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Prolonged Apoptotic Cell Accumulation in Germinal Centers of Mer-Deficient Mice Causes Elevated B Cell and CD4+ Th Cell Responses Leading to Autoantibody Production

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Efficient clearance of apoptotic cells (ACs) generated systemically in numerous ways appears to be an essential step for the immune system to maintain tissue homeostasis (1). Clearance of ACs generated in germinal centers (GCs) as a result of negative selection of low-affinity and autoreactive B cells may also be necessary to regulate B and/or T cell responses against foreign Ag, as well as to maintain immune tolerance to self-Ag. Inefficient clearance of ACs results in the development of autoimmunities (2–5). We recently showed that tingible body macrophages (TBM) in GCs are the primary phagocytes that express Mer and are associated with the clearance of ACs generated within GCs (6).

Several studies in mice have suggested the role of TBM in regulating the GC response and in maintaining peripheral B cell tolerance (2, 6–8). The presence of ACs during monocyte activation increases their secretion of the proinflammatory cytokine IL-10 and decreases secretion of the proinflammatory cytokines TNF-α, IL-1, and IL-12 (9, 10). Likewise, the phagocytosis of ACs by TBM in GCs may potentially mediate the induction of anti-inflammatory cytokines IL-10 and TGF-β, which, in turn, may help negatively regulate B cells, including autoreactive cells that are present in GCs. In contrast, in the absence of efficient clearance, ACs in GCs may progress to the late stage of apoptotic cell death (termed secondary necrosis) and pose a threat to immune regulation and tolerance by promoting the production of proinflammatory cytokines. Whereas TBM regulate GC response and tolerance by clearing ACs generated in GCs, the phagocytosis of injected ACs by marginal zone (MZ) macrophages was previously shown to maintain MZ B cell tolerance as deletion of MZ macrophages led to proinflammatory cytokine production and accelerated autoimmune disease progression in lupus-prone mice (11, 12).

A number of receptors that mediate phagocytosis and clear ACs include TAM: Tyro-3, Axl, and Mer/Mer receptor tyrosine kinase (MerTK) subfamily of tyrosine kinase receptors, αβ integrin, T cell Ig-4 (Tim-4), and CD36 (1, 13–15). Several soluble dual-function bridging proteins, including milk fat globule epidermal growth factor (MFG-E8), growth arrest–specific 6 and protein S (16–18), their corresponding receptors expressed on phagocytes. TAM receptors primarily use growth arrest–specific 6 and protein S (16–18). Whereas Tim-4 directly binds phosphatidylserine exposed on the surface of ACs and their corresponding receptors expressed on phagocytes. TAM receptors use MFG-E8 for engulfing ACs (19).

MerTK or Mer expression on phagocytes (i.e., macrophages and dendritic cells [DCs]) facilitates macrophage and DC clearance of ACs (16, 18, 20, 21). Mer appears to be necessary for the maintenance of self-tolerance, as a lupus-like syndrome is observed in mice lacking the Mer receptor (Mer−/−) (3). The development of autoimmunity in these mice is believed to be due to the delayed clearance of ACs and dysregulated cytokine production by macrophages and DCs (22). Recent studies by the Cohen group (23)
have an enhanced MZ B cell response to type II T-independent Ag in Mer-/- mice. Loss of tolerance of DNA-specific IgH transgenic 3H9 B cells that primarily develop into MZ B cell phenotype has also been described in Mer-/- mice (23). Recently, in an analysis of the early immune response (9, 14, and 21 d postimmunization) against the T cell–dependent Ag (TD-Ag) (4-hydroxy-3-nitrophenyl) acetyl–g- globulin, we showed enhanced Ab-forming cell (AFC), GC, and IgG2 Ab responses in Mer-/- mice compared with control animals (6). We also observed an accumulation of ACs in GCs at this time point due to lack of Mer expression on TBMs (6). Whereas impaired clearance of ACs in GCs appeared to elevate AFC, GC, and IgG2 Ab responses at earlier time points (6), the role for the GC as a site of long-term accumulation of uncleared apoptotic debris that may lead to a break in peripheral B cell tolerance and production of anti-nuclear Abs (ANA) in Mer-/- mice is not clear.

In this study, we determined whether AC accumulation in GCs and elevated AFC, GC, and IgG2 Ab responses caused by Mer deficiency sustained for an extended period of time, which consequently harbored the generation of autoreactive B cells and production of high titers of ANA. We also evaluated whether enhanced responses and AC accumulation in GCs of Mer-/- mice were a result of increased B and Th cell responses, especially GC B cells and T follicular helper (Tfh) cells. We observed high titers of ANAs in Mer-/- mice compared with wild-type (WT) controls, indicating a break in B cell tolerance in the presence of AC accumulation in Mer-/- GCs. We found significantly increased activation and proliferation of GC B cells and CD4+ effector Th cell responses, including Tfh cells, that were associated with AC accumulation in Mer-/- GCs compared with WT controls. Mer deficiency also resulted in a significant increase in the generation of long-lived primary (IgG2a AFC, GC, Tfh cell, IgG, and IgG2 Ab) and memory (IgG2a AFC, IgG, and IgG2 Ab) responses compared with WT controls. Finally, we observed a significant increase in IFN-g production by CD4+ Th cells obtained from 4-hydroxy-3-nitrophenyl) acetyl-OVA (NP-OVA)–immunized Mer-/- mice compared with WT controls. These data reinforce a crucial role of Mer-mediated clearance of ACs from GCs in regulating peripheral B cell, CD4+ Th cell, and autoantibody responses, and, therefore, having major implications in loss of peripheral B cell tolerance and the induction of autoimmunity.

Materials and Methods

Mice

Mice deficient in Mer/MerTK on a F2 hybrid background of C57BL/6 (B6) and 129 mice (B6.129Mer(-/-)) (designated Mer-/-) and wild-type (WT) controls with identical background (B6.129F2J) were purchased from The Jackson Laboratory (Bar Harbor, ME). Subsequently, F3 mice of both Mer-/- and WT controls were generated by crossing F2 males and females of each genotype and maintained in a pathogen-free facility. All experimental procedures performed on these animals were conducted according to the guidelines of our Institutional Animal Care and Use Committee. All mice were 7–9 wk old at the time of immunization.

Immunization procedure

T-dependent Ag NP-OVA (Biosearch Technologies, Novato, CA) was precipitated with 10% alum. Mer-/- and WT control mice were immunized (i.p.) twice with NP-OVA/alum: on day 0 (with 100 mg per mouse) and then again on day 7 (with 50 mg). At multiple time points postimmunization, spleen and bone marrow were harvested for various analyses. For measuring Ab titers, serum samples were collected from these mice on days 0, 14, 21, 28, 45, 60, and 80 postimmunization. To study secondary responses, mice were rested for 80 d postimmunization and analyzed 4 d after boosting with 50 mg NP-OVA in PBS.

Reagents and Abs for flow cytometry

Pacific blue anti-B220 (RA3-6B2); PECy7 anti-CD95 (Fas) (Jo2); Alexa Fluor 700 anti-CD4 (RM4-5); PE anti-CD8-PE (J34); biotin anti-CXCR5 (2G8) (BD Pharmingen, San Diego, CA); PerCP Cy5.5 anti-CD69 (H1.253); PE Cy5 anti-CD86 (GL1); allophyocyanin anti-CD44 (IM7); PE Cy7 anti-CD62L (MEL-14); PE Cy5 streptavidin (SA); PE anti-CD80 (15–10A1); allophyocyanin Cy7 anti-CD25 (PC61); FITC anti–IFN-gamma (XMGl.2); allophyocyanin anti–IL-4 (11B11) (eBioscience, San Diego, CA); FITC peanut agglutinin (PNA; Sigma–Aldrich, St. Louis, MO).

Reagents and Abs for immunohistochemistry

FITC–GL7; biotin rat anti-mouse IgD (11–26c; Southern Biotech, Birmingham, AL); biotin anti–BrDU (Bu20a); purified anti-mouse Tim-4 (RMT4-54) (BioLegend, San Diego, CA); HRP PNA (Sigma–Aldrich); biotin mouse anti-rat IgG; Alexa Fluor 488 anti-Armenian hamster IgG (Jackson ImmunoResearch Laboratories, West Grove, PA); alkaline phosphatase (AP–SA); AP Blue substrate kit III; Vector NovaRED substrate kit (Vector Laboratories, Burlingame, CA); red PE and Alexa Fluor 647 anti–CD68 (AbD Serotec, Raleigh, NC); affinity-purified anti-mouse Tyro-3 and Axl (R&D Systems, Minneapolis, MN); biotin rat anti-mouse MOMA-1 (Abcam, Cambridge, MA); purified rat anti-mouse Ki67 (ImmunoKontact, Abingdon, U.K.).

Flow cytometry

Multicolor flow cytometric analysis was performed on single-cell suspensions prepared from spleens of immunized mice stained with different combinations of the Abs listed above. Biotinylated Abs were detected with SA-conjugated fluorochromes. Stained cells were analyzed using the BD LSRII analyzer. Data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Immunohistology and TUNEL assay

Preparation of spleen cryostat sections (5–6 mm) and immunohistological analysis were performed using the Abs and reagents, as previously described (24). TUNEL kit and Apoptag Peroxidase In Situ Apoptosis detection Kit (Millipore, Temecula, CA) were used to perform apoptosis detection assays on the spleen sections following the manufacturer’s instructions. The stained spleen sections using both the kits were analyzed using a fluorescent microscope (Leica Microsystems, Buffalo Grove, IL), and images were captured, as described (6). The color intensity of the images was slightly enhanced by Adobe Photoshop (Adobe Systems, San Jose, CA), which was necessary for improved visualization, and was carried out consistently between Mer-/- and WT controls, while maintaining the integrity of the data. The original magnification of the images was ×50, ×100, or ×200, as indicated in the figure legends.

In vivo B cell proliferation assay

B cell proliferation in GCs was examined using two different methods, as follows: 1) in situ BrdU proliferation assay and 2) in situ intracellular staining of B cells for Ki67. The in situ BrdU proliferation assay was performed using a kit (BD Biosciences, Franklin Lakes, NJ). Mice were immunized with NP-OVA, as described above. On day 21 post-first immunization, BrdU (1 mg/mouse) was administered i.p. 1–2 h prior to sacrificing and freezing spleens. One of two consecutive spleen sections (5–6 mm) was stained with anti-IgD and PNA. AP-conjugated IgD and HRP-labeled PNA were developed using the Blue Alkaline Phosphatase Substrate Kit III and NovaRed Substrate Kit (both from Vector Laboratories), respectively. BrdU uptake was detected on the other section following manufacturer’s instructions. BrdU+ cells in GCs were counted by two individuals with randomly picked GCs from several WT and Mer-/- mice. A two-color immunofluorescent staining with anti-IgD and anti-CD25 revealed that GCs (a proliferation marker) and GC B cell marker GL7 was performed on spleen sections obtained on day 21 post–NP-OVA immunization, as described above.

ELISPOT assays

ELISPOT assays were performed, as described (6). Briefly, splenocytes and/or bone marrow single-cell suspensions from NP-OVA–immunized WT and Mer-/- mice were plated at 1 × 10^6 cells/well in 96-well plates (Millipore, Bedford, MA) for 6 h at 37°C and 4% CO2. NP-specific IgM Abs produced by AFCs were detected using biotinylated anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) and SA-AP (Vector Laboratories). NP-specific IgG Abs produced by AFCs were detected using AP-conjugated IgG (Molecular Probes, Eugene, OR). 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, as described (25). To measure NP-specific total serum Ab titers of different isotypes and subtypes (such as IgM [Jackson ImmunoResearch Laboratories], IgG [BioLegend], IgG1 [Molecular Probes], and IgG2 [Sigma-Aldrich]), ELISA plates were coated with NP/C-BSA (10 μg/ml). To measure ANA titers, plates were coated with dsDNA (20 μg/ml), histone (10 μg/ml), or nucleosome (mixture of dsDNA and histone). Biotinylated Abs were detected by SA-AP (Vector Laboratories). The plates were developed with p-nitrophenyl phosphate, disodium salt (Thermo Fisher Scientific, Rockford, IL) substrates for AP. Serum samples were first diluted 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 (OriginLab, Northampton, MA), as described (6).

Ex vivo B and T cell proliferation assay

Naive B and T cells were purified from splenocytes of WT and Mer−/− mice 10 d after immunization with NP-OVA, by negative selection using mouse Pan T Cell Isolation Kit II and anti-CD43 (Ly-48) microbeads, respectively, using magnetic cell separation (Miltenyi Biotec). Purified cells were intracellularly stained with 3 μM CellTrace Violet (Invitrogen, Carlsbad, CA), as per manufacturer’s instructions. Stained T cells were then cultured with 10 μg/ml plate-bound anti-CD3 and 2 μg/ml soluble anti-CD28 Ab (BioLegend) in RPMI 1640 supplemented with 10% FBS, whereas stained B cells were cultured with 25 μg/ml soluble anti-IgM (Jackson ImmunoResearch Laboratories) and 5 μg/ml soluble anti-CD40 Ab (BioLegend) in a round-bottom 96-well plate for different time periods. CellTrace-stained or unstained, unstimulated plates were coated with dsDNA (20 μg/ml) and not in the T cell zone, MZ, or red pulp area of the spleen in Mer−/−GCs, we determined the expression of Axl and Tyro-3 in GCs at least up to day 80.

Cytokine production. Stained cells were analyzed using a BD LSR II (BD Biosciences) analyzer. Data were analyzed using FlowJo software (Tree Star). Proliferating cells were scored by quantifying the reduction in CellTrace Violet fluorescence intensity in dividing cells as compared with stained unactivated control cells.

Ex vivo T cell stimulation and intracellular staining for cytokines

The 7- to 9-wk-old WT and Mer−/− mice were immunized with NP-OVA, as described above. Splenocytes were harvested on days 10 and 15 postimmunization. Cells were plated at 0.5 × 106 cells/well in 96-well plate and stimulated with PMA (10 ng/ml) and ionomycin (0.5 μg/ml). GolgIStop (BD Biosciences) was added according to manufacturer’s guidelines. Cells were harvested at 0, 4, and 8 h poststimulation and stained intracellularly to measure cytokine production. Stained cells were analyzed using a BD LSR II (BD Biosciences) analyzer. Data were acquired using FACS Diva software (BD Biosciences, San Jose, CA) and analyzed by FlowJo software (Tree Star). Proliferating cells were scored by quantifying the reduction in CellTrace Violet fluorescence intensity in dividing cells as compared with stained unactivated control cells.

Determination of Th1, Th2, and proinflammatory cytokine levels

Purified T cells (2.5 × 106/ml) obtained from NP-OVA-immunized WT and Mer−/− mice 10 d after immunization were activated with PMA (10 ng/ml) and ionomycin (0.5 μg/ml) in RPMI 1640 supplemented with 10% FBS in 96-well plates. The supernatant from the activated cells was harvested at 24 and 48 h poststimulation and used for the quantification of secreted cytokines. Levels of Th1/Th2/Th17 cytokines were determined by flow cytometry using the BD Cytometric Bead Array mouse Th1/Th2/Th17 cytokine kit (BD Biosciences), following manufacturer’s instructions. The cytokine concentrations were quantified by standard curves plotted using a five-parameter logistic curve-fitting model, using BD FCAP Array software (Soft Flow Hungary Ltd. for BD Biosciences). Samples with cytokine concentration below the detection limit for the assay have been arbitrarily assigned a value of 1 pg/ml.

Statistical analysis

Statistical analysis was performed using Student t test. The p values <0.05, <0.01, and <0.001 are depicted as *, **, and ***, respectively. The p values <0.05 were considered significant.

Results

Long-term accumulation of ACs occurs predominantly in GCs and not in the T cell zone, MZ, or red pulp area of the spleen in Mer−/− mice

By evaluating an earlier time point (day 14) of the GC response against TD-Ag, we have recently shown that ACs accumulate in GCs of Mer−/− mice in the absence of Mer-mediated clearance of dead and/or dying cells by TBM (6). However, it is not clear whether ACs continue to accumulate in GCs of Mer−/− mice over time, which, in turn, may alter peripheral B cell tolerance at the GC checkpoint that leads to autoreactive body production in Mer−/− mice. To study potential accumulation of ACs in Mer−/− GCs over an extended period of time, we immunized Mer−/− mice and their WT counterparts with the TD-Ag NP-OVA. We used a modified version of the repeated immunization protocol (described in Materials and Methods) previously shown to induce a robust GC response (2). According to this protocol, mice were immunized (i.p.) with 100 μg at day 0 and again 1 wk later with 50 μg NP-OVA in alum. Spleens from Mer−/− and WT control mice were harvested at 21 and 80 d after first immunization.

Spleen sections of NP-OVA–immunized WT mice stained with GL7 (green, GC B cell marker), anti-CD68 (red, a marker for TBM), and TUNEL (blue, apoptotic cell detection assay) exhibited very few TUNEL+ ACs in GCs (defined by dashed lines) and 21 and 80 d after immunization (second and third panels in first and third rows, Fig. 1A, 1B). In contrast, we found a marked increase in TUNEL+ ACs in GCs of Mer−/− mice up to day 80 (second and third panels in second and fourth rows, Fig. 1A, 1B). The TUNEL+ ACs continued to be predominantly localized outside CD68+ TBM within Mer−/− GCs at least up to day 80.

The B cell follicle, T cell zone, MZ, and red pulp are four distinct sites in the spleen where potential AC accumulation in the absence of Mer may influence B cells, T cells, and APCs. Therefore, to study the overall distribution of AC accumulation in NP-OVA–immunized Mer−/− spleen, we performed immunohistology on two consecutive spleen sections obtained on day 21 postimmunization of WT and Mer−/− mice. One was stained with anti-IgD and TUNEL (first and third panels, Fig. 1C), and the other was stained with MOMA-1 and TUNEL (second and fourth panels, Fig. 1C). GCs (highlighted by the dashed yellow lines) are defined as IgDnegative. MOMA-1 stains for macrophages, which defines the border between B cell follicles and MZ. Low original magnification (×100) representative images in this analysis revealed very few TUNEL+ cells outside GCs (i.e., red pulp, MZ, or T cell zones) in Mer−/− spleen (Fig. 1C, right), indicating predominant accumulation of ACs over time in GCs and not in the T cell zone, MZ, and red pulp of the spleen in the absence of Mer-mediated clearance. In contrast, very low number of TUNEL+ cells was observed scattered over/in all areas of WT control spleen, including GCs (Fig. 1C, left).

Tyro-3 and Axl expression was not compromised in Mer−/− mice

Axl, Mer, and Tyro-3 are three members of a subfamily of receptors involved in the clearance of ACs (16, 26). Whereas we show Mer expression on TBM in GCs, the expression of Axl and Tyro-3 in GCs has never been examined. Given our current data showing long-term accumulation of ACs in Mer−/− GCs, we determined whether Axl and Tyro-3 were expressed on TBM and whether Mer deficiency somehow altered the expression of these two receptors on TBM in Mer−/− GCs. Spleen sections obtained from Mer−/− and WT control mice at day 14 postimmunization were stained with either anti–Tyro-3 (second column, Fig. 2A) or anti-Axl (second column, Fig. 2B) in combination with PNA (first column, Fig. 2A, 2B) and anti-CD68 (third column, Fig. 2A, 2B). We found that these two receptors were expressed on CD68+ TBM in GCs as evidenced by overlapped purple staining in overlay images (fourth column, Fig. 2A, 2B) with no evident differences in expression between WT control and Mer−/− mice (Fig. 2). In a similar analysis, we also determined the expression of two other molecules (MFG-E8 and Tim-4) involved in phagocytosis.
and found no difference between WT and Mer−/− mice (data not shown). Together, these data indicate that accumulation of ACs in Mer−/− GCs is primarily due to Mer deficiency and not due to reduced expression of Axl, Tyro-3, MFG-E8, and Tim-4 in the absence of Mer.

Long-term accumulation of ACs in Mer−/− GCs led to enhanced long-lasting GC, AFC, and IgG2 Ab responses

Next, we evaluated whether long-term accumulation of ACs in GCs of Mer−/− mice led to elevated long-lasting GC, AFC, and IgG2 Ab responses in Mer−/− mice. The anti-NP GC response in WT and Mer−/− mice was analyzed on days 21 and 80 post-first immunization. The GC response was examined by flow cytometric analysis in which splenocytes from NP-OVA–immunized mice were stained with GC B cell markers (B220, PNA, and anti-Fas/CD95 Ab). We found significantly higher percentage of B220+PNAhighFashigh GC B cells in Mer−/− mice compared with WT controls on days 21 (Fig. 3A) and 80 (Fig. 3B). We also performed immunohistological analysis in which spleen sections obtained from these mice on day 21 and 80 postimmunization were stained with anti-IgD (blue) and PNA (red). Consistent with flow cytometry data, we found increased frequency of predominantly large GCs in Mer−/− mice on both days 21 (Fig. 3D) and 80 (Fig. 3F) compared with sparse and relatively smaller GCs in WT controls (Fig. 3C, 3E). Enhanced GC response
FIGURE 3. Augmented primary GC and AFC responses in the absence of Mer over time. Flow cytometric analysis was performed on splenocytes obtained from WT and Mer−/− mice on days 21 (A) and 80 (B) postimmunization with NP-OVA. Splenocytes were stained with GC B cell markers (B220, PNA, and anti-CD95/Fas). Representative contour plots obtained from WT (upper panel) and Mer−/− (lower panel) mice on day 21 show B220+PNAhigh Fashigh GC B cells in rectangular gates (A, left panel). The percentage of B220+PNAhigh Fashigh GC B cells in WT (open circle) and Mer−/− (closed circle) mice is shown in scatter plots (A, right panel). Analysis of samples obtained on day 21 was performed on individual mice, and on day 80 was performed on pooled samples of seven WT control (top) and four Mer−/− (bottom) mice in which B220+PNAhigh Fashigh GC B cells are shown in rectangular gates only (B). Spleen sections obtained from WT (C and E) and Mer−/− mice (D and F) on days 21 and 80 post–NP-OVA immunization were stained with anti-IgD (blue) and PNA (red). Low (×50) and high original magnification (×200) representative images are shown. The number of short-lived splenic NP-specific IgM (G) and IgG (H)–secreting AFCs was measured by ELISPOT at indicated days after immunization of WT (open circle) and Mer−/− (closed circle) mice with NP-OVA. The number of long-lived splenic (I) and bone marrow–derived (J) NP-specific IgG-producing AFCs was measured by ELISPOT 80 d postimmunization. Each circle represents the number of AFCs per 1 × 10^6 splenocytes obtained from an individual mouse. Horizontal bars represent the mean values. These data were obtained from age- and sex-matched four to seven mice per genotype.
and AC accumulation in GCs in Mer<sup>−/−</sup> mice also resulted in augmented NP-specific primary AFC response evaluated by ELISPOT assay 21 d postimmunization (Fig. 3G, 3H). Long-lived IgG-producing AFCs in both spleen (Fig. 3I) and bone marrow (Fig. 3J) from Mer<sup>−/−</sup> mice (closed circle) were also significantly higher compared with WT controls (open circle) 80 d after NP-OVA immunization.

We extensively analyzed whether elevated long-term GC and AFC responses associated with accumulation of ACs in Mer<sup>−/−</sup> GCs translated to high titers of serum Abs in Mer<sup>−/−</sup> mice compared with WT controls over an extended period at multiple time points after NP-OVA immunization. The T cell–dependent anti-NP response predominantly generates Abs of Th2-type IgG1 Abs in B6 mice (27). We found significantly higher titers of NP-specific total IgG (Fig. 4B) and IgG2 (Fig. 4D) Abs in Mer<sup>−/−</sup> mice compared with WT controls on days 21, 28, 45, 60, and 80 post–NP-OVA immunization with the peak response at day 60. This difference was also significant at day 14 in the IgG2 titers (Fig. 4D). We did not observe any significant difference in anti-NP total IgM (Fig. 4A) and IgG1 (Fig. 4C) serum Ab levels. Due to the mixed background (B6 and 129) of Mer<sup>−/−</sup> and WT control mice in which both IgG2a (derived from 129 allele) and IgG2c (B6) can potentially be produced, we measured total IgG2 Ab levels (Fig. 4D). No significant difference was observed in IgG3 levels between Mer<sup>−/−</sup> and WT controls (data not shown).

We further analyzed affinity maturation of anti-NP serum IgG1 (Th2-biased) and IgG2 (Th1-biased) Abs by ELISA from serum samples obtained from WT and Mer<sup>−/−</sup> mice on days 14, 21, 28, 45, 60, and 80 post–NP-OVA immunization. No significant differences in Ag affinity of both IgG1 and IgG2 Abs at any time point were revealed between WT and Mer<sup>−/−</sup> mice (data not shown).

Prolonged accumulation of ACs in Mer<sup>−/−</sup> GCs and enhanced AFC, GC, and Ab responses led to high titers of ANAs

Even though delayed clearance of ACs is thought to contribute to ANA production in Mer<sup>−/−</sup> mice (3), the site where ACs may accumulate and influence ANA production in Mer<sup>−/−</sup> mice is not clear. To study the role of GC as a site of AC accumulation in Mer<sup>−/−</sup> mice, which may lead to ANA production, we measured the titers of dsDNA–, histone–, and nucleosome–specific Abs by ELISA in serum samples obtained from Mer<sup>−/−</sup> and WT control mice before (day 0) and after (days 28, 60, and 80) NP-OVA immunization. We observed significantly higher anti-dsDNA (Fig. 5A), anti-histone (Fig. 5B), and anti-nucleosome Abs (Fig. 5C) in Mer<sup>−/−</sup> mice (closed circle) compared with WT controls (open circle) on days 28, 60, and 80 postimmunization, whereas no significant difference in ANA titers was found between the two strains on day 0 (Fig. 5). These ANA titers are in concordance with accumulation of ACs in GCs and enhanced GC, AFC, and Ab responses in Mer<sup>−/−</sup> mice.

Elevated AFC, GC, and IgG2 Ab responses in Mer<sup>−/−</sup> mice were due to increased activation and proliferation of B cells

Next, we evaluated whether the enhanced AFC, GC, and IgG2 Ab responses apparently resulting from AC accumulation in Mer<sup>−/−</sup> GCs were due to increased activation of B cells in Mer<sup>−/−</sup> mice. Flow cytometric analysis of spleen cells obtained from Mer<sup>−/−</sup> and WT mice on day 14 postimmunization revealed significantly higher percentage of activated B cells, as evidenced by the expression of activation markers CD80 (left two panels, Fig. 6A) and CD86 (right two panels, Fig. 6A) in Mer<sup>−/−</sup> mice compared with WT controls. Although we found no significant difference in CD80<sup>+</sup> cells between Mer<sup>−/−</sup> and control mice (left two panels, Fig. 6B), the percentage of CD86<sup>+</sup> cells remained significantly higher in Mer<sup>−/−</sup> mice compared with controls 21 d postimmunization (right two panels, Fig. 6B).

![FIGURE 4](http://www.jimmunol.org/Downloadedfrom) Augmented IgG and IgG2 Ab responses in Mer<sup>−/−</sup> mice. Anti-NP IgM (A), IgG (B), IgG1 (C), and IgG2 (D) Ab titers were measured by ELISA in WT (open circle) and Mer<sup>−/−</sup> (closed circle) serum samples obtained on multiple time points (days 14, 21, 28, 45, 60, and 80) postimmunization of these mice with NP-OVA. The dashed lines represent WT, and solid lines represent Mer<sup>−/−</sup> mice. Each circle represents an individual mouse, and horizontal bars represent the mean values. The p values <0.05 and <0.01 are depicted as * and **, respectively. These data were obtained from age- and sex-matched five to six mice of each genotype at each time point.
To study whether AC accumulation in GCs and elevated GC, AFC, and IgG2 Ab responses in Mer\(^{-/-}\) mice were due to increased B cell proliferation, we performed an in vivo BrdU proliferation assay in which we injected BrdU (i.p.) into Mer\(^{-/-}\) and WT control mice 1–2 h before harvesting spleens from these animals on day 21 post–NP-OVA immunization. Immunohistological analysis was conducted on two consecutive spleen sections obtained from these mice. One was stained with anti-IgD (blue) and PNA (red), whereas the other one was stained with anti-IgD (blue) and anti-BrdU (brown, Fig. 6C). Both low (×50, left panels) and high (×200, right panels) original magnification images are shown (Fig. 6C). We found significantly higher numbers of BrdU\(^+\) cells in GCs and the red pulp areas of Mer\(^{-/-}\) mice (bottom row, second and fourth panels, Fig. 6C) compared with WT controls (upper row, second and fourth panels, Fig. 6C). By performing a semiquantitative analysis, in which we counted BrdU\(^+\) cells in 45–50 representative GCs from seven mice of each genotype, we found the number of BrdU\(^+\) cells in Mer\(^{-/-}\) GCs (brown) to be significantly higher compared with WT controls (blue) (Fig. 6D).

Additionally, when we stained spleen sections from Mer\(^{-/-}\) and control mice with GL7 (green) and Ki67 (red), a cell proliferation marker, we found analogous results as in Fig. 6C, showing increased numbers of Ki67\(^+\) cells in Mer\(^{-/-}\) GCs (right two panels, Fig. 6E) compared with WT controls (left two panels, Fig. 6E).

**Increased CD4\(^+\) Th cell responses in Mer\(^{-/-}\) mice with elevated AFC, GC, and IgG2 Ab responses**

We observed an enhanced IgG2 Ab response in Mer\(^{-/-}\) mice compared with WT controls (Fig. 4). Therefore, we analyzed whether AC accumulation in GCs and elevated GC, AFC, and IgG2 Ab responses in Mer\(^{-/-}\) mice were correlated with enhanced CD4\(^+\) Th cell activation and differentiation into effector cells, including T\(_{FH}\) cells. Splenocytes obtained from Mer\(^{-/-}\) and WT control mice 14 and 21 d postimmunization were stained with Abs against CD4, CD44, CD25 (IL-2R\(\alpha\)), CD62L, PD-1, and CXCR5. Flow cytometric analysis of stained spleen cells at both time points revealed significantly higher percentages of activated CD4\(^+\) CD44\(^{high}\)CD62L\(^{low}\) short-lived (middle panels, Fig. 7A, 7B) and CD4\(^+\)CD44\(^{high}\)CD62L\(^{high}\) memory (right panels, Fig. 7A, 7B) effector Th cells in Mer\(^{-/-}\) mice compared with WT controls.

Mer\(^{-/-}\) mice also had increased percentages of CD4\(^+\)CD25\(^+\) cells compared with WT controls on both day 14 and 21 time points (left and middle panel, Fig. 7C, 7D). In addition, the expression of CD25 on CD4\(^+\) cells was increased in Mer\(^{-/-}\) mice compared with WT controls (shown in histograms overlay; right panels, Fig. 7C, 7D). This increased percentage of CD4\(^+\)CD25\(^+\) cells in Mer\(^{-/-}\) mice was associated with elevated CD4\(^+\)PD-1\(^{high}\)CXCR5\(^{high}\) T\(_{FH}\) cells in Mer\(^{-/-}\) mice compared with WT controls (Fig. 7E, 7F). We also evaluated the T\(_{FH}\) cell response in these mice 80 d postimmunization. CD4\(^+\)PD-1\(^{high}\)CXCR5\(^{high}\) T\(_{FH}\) cell response in Mer\(^{-/-}\) mice was on average 3-fold higher compared with WT controls at day 80 time point (Fig. 7G). We did not find any difference in CD4\(^+\)CD25\(^+\)Foxp3\(^{-}\) T regulatory (Treg) cells between these two strains at any time point (data not shown).

**B cells and CD4\(^+\) Th cells from immunized Mer\(^{-/-}\) mice proliferated at faster rate than WT control cells upon ex vivo stimulation**

Using the BrdU proliferation assay, we observed significantly higher number of proliferating B cells in Mer\(^{-/-}\) GCs compared with WT controls (Fig. 6). To further delineate whether the microenvironment created in the GCs of Mer\(^{-/-}\) mice intrinsically primed B and T cells to proliferate rapidly upon stimulation, we used an ex vivo cell proliferation assay for in vivo primed cells. MACS-purified B cells obtained from Mer\(^{-/-}\) and WT control mice 10 d post–NP-OVA immunization were labeled with CellTrace Violet, followed by stimulation with anti-IgM and agonistic anti-CD40 Ab. By performing flow cytometric analysis, we observed an increased rate of proliferation of B cells from Mer\(^{-/-}\) mice compared with WT controls at both 72 and 96 h poststimulation, as evident by the higher percentage of dividing B cells with lower CellTrace Violet intensity in Mer\(^{-/-}\) mice (Fig. 8A).

Similarly, we evaluated whether increased percentages of subsets of CD4\(^+\) Th cells (i.e., short-lived and memory effector cells as well as T\(_{FH}\) cells) in Mer\(^{-/-}\) mice were due to enhanced proliferation of CD4\(^+\) Th cells in the presence of AC accumulation in GCs. MACS-purified CD4\(^+\) Th cells from Mer\(^{-/-}\) and WT control mice 10 d post–NP-OVA immunization were stained with Cell-
Trace dye and then stimulated ex vivo with anti-CD3 and anti-CD28 Abs. The rate of proliferation of CD4+ Th cells was evaluated in a flow cytomteric analysis by determining the percentage of dividing cells. We found significantly higher percentage of cells in successive peaks with reduced CellTrace Violet fluorescence, signifying more proliferating CD4+ Th cells in Mer2/2 mice compared to WT mice.

FIGURE 6. Increased percentage of activated and proliferating B cells in Mer2/2 mice. B cell activation was evaluated by flow cytometric analysis of spleen cells obtained at days 14 (A) and 21 (B) postimmunization of WT and Mer2/2 mice with NP-OVA. Spleen cells were stained with Abs against B cell activation markers CD80 (A and B, left two panels) and CD86 (A and B, right two panels). The percentage of B220+CD80+ (left panels) and B220+CD86+ (right panels) cells in WT (open circle) and Mer2/2 (closed circle) mice is shown in scatter plots. (C) Immunohistological analysis was performed on two consecutive spleen sections obtained from WT (upper row) and Mer2/2 (lower row) mice on day 21 after NP-OVA immunization. One section (first and third columns) was stained using anti-IgD (blue) and PNA (red), and the other (second and fourth columns) was stained with anti-IgD (blue) and anti-BrdU (brown). The high original magnification (×200) images shown in third and fourth columns are obtained from low original magnification (×50) GCs delineated by rectangles in the first and second columns. (D) Semiquantitative analysis of BrdU+ B cells per GC was performed by counting BrdU+ cells in 45–50 representative GCs from seven WT (blue) and eight Mer2/2 mice. (E) Spleen sections obtained from WT (left two panels) and Mer2/2 (right two panels) mice on day 21 after NP-OVA immunization were stained with GL7 (green) and anti-Ki67 (red). Original magnification ×200. Two representative images from each strain are shown. These data represent at least seven mice of each genotype.
pared with WT controls at both 72 and 96 h after stimulation (Fig. 8B).

CD4+ Th cells from immunized Mer−/− mice produced increased levels of Th1 and proinflammatory cytokines

We observed significantly higher Th1-biased IgG2 Abs in Mer−/− mice compared with WT controls. IgG2 Abs are believed to be driven by Th1 cytokines (i.e., IFN-γ). Therefore, we examined whether immunized Mer−/− mice had increased frequency of CD4+ IFN-γ+ Th cells. Splenocytes obtained from Mer−/− and control mice 10 and 15 d after NP-OVA immunization were stimulated ex vivo with PMA and ionomycin. IFN-γ- and IL-4-producing CD4+ Th cells were determined by intracellular staining with anti–IFN-γ and anti–IL-4 Abs through flow cytometry analysis at 0, 4, and 8 h poststimulation. We found that Mer−/− mice had significantly higher percentage of CD4+IFN-γ+ T cells compared with WT controls at both 24- and 48-h time points.

We further determined levels of cytokines produced by CD4+ Th cells in Mer−/− mice. Purified T cells obtained from Mer−/− and WT control mice 10 d post–NP-OVA immunization were stimulated ex vivo with PMA and ionomycin, and supernatants were collected at 24 and 48 h poststimulation. Levels of cytokines were measured by flow cytometry using the BD Cytometric Bead Array Th1/Th2/Th17 cytokine kit. Consistent with increased frequency of CD4+IFN-γ+ T cells in Mer−/− mice (Fig. 9A, 9B), CD4+ Th cells from Mer−/− mice produced significantly elevated levels of IFN-γ compared with WT controls at both 24- and 48-h time points.
points (Fig. 9C). In addition, we observed increased levels of TNF (both 24 and 48 h) and IL-2 (24 h) in Mer<sup>2/2</sup> mice compared with WT controls (Fig. 9C). Albeit at low titers, levels of IL-6 and IL-17 (IL-17A) were also at least 2-fold higher in Mer<sup>2/2</sup> mice compared with WT controls at 24-h time point. Interestingly, we found significantly reduced IL-10 production in Mer<sup>2/2</sup> mice compared with WT controls (Fig. 9C). Finally, we observed similar levels of secreted IL-4 in both Mer<sup>2/2</sup> and control mice (data not shown).

Enhanced GC response and accumulation of ACs in Mer<sup>2/2</sup> GCs resulted in augmented memory B cell response in Mer<sup>2/2</sup> mice

GCs are microenvironments in which B cells with high affinity to foreign Ag are positively selected into memory B cells. We determined whether long-term accumulation of ACs in GCs and dysregulated GC response led to elevated memory B cell response in Mer<sup>2/2</sup> mice, which, in turn, may harbor autoreactive B cells. Mer<sup>2/2</sup> and WT control mice were immunized with NP-OVA, as described earlier, rested for 80 d, and then boosted (i.p.) with 50 μg NP-OVA in PBS. Memory B cell response was indirectly evaluated by analyzing secondary AFCs 4 d after the boost. IgG-producing secondary AFCs in Mer<sup>2/2</sup> spleens (closed circle) were augmented compared with WT controls (open circle, Fig. 10A). These elevated secondary IgG AFC responses resulted in increased anamnestic anti-NP total IgG (Fig. 10B) and IgG2 (Fig. 10D) serum Ab titers in Mer<sup>2/2</sup> mice compared with WT controls. Analogous to the primary response, the secondary anti-NP IgG1 Ab responses remained comparable between Mer<sup>2/2</sup> and WT control mice (Fig. 10C).

Discussion

The development of autoimmunity in aged Mer<sup>2/2</sup> mice is attributed to delayed clearance of ACs over time (3). However, the site where delayed clearance of ACs may occur in Mer<sup>2/2</sup> mice and alter B cell tolerance leading to ANA production is unclear. Because many B cells undergo apoptotic cell death in GCs due to clonal selection as a function of GC homeostasis, we propose that GCs are the primary site where ACs accumulate in the absence of Mer-mediated clearance. Consistent with this idea, we recently showed that ACs accumulate in GCs in the absence of Mer<sup>2/2</sup> mice (4).

Although we previously showed AC accumulation in GCs and the enhanced AFC, GC, and IgG2 Ab responses in Mer<sup>2/2</sup> mice at earlier time point (6), it was not clear whether ACs continue to accumulate over time even in the presence of several other receptors and molecules (i.e., Axl, Tyro-3, MFG-E8, Tim-4, C1q, FIGURE 8. Mer deficiency drives faster proliferation in lymphocytes upon ex vivo stimulation. Representative flow cytometric histograms showing the quantification of proliferating cells from WT and Mer<sup>2/2</sup> mice by CellTrace Violet dilution (x-axis) in B cells stimulated with anti-IgM and anti-CD40 Abs (A), and T cells stimulated with anti-CD3 and anti-CD28 Abs (B) at 72 h (upper panels, A and B) and 96 h (lower panels, A and B). Gate and numbers in each panel indicate the percentage of cells with decreased CellTrace Violet fluorescence intensity indicating proliferation. Solid gray line represents WT; solid black line, Mer<sup>2/2</sup>; dashed gray line, unstimulated cells. An overlay of these three histograms is shown in the right panel. These data are representative of two independent experiments each performed using cells pooled from at least five mice per group.
FIGURE 9. Increased IFN-γ production by CD4+ Th cells in NP-OVA–immunized Mer−/− mice. (A and B) Intracellular cytokine flow cytometric analysis of CD4+ Th cells obtained from NP-OVA–immunized 7- to 9-wk-old WT and Mer−/− mice for IFN-γ (left panels) and IL-4 (right panels) at indicated days after immunization. Splenocytes were stimulated with PMA and ionomycin for 0, 4, or 8 h ex vivo after isolation on days 10 and 15 postimmunization. Mean values are shown in bars (WT, open; Mer−/−, solid gray), and each circle overlayed on the bars represents an individual mouse. (C) Flow cytometric quantification of indicated cytokines in culture supernatants of purified T cells pooled from NP-OVA–immunized five WT (open bars) and five Mer−/− (solid bars) mice, stimulated with PMA and ionomycin for 24 and 48 h. Concentration in pg/ml reflects the mean of at least two dilutions per sample. T cells were purified from Mer−/− and WT control mice 10 d postimmunization. *p < 0.05, **p < 0.01, ***p < 0.001.
The increased frequency of GC B cells and effector Th cells in controls. These elevated responses and AC accumulation in Mer numbers of activated effector CD4+ Th cells, including TFH cells. and proliferation of total and GC B cells, T cells, and greater in our studies were a result of significantly increased activation GC, and IgG2 Ab responses in Mer DEFICIENCY AFFECTS B AND Th CELL RESPONSES the thymus (31), which may give rise to autoreactive T cells. Subsequently, abnormal help from GC Tfh cells could facilitate enhanced GC B cell responses and the process of breaking B cell tolerance. The evidence of loss of AC-induced T cell tolerance in the absence of Mer was previously described (32). In accordance with our results, a potential role for defective TFH cell compartment in breaking GC tolerance and promoting autoimmunity has also been described in a number of autoimmune mouse models, including Roquin−/− (sanroque) (33) and BXSB.yaa mice (34, 35).

An increasing body of evidence from literature suggests that a failure to clear ACs results in autoimmunity (2–5, 36–38). In addition to Mer and MFG-E8 deficiency, mice deficient in several other molecules involved in the phagocytosis and clearance of and CD36), which may clear dead and/or dying cells from GCs at later time points after the initial peak GC response wanes. This is particularly important to study because long-term accumulation of ACs in GCs in Mer−/− mice in the presence of other phagocytic receptors can further highlight the significant role played by Mer in clearing ACs in GCs and have major implications in breaking B cell tolerance leading to ANA production in Mer−/− mice. Our analysis of the later time points (up to 80 d) indeed revealed long-term AC accumulation in GCs leading to steady state elevated AFC. These elevated responses and AC accumulation in Mer−/− GCs were tightly linked to significantly higher titers of ANAs in Mer−/− mice compared with WT controls, indicating a break in peripheral B cell tolerance due to delayed clearance of ACs in GCs.

The enhanced AFC, GC, and IgG2 Ab responses in Mer−/− mice in our studies were a result of significantly increased activation and proliferation of total and GC B cells, T cells, and greater numbers of activated effector CD4+ Th cells, including Tfh cells. The increased frequency of GC B cells and effector Th cells in Mer−/− mice was also associated with significantly elevated long-lived primary and memory responses compared with WT controls. We did not, however, observe any differences in affinity maturation of both Th2/IgG1 and Th1/IgG2 Ab responses between WT control and Mer−/− mice. Importantly, we ruled out the contribution of the potentially reduced expression of several other bridging molecules/receptors (i.e., Axl, Tyro-3, MFG-E8, and Tim-4) to AC accumulation in Mer−/− GCs. These data demonstrate that accumulation of ACs in Mer−/− GCs is primarily due to a deficiency in Mer expression and not due to altered expression of Axl, Tyro-3, MFG-E8, and Tim-4, potentially induced by Mer deficiency.

We observed enhanced long-lasting AFC, GC, and IgG2 Ab responses in Mer−/− mice compared with WT controls. In contrast, the Cohen group (23) previously reported no difference in T-dependent Ab responses between Mer−/− and control mice by measuring only serum OVA-specific total IgG and IgG1 (Th2) Ab titers 14 d after immunizing these mice with OVA. The same group (28) in a more recent publication showed decreased GC response in Mer−/− mice in response to chronic graft-versus-host response mediated by allogenic bm12 cells transferred into Mer−/− mice. Although both these reports by the Cohen group (23, 28) used different models to determine the role of Mer in regulating MZ B cell response and tolerance, these data may not be extrapolated to assign a role for Mer-mediated clearance of ACs from GCs in regulating follicular GC B cell and Ab responses against various T-dependent Ags. Our results at day 14 (Fig. 3B, 3C) are analogous to the data published by the Cohen group (23) in which they reported no differences in total OVA-specific IgG1 Ab titers between WT control and Mer−/− mice 14 d after immunization. However, when we measured serum Ab titers at multiple later time points over a period of 80 d post-NP-OVA immunization, we observed significantly higher total NP-specific IgG1 titers in Mer−/− mice compared with WT controls starting from day 21 through day 80. Th1-biased IgG2 Ab response was significantly higher in Mer−/− mice compared with WT controls starting from day 14 through day 80, whereas Th2–IgG1 response remained similar between the two strains during this period, indicating enhanced steady state Th1 cytokine-driven IgG2 Ab responses in Mer−/− mice in the presence of AC accumulation in GCs.

In the absence of efficient removal of ACs, uncleared ACs in Mer−/− GCs can undergo necrotic cell death (termed secondary necrosis), allowing the rupture of cellular membranes and release of danger-associated molecular patterns (DAMPs) (29, 30). DAMPs released from these cells can then stimulate both phagocytes (i.e., TBMφs) and B cells via intracellular or extracellular DAMP receptors such as TLR and NOD-like receptors expressed by these cells. The proinflammatory conditions generated through DAMP receptor stimulation of TBMφ and B cells may drive differentiation of Th cells toward Th1 and may lead to a break in T cell tolerance. Consistent with this idea, we find increased levels of Th1 (i.e., IFN-γ and IL-2) and proinflammatory (i.e., TNF and IL-6) cytokines in Mer−/− CD4+ T cells compared with WT controls. Mer−/− mice also have a defect in AC clearance in the thymus (31), which may give rise to autoreactive T cells. Subsequently, abnormal help from GC Tfh cells could facilitate enhanced GC B cell responses and the process of breaking B cell tolerance. The evidence of loss of AC-induced T cell tolerance in the absence of Mer was previously described (32). In accordance with our results, a potential role for defective Tfh cell compartment in breaking GC tolerance and promoting autoimmunity has also been described in a number of autoimmune mouse models, including Roquin−/− (sanroque) (33) and BXSB.yaa mice (34, 35).

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ACs such as C1q (C1q−/−) and Tim-4 (Tim-4−/−) all developed autoimmunity (4, 5). Synergistic AC administration or blocking of phosphatidylerine-mediated phagocytosis of ACs results in the production of ANAs and IgG deposition on kidney glomeruli (39, 40). Impaired phagocytosis of ACs by peritoneal macrophages from autoimmune-prone mice has been described (41, 42). Macrophages from human systemic lupus erythematosus patients have a defect in phagocytosis of ACs (36–38). Systemic lupus erythematosus patients and lupus-prone (SWR+/- CD4−/−) mice have a marked increase in the frequency of peripheral lymphocyte apoptosis (43–45). These data clearly indicate that a defect in AC clearance or increased rate of apoptosis can both potentially contribute to AC accumulation and loss of immune regulation. Our data from the current study suggest that GCs are the primary site where ACs accumulate in the absence of Med-mediated clearance. This AC accumulation in Mer−/− mice may play a role in the formation of immune complexes and activation of innate immune cells. In addition, the presence of apoptotic ACs in GCs may provide altered signaling leading to dysregulation of GC homeostasis and functions, which may contribute to a break in peripheral immune tolerance and induction of autoimmunity.

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