonstrated the ability of the omentum to collect peritoneal antigens and support TD immune responses, in the absence of secondary lymphoid organs (21). Those studies detailed isotype switching and affinity maturation among antigen-specific B cells, and these responses were initiated within germinal centers located in omental milky spots. The TI nature of the ehrlichial IgM response suggests that omental GCs play a minimal role in the antibody response in our infection model, although lymphocyte recruitment likely occurs through similar mechanisms. For example, B cells may be recruited from the blood into the omentum in response to CXCL13 signaling via CXCR5, as well as via $\alpha_4\beta_7$ integrin expression (16, 19, 43). In addition to chemokine receptor- and integrin-mediated entry into the omentum, Toll-like receptor (TLR) signaling is known to contribute to rapid egress of B-1 cells from the peritoneal cavity to the omentum (17). The ehrlichiae, however, do not encode canonical TLR ligands, with the potential exception of CpG DNA (44).

As the omentum is known to support B lymphopoiesis throughout adult life (45), an alternate explanation for our observations is that ehrlichial infection drives B lymphopoiesis in the omentum during intraperitoneal infection, as a means to rapidly recruit B cells into the humoral immune response. Indeed, IgM production in the omentum was elicited following peritoneal but not intravenous infection, which suggests that the omental B cells interact directly with the bacteria in that tissue and that the response develops *in situ*. This interpretation suggests that there exist two distinct populations of B cells capable of responding to TI antigens during ehrlichial infection: the first, located in the omentum, are perhaps B-1 cells generated *de novo* by B lymphopoiesis, and a second is a population of canonical spleen extrafollicular B cells generated in the bone marrow. Thus, our data suggest a layered organization of secondary lymphoid tissues, including the omentum, that can generate compensatory antibody responses to pathogens in a route-dependent manner.

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