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Impaired Apoptotic Cell Clearance in the Germinal Center by Mer-Deficient Tingible Body Macrophages Leads to Enhanced Antibody-Forming Cell and Germinal Center Responses

Ziaur S. M. Rahman,* Wen-Hai Shao,† Tahsin N. Khan,* Yuxuan Zhen,‡ and Philip L. Cohen†

Germinal centers (GCs) are specialized microenvironments that generate high-affinity Ab-forming cells (AFCs) and memory B cells. Many B cells undergo apoptosis during B cell clonal selection in GCs. Although the factors that regulate the AFC and GC responses are not precisely understood, it is widely believed that dysregulated AFCs and GCs contribute to autoimmunity. The Mer receptor tyrosine kinase (Mer) facilitates macrophage clearance of apoptotic cells. The Tyro-3, Axl, and Mer receptors, including Mer, suppress TLRs and cytokine-mediated inflammatory responses. We report in this study that tingible body macrophages (TBMs) in GCs express Mer. Compared to C57BL/6 (B6) controls, Mer-deficient (Mer<sup>-/-</sup>) mice had significantly higher AFC, GC, and Th1-skewed IgG2 Ab (especially IgG2c) responses against the T cell-dependent Ag (4-hydroxy-3-nitrophenyl) acetyl-chicken γ globulin. Mer<sup>-/-</sup> mice had a significantly higher percentage of GC B cells on days 9, 14, and 21 postimmunization compared with B6 controls. Significantly increased numbers of apoptotic cells accumulated in Mer<sup>-/-</sup> GCs than in B6 GCs, whereas the number of TBMs remained similar in both strains. Our data are the first, to our knowledge, to demonstrate a critical role for Mer in GC apoptotic cell clearance by TBMs and have interesting implications for Mer in the regulation of B cell tolerance operative in the AFC and GC pathways.


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Abbreviations used in this paper: AFC, Ab-forming cell; AP, alkaline phosphatase; CGG, chicken γ globulin; DC, dendritic cell; FDC, follicular dendritic cell; FO, follicular; Gas6, growth arrest-specific 6; GC, germinal center; Mφ, macrophage; Mer, Mer receptor tyrosine kinase; Mer<sup>-/-</sup>, Mer-deficient; MFG-E8, milk fat globule epidermal growth factor 8; MZ, marginal zone; NP, (4-hydroxy-3-nitrophenyl) acetyl-tyr; PNA, peanut agglutinin; PR, red pulp; SA, streptavidin; SLE, systemic lupus erythematosus; T, transitional type; TAM, Tyro-3, Axl, and Mer; TBM<sub>s</sub>, tingible body macrophage; TD, T cell-dependent; WF, white pulp; WT, wild-type.

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Mer receptor tyrosine kinase (Mer) belongs to the TAM subfamily of TAM receptors (33). The receptors in this subfamily have important immunoregulatory roles. Whereas TAM double (Tyro3/−/−, Axl/−/−, Mer−/−, and Tyro3/−/−Mer−/−) and triple (Tyro3/−/−Axl/−/−Mer−/−) mutants suffer from more severe disease than the single mutants (35, 36), mice lacking Mer alone (Mer−/−) develop lupus-like autoimmunity (26). In addition, expression of Mer on phagocytes (i.e., Mψs and DCs) has been described to facilitate the engulfment of apoptotic cells (34, 37, 38).

We recently observed an enhanced marginal zone (MZ) B cell response to type II T-independent Ag in Mer−/− mice (W.-H. Shao, unpublished observations). DNA-specific Ig H chain transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred onto H-2b knock-in mice (W.-H. Shao, unpublished observations).

Furthermore, strains of mice that typically develop autoimmune SLE-like illness (NZB/W F1 and MRL/Ipr) and autoimmune diabetes (NOD mice) exhibit spontaneous GC formation in the spleen by 1 to 2 mo of age in the absence of immunization or infection (39). Although Mer function has been implicated in maintaining immune tolerance, including T cells (21), the immunoregulatory role of Mer in peripheral B cell tolerance is not well defined. To understand the role of Mer in regulation of B cell responses to exogenous Ag, we performed an in-depth analysis of the immune response of Mer−/− mice to the TD Ag (4-hydroxy-3-nitrophenyl) acetyl (NP)-chicken γ globulin (CGG). We observed enhanced primary AFC, GC, and Ab responses in Mer−/− mice compared with B6 controls. We also found that the expression of Mer on TBMds was critical for the clearance of apoptotic cells in GCs. These data indicate a vital role of Mer in clearing apoptotic cells in GCs by TBMds, which may, in turn, maintain peripheral AFC and GC tolerance.

**Materials and Methods**

**Mice**

Mer−/− mice were originally generated on a F1 hybrid background of C57BL/6 (B6) and 129 mice (40). Subsequently, these were backcrossed onto the B6 background for 10 generations. B6 wild-type (WT) control mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and then bred in house. All mice were 7–9 wk old when used in experiments. All experimental procedures performed on these animals were conducted according to the guidelines of our Institutional Animal Care and Use Committee.

**Abs and other reagents**

Abs and other reagents used for flow cytometry and immunohistochemistry included: FITC-GL7, Alexa Fluor 647-anti-C3d, streptavidin-CyChrome, PE-anti-CD23 (B1B4), purified follicular DC (FDC)-M1, and FITC-anti-FcγRII/III (2.4G2, Applied Biosystems, Foster City, CA/BD Pharmingen, San Diego, CA); FITC-MOMA-1, red PE, and Alexa Fluor 647-anti-CD68 (AbD Serotec, Raleigh, NC); PE, FITC, and biotin-anti-B220 (RA3-6B2), PE-anti-CD3, FITC- and PE-anti-CD21/35 (4E3), and biotin-anti-IgD (11–26) (ebioscience, San Diego, CA); streptavidin (SA)-PE, SA-Alexa Fluor 633, SA-Alexa Fluor 488, biotin-anti-mouse κ, alcaline phosphatase (AP)-anti-mouse IgG, and biotin-anti-mouse IgGl ( Molecular Probes, Eugene, OR); FITC- and HRP-peroxidase (PNA; Sigma-Aldrich, St. Louis, MO); biotin-anti-mouse IgM, biotin-mouse anti-rat, and FITC-donkey anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA); AP-anti-mouse IgG2c and biotin-anti-mouse IgG2b (affinity-purified goat polyclonal) were from Southern Biotechnology Associates (Birmingham, AL), and biotin-anti-mouse Mer was from R&D Systems (Minneapolis, MN).

**Immunization procedure**

TD Ag NP6-CGG (Biosearch Technologies, Novato, CA) was precipitated with 10% alum and injected i.p. into Mer−/− and WT mice (100 µg in 200 µl 1× PBS per mouse). On days 9, 14, and 21 postimmunization, mouse spleens were removed, frozen in OCT medium, and stored at −80˚C. Serum samples were collected from these mice on the day (day 9, 14, and 21) animals were sacrificed.

**Immunohistology and TUNEL assay**

Spleen cryostat sections (5–6 µm) were prepared as described (41). Immunohistology was performed using the Abs listed above. The TUNEL assay was performed on the spleen sections using a TUNEL apoptosis detection kit (Millipore, Temecula, CA) following the manufacturer’s instructions. The stained sections were analyzed using a fluorescence microscope (Leica Microsystems, Deerfield, IL), and images were captured as described (42). The color intensity of the image was slightly enhanced by Photoshop (Adobe Systems, San Jose, CA). This manipulation was necessary for better visualization, which was carried out consistently between Mer−/− and B6 controls while maintaining the integrity of the data. The magnification of the image was ×100, ×200, or ×320 as indicated in the figure legends. TUNEL+ and CD68+ cells were counted by two individuals in randomly picked GCs from four to five B6 and Mer−/− mice.

**Flow cytometry**

Three- and four-color flow cytometric analysis was done on cell suspensions prepared from spleens of naive and immunized mice stained with multiple combinations of the Abs listed above. Biotinylated Abs were detected with streptavidin-CyChrome. Stained cells were analyzed using a Coulter Epics XL/MCL analyzer (Beckman Coulter, Fullerton, CA). Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

**ELISPOT assays**

Splenocyte suspensions from NP-CGG immunized B6 and Mer−/− mice were plated at 1×10^6 cells/well and diluted serially (1:2) in NP-μ-BSA coated multiscreen 96-well filtration plates (Millipore, Bedford, MA) for 6 h at 37˚C. NP-specific IgM Abs produced by AFCs were detected using biotinylated anti-mouse IgM (Jackson ImmunoResearch Laboratories) and SA-AP (Vector Laboratories, Burlingame, CA). NP-specific IgG Abs produced by AFCs were detected using AP-conjugated IgG (Molecular Probes). Plates were developed using the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). ELISPOTs were counted using a computerized imaging video system (Cellular Technology, Cleveland, OH).

**ELISA**

NP-specific serum Abs were measured in sera from immunized mice by solid-phase ELISA on 96-well plates (Immulon-4; Thermo Electron, Thermo Fisher Scientific, Waltham, MA) as previously described (41). To measure the total serum Ab titers of different isotypes and subtypes (such as IgM, IgG1, IgG2c, and -2b), ELISA plates were coated with NP11-BSA. Biotinylated Abs were detected by SA-AP (Vector Laboratories). The plates were developed by the p-nitrophenyl phosphate, disodium salt substrates for AP. Serum samples were first diluted (1/100) in PBS and then subsequently 3-fold serial dilution was carried out for each sample. The dilution factor for each sample was generated in a logarithmic scale via the software named “Origin” based on the different OD values of 0.8, 0.9, or 1.0 (at 405 nm) set for different isotype-specific ELISA. The OD values of 0.8, 0.9, or 1.0 were determined based on the linear distribution of most of the samples in any given ELISA. In this way, a dilution factor of 0.1 would indicate that this particular serum sample needed to be diluted 400 times to obtain an OD value of 1.0 at 405 nm. In contrast, a serum sample with a dilution factor of 150 needed to be diluted 150 times to obtain the same OD value. Therefore, the higher the dilution factor in an individual mouse the higher the Ab titers for that particular mutant.

**Statistical analysis**

Statistical analysis was done using Student t test, p values <0.05 were considered significant.

**Results**

Primary development of B cells is not overtly affected in Mer−/− mice

To study the role of Mer in regulating AFC, GC, and Ab responses, first we evaluated whether primary development of B cells was altered in the absence of Mer using Mer−/− mice (40). Mer−/− mice
mice used in the current studies were backcrossed for 10 generations onto the C57BL/6J (B6) background. By performing flow cytometric analysis on splenocytes obtained from age (8–10 wk old) and sex-matched naïve mice, we found that the total number and percentage of B220+ cells in Mer-/- mice were comparable to that observed in B6 controls (Fig. 1A). Staining with the anti-CD93 mAb specific for the C1qRp molecule expressed on the immature and transitional stages of follicular (FO) B cell development, we analyzed mature (B220+ CD93neg) and immature (B220+ CD93+) B cell populations (Fig. 1B, top panel) in the spleen. The ratios of B cells expressing and lacking this marker were comparable in both strains (data not shown).

Further subdivision of the CD93+ population using peripheral B cell lineage markers CD23 and surface IgM revealed a ~2-fold increase in the percentage of CD93negCD23neg IgMlow marginal zone (MZ) B cells and, on average, a 20% decrease in the percentage of CD93negCD23+ IgMlow mature FO B cells in Mer-/- mice (Fig. 1B, bottom left panel) compared with B6 controls (Fig. 1B, middle left panel). Our finding of this increased percentage of MZ B cells in Mer-/- mice is consistent with our recent observation (W.-H. Shao, unpublished observations). Similar analysis of the immature (B220+ CD93+) transitional B cell population revealed no differences in the percentage of CD93negCD23+ IgMhigh transitional type (T1), CD93-CD23+IgMhigh T2, and CD93+CD23+IgMlow T3 subsets (Fig. 1B, middle and bottom right two panels) between B6 and Mer-/- mice.

Additionally, we performed immunohistological analysis of spleen sections obtained from naïve mice. By staining with B220 (green) and anti-CD3 (red), we observed no significant differences in the splenic architecture, organization of B (green) and T (red) cell areas in Mer-/- mice (Fig. 1C, right four panels) compared with that observed in B6 controls (Fig. 1C, left four panels). As shown in Fig. 1D, next we performed a similar analysis in which we stained with B220 (red) and MOMA-1 (green). MOMA-1 stains for metallophilic macrophages located at the border of follicles and the MZ. Consistent with the flow cytometry data, we observed an increase in the MZ B cell population outside the MOMA-1 border in Mer-/- mice (Fig. 1D, right two panels) compared with B6 controls (Fig. 1D, left two panels). The surface levels of the activation/costimulatory markers CD69, CD80, and CD86 on B cells from 8–10-wk-old Mer-/- naïve mice were comparable to age-matched B6 controls (data not shown). These data indicate that Mer deficiency does not overtly alter the development and maturation of B cells.

Enhanced primary (short-lived) AFC responses of Mer-/- mice

To study whether the absence of Mer led to an enhanced primary AFC response, we immunized Mer-/- mice with the TD Ag NP-CGG in alum. We used B6 mice as controls. Spleens were harvested, and anti-NP IgM- and IgG-producing AFCs were measured by ELISPOT assay on days 9, 14, and 21 postimmunization. Whereas IgM-producing AFCs remained similar between B6 and Mer-/- mice for all three time points (Fig. 2A), we found significantly higher NP-specific IgG-producing AFCs on day 14 and 21 postimmunization in Mer-/- mice compared with B6 controls (Fig. 2B). A representative image from day 14 of the primary response showing significantly higher number of IgG-producing AFCs in Mer-/- mice compared with B6 controls is shown in Fig. 2C.

**FIGURE 1.** Primary development of B cells in the presence or absence of Mer. A and B, Quantitative and four-color flow cytometric analysis was performed on spleen cells obtained from B6 and Mer-/- mice poststaining with B220, anti-CD93, anti-IgM, and anti-CD3. A, Total number of B cells (left panel) and the percentage of B220+ cells (right panel) are shown in C57BL/6 (open bar) and Mer-/- (shaded bar) sex matched 8–10-wk-old mice. B, The percentage of mature (B220+CD93neg) and immature (B220+CD93+g) B cells are shown in the top panel. Subdivision of mature and immature B cells into MZ B, FO B, and T1, T2, and T3 populations are shown by oval and rectangular gates, respectively, in the lower panels. The percentage of each B cell population in the spleens of B6 (middle panels) and Mer-/- (bottom panels) mice is indicated next to each gate. C, Spleen sections obtained from naïve mice of the indicated genotypes were stained with B220 (green) and anti-CD3 (red) and images captured by fluorescence microscopy. Original magnification of images was ×100 (upper panels) and ×200 (lower panels). D, Similar analysis described in C was done in which spleen sections were stained with B220 (red) and MOMA-1 (green). Original magnification of images was ×320. All data are representative of at least three mice per group.
FIGURE 2. Primary IgM and IgG AFC responses in the presence or absence of Mer. The number of splenic NP-specific IgM- 
( ) and IgG-secreting 
( ) AFCs were measured by ELISPOT assay. Each circle represents the number of AFCs per 1 × 10⁶ splenocytes obtained from an individual mouse. Open and closed circles represent data from B6 and Mer⁻/⁻ mice, respectively. Horizontal bars represent the average number of AFCs. Statistical analysis was performed by Student t test. A, A representative image of an ELISPOT assay obtained on day 14 of the anti-NP response in six B6 (left panel) and six Mer⁻/⁻ (right panel) mice is shown. As described in Materials and Methods, one million (1 × 10⁶) splenocytes were plated in the first row and subsequently 2-fold serial dilution was carried out for each sample (indicated in the left side of the image as 1, 1/2, and 1/4). These data were obtained from five to six age- and sex-matched mice of each genotype.

The anti-NP GC response is augmented in Mer⁻/⁻ mice

To study the influence of Mer deficiency on the GC response, Mer⁻/⁻ and B6 control mice were immunized with NP-CGG in alum. Flow cytometry analysis of splenocytes obtained on days 9 and 14 postimmunization revealed a significant increase in the percentage of B220⁺PNA⁺ GC B cells in Mer⁻/⁻ mice compared with B6 controls (Fig. 3A). Analogous results were obtained when we used a different GC B cell marker GL7 (data not shown). We next performed semiquantitative analysis of the number of splenic GCs in Mer⁻/⁻ and B6 control mice on days 9 and 14 by immunostaining spleen sections with anti-B220 and the GC B cell marker PNA. A representative image of an immunostained section is shown in Fig. 3B. In both panels, open circles and closed circles represent data from B6 and Mer⁻/⁻ mice, respectively. In B6 mice, GCs are predominantly localized in the white pulp areas of spleen, whereas in Mer⁻/⁻ mice, GCs are also found in both the red and white pulp areas of spleen.

Enhanced GC responses of B cells deficient in Mer

To evaluate whether the augmented anti-NP AFC and GC responses in Mer⁻/⁻ mice led to an increased titer of serum Abs, we examined the kinetics of the anti-NP Ab responses by measuring the levels of NP-specific Abs in sera obtained from B6 and Mer⁻/⁻ mice on days 9, 14, and 21 postimmunization with TD-Ag NP-CGG. The TD anti-NP response predominantly generates Abs of IgG1 isotype in B6 mice (43). Although we did not observe any significant difference in anti-NP total IgM, IgG, and IgG1 anti-NP Ab levels, we were surprised to find significantly higher titers of IgG2, especially IgG2c in Mer⁻/⁻ mice (44). To explain this unexpected observation, we next examined whether Mer expression on spleen cells that might regulate AFC, GC, and Ab responses was altered in Mer⁻/⁻ mice. We performed a detailed additional experiment in which the GC responses were analyzed on days 9, 14, and 21 postimmunization. Flow cytometry analysis of the GC response on these three time points showed a significantly higher number of B220⁺PNA⁺ GC B cells in Mer⁻/⁻ mice than that observed in B6 controls (Fig. 3C).

Elevated Th1-skewed IgG2 Ab responses in Mer⁻/⁻ mice

To evaluate whether the augmented anti-NP AFC and GC responses in Mer⁻/⁻ mice led to an increased titer of serum Abs, we examined the kinetics of the anti-NP Ab responses by measuring the levels of NP-specific Abs in sera obtained from B6 and Mer⁻/⁻ mice on days 9, 14, and 21 postimmunization with TD-Ag NP-CGG. The TD anti-NP response predominantly generates Abs of IgG1 isotype in B6 mice (43). Although we did not observe any significant difference in anti-NP total IgM, IgG, and IgG1 anti-NP Ab levels, we were surprised to find significantly higher titers of IgG2, especially IgG2c in Mer⁻/⁻ mice (44) compared with B6 controls ( ) on days 9 (Fig. 4, top panels), 14 (Fig. 4, middle panels), and 21 (Fig. 4, bottom panels).

Mer-expressing cells are localized in both the red and white pulp areas of spleen

Having observed enhanced AFC, GC, and Ab responses in Mer⁻/⁻ mice, we next examined Mer expression on spleen cells that might regulate AFC, GC, and Ab responses. We performed a detailed additional experiment in which the GC responses were analyzed on days 9, 14, and 21 postimmunization. Flow cytometry analysis of the GC response on these three time points showed a significantly higher number of B220⁺PNA⁺ GC B cells in Mer⁻/⁻ mice than that observed in B6 controls (Fig. 3C).
analysis of the anatomical location of cells expressing Mer in the spleen by performing three color immunofluorescence staining of spleen sections obtained from naive B6 mice (Fig. 5, top row). By staining with MOMA-1 (green) and mAbs specific for Mer (red) and CD3 (blue), we showed that cells expressing high levels of Mer were localized in the red pulp (RP) areas of the spleen outside of MOMA-1 border (Fig. 5, top row, second and fourth column). Compared to this population, cells expressing relatively low levels of Mer were localized in the white pulp (WP) area, predominantly within the T cell zone (Fig. 5, overlay, top row), whereas Mer expression was completely abolished in these two areas of spleen in Mer<sup>−/−</sup> mice (Fig. 5, bottom row). Consistent with previously published data by us (34) and others (35), T and B cells did not appear to express Mer as evidenced by the absence of overlap staining of Mer and CD3 in the T cell zone and the absence of Mer staining on B cells within the follicles inside the MOMA-1 border (Fig. 5, top row).

Mer-expressing cells within GCs are not FCDs, and Mer deficiency does not alter the development of the FDC network in GCs

Next, we evaluated whether GCs contained any Mer-expressing cells, which may regulate GC responses. Spleen sections, obtained from B6 and Mer<sup>−/−</sup> mice on day 14 postimmunization, were stained with the GC B cell marker PNA (green), anti-Mer (red), and anti-CD3 (blue). Although most of the Mer<sup>+</sup> cells in the WP area were localized within the T cell zone, we also observed Mer<sup>+</sup> cells within B6 GCs as evidenced by the red staining within the GC defined by the green area (Fig. 6A, top row, second panel from left). These Mer<sup>+</sup> cells in GCs did not colocalize with CD3<sup>+</sup> T cells within GC and PNA<sup>+</sup> GC B cells (Fig. 6A, top row, fourth and fifth panels from left).

FDCs are the major stromal elements within GCs that are thought to regulate the GC response. By immunohistochemical analysis of spleen sections from immunized mice, we previously showed that only FDCs within GCs (referred to as secondary FDCs) expressed high levels of CD21/35 (complement receptor 1/2), inhibitory FcRIIB, and FDC-specific marker FDC-M1 (42). We used these markers in this study to detect CD21/35<sup>high</sup> FDC-M1<sup>+</sup> secondary FDCs, allowing us to study whether FDCs in GCs expressed Mer. We stained two parallel sections, obtained from B6 and Mer<sup>−/−</sup> mice on day 14 postimmunization, one with anti-CD21/35 (CR2/CR1, green) and anti-Mer (red) and the other with anti-CD21/35 (green) and FDC-M1 (4C11, red). In this way, we showed that although higher intensity CD21/35 staining overlapped with FDC-M1 (Fig. 6B, top rightmost panel), CD21/35<sup>high</sup> FDC-M1<sup>+</sup> secondary FDCs did not express Mer because Mer staining was only restricted to individual cells near or within the FDC network (Fig. 6B, top left two panels). We performed similar experiments as in Fig. 6B in which we replaced CD21/35 staining with 2.4 G2 (FcγRII/III), which is also highly expressed.
FIGURE 6. Mer-expressing cells within GCs do not colocalize with FDCs. A. Immunohistological analysis of spleen sections, obtained from B6 (top row) and Mer−/− mice (bottom row) on day 14 after NP-CGG immunization, was performed using PNA (green), anti-Mer (red), and anti-CD3 (blue). In the first column, PNA+ (green) GC area is shown in dotted red line. In the second column, the localization of Mer-expressing cells within the GC marked by the dotted green line is shown. In the third column, the presence of FO Th cells within the GC is also shown in a similar green area. In the fourth column, two-color overlay image of anti-Mer (red) and anti-CD3 (blue) staining is shown. In the fifth column, the distribution of Mer-expressing cells in GCs, T cell zone, and RP areas is shown in the three-color overlay image. Arrow indicates the accumulation of Mer+ cells at the border between GC and T cell zone. B. Similar immunohistology analysis as in A was performed in which two adjacent spleen sections were analyzed. One section was costained with anti-CD21/35 (green) and anti-Mer (red), and the other was stained with anti-CD21/35 (green) and FDC marker FDC-M1 (red). C. Similar analysis as in B was performed in which anti-CD21/35 (green) staining was replaced by 2.4G2 (FcyRII/III). Original magnification of images was ×200. These data were obtained from four to five mice of each genotype.

Mer-expressing cells in GCs are TBMΦs

By staining spleen sections from NP-CGG–immunized B6 mice with Abs against a number of myeloid cell markers, such as CD11b, CD11c, F4/80, Gr-1, and CD68, we found that CD11c+ DCs and F4/80+ and CD68+ macrophages expressed Mer (Table I). Of these three myeloid cell populations expressing Mer, only CD11c+ DCs and CD68+ TBMΦs were localized in both the WP and RP areas of the spleen (Fig. 7, Table I). F4/80+ macrophages were only localized in the RP areas of the spleen (Table I). TBMΦs are considered to play an important role in regulating GC reaction and tolerance by clearing apoptotic cells (17). We next examined whether Mer-expressing cells within GCs were TBMΦs.

As shown in Fig. 7A, we stained spleen sections from B6 and Mer−/− mice 14 d after NP-CGG immunization with PNA (green), anti-Mer (red), and anti-CD68 (blue). We observed that the majority of CD68+ TBMΦs in both the red and WP areas expressed Mer as evidenced by the purple overlap staining of anti-Mer (red) and anti-CD68 (blue) in these areas (Fig. 7A, top row, second panel from right). Interestingly, we also found CD68+Mer+ TBMΦs within B6 GC as judged by the purple overlap staining within the GC area defined by the green dotted line (Fig. 7A, top row, second panel from right). Similar analysis on Mer−/− mice showed the presence of CD68+ cells in Mer−/− GCs without the expression of Mer, indicating that the expression of Mer on TBMΦs does not dictate the migration or retention of these cells within GCs.

We performed similar experiments in Fig. 7B as shown in Fig. 7A in which we investigated the localization of CD11c+ DCs in the spleen that expressed Mer. Similar to that shown in Fig. 7A, we observed Mer+ cells in GC area defined by the green dotted line (Fig. 7B, top row, second panel from left). In contrast to CD68+ cells, a very few CD11c+ cells were observed either in B6 or Mer−/− GCs (Fig. 7B, middle panels). In addition, the few Mer+ cells seen in B6 GC did not colocalize with CD11c (Fig. 7B, top row, fourth panel from the left). Overall, a very small percentage of CD11c+ cells in B6 spleen expressed Mer, consistent with our recently published data (34). Together, these data indicate that CD68+ TBMΦs are the major phagocytes located in the GCs that express Mer.

<table>
<thead>
<tr>
<th>Cell Typesa</th>
<th>Mer Expression in the WPb</th>
<th>Mer Expression in the RPc</th>
<th>Mer Expression in the GCd</th>
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<tr>
<td>CD11b+ leukocytes</td>
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<td>+</td>
<td>+</td>
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<tr>
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<td>CD3+ T cells</td>
<td>−</td>
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*aMer expression on different cell types in the spleen tissue of immunized mice was determined by immunohistology in which spleen sections obtained on day 14 postimmunization were costained with anti-Mer and different cell-type specific markers.

bLocalization of Mer-expressing cell types in the WP area of the spleen.

cLocalization of Mer-expressing cell types in the RP area of the spleen.

dLocalization of Mer-expressing cell types within the germinal centers formed in the WP area.

+ and − symbols indicate the presence and absence of a particular cell type expressing Mer.

Significantly increased number of apoptotic cells seen in the FDC areas of Mer−/− GCs

GCs are a microenvironment in which many B cells, including autoreactive ones, undergo negative selection via apoptosis (9, 10).
As Mer expression on macrophages has been postulated to maintain tolerance by facilitating clearance of apoptotic cells, we next examined whether the enhanced GC and Ab responses in Mer−/− mice was associated with inefficient clearance of apoptotic cells generated in GCs by Mer−/− CD68+ TBM. By staining the spleen sections of NP-CCG–immunized B6 and Mer−/− mice with PNA (green), anti-CD68 (red), and anti-TUNEL (blue), we showed that in the presence of Mer, CD68+ cells in B6 GCs could engulf and clear the apoptotic cells efficiently as very few apoptotic cells were observed in GCs (Fig. 8A, top two rows). In addition, most of the apoptotic cells seen in B6 GCs (Fig. 8A, top two rows, middle panel) were already engulfed by the TBM as evidenced by the absence of apoptotic cells outside of CD68+ cells in B6 GCs (Fig. 8A, top two rows, second panel from right). Conversely, compared with B6 GCs, a significantly increased number of apoptotic cells accumulated in Mer−/− GCs (Fig. 8A, bottom row, middle panel). Most of these apoptotic cells were seen outside of CD68+ TBM in Mer−/− GCs (Fig. 8A, bottom row, second panel from right).

By immunohistological analysis of spleen sections from NP-CCG–immunized B6 and Mer−/− mice with PNA (green), anti-CD21/35 (red), and TUNEL (blue), we further showed that the TUNEL+ apoptotic cells in Mer−/− mice were localized in the FDC area (CD21/35high) of GCs (Fig. 8B, bottom row, third and fourth panels from left), the presumed site of negative selection of GC B cells with low affinity for Ag on FDCs or B cells with autoreactivity. On the contrary, the TUNEL+ cells in B6 GCs were spread throughout the GC (Fig. 8B, top row, third and fourth panels from left), apparently due to the movement of TBM in GCs after they engulfed the apoptotic cells. Next, we performed semiquantitative analysis in which we counted the number of TUNEL+ cells in randomly picked small and medium GCs from 4 to 5 B6 and Mer−/− mice. Because we did not observe large GCs in B6 mice, in this analysis, we excluded the large GCs observed in Mer−/− mice. The number of TUNEL+ cells in Mer−/− GCs (Fig. 8C, red circles) was significantly higher than those in B6 GCs (Fig. 8C, blue circles). These differences were much higher when we counted the number of TUNEL+ cells that were outside of CD68+ TBM (data not shown), as very few uninested TUNEL+ cells were seen in B6 GCs. The increased number of TUNEL+ cells in Mer−/− mice was due to the reduced number of CD68+ TBM in Mer−/− GCs, as equal frequency of CD68+ cells was observed in both B6 and Mer−/− GCs (Fig. 8D).

Discussion
The GC reaction involves a complex mechanism of cellular proliferation, apoptosis, and selection in response to foreign Ags. To...
ensure selection of the quality-controlled B cell clone for Ab-mediated immunity, multiple molecular signals and cellular actions synchronize in the GC. We previously showed an increased MZ B cell population and spontaneous autoreactive MZ B cell generation from Mer−/− mice on an anti-dsDNA knockout background (W.-H. Shao, submitted for publication). Although specific mechanisms by which MZ B cells might contribute to augmented GC response observed in Mer−/− mice are not clear, these cells have been described to bridge the innate and adaptive immune response (44, 45). In this report, we investigated the role of Mer in regulating AFC, GC, and Ab responses by clearing apoptotic cells in GCs induced by the TD Ag NP-CGG. Our data showed an overall hyperactivity in the AFC and GC B cell responses in the absence of Mer that led to elevated anti-NP Ab responses from Mer−/− mice compared with WT controls.

The development and homeostasis of the immune system is maintained through elimination of mature immune cells that undergo apoptosis or cell death. Mer plays a pivotal role in apoptotic cell clearance by macrophage and DC engulfment and is thought to mediate immune tolerance (21, 35, 46, 47). Mer-dependent immunoregulation is mediated through inhibition of both TLR and TLR-induced inflammatory cytokine pathways (48). Recently, pretreatment of DCs with apoptotic cells in vitro was shown to induce Mer-mediated inhibition of TLR-stimulated PI3K/AKT and NF-κB activation (20). B cell clonal selection in GCs also results in an accumulation of apoptotic B cells. Rapid clearance of these apoptotic cells may be important to maintain peripheral tolerance, as delayed clearance of such cells can trigger inflammation and autoimmune responses against intracellular materials (11, 29, 38, 49–52). TBMs are the primary phagocytes in GCs, which are thought to regulate GC response (17). The Nagata group (18) has recently described a critical role of MFG-E8–mediated engulfment of apoptotic cells by TBMs in GCs. Inadequate clearance of apoptotic cells in MFG-E8−deficient (MFG-E8−/−) mice was shown to be associated with the development of autoimmune disease (18). Using the same mouse model, Kranich et al. (19) showed that FDCs in GCs determine the engulfment of apoptotic cells by secreting MFG-E8. They proposed a model for integrin-mediated phagocytosis of apoptotic bodies by TBMs. This group further showed that MFG-E8−/− mice were deficient in the development of FDCs, resulting in defective apoptotic cell clearance. Whereas a deficiency in FDCs might play a vital role in breaking peripheral B cell tolerance, leading to the development of lupus-like disease in MFG-E8−/− mice, we showed an intact FDC compartment in Mer−/− mice.

The Lemke group (35) previously reported the expression of Mer mRNA in lymphoid tissues, including GCs. However, the Mer-expressing cell type in GCs was not defined. We previously reported the expression of Mer on TBMs in the spleen (34). We also reported expression of Mer on platelets and on a sizeable fraction of those macrophages with characteristics of splenic MZ macrophages. It is likely that the MZ macrophages, as they are exposed to circulating apoptotic debris entering the spleen via the splenic artery and its branches, are equipped to deal with apoptotic debris from distant sources and that Mer serves as an important receptor to aid them in binding to and ingesting apoptotic cells and fragments. The role of Mer in platelets remains controversial, but does not exclude a function of platelets in binding to cellular debris and subsequently adhering to mononuclear phagocytes.

In the current studies, consistent with our prior macrophage phenotype inferences, we show directly that Mer-expressing TBMs are localized in the RP area, T cell zone, and within GCs of B6 WT controls. Compared to B6 controls, we observed larger GCs and an accumulation of apoptotic cells outside of TBMs in the splenic GCs of Mer−/− mice 14 d after NP-CGG immunization. Our data indicate a GC defect in apoptotic cell clearance by TBMs in the absence of Mer. This apoptotic debris may differ from that ingested by MZ macrophages and may include locally generated apoptotic cells, including B cells. Although it is not clear which ligand(s) the TBMs might use in the engulfment and clearance of apoptotic cells in GCs, based on our current data, this process appears to be primarily dependent on Mer, as Mer−/− mice have an intact MFG-E8–integrin pathway. In contrast, MFG-E8−/− mice also had a defect in apoptotic cell clearance in the presence of Mer, indicating the importance of both Mer- and MFG-E8–mediated removal of apoptotic cells in GCs. The relative contributions and the mechanisms of these two pathways in clearance of apoptotic cells and the maintenance of immune tolerance can only be deduced by comparative studies of Mer−/− versus MFG-E8−/− GCs.

Multiple studies highlighted the importance of cytokines in modulating GC responses. Cytokines can affect many aspects of GC B cell development. For instance, IL-4 induces isotype switch, IL-6 drives B cell differentiation into Ab-secreting cells, Bcl-2 and BAFF promote B cell survival, whereas TNF stimulates B cell apoptosis (53). Without Mer, M6 produce a greater amount of TNF-α in the presence of apoptotic cells or TLR stimuli (40). Mer-mediated M6 engulfment of apoptotic cells is accompanied by active production of anti-inflammatory cytokines, such as TGF-β and IL-10, and by downmodulation of proinflammatory cytokines, such as TNF-α and IL-12 (47, 54). The antiapoptotic effect of Gas6 (a ligand for Mer) involves survival pathways including PI3K/AKT and NF-κB stimulation (55). In vitro data from the Matsushima group (56) showed that apoptotic cells and LPS-stimulated bone marrow–derived DCs from Mer−/− mice contained an elevated number of BAFF-secreting cells. Although Mer bearing TBMs has the potential to affect GC B cell development through several mechanisms, elucidation of the precise pathway will require further studies.

The most striking observation in our current studies is the accumulation of unengested apoptotic cells in the FDC area of Mer−/− GCs, apparently due to an inadequate clearance of these cells by TBMs in the absence of Mer. Similar features were described recently in studies analyzing lymph nodes of human patients with SLE (23). Although we did not explore the possibility for apoptotic cells to be bound on the surface of FDCs, the Herrmann group (23) found TUNEL+ apoptotic materials from patients with SLE to be associated with the surface of FDCs. FDCs are the major stromal cell type in GCs that can trap Ags on their surface in the form of immune complexes via complement receptors (57–59) and FcRs (60–62). It is not clear whether TUNEL+ cells in Mer−/− GCs are captured on the FDCs or simply localized there after B cells undergo negative selection at that site. However, the localization of TUNEL+ cells in the FDC area of Mer−/− GCs may have implications in breaking peripheral AFC and GC tolerance. Unprocessed apoptotic cells in GCs may release intracellular or nuclear materials at the late stage of apoptosis or during secondary necrosis that can potentially serve as immunogens to stimulate autoreactive B cells. This, in turn, may lead to the development of autoimmunity (11, 28). Impaired phagocytosis of apoptotic cells by peritoneal macrophages from autoimmune-prone mouse has been described in vitro (24, 25). Delayed apoptotic cell clearance in Mer−/− mice was shown to be associated with lupus-like autoimmunity (26). Lupus-prone (MRL.lpr) as well as normal mice injected with irradiated apoptotic cells also develop autoantibodies (63). Whether impaired apoptotic cell clearance in spontaneously formed GCs in lupus mice and human patients with SLE is associated with the defect in...
Mer or other Tyro3 family receptor (such as Axl and Tyro3) expression or function is not clear, and this area is under active investigation.

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Disclosures

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References


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