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CD11c Expression Identifies a Population of Extrafollicular Antigen-Specific Splenic Plasmablasts Responsible for CD4 T-Independent Antibody Responses during Intracellular Bacterial Infection

Rachael Racine,† Madhumouli Chatterjee,* and Gary M. Winslow2*†

Although T-independent immunity is known to be generated against bacterial capsular and cell wall polysaccharides expressed by a number of bacterial pathogens, it has not been studied in depth during intracellular bacterial infections. Our previous study demonstrated that Ehrlichia muris, an obligate intracellular tick-borne pathogen, generates protective classical TI responses in CD4 T cell-deficient C57BL/6 mice. We found that E. muris T-independent immunity is accompanied by the expansion of a very large extrafollicular spleen population of CD11clow-expressing plasmablasts that exhibit characteristics of both B-1 and marginal zone B cells. The plasmablasts comprised up to 15% of the total spleen lymphocytes and ~70% of total spleen IgMhighIgDlow cells during peak infection in both wild-type and MHC class II-deficient mice. The CD11c+ cells exhibited low surface expression of B220, CD19, and CD1d, high expression of CD11b, CD43, but did not express CD5. Approximately 50% of the CD11c+ cells also expressed CD138. In addition to CD11b and CD11c, the plasmablasts expressed the β1 (CD29) and α4 (CD49d) integrins, as well as the chemokine receptor CXCR4, molecules which may play roles in localizing the B cells extrafollicular region of the spleen. During peak infection, the CD11c+ cells accounted for the majority of the IgM-producing splenic B cells and nearly all of the E. muris outer membrane protein-specific IgM-secreting cells. Thus, during this intracellular bacterial infection, CD11c expression identifies a population of Ag-specific spleen plasmablasts responsible for T-independent Ab production. The Journal of Immunology, 2008, 181: 1375–1385.

B cell responses T-independent (TI) Ags are well known to contribute to immunity during a number of bacterial and viral infections (1). TI B cell Ags are typically capsular polysaccharides or highly repetitive protein Ags, such as outer membrane proteins (reviewed in Ref. 2). Much of our knowledge of TI immunity, however, has been obtained from studies that have used well-defined hapten systems (3, 4–6). T-independent type II Ab responses have been studied following immunization with polysaccharide-conjugated hapten such as (4-hydroxy-3-nitrophenyl)-acetyl (7–9), but also in bacterial and viral infection models that elicit TI immunity (10–13). This work has identified two major B cell subset cells known to be involved in TI immunity, B-1 and marginal zone (MZ) B cells. B-1 cells are found primarily in the peritoneal and pleural cavities and include the B-1a (B220low, IgMhigh CD11b, CD5+) and B-1b (B220low, IgMhigh CD11b, CD5−) subsets (reviewed in Ref. 14). The former are responsible for the production of natural Abs (15, 16) and the latter can undergo expansion in response to specific Ags or pathogens (12, 17). B-1 cells have also been reported in the spleen, although they are typically found there at very low frequencies (18, 19). In contrast, MZ B cells are located in the MZ of the spleen and are ideally suited to encounter blood-borne bacterial Ags (20, 21). Consequently, MZ B cells often play a role in immunity early during blood-borne bacterial infections (3, 22).

Although considerable progress has been made in our understanding of TI immunity using highly defined Ags and extracellular bacteria, TI immunity has only begun to be investigated during intracellular bacterial infections (23), perhaps in part because until relatively recently Abs had not been considered to be effective against such agents (24). Our previous study demonstrated that CD4 TI immunity could provide protection against fatal ehrlichial infection (25). The ehrlichiae are obligate intracellular rickettsiae that reside in endosomal compartments, primarily in macrophages, and are the etiologic agents of human ehrlichioses. Ehrlichioses are tick-borne diseases caused by infections of a number of related human and animal pathogens (26). Ehrlichia chaffeensis is the etiologic agent of human monocytotropic ehrlichiosis (HME), a disease that is prevalent in the Southern and South-Central United States. HME is an acute febrile illness characterized by a number of hematologic abnormalities, including lymphopenia and thrombocytopenia. E. chaffeensis causes a mild, largely asymptomatic infection in immunocompetent mice, but is fatal in severely immunocompromised strains. Passive Ab transfer can be highly effective in SCID mice, even when performed during late stages of infection (27). In these studies, high-affinity T-dependent Abs were very effective, although possible contributions of TI Abs were not evaluated.

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1Abbreviations used in this paper: TI, T independent; MZ, marginal zone; HME, human monocytotropic ehrlichiosis; IOE, Ixodes ovatus ehrlichia; OMP, outer membrane protein; rt, room temperature; DC, dendritic cell; MFI, mean fluorescence intensity; FO, follicular.

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Our more recent experimental work has used two ehrlichiae, in addition to *E. chaffeensis*, *E. muris* and ehrlichia from *Ixodes ovalus* (IOE). Although the three ehrlichiae are phylogenetically closely related, they induce different diseases in laboratory mice. Histopathologic features of *E. muris* infection have been described previously in BALB/c, AKR, and C57BL/6 mice (28). These include splenomegaly, anemia, and spleen follicular (FO) hyperplasia, and infected mice subsequently develop a low-level persistent infection (29). IOE, in contrast, causes a acute dose-dependent infection in inbred mice that exhibits many pathologic features of HME (30). Although low-dose IOE inoculation does not generate protection against fatal high-dose IOE challenge (31), immunity against high-dose fatal IOE infection was achieved following heterologous *E. muris* infection (25, 30). Remarkably, immunity in this model was found to occur independent of CD4 T cells and type I immunity, but required instead B cells and/or Ab (25). The B cell population(s) responsible for T1 ehrlichial immunity had not been identified or characterized. In this study, we have identified a population of extrafollicular plasmablasts that are responsible for the production of T1 IgM during ehrlichia infection. The B cells are found at frequencies as high as 15% in the spleen and exhibit a number of novel properties. These include the expression of several integrins, notably notably CD11c, often considered a definitive marker for dendritic cells (DCs). Within the plasmablast population, CD11c expression identifies a population of Ag-specific Ab-secreting cells. These studies demonstrate that intracellular bacterial infection can generate a large T1 plasmablast response with unusual characteristics that can make a major contribution to humoral immunity.

Materials and Methods

**Mice**

The mice used in these studies were obtained from The Jackson Laboratory or were bred in the animal care facility at the Wadsworth Center under microisolator conditions, in accordance with institutional guidelines for animal welfare. Mice were gender matched for each experiment and were 6–12 wk in age. The studies were performed in C57BL/6 and MHC class II-deficient (B6.129-H*2*Ab1−1*ElajJ*) mice. Institutional Animal Care and Use Committee guidelines do not permit the use of death as an experimental end point in animal studies; therefore, moribund animals that were judged to be incapable of surviving infection were humanely sacrificed.

**Bacterial infections**

Details regarding the bacterial strains and infection protocols have been described previously (25). *E. muris* was passaged in mice by serial transfer of homogenized spleen tissue. Experimental infections were performed via the peritoneum, and spleen suspension from infected mice was injected at 37°C with Fc blocking solution containing 10% normal rabbit serum. The cells were depleted of T cells using anti-Thy1–1.2 (source) and rabbit serum/HBSS/0.1% sodium azide (Fc blocking solution). The cells were stained with mAbs and analyzed without fixation. Negative controls were used to set the flow cytometer photomultiplier tube voltages, and single-color positive controls were used to adjust instrument compensation settings. Data from stained samples were acquired using a FACSCalibur flow cytometer equipped with CellQuest software (BD Biosciences) and were analyzed using FloJo software (Tree Star).

**Flow cytometric sorting of B cells**

Spleens from mock-infected mice and infected mice were harvested aseptically. Single-cell suspensions were prepared as described above, except the splenocytes were resuspended in HBSS without FBS. Single-cell suspension was pooled and incubated with mAbs directed against IgD, IgM, and CD19. The IgD−IgM−IgD+IgM+ populations were sorted from mock-infected and day 10-infected splenocytes using a FACSAria cell sorter (BD Biosciences). The IgD−IgM−ab population from the infected mice underwent an additional round of sorting to separate cells on the basis of CD11c expression.

**ELISPOT analyses**

Detection of IgM- and outer membrane protein (OMP)-specific IgM-producing spleen cells was conducted using an ELISPOT assay. Nitrocellulose plates (Multiscreen-HA; Millipore) were coated overnight at 4°C with a polyclonal IgM capture Ab (Southern Biotechnology Associates), or purified recombinant *E. muris* OMP-19 (10 μg/ml) (32). The plates were then incubated in blocking solution (IMDM supplemented 10% FBS) for 2–3 h at 37°C. Flow cytometrically sorted B cells were cultured in IMDM containing 10% FBS at a concentration of 1 × 10⁵ cells/ml. After 18 h incubation at 37°C in 5% CO₂, bound IgM was detected using goat anti-mouse IgM-conjugated to alkaline phosphatase (Southern Biotechnology Associates), followed by 5-bromo-4-chloro-3-indol phosphate/NBT (Sigma-Aldrich) as the substrate. Spot-forming cells were enumerated using a CTL-ImmuNoSpot S5 Core Analyzer, and the data were analyzed using CTL ImmunoSpot software (Cellular Technology). The frequency of Ab-secreting cells within each population and the frequency of the population within the spleen was determined and used to calculate the number of Ab-secreting cells per organ. Spots produced using IgM⁹⁹⁹ or IgD⁹⁹⁹ cells obtained from uninfected mice were subtracted from the responses of the populations obtained from infected mice.

**ELISA**

IgM serum Ab titers were determined by ELISA using purified recombinant *E. muris* OMP-19 as described previously (25).

**Immunohistochemistry**

To detect B cells in situ, spleens were embedded in Neg-50 medium (Richard-Allan Scientific), frozen on dry ice, and stored at −80°C. Cryostat sections (7 mm) were fixed in ice-cold acetone for 15 min and rehydrated in PBS. To block nonspecific binding, the sections were treated for 30 min at 37°C with Fc blocking solution containing 10% normal rabbit serum. The sections (see Fig. 5A) were stained in succession with rat anti-mouse MOMA1 (overnight; AbD Serotec), biotinylated rabbit anti- rat IgG (Vector Laboratories (60 min at room temperature (tr)), streptavidin-Alexa Fluor 480 (20 min; Invitrogen), washed extensively in PBS, and blocked with 2% BSA for 60 min at rt. The sections were next incubated with rat anti-mouse CD138 (2 h at rt; BD Biosciences), Texas Red-conjugated goat anti-rat F(ab)₂ (60 min; Southern Biotechnology Associates), biotinylated hamster anti-mouse CD11c (BD Biosciences), and streptavidin-Alexa Fluor 488 (30 min; Invitrogen). For the detection of T cells (see Fig. 5B), rat anti-mouse Thy1.2 (overnight at 4°C; BD Biosciences) was used and was detected with biotinylated rabbit anti-rat IgG (60 min at rt; Vector Laboratories) followed by streptavidin-Alexa Fluor 488 (30 min; Invitrogen). B cells were detected in the same sections using biotinylated rat anti-mouse B220 (1 h at rt; BD Biosciences) followed by streptavidin-Alexa Fluor 488 (30 min; Invitrogen). For detection of *E. muris* (see Fig. 5C) biotinylated Ec18.1 was used in place of anti-CD11c. When using biotinylated Abs, a streptavidin-biotin blocking kit (Vector Laboratories) was used between each of the Ab incubations. The stained sections were mounted in the E. muris mounting media (Low-Fade Gold; Invitrogen). Images were acquired using an Epi-fluorescence microscope (Zeiss Axioplan2) equipped with a Hamamatsu camera and were processed using OPENLAB software (Zeiss).
Statistical analyses

Statistical analysis of Ab-secreting cells in the ELISPOT assays were performed using an unpaired Student’s t test.

Results

Features of E. muris infection in the mouse

E. muris infection induces protective immunity to fatal challenge infection with IOE (30). Our recent studies have demonstrated that protective immunity can be achieved independent of CD4 T cells; therefore, our initial objective was to identify features of E. muris lymphocyte responses that contribute to classical TI immunity. As reported previously (28), we observed marked splenomegaly in C57BL/6 mice following i.p. infection with $5 \times 10^4$ E. muris, as reflected in changes in both spleen weight and cellularity (Fig. 1, A and B). Marked lymphopenia and thrombocytopenia were also noted, beginning on about day 7 postinfection (data not shown). Splenomegaly was most pronounced on day 17 postinfection, which was several days later than peak bacterial infection, which occurred on day 9 (Fig. 1C). Similar findings were observed previously in BALB/c mice (29). Bacterial numbers declined after day 9 postinfection, although low-level persistent infection was established in several tissues (data not shown and Ref. 28) These data reveal that although E. muris does not cause fatal disease, infection is associated with organ pathology and perturbations in cellular responses.

Detection of an unusual cell population in spleen

Our initial studies led us to monitor the frequency of spleen APCs, including DCs, using Abs that recognized CD11c and class II MHC IA<sup>b</sup>. Beginning on day 9 postinfection, we observed a distinct population of class II MHC IA<sup>b</sup> high-expressing, CD11clow cells (Fig. 2A). These cells were largely absent from uninfected mice, were detected at frequencies constituting as high as 15% of spleen mononuclear cells, and represented as many as $3 \times 10^7$ splenocytes (Fig. 2, B and C). The cells were detected in the spleen for as long as 9 days (until day 18 postinfection). The CD11clow cell population also exhibited low surface expression of B220 relative to CD11c<sup>high</sup>IA<sup>b</sup>-expressing cells (Fig. 2D; cf R1 and R3 in day 9-infected mice). CD11clowB220<sub>low</sub> cells were not detected in mock-infected mice (cf R1 and R2 in the mock-infected mice in Fig. 2D), indicating that they are not found in spleens of normal mice.

To characterize the cell population in greater detail, we next stained the B220<sub>low</sub>CD11clow splenocytes from infected mice with Abs that recognize various cell lineages, including Gr-1 (Ly6-C; for neutrophils), NK1.1 (NK and NKT cells), CD3 (T cells), and CD19 and IgM (B cells; Fig. 3, A–F). The B220<sub>low</sub>CD11clow cells did not express any cell lineage marker other than CD19 and IgM; expression of CD19 was low relative to conventional B cells (mean fluorescence intensity (MFI) = 199 ± 12.5 vs MFI = 233 ± 11.1). These data suggested that the cell population was not...
FIGURE 3. Flow cytometric characterization of the CD11c<sub>low</sub>-expressing splenocytes. A, Splenocytes from infected mice (day 9 postinfection) were stained with CD11c and B220, and the indicated populations (R1–R3) were analyzed for expression of Gr-1 (Ly6G; B), CD3 (C), NK1.1 (D), CD19 (E), and IgM (F). CD19 and IgM were the only lineage markers expressed by the B220<sup>+</sup>CD11c<sub>low</sub> population (R1; MFI = 119 ± 12.5 and 107 ± 4.7, respectively). The inset in D shows NK1.1 staining in mock-infected mice. G, IgM<sup>high</sup> (R1) and IgM<sup>low</sup> (R2) populations from mock- and day 9-infected spleens were analyzed for CD11c expression (lower panels). MFI values were determined for the populations from the mock-infected (R1 = 24 ± 8.9; R2 = 17.7 ± 6.9; positive population only) and day 9-infected splenocytes (R1 = 113.0 ± 23.5; R2 = 18.1 ± 2.7). CD19-negative cells were omitted from the analyses. H, The B220<sup>low</sup>CD11c<sub>low</sub> population was monitored on day 9 postinfection following mouse infection via the i.v. (IV) or i.p. (IP) routes.

The CD11c<sub>low</sub> cells exhibit characteristics of both MZ and B-1b plasmablasts

Three populations of mature B cells are present in normal spleens: classical B-2 FO B cells, MZ B cells, and small populations of B-1 B cells (reviewed in Ref. 33). These populations can be distinguished in part on the basis of CD21 and CD23 expression, where FO cells are typically found to be CD21<sub>low</sub>CD23<sub>high</sub>, MZ cells are CD21<sub>high</sub>CD23<sub>low</sub>, and B-1 cells are CD21<sub>neg</sub>CD23<sub>neg</sub>. During E. muris infection, however, both CD21 and CD23 expression was low on all B-lineage spleen cells (Fig. 4A), perhaps due to the action of ADAM-10 proteases secreted during ehrlichia infection (34). These observations limited the use of these markers for phenotype analysis. Therefore, other B cell markers were evaluated on different spleen cell populations in day 9-infected and mock-infected mice (the flow cytometry gating used for the analyses of the infected and control mice are indicated in Fig. 4, B and C, respectively). The analyses of the markers in the mock-infected mice were used as a basis for comparison of splenocytes from infected and normal mice.

CD11b (Mac-1) and CD43, markers characteristic of B-1 cells, were highly expressed by the CD11c<sub>low</sub> cell population in the infected mice (Fig. 4D). Although CD11b is not typically expressed on resident spleen B-1 cells, it is expressed transiently following the immigration of B-1 cells to the spleen (35). The CD11c<sub>low</sub> cells elicited during infection also exhibited lower surface expression of CD1d relative to IgM<sup>high</sup> MZ cells from uninfected C57BL/6 mice (Fig. 4D; MFI = 131.3 ± 7.6 vs 441 ± 7.0, respectively) (36, 37).

Because the CD11c<sub>low</sub> cells exhibited characteristics of B-1 cells (i.e., expression of CD11b), we also examined CD5 expression on the CD11c<sub>low</sub> population. B-1 cells are comprised of two subsets, B-1a and B-1b cells, which are distinguished by expression of CD5. The spleen CD11c<sub>low</sub> cells were largely CD5 negative, another characteristic of B-1 cells (Fig. 4D and Ref. 38). B-1b cells normally comprise only ~0.5% of spleen mononuclear cells (39) but it is possible that resident B-1b CD11c<sub>low</sub> cells underwent expansion within the spleen, or emigrated there from other sites. The CD11c<sub>low</sub> B cells also expressed CD9, a member of the tetraspanin protein family, which is expressed on both B-1 and MZ, but not FO B cells, and is up-regulated during plasma cell differentiation (40). The CD11c<sub>low</sub> cells exhibited an activated phenotype, as revealed by bimodal or high surface expression of CD40, CD80, and CD44 and low expression of CD62L relative to the other cell populations in the infected mice and to B cells in mock-infected splenocytes (Fig. 4E).

Because activation of B-1 and MZ B cells precedes their differentiation into plasma cells, we next examined the expression of plasma cell surface markers on the CD11c<sub>low</sub> population. CD138 (syndecan-1), a proteoglycan that is expressed on plasmablasts and plasma cells in secondary lymphoid organs (41), was expressed on ~50% of the CD11c<sub>low</sub> cells (Fig. 4F). In contrast, CD38, an ectoenzyme that is highly expressed on all B cells but is down-regulated during plasma cell differentiation (42) was also expressed on the CD11c<sub>low</sub> cells, suggesting that the CD11c<sub>low</sub> cells were not fully differentiated plasma cells.

As the CD11c<sub>low</sub> B cells were identified by the expression of an integrin not usually associated with B cells, expression of other integrins was evaluated. The CD11c<sub>low</sub> B cells also exhibited high expression of the VLA-4 component CD29, and similar CD49d expression, relative to FO B cells (Fig. 4G). VLA-4 expression by extrafollicular B cells has been reported during mouse mammary tumor virus infection and is thought to play a role in regulating cell migration (43). Migration of splenic B cells to extrafollicular regions...
is also regulated by chemokines, in particular CXCL12, the ligand for CXCR4 (44–46). The CD11clow-expressing cells exhibit high expression of CXCR4 relative to FO B cells (Fig. 4G; MFI = 247.3 ± 10 vs 118 ± 24), suggesting that CXCL12 may play a role in recruitment to, or localization within, the spleen. Finally, the CD11clow cells exhibited high forward and side scatter characteristics, relative to B220high B cells, a feature that is consistent with their differentiation into plasmablasts (Fig. 4H). The phenotypic analyses, taken together, reveal that the CD11clow B cells are differentiating plasmablasts, although it was not possible to establish the B cell lineage. A summary of the cell surface phenotype and the MFI values of the CD11clow cells is available in Table I.

**FIGURE 4.** The CD11c<sup>low</sup>-expressing cells exhibit characteristics of plasmablasts. *A*, Splenocytes from mock-infected and day 9-infected mice were analyzed for cell surface expression of CD23 and CD21. *B*, Gating strategy for phenotypic analyses of the day 9-infected mice shown in *D*. The B220<sup>high</sup>CD11c<sup>low</sup> (R1), B220<sup>+</sup>CD11c<sup>−</sup> (R2), and B220<sup>−</sup>CD11c<sup>−</sup> (R3) populations are indicated. *C*, Regions used for the analysis of IgM<sup>+</sup> (R4) and IgD<sup>+</sup> (R5) B cell populations from a mock-infected mouse. The cell populations indicated in *B* and *C* were analyzed using panels of Abs specific for various B cell subsets (*D*), cell activation status (*E*), plasma cell differentiation (*F*), and integrin and chemokine expression (*G*). The analysis of the cells from the mock-infected mice was provided as a basis of comparison. Surface staining with isotype-matched control Abs was in all cases within the first decade of the histograms and was omitted for clarity. *H*, Forward and side scatter analysis of the CD11c<sup>low</sup>B220<sup>high</sup> and B220<sup>high</sup> populations from an infected mouse are shown (equivalent to R1 and R2 in *B*, respectively). *I*, Expression of CD11c and CD138 on splenocytes from a day 8-infected mouse (encircled).
Stained B220/CD138-positive cells. CD138 B220/MERGEThy1.2. In E. muris-infected mice (bottom row) were stained with the metallocophilic macrophages (48), was used to delineate the spleen from mock-infected controls (top row) and day 8 E. muris-infected mice (bottom row) were stained with the indicated Abs. Double-positive CD11c/CD138 cells can be seen in yellow in the merged image. A. Studies similar to A were performed to define the spatial relationship between the T and B cells and the CD138 plasmablasts. The arrow in the lower right panel indicates double-stained B220/CD138-positive cells. C. Location of E. muris relative to the CD138 plasmablasts. Arrows in the bottom right panel indicate that most bacteria were observed in the extrafollicular region (yellow arrow), but bacteria could also be found in MOMA-1 macrophages (orange arrow) and, on occasion, in the follicle (green arrow).

**Table I. Flow cytometry MFI values**

<table>
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<tr>
<th>Ab</th>
<th>CD11c&lt;sup&gt;low&lt;/sup&gt; B Cells (R1)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>B Cells (R2)</th>
<th>MZ B Cells (R&lt;sup&gt;+&lt;/sup&gt;)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FO B Cells (R&lt;sup&gt;5&lt;/sup&gt;)</th>
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<tr>
<td>CD11b</td>
<td>94.9 ± 3.0</td>
<td>6.60 ± 0.3</td>
<td>13 ± 4.2</td>
<td>4 ± 1.4</td>
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<td>CD43</td>
<td>235 ± 15.2</td>
<td>144 ± 14.4</td>
<td>41 ± 3.4</td>
<td>13.2 ± 0.4</td>
</tr>
<tr>
<td>CD1d</td>
<td>131 ± 7.6</td>
<td>72.0 ± 4.4</td>
<td>320 ± 11.3</td>
<td>119 ± 1.4</td>
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<tr>
<td>CD5</td>
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<td>165 ± 7.7</td>
<td>120 ± 4.1</td>
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<td>CD138</td>
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<td>31.5 ± 5.3</td>
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<td>CD9</td>
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<td>197 ± 10.6</td>
<td>73.5 ± 9.2</td>
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<tr>
<td>CD38</td>
<td>413 ± 27.0</td>
<td>507 ± 56.5</td>
<td>1912 ± 227</td>
<td>896 ± 228</td>
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<tr>
<td>CD40</td>
<td>118 ± 26.5</td>
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<td>72.3 ± 3.7</td>
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<td>CD80</td>
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<td>CD86</td>
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<td>CD62L</td>
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<td>CD29</td>
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<td>CD49d</td>
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<td>9.1 ± 0.3</td>
<td>36.1 ± 0.6</td>
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<tr>
<td>CXCR4</td>
<td>247 ± 10</td>
<td>118 ± 24</td>
<td>132 ± 19.1</td>
<td>58.5 ± 0.7</td>
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<sup>a</sup>The values represent data from Fig. 4, b–g, and indicate the MFI values in the major cell population within each of the histograms. The mean and SD are shown; three mice were used per group. Statistically significant differences (p < 0.001) between the CD11c<sup>low</sup> B cells and B cells are indicated in bold, as determined using an unpaired Student t test.

<sup>b</sup>The flow cytometry gates, indicated in parentheses, are from Fig. 4, b and c.

**Extral follicular distribution of the plasmablasts**

Since integrin expression may regulate migration of the plasmablasts to extrafollicular or peripheral sites (43) and TI B cell responses are typically localized to extrafollicular regions in the spleen (3, 7, 47), we next addressed whether the CD11c<sup>low</sup> plasmablasts were localized to the extrafollicular area during *E. muris* infection. To accomplish this, spleens were stained with Abs to MOMA-1, CD11c, and CD138. MOMA-1, which recognizes MZ metallophilic macrophages (48), was used to delineate the spleen follicles. In addition to CD11c, CD138 was used as a marker for the CD11c<sup>low</sup> plasmablasts, since nearly all CD138-positive cells exhibited low expression of CD11c in infected mice (Fig. 4I). Large numbers of elicited plasmablasts, identified by coexpression of CD11c and CD138, as well as CD11c<sup>low/CD138<sup>neg</sup></sup> cells, were found nearly exclusively in the extrafollicular areas of the spleen, distinct from FO B and T cells (Fig. 5, A and B), consistent with the role of the cells in extrafollicular TI B cell responses. The CD11c<sup>low</sup> plasmablasts were sometimes found in or adjacent to the...
splenic MZ (Fig. 5B, day 8, arrows). The ehrlichiae in the spleen on day 8 postinfection were also found primarily in the red pulp, although bacteria were occasionally detected in MOMA-1 macrophages and within the follicles (Fig. 5C, yellow, orange, and green arrows, respectively).

**CD11c expression on plasmablasts is a marker for Ag-specific IgM-secreting cells**

The characteristics of the CD11clow plasmablasts suggested that they were responsible for the production of CD4 T1 IgM during infection. Accordingly, the accumulation and decline of serum *E. muris* OMP-19-specific IgM mirrored spleen CD11clow plasma-blast frequencies (Fig. 6A; cf with Fig. 2B). To directly address whether the CD11clow plasmablasts were responsible for the production of IgM, ELISPOT analyses were used to enumerate total IgM- and OMP-19-specific IgM-secreting B cells on day 10 postinfection. Spleen IgDhighIgMlow and IgDlowIgMhigh cells were separated by flow cytometric cell sorting, and the IgDlowIgMhigh cells underwent an additional round of cell sorting to separate the CD11clow and CD11cneg subsets (Fig. 6B). Very low frequencies

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**FIGURE 6.** IgMhighCD11clow cells secrete the majority of IgM and nearly all of the Ag-specific IgM. A, Serum *E. muris* OMP-19-specific IgM was detected by ELISA at the indicated days postinfection. B, Spleen IgDhighIgMlow and IgDlowIgMhigh cells from *E. muris*-infected (day 10) and mock-infected control mice were separated by flow cytometric cell sorting. IgDlowIgMhigh cells from infected mice were further separated into the CD11clow and CD11cneg subsets. The frequency and total number of IgM (C)- and OMP-19-specific IgM (D)-secreting cells were determined by ELISPOT. Spots produced by the mock-infected controls (which were negligible) were subtracted from the corresponding populations obtained from the infected mice. Data are representative of four independent experiments. E, Kinetic analysis of the splenic IgM- and OMP-specific IgM-secreting cells. ELISPOTs were performed as in C, except that T cell depletion was performed instead of flow cytometric cell sorting. The differences between the CD11clow and CD11cneg populations in C and D were statistically significant (*p < 0.001, indicated by the brackets), as determined using the unpaired Student’s *t* test). Error bars, SDs.
of IgM- or OMP-19 IgM-secreting cells were detected within the IgM<sup>high</sup> population from mock-infected mice (0.1 and 0.03%, respectively; data not shown) or within the IgM<sup>low</sup>IgD<sup>high</sup> population from infected mice (Fig. 6C). In contrast, ~40% of the IgM<sup>low</sup> cells isolated following infection were found to secrete IgM and, within the IgM<sup>low</sup> population, the majority of the IgM-secreting cells were found in the CD11c<sup>low</sup> cell subset (Fig. 6C). Similarly, the OMP-19-specific IgM-secreting cells were found nearly exclusively in the IgM<sup>high</sup> population and, within the this population, the CD11c<sup>low</sup> cells accounted for the production of nearly all of the Ag-specific IgM (Fig. 6D). The CD11c<sup>low</sup>IgM<sup>high</sup> subset was not responsible for Ag-specific IgG production elicited during E. muris infection (25), as few, if any, IgG<sub>3</sub>, IgG<sub>2b</sub>, or IgG<sub>2c</sub>-secreting B cells were found within any of the populations shown in the studies described in Fig. 6 (data not shown). As high as 10% of the CD11c<sup>low</sup> IgM-producing cells were specific for OMP-19, which supports previous studies that have demonstrated that the ehrlichia OMPs are immunodominant Ags (27, 49). The frequency of total and OMP-19-specific IgM-producing cells was highest on days 10–12 postinfection, and IgM-producing cells were detected for at least as long as 28 days postinfection (Fig. 6E). Thus, CD11c expression identifies a population of Ag-specific plasmablasts responsible for the majority of TI IgM production during acute ehrlichial infection.

**CD11c<sup>low</sup> cells are elicited in the absence of CD4 T cells and only under conditions that generate protective immunity**

Although the CD11c<sup>low</sup> plasmablasts were associated with TI responses, it was not known whether they were elicited in the absence of CD4 T cells. Indeed, the CD11c<sup>low</sup>B220<sup>low</sup> population was present in infected MHC class II-deficient mice (Fig. 7A). Moreover, CD11c<sup>low</sup> cells isolated from the MHC class II-deficient mice on day 10 postinfection produced IgM- and OMP19-specific IgM, demonstrating that Ab production by these cells was CD4 T independent (Fig. 7B). The CD11c<sup>low</sup> population was not elicited following low-dose IOE or E. chaffeensis infection (Fig. 7C) or using heat-killed E. muris (Fig. 7D), both conditions which failed to generate protective immunity to high-dose IOE challenge (50). Thus, although evidence that the plasmablast population is essential for TI immunity during fatal IOE challenge is not yet available, the data suggest that the cells and/or their products will play an important role during infection.

**Discussion**

**CD11c identifies Ab-secreting plasmablasts**

Although CD11c is often considered to be a definitive marker for DCs (51, 52), our studies reveal an unexpected utilization of this α<sub>x</sub> integrin by a population of CD11c<sup>low</sup>-expressing plasmablasts during intracellular bacterial infection. Although CD11c-positive plasmacytoid DCs also express B220 (53), the cells we have characterized are not DCs, as the plasmablasts expressed definitive B cell markers such as CD19 and surface IgM, which are uniquely expressed by B cells. A previous study has reported activity of the CD11c promoter in a population of Ab-secreting murine plasmablasts in immunized CD11c-diphtheria toxin receptor transgenic mice, although surface expression of CD11c was not detected on B cells (54). A second study identified a population of low-density CD19<sup>+</sup>CD11c<sup>low</sup>B220<sup>+</sup> cells in BALB/c mice that increased in frequency following a maturational stimulation (55). These cells were characterized as DC-like B cells, although it is possible that they are resident CD11c<sup>low</sup> plasmablasts equivalent to the cells we have described. We were unable to identify CD11c<sup>low</sup> cells within the IgM<sup>high</sup> population in naïve mice (Fig. 3G), suggesting that...
CD11c<sup>low</sup> B cells are absent or rare in naive spleens. Cell surface expression of CD11c has also been reported on activated human B cells (56) and has been used as a diagnostic marker for hairy cell and B cell chronic leukemias (57, 58), indicating that CD11c expression may be associated with proliferation and activation of human B cells, perhaps in response to TI Ags.

Our studies reveal that CD11c expression identifies Ab-secreting cells within the IgM<sup>high</sup>IgD<sup>high</sup> splenic B cell population during E. muris infection. In this respect, CD11c expression defines a plasmablast population also identified by CD138 (sydlec-1), a marker commonly used to identify Ab-secreting cells (41). However, although nearly all CD138-positive cells were CD11c<sup>low</sup> (see Fig. 4), not all CD11c<sup>low</sup> cells expressed CD138, indicating that the CD138<sup>low</sup> populations are not equivalent. Thus, although the majority of IgM-secreting cells were found in the CD11c<sup>low</sup> population, the IgM<sup>high</sup> population also contains a proportion of CD138-negative, non-Ab-secreting B cells (data not shown). Therefore, CD11c expression may precede that of CD138, and may identify a developmental precursor in the plasma cell differentiation pathway (59). Although the CD11c<sup>low</sup> plasmablasts secreted the majority of TI IgM, it is also possible that they play additional roles in immunity. Indeed, the plasmablasts exhibited high surface expression of MHC class II, CD40, CD80, and CD86 molecules expressed by APCs, including MZ B cells, that facilitate T cell activation (60).

Origin of the CD11c<sup>low</sup> plasmablasts

It has not been possible to resolve the origin of the CD11c<sup>low</sup> plasmablasts, because the expression of markers commonly used to define B cell lineages in naive mice (i.e., CD21, CD23, CD11d) were apparently down-regulated during acute E. muris infection. We cannot rule out the possibility that the CD11c<sup>low</sup> cells are differentiated FO cells that have down-regulated IgD following activation. However, we favor the notion that the plasmablasts are MZ- or B-1-derived, because they exhibit cell surface expression of several markers characteristic of innate-like MZ and/or B-1 cells (i.e., CD9, CD38, IgM), as well as activation markers (i.e., CD80, CD86), and can rapidly differentiate into plasmablasts (61). The CD11c<sup>low</sup> plasmablasts were elicited following both i.p. and i.v. inoculation using relatively low numbers of bacteria. Although MZ B cell responses are typically only detected following blood-borne infections (3), E. muris colonizes the spleen following either inoculation route (25); therefore, it is possible that MZ B cells responses are elicited during E. muris infection independent of inoculation route. On the other hand, expression of CD11b and CD43 on the CD11c<sup>low</sup> plasmablasts supports their B-1b origin. CD11b expression on MZ B cells has not previously been described, although CD43 is induced during plasmablast differentiation (62). Although it was believed that CD11b expression was limited to peritoneal B-1 cells, it has been reported that CD11b can also be expressed on splenic B-1 cells (63). TI B-1 cells have been shown to be elicited in other bacterial infection models. Alugupalli et al. (12, 64) observed that peritoneal B-1b cells underwent expansion following Babesia hermsii infection, and the cells were associated with protective immunity. B-1 cells were also detected in the spleen following Streptococcus pneumoniae infection (3). Preliminary studies have suggested that the CD11c<sup>low</sup> plasmablasts are elicited in xid mice (R. Racine and G. M. Winslow, unpublished data). xid mice, which lack activity of Bruton’s tyrosine kinase, due to a point mutation in the pleckstrin homology domain, are deficient in B-1b cells and do not produce IgM in response to TI Ags (65). However, peritoneal B-1b cells can undergo expansion in xid mice following infection (12), and recent data suggest that an alternative pathway of activation, via MyD88, can lead to the development of B-1 cells in xid mice (66). Thus, none of our data definitively establish the origin of the CD11c<sup>low</sup> cells.

A striking observation was the sudden appearance of the CD11c<sup>low</sup> cells in the spleen between days 8 and 9 postinfection. Although it is possible that CD11c expression was induced on resident spleen B cells, we consider this unlikely, especially considering the massive increase in spleen cellularity occurring at this time. A second possibility is that the CD11c<sup>low</sup> population resulted in part following the emigration of B cell precursors that underwent rapid differentiation in the spleen. Inflammation has been shown to mobilize developing B lymphocytes from the bone marrow followed by the expansion of some B cell subsets in the spleen (67). Such a population may include transitional/immature (TI) B cells that result from extramedullary hematopoiesis. These cells have been shown to differentiate into plasmablasts in response to TLR ligands during infection (68). Similarly, it is possible that CD11c<sup>low</sup> plasmablasts of B-1 origin arose following immigration of peritoneal B-1 cells to the spleen, as we have observed a 2- to 3-fold decrease in the frequency of peritoneal B cells on day 8 postinfection (R. Racine and G. M. Winslow, unpublished data). Another explanation is that B-1-derived CD11c<sup>low</sup> plasmablasts arise from uncommitted FO splenic B cells following Ag-driven selection (the induced differentiation hypothesis (69, 70)). Additional studies will be required to resolve these possibilities.

Role in TI responses

Although B-1 and MZ cells are well known to participate in TI responses, we have shown that the CD11c<sup>low</sup> plasmablasts are the principal cells involved in CD4 TI humoral immunity following E. muris infection. The CD11c<sup>low</sup> cells were present in CD4 T cell-deficient mice and were responsible for nearly all of the IgM produced in the spleen during acute infection. Moreover, their extrafollicular location was consistent with a TI response. Definitive evidence that the CD11c<sup>low</sup> plasmablasts are essential for the TI protection is not yet available, but the cells were elicited only under conditions that conferred protection against subsequent fatal IOE challenge. Our previous studies in E. chafeensis-infected SCID mice did not reveal a protective role for IgM, but it is possible that IOE is susceptible to IgM-mediated complement fixation, as complement-depleted mice, unlike untreated mice, were susceptible to low-dose IOE infection (71).

Although both B-2 and B-1 cells are a source of Abs during influenza infection (11), during E. muris infection IgM is produced in the spleen nearly exclusively by the CD11c<sup>low</sup> plasmablasts, because few if any IgD-positive FO splenic B cells were found to produce IgM. Our data suggest that the robust CD11c<sup>low</sup> plasmablast responses occur at the expense of the FO B-2 responses, as we have observed a general breakdown of the FO organization of the spleen and loss of germinal centers by day 10 postinfection (G. M. Winslow and M. Chatterjee, unpublished data). This observation is perhaps similar to the loss of the germinal center response that has been observed in attenuated Salmonella typhimurium infection (72). High Ag levels during early infection may drive germinal center B cells to undergo apoptosis (73–75). In this regard, CD11c<sup>low</sup> plasmablasts declined in frequency after peak expansion on day 10, consistent with their rapid elimination by apoptosis (76–78).

A large proportion (~10%) of the IgM produced by the CD11c<sup>low</sup> plasmablasts was directed toward E. muris OMP-19, indicating that the OMPs are major TI Ags, consistent with the immunodominant responses characterizedly observed against ehrlichia OMPs (27, 49). It is likely that the Abs recognize protein, not associated carbohydrate, as the OMP-19 used in the study was generated in recombinant form in Escherichia coli, and previous studies demonstrated that most OMP-19 Abs recognized a linear
peptide epitope within the OMP-19 hypervariable region 1 (27). These data suggest that the ehrlichia OMPs are presented on the bacterial surface as repetitive arrays that are sufficient to trigger TI responses. The role of other bacterial factors in assisting this TI response are not yet known, but TLR ligands are obvious candidates (66). It is unclear why recognition of OMPs did not induce, in addition, a T-dependent germline center-driven response from FO B cells during acute infection. One possibility is that germinal center responses are inhibited in the spleen by the massive ongoing TI response.

**Generation of the plasmablast response by E. muris**

Although early and robust TI plasmablast responses have been observed in MZ and B-1 cells following immunization and infection (3, 13), the magnitude of the response during *E. muris* infection was striking. Why *E. muris*, and not even other ehrlichiae, elicits such a large plasmablast response is not known. B-1 cells respond to TLR agonists (79) and can be induced to migrate from the peritoneal to the spleen where they undergo plasma cell differentiation following TLR ligation (80). However, the ehrlichiae have not been reported to express TLR ligands such as LPS or peptidoglycan (81), although we have observed that MyD88- and TLR2-deficient mice are susceptible to low-dose IOE infection. (G. M. Winslow, unpublished data). *E. muris* has been shown to express lipid Ags and to trigger NKt cells via CD1 responses (82). A population of CD11clow blood DC can promote differentiation of B-1 cells to plasmablasts (6, 13, 83), suggesting another possible mechanism.

**Role of integrins and chemokine receptors in extrafollicular plasmablast responses**

The CD11c<sup>low</sup> plasmablasts we have described expressed the integrins VLA-4, CD11c, and CD11b. VCAM-1 and fibronectin, both of which are believed to be involved in localization of plasmablasts to the extrafollicular space (43, 84). VLA-4 is also a predominant integrin which is also involved with the migration of plasmablasts to the ex-...


