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Infection-Induced Marginal Zone B Cell Production of *Borrelia hermsii*-Specific Antibody Is Impaired in the Absence of CD1d

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Ab that arise in the absence of T cell help are a critical host defense against infection with the spirochetes *Borrelia burgdorferi* and *Borrelia hermsii*. We have previously shown that CD1d-deficient (CD1d<sup>−/−</sup>) mice have impaired resistance to infection with *B. burgdorferi*. In mice, CD1d expression is highest on marginal zone B (MZB) cells, which produce Ab to blood-borne Ag. In this study we examined MZB cell activation and Ab production in mice infected with *B. hermsii*, which achieve high levels of bacteremia. We show by flow cytometry that MZB cells associate with *B. hermsii* and up-regulate the activation markers syndecan 1 and B7.1 within 16 h of infection. By 24 h, MZB cells secrete *B. hermsii*-specific IgM, coinciding with the loss of activation marker expression and the reduction in spirochete burden. In contrast, MZB cells from CD1d<sup>−/−</sup> mice remain activated for at least 96 h of infection, but produce only minimal *B. hermsii*-specific IgM in vivo and ex vivo; pathogen burden in the blood also remains elevated. Wild-type mice depleted of MZB cells using mAb to LFA-1 and α<sub>4</sub>β<sub>1</sub> integrin have reduced serum levels of *B. hermsii*-specific IgM and increased pathogen burden, similar to *B. hermsii*-infected CD1d<sup>−/−</sup> mice. Passive transfer of immune mouse serum, but not naive mouse serum, into infected CD1d<sup>−/−</sup> mice leads to down-regulation of activation markers and clearance of *B. hermsii* from the MZB cells. These results demonstrate that blood-borne spirochetes activate MZB cells to produce pathogen-specific IgM and reveal a role for CD1d in this process. The Journal of Immunology, 2005, 174: 5681–5686.

Spirochetes of the genus *Borrelia* are arthropod-borne agents of both human and domestic animal diseases and are transmitted to vertebrate hosts during vector feeding (1). *Borrelia* species, including the Lyme disease spirochete, *Borrelia burgdorferi*, and the agent of relapsing fever, *Borrelia hermsii*, have distinct blood-borne phases of infection within the vertebrate host. Blood-borne dissemination may facilitate arthropod acquisition of the pathogen during vector feeding, but secondarily can cause systemic disease. Control of the pathogen during the hematogenous stage of infection is an important host defense against pathologic sequelae.

Humoral immunity is an essential mammalian defense against *Borrelia* organisms. Although pathogen-specific Ab produced by follicular B cells can protect mice against *B. burgdorferi* infection, protective Ab can also arise in mice in the absence of T cells, MHC class II, or CD40, a molecule required for T cell-dependent B cell responses (2, 3). Similarly, T cells are not required for the production of disease-remitting *B. hermsii*-specific Ab, because nude mice, which lack functional T cells, and IL-7-deficient mice, which lack T cells and follicular B cells, eliminate blood-borne spirochetes with the same efficiency as wild-type (WT)<sup>3</sup> mice (4, 5). These findings underscore the importance of pathogen-specific IgM in the clearance of *Borrelia* spirochetes and suggest that B cell subsets other than follicular B cells contribute to protective immunity against infection with these organisms. In this regard, the B1b cell subset has been shown to be a source of protective Ab against *B. hermsii*, but only after priming by infection (6). Naive B1b cells, in contrast, produce Ab that can clear attenuated (high passage) *B. hermsii*, but are unable to clear the first episode of spirochetal infection in primary infection of mice with virulent (low passage) *B. hermsii* (6). Together these findings suggest that other B cell subsets, such as marginal zone B (MZB) cells, may be an additional source of early protective Ab against *B. hermsii*.

MZB cells comprise a B cell subset localized in mice to the marginal sinuses of the spleen, where they provide early immune responses to blood-borne particulate Ag (7–10). Like B1b cells, these cells bridge the innate and adaptive immune systems, because they have the capacity to respond to specific foreign Ag more rapidly than conventional T and B lymphocytes (10). MZB cells can be distinguished from follicular B cells by their complement receptor 2 (CD21)<sup>high</sup>/CD1d<sup>high</sup>/CD23<sup>low</sup> phenotype (11, 12) and from splenic B1 B cells by their CD21<sup>high</sup>/CD43<sup>low</sup> expression (13). Based on studies using inert particles and heat-killed bacteria, it has been postulated that reduced CD23 expression, a negative regulator of B cells, may allow MZB cells to produce a more rapid Ab response compared with follicular B cells, and that CD21<sup>high</sup> expression facilitates MZB cell interaction with complement-coated blood-borne Ag (14). The relevance of elevated CD1d expression on MZB cells compared with other CD1d-expressing cells remains unknown.

CD1d is a nonclassical MHC molecule that presents foreign and self-lipid Ags to T cells with limited Ag receptor diversity (15, 16). We have previously shown that mice deficient in CD1d exhibit impaired resistance to infection with *B. burgdorferi* (17). After intradermal inoculation into mice, *B. burgdorferi* can be detected earlier in a site representative of blood-borne dissemination in CD1d<sup>−/−</sup> mice, the urinary bladders, compared with similarly infected WT mice. Because MZB cells have been implicated in the production of Ab to blood-borne Ags, we questioned whether...
Mice were housed in filter-frame cages and administered food and water ad libitum according to Yale University animal care and use guidelines. Mice were obtained from The Jackson Laboratory. Mice provided by Dr. A. Bendelac (University of Chicago, Chicago, IL) (1). All mice backcrossed eight times onto B6 had been previously provided by Dr. A. Bendelac (University of Chicago, Chicago, IL) (1). All knockout mice were bred as homozygotes in our animal facility. B6.129 SF2/J and B6 WT mice were obtained from The Jackson Laboratory. Mice were housed in filter-frame cages and administered food and water ad libitum according to Yale University animal care and use guidelines. Mice were euthanized by carbon dioxide asphyxiation. The Yale animal care and use committee approved all studies involving mice.

Infection of mice

The DAH strain of B. hermsii (5) was a gift from Dr. J. Leong (University of Massachusetts School of Medicine, Worcester, MA). Frozen aliquots of DAH passage 1 were grown to logarithmic phase in BSK-H medium (Sigma-Aldrich) and enumerated by darkfield microscopy using a Petroff Hauser chamber before injection into mice. Each mouse was inoculated i.p. with 10⁶ spirochetes in 100 μl of BSK-H. At the time of death, infection was confirmed by documenting the presence of motile spirochetes in peripheral blood smears using darkfield microscopy.

Flow cytometry

Splenocytes were isolated from mice at the time that mice were killed. After RBC lysis and washing, splenic MZB cells were identified and analyzed by flow cytometry using four-color immunofluorescence staining. FITCs were first labeled with unconjugated anti-CD16/32 mAb before labeling of splenocytes with allophycocyanin-conjugated anti-B220, PE-conjugated anti-CD23, and either biotin- or FITC-conjugated anti-CD21/CD35 mAb to define MZB and follicular B cells (BD Pharmingen). Activation markers were identified with FITC conjugates of goat anti-mouse B7.1 or CD25 or with biotinylated syndecan 1 (CD138; BD Pharmingen). The percent B220lowCD23low/negCD43high (BD Pharmingen) phenotype in this process. MZB cells were depleted from 6-wk-old mice with a single i.p. treatment of Click’s medium (Irvine Scientific) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 24-well tissue culture plates for subsequent ELISA determination of Ab secretion.

ELISA and ELISPOT assay

For analysis of Ab production, MZB cells from infected and uninfected mice were enriched by negative selection. Briefly, splenocytes were incubated with biotinylated anti-Thy-1.2, anti-NK-1.1, anti-CD11b, anti-CD43, anti-Ly6C, and anti-CD23 mAb (BD Pharmingen), and the unlabelled MZB cell population was separated using anti-biotin-coated magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec). The percent-age of MZB cells in each sample was determined by flow cytometry, and the same number of MZB cells was cultured for each sample. Approximately 10% of the cells in the enriched population were contaminating CD21⁺/CD23neg B cells, consistent with an immature phenotype. Enriched MZB cells were then used directly in ELISPOT assays. Alternatively, they were cultured for 3 d at a density of 1 × 10⁶ cells/ml in 1 ml of Click’s medium (Irvine Scientific) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) in 24-well tissue culture plates for subsequent ELISA determination of Ab secretion.

ELISA was performed using the culture supernatants or serum from infected or immunized mice. Ninety-six-well microtiter plates were coated with B. hermsii lysate (3 μg in 50 μl of 1% ethanol/well) by overnight incubation at 4°C. For detection of anti-nitrophenylacetyl (anti-NP) Ab, plates were coated overnight at 4°C with 500 ng/well NP-BSA (Biosearch Technologies) in carbonate buffer (pH 9.6). After blocking in PBS containing 5% BSA, serial 2-fold dilutions of culture supernatants or serum were added to the wells and incubated for 2 h at room temperature. Secondary biotinylated anti-mouse IgM (Vector Laboratories) was used at a 1/1000 dilution, and bound Ab was detected with the ABC Elite peroxidase detection and ABTS substrate kit (Vector Laboratories).

For ELISPOT assays, 200-μl aliquots of the enriched MZB cells were aliquoted onto 96-well ELISA plates previously coated with B. hermsii lysate and incubated for 16 h at 37°C in a 5% CO₂ humidified chamber. Plates were coated with 5% BSA, serial 2-fold dilutions of culture supernatants or serum were added to the wells and incubated for 2 h at room temperature. Secondary biotinylated anti-mouse-IgM (I/I1000 dilution; Vector Laboratories), and bound Ab was detected with the ABC Elite peroxidase detection and tetramethylbenzidine substrate kits (Vector Laboratories). Spots were enumerated from three wells in 10 microscope fields/well using ×200 magnification. The number of spots was normalized to the plating density of cells per well.

Quantitative PCR of B. hermsii DNA

DNA was isolated from 100 μl of whole blood using the Isoquick DNA isolation kit (ORCA Research). A known quantity of human DNA was added to each blood sample before DNA isolation for use as an internal control for DNA recovery and for data normalization. The level of B. hermsii DNA in each sample was determined by quantitative PCR of the plasmid gene using an (5'-GACTGAAAGCTTGGTACA-3') primer and a 60°C annealing temperature were used. Standard curves were generated for both PCRs using known quantities of DNA. Reactions were performed in duplicate, and the quantities of PCR products were determined from the standard curves.

Immunization of mice

Groups of five WT and CD1d⁻/- mice were infected as described above. At 72 h after infection, one group of CD1d⁻/- mice received i.p. inoculations of 300 μl of serum from 72-h-infected WT mice. A control group of CD1d⁻/- mice was passively immunized with 300 μl of serum from uninfected WT mice. Twenty-four hours after immunization, mice were killed, and MZB cells were analyzed for spirochete association and activation marker expression by flow cytometry as described above.

For immunization with a type II T-independent (TI-2) Ag, mice were inoculated i.p. with 20 μg of NP-conjugated Ficoll (NP-Ficoll; Biosearch Technologies) in 200 μl of sterile PBS. Five and 9 days after immunization, serum was obtained by tail vein puncture and analyzed for the presence of anti-NP IgM by ELISA.

Depletion of MZB cells

Mice infected with a type II T-independent (TI-2) Ag, mice were inoculated with 20 μg of NP-conjugated Ficoll (NP-Ficoll; Biosearch Technologies) in 200 μl of sterile PBS. Five and 9 days after immunization, serum was obtained by tail vein puncture and analyzed for the presence of anti-NP IgM by ELISA.
numbers at 96 h; in contrast, spirochetes could be detected in association with CD1d\(^{-/-}\) MZB cells at all time points analyzed (Fig. 1A). We did not, however, detect spirochetes in association with splenic follicular or B1 B cells at any of the time points analyzed (data not shown). The level of spirochete binding to CD1d\(^{-/-}\) MZB cells increased over time, suggesting that the pathogen burden was higher in CD1d\(^{-/-}\) mice.

**B. hermsii infection activates MZB cells**

In mice, MZB cells are reported to be activated and up-regulate syndecan I, a marker of plasma cells, 24 h after i.v. injection of particulate Ag (20). We found increased expression of syndecan I on WT MZB cells as early as 16 h after infection, but by 24 h, this marker was no longer detected (Fig. 1B). At 96 h, a subset of the MZB cells had increased levels of syndecan I (Fig. 1B), which may represent their response to a second episode of spirochetemia captured in some WT mice at 96 h (Fig. 1A). In contrast, MZB cells from CD1d\(^{-/-}\) mice exhibited a delay in syndecan I up-regulation, but unlike WT MZB cells, syndecan I expression was detected at all subsequent time points analyzed. At 16 h of infection, greater syndecan I expression was observed on WT MZB cells compared with CD1d\(^{-/-}\) MZB cells (Fig. 1B) even though similar levels of spirochetes were detected on MZB cells from WT and CD1d\(^{-/-}\) mice (Fig. 1A). These findings were unique to infection, because i.v. injection of heat-killed spirochetes into mice resulted in equivalent up-regulation of syndecan I on MZB cells from WT and CD1d\(^{-/-}\) mice 2 h postinjection (data not shown).

Expression of the T cell costimulatory molecule B7.1 was increased on both WT and CD1d\(^{-/-}\) MZB cells at 16 h of infection (Fig. 1C), but, as with syndecan I, B7.1 was not detected on WT MZB cells at the later time points. B7.1 expression remained elevated, however, on CD1d\(^{-/-}\) MZB cells for at least 96 h (Fig. 1C). CD25 was similarly up-regulated on CD1d\(^{-/-}\) MZB cells, but we were unable to detect significant changes in the expression of this marker on WT cells at any of the four time points.

**MZB cells produce B. hermsii-specific Ab, but levels are reduced in the absence of CD1d**

*B. hermsii*-specific IgM is essential for clearance of spirochetes from infected mice (21). After 48 h of infection, WT, but not CD1d\(^{-/-}\), MZB cells cultured ex vivo without Ag stimulation secreted *B. hermsii*-specific IgM (Fig. 2A). ELISPOT assays performed with MZB cells from mice infected for only 24 h showed that a higher number of WT MZB cells secreted *B. hermsii*-specific IgM compared with CD1d\(^{-/-}\) MZB cells (Fig. 2B). At 72 h, the first time point when *B. hermsii*-specific Ab could be detected in serum, WT mice had at least 5-fold higher anti-*B. hermsii* IgM titers than CD1d\(^{-/-}\) mice (Table I). Thus, even though CD1d\(^{-/-}\) MZB cells up-regulated B7.1, CD25, and syndecan I to levels that surpassed those observed on WT cells and maintained their activated state for up to 96 h, their production of *B. hermsii*-specific Ab was impaired. Similar results were obtained with *B. hermsii*-infected B6 WT and CD1d\(^{-/-}\) mice, but specific IgM levels were lower than those seen in B6.129 mice (Table I). We detected significantly fewer MZB cells in uninfected B6 mice compared with B6.129 mice (Table I; \(p = 0.014\), by unpaired Student’s t test), which may explain the differences in pathogen-specific IgM levels detected in the two strains. The defect in *Borrelia*-specific Ab production in CD1d\(^{-/-}\) mice cannot be explained by a general deficiency in MZB cell production of Ab, because WT and CD1d\(^{-/-}\) mice immunized with the T-independent Ag NP-Ficoll produced

**FIGURE 1.** MZB cells from *B. hermsii*-infected mice are activated early in infection. Pooled splenocytes from three mice infected for 16, 24, 48, or 96 h were stained with mAb to B220, CD23, and CD21 and anti-*Borrelia* polyclonal sera (A), anti-CD138 (B), anti-CD80 (C), or anti-CD25 (D). Gates were set on the MZB cell population (B220\(^{-}\)CD22\(^{lo}\)CD23\(^{hi}\)CD21\(^{hi}\)). The results shown are representative of three to five separate experiments performed at each time point. A, Cells from uninfected controls; B, cells from infected WT mice; C, cells from infected CD1d\(^{-/-}\) mice. At the 96 h point, the level of surface marker expression on uninfected cells was slightly elevated due to experimental variation, but the median fluorescent intensities were not statistically different from those at the other time points. The results depicted are those for B6.129 WT and CD1d\(^{-/-}\) mice and were representative of those obtained in B6 CD1d\(^{-/-}\) mice, which also showed elevated levels of activation markers for at least 96 h compared with B6 WT mice (date not shown).

**FIGURE 2.** MZB cells from CD1d\(^{-/-}\) mice produce less *B. hermsii*-specific Ab. A, Reciprocal end-point positive ELISA titers for *B. hermsii*-specific IgM in supernatants of in vitro cultured MZB cells isolated from 48-h-infected mice. B, ELISPOT assay of *B. hermsii*-specific IgM produced by in vitro cultured MZB cells isolated from 24-h-infected mice. Inf., infected; Uninf., uninfected.
We have previously demonstrated that from 72-h-infected mice. Results represent the average of values obtained from three control mAb were without effect (Fig. 3).

Passive immunization of infected CD1d

Passive immunization of WT mice (17). By quantitative PCR of spirochete DNA isolated from peripheral blood specimens, we found that infected CD1d

Passive immunization of WT mice (Fig. 4). Together with the increased spirochete binding to the MZB cells (Fig. 1A), these data confirm that elimination of blood-borne spirochetes is impaired in CD1d

Depletion of MZB cells leads to a decrease in B. hermsii-specific IgM titers

To further implicate MZB cells as a source of pathogen-specific IgM in B. hermsii infection, we depleted MZB cells from WT mice using mAb to LFA-1 and $\alpha_4\beta_1$ integrins (19). This treatment results in selective depletion of MZB cells without affecting other lymphocyte populations in the spleen, and thus has an advantage over mutant mouse strains noted to lack MZB cells in the setting of other immunologic defects (22). In addition, mAb depletion of MZB cells retains normal trafficking of B. hermsii through the spleen, in contrast to the altered physiology resulting from splenectomy. B. hermsii are known to bind to platelets via $\alpha_{IIb}\beta_3$ integrins, but there is no evidence that they interact with the LFA-1 and $\alpha_4\beta_1$ integrins (23, 24). mAb depletion of MZB cells from WT mice before infection resulted in a decrease in serum B. hermsii-specific IgM titers commensurate with the reduction in the percentage of MZB cells (Fig. 3). The pathogen burden was also increased from a mean of 20 ng of B. hermsii flagellin DNA/μg β-actin DNA in control B6.129 mice to 400 ng in MZB cell-depleted B6.129 mice, and from a mean of 33 ng in control B6 WT mice to 1050 ng in MZB cell-depleted B6 mice. No significant effect of MZB cell depletion on B. hermsii infection and Ab response could be detected in CD1d

CD1d

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FIGURE 3. Depletion of MZB cells reduces B. hermsii-specific IgM titers in serum at 72 h of infection. WT and CD1d

FIGURE 4. Quantitative PCR of B. hermsii DNA in blood samples from infected mice. The amount of B. hermsii DNA present in 100 μl of blood was quantified by real-time PCR of the B. hermsii flagellin gene as described in Materials and Methods. Each data point represents results from an individual mouse (four mice per group per time point), and is the average of duplicate determinations performed in two separate assays. The $p$ values for 16, 24, 48, 72, and 96 h were 0.0006, 0.0001, 0.0902, <0.0001, and 0.693, respectively (by unpaired Student’s $t$ test).

**Table I. Reduced B. hermsii-specific IgM in the absence of CD1d**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Group</th>
<th>MZB (%) of B cells</th>
<th>B. hermsii-Specific IgM Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6.129</td>
<td>WT</td>
<td>9.1 ± 1.2</td>
<td>2560 ± 50</td>
</tr>
</tbody>
</table>
| B6.129 | CD1d

<table>
<thead>
<tr>
<th>B6</th>
<th>WT</th>
<th>3.8 ± 0.5</th>
<th>1040 ± 344</th>
</tr>
</thead>
</table>
| B6    | CD1d

|       | WT    | 4.9 ± 1.2          | 486 ± 304d                    |

* Percentages of MZB cells are derived from uninfected mice included as controls in three to six separate experiments. Values represent the percentage of CD21high/CD21low cells within B220+ splenocytes.

† Reciprocal end-point positive titers of B. hermsii-specific IgM in serum obtained from 72-h-infected mice. Results represent the average of values obtained from three to six separate experiments using two to five mice per group.

‡ Significantly different from WT B6 values ($p = 0.0001$, by unpaired Student’s $t$ test).

§ Significantly different from WT B6 values ($p = 0.026$, by unpaired Student’s $t$ test).
tion molecules. Because normal mouse serum contains natural Ab dominant vmp expressed by spirochetes with each episode of bac-
spirochetes, and that this response is impaired in the absence of serum IgM and IgG1, and providing early cytokines for inducing Th cell differentiation (14, 16, 27, 28). In our study we found that MZB cells from B. hermsii-infected CD1d−/− mice retained an activated phenotype for longer periods than those from WT mice, consistent with an absence of immune regulation. However, B. hermsii-specific IgM was sufficient to reduce MZB cell activation in this model, suggesting that the pathogen itself was driving the prolonged MZB cell activation, not the absence of NK T cells. Although CD1d expression and up-regulation of T cell costimulatory molecules by MZB cells may permit interaction with NK T cells, these events are not thought to be necessary for MZB cell production of B. hermsii-specific IgM, because protective IgM arises in the absence of all T cells. We also did not observe any differences in spirochete binding or activation marker expression between MZB cells from WT BALB/c mice and CD1d−/− BALB/c mice, which lack a major subset of CD1d-restricted NK T cells (data not shown).

CD1d−/− mice have impaired production of pathogen-specific Ab after infection, but not Ab to the TI-2 Ag NP after immuniza-
tion with NP-Ficoll. Consistent with an intact response to TI-2 Ag, the expression of TACI, a molecule required for Ab production to TI-2 Ag (29), was similar on WT and CD1d−/− MZB cells (data not shown). TACI is primarily considered a negative regulator of B cells, because TACI−/− mice have expanded B cell populations and spontaneous autoantibody production (30). We found that both WT and CD1d−/− MZB cells down-regulate TACI expression equally by 72 h of infection, indicating that the impaired pathogen-specific Ab production we observed in CD1d−/− mice is not a consequence of aberrant TACI expression by MZB cells.

One explanation for our findings is that the CD1d molecule itself contributes an intracellular signal that enhances MZB cell production of Ab. The cytoplasmic tail of the CD1d molecule con-
tains a putative tyrosine-dependent internalization signal, YXXZ, which was found to play a critical role in autocrine signaling (31). CD1d expressed on the surface of intestinal epithelial cells was readily activated by Ab cross-linking, as evidenced by tyrosine phosphorylation and IL-10 up-regulation, but signaling was abol-
ished when the cytoplasmic tail of the CD1d molecule was re-
moved (31). It was postulated that in vivo, intraepithelial lympho-
cyes could cross-link CD1d to elicit these events. Our data suggest that if CD1d on MZB cells can provide an autocrine signal for production of pathogen-specific Ab, T cells are not necessary. The high level of CD1b on MZB cells may permit its cross-linking along with other cell surface receptors in the absence of a specific interaction with CD1d-restricted T cells.

Our study does not preclude a role for CD1d on other APC, such as dendritic cells or macrophages, in promoting MZB cell produc-
tion of pathogen-specific Ab. Dendritic cells can capture Ag and transport them to the spleen where they interact with MZB cells to initiate T-independent immune responses (20). However, marginal zone macrophages probably play a lesser role, because their de-
pletion has been shown to increase humoral responses to TI-2 Ag (9).

In summary, our studies show that MZB cells directly associate with live bacteria and are activated in vivo during the acute stage of infection with a blood-borne pathogen to produce pathogen-specific Ab. The lack of CD1d expression does not interfere with the ability of MZB cells to interact with spirochetes, but does result in prolonged activation of these cells. Despite apparent activation,
MZB cell production of B. hermsii-specific Ab was compromised in CD1d−/− mice, implicating CD1d expression in the production of pathogen-specific IgM at the earliest stages of infection. It is possible that impaired MZB cell production of protective Ab accounts for our previous observation that B. burgdorferi disseminate more quickly after intradermal inoculation into CD1d−/− mice compared with controls. Studies are in progress to further define the role of CD1d in Ab production by MZB cells.

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Disclosures
The authors have no financial conflict of interest.

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