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Cutting Edge: Defective Follicular Exclusion of Apoptotic Antigens Due to Marginal Zone Macrophage Defects in Autoimmune BXD2 Mice

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Marginal zone macrophages (MZMs) act as a barrier to entry of circulating apoptotic debris into the follicles of secondary lymphoid organs. In autoimmune BXD2 mice, there is a progressive reduction in the function and numbers of MZMs. Absence of MZMs results in retention of apoptotic cell (AC) debris within the marginal zone (MZ) and increased loading of AC Ags on MZ B cells and MZ-precursor (MZ-P) B cells. The MZ-P B cells are capable of translocating the AC Ags to the follicular zone and stimulating T cells. Both MZMs and MZ-P B cells from BXD2 mice express low levels of tolerogenic signals and high levels of inflammatory signals. Thus, the current study suggests a multifaceted mechanism in which MZMs maintain tolerance to apoptotic autoantigens and suppress their translocation to follicles. Lack of clearance of apoptotic debris by MZMs drives follicular Ag-transportation by MZ-P B cells to stimulate an autoimmune response. *The Journal of Immunology*, 2013, 190: 000–000.

Apoptotic cells (ACs) contain “blebs” that are highly enriched with autoantigens, including nucleosomal DNA and small nuclear riboproteins (1, 2). Normally, the corpses of the ACs are removed locally by tissue macrophages (3). ACs and debris that escape this clearance mechanism are prevented from entering the follicles of secondary lymphoid organs by the marginal zone (MZ). There is considerable evidence that loss of integrity of the MZ barrier that results in the lack of clearance of AC autoantigens can contribute to development of autoimmune disease (4–8). The MZ macrophages (MZMs), which express the scavenger receptor SR-A and MARCO to sense and remove waste materials (8, 9), are a critical component of this barrier. In mice, autoimmune responses can be induced or promoted by either

targeted deletion of SH2 domain-containing inositol 5'-phosphatase 1 (SHIP-1) in myeloid cells (9), which results in dislocations of MZMs, or treatment with an appropriate dose of clodronate liposome (CL), which results in loss of MZMs (5, 6).

BXD2 mice develop a lupus-like autoimmune disease (10). The generation of the high-affinity pathogenic autoantibodies is associated with the development of large spontaneous germinal centers (GCs) in the spleen (11–13). However, the mechanisms that permit the initial exposure of the B cells to the autoantigens have not been elucidated. The production of autoantibodies in BXD2 mice with specificity for Ags derived from a wide range of cellular compartments, including filament proteins, chromatin, and ribonuclear components (14), is consistent with the concept that autoantigens can be derived by proteolytic cleavage during the process of apoptosis (1). The present results confirm that defective clearance of apoptotic debris by MZMs and the associated transport of AC Ags by B cells can provoke a T cell response in BXD2 mice.

Materials and Methods

Mice

Wild-type C57BL/6 (B6), C57BL/6-Tg(Tcr α Tcr β)425Cbn/J (OT-II TCR transgenic [Tg]), B6-*Rag2*^{-/-}, C57BL/6-Tg(CAG-OVA)916Jen/J, transgenic mice expressing the membrane-bound chicken OVA on all cell surfaces (mOVA Tg), and BXD2 recombinant inbred mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6-*Rag2*^{-/-} or B6-OT-II TCR Tg mice were backcrossed with BXD2 mice for >10 generations to generate BXD2 OT-II TCR Tg or BXD2-*Rag2*^{-/-} mice, which were further intercrossed to generate BXD2 OT-II TCR Tg *Rag2*^{-/-} mice.

Apoptotic cells

For in vivo administration of ACs, mice were injected i.v. with 2×10^7 apoptotic thymocytes. For depletion of MZMs, mice were treated i.v. with 150 μ g CL (Encapsula NanoSciences, Nashville, TN) 4 h before AC transfer, as described (5). Control mice were treated with PBS liposomes. The dose for selective delivery to MZMs was optimized using Fluoroliposome (Encapsula NanoSciences).

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Abbreviations used in this article: AC, apoptotic cell; CL, clodronate liposome; FO, follicular; GC, germinal center; mOVA, membrane-bound chicken OVA; MZ, marginal zone; MZM, marginal zone macrophage; MZ-P, marginal zone precursor; RPM, red pulp macrophage; SHIP-1, SH2 domain-containing inositol 5'-phosphatase 1; Tg, transgenic.

CD4 T cell stimulation

Splenic CD4 cells from BXD2 OT-II TCR Tg *Rag2*^{-/-} mice were labeled with CFSE (5 μ M) and transferred i.v. into recipient mice (2 \times 10⁶/mouse). Recipient mice were administered apoptotic thymocytes prepared from mOVA Tg mice 12 h later and sacrificed after an additional 72 h.

In some experiments, red pulp macrophages (RPMs) (F4/80⁺CD11b^{lo}) or follicular (FO; CD19⁺IgM^{lo}CD21^{lo}CD23^{hi}), MZ (CD19⁺IgM^{hi}CD21^{hi}CD23^{lo}), or MZ-precursor (MZ-P; CD19⁺IgM^{hi}CD21^{hi}CD23^{hi}) B cells were FACS sorted from mice that were injected with mOVA⁺ ACs 30 min prior to sacrifice. In other experiments, sorted RPMs or FO, MZ, or MZ-P B cells (5 \times 10⁵ each) from naive 2-mo-old BXD2 mice were cocultured with purified and CFSE (1 μ M)-labeled OT-II CD4 T cells (2 \times 10⁵) in the presence or absence of either OVA protein or OVA₃₂₃₋₃₃₉ peptide, as described previously (15). In all in vitro experiments, CD4 T cell-proliferative response was determined by the reduction in CFSE intensity by flow cytometry 3 d after coculture.

Reagents and analyses

Flow cytometry and immunofluorescence microscopy were carried out as previously described (12). Real-time quantitative PCR was carried out, as described (12), using the following primers: *Il6*, 5'-TCCAGTTTGGTACATCCATC-3' (forward), 5'-CCGGAGAGGAGACTTCACAG-3' (reverse); *Il10*, 5'-AATTCCCTGGGTGAGAAG-3' (forward), 5'-TGCAGTTGATGATGAAGATGTC-3' (reverse); and *Tgfb*, 5'-CTACTATGCTAAAGAGGTCAC-3' (forward), 5'-CATGTTGCTCCACACTTG-3' (reverse). For Western blot analysis, anti-Btk (Santa Cruz), anti-IDO (Abcam), anti-GAPDH, anti-SHIP1, and anti-rabbit HRP-conjugated Ab (Cell Signaling Technology) were used.

Results and Discussion

Reduction of MZMs in BXD2 mice

Confocal imaging (Fig. 1A) and FACS analysis (Fig. 1B) were used to determine the integrity and enumerate the percentage of SIGN-R1⁺ MZMs in the spleens of autoimmune BXD2 mice. There was an age-dependent loss of MZMs in the BXD2 mice but not in the B6 mice (Fig. 1A, 1B). The total numbers of F4/80^{neg}CD11b^{lo}I-A^bnegSIGN-R1⁺ MZMs (16) were equivalent in 3-mo-old B6 and BXD2 mice, but there was a dramatic loss of these cells in 9-mo-old BXD2 mice but not B6 mice (Fig. 1B). There was an age-related defect in the clearance of ACs, as indicated by an increase in the number of TUNEL⁺ ACs found near the MZMs and inside areas of the follicle distinct from the GCs in BXD2 mice (Fig. 1C). Consistent with these, there were age-related increases in the levels of circulating autoantibodies for MARCO, SR-A, and DNA in BXD2 mice (Fig. 1D). Elevation of IgG anti-MARCO and anti-SR-A was found in the serum of lupus patients and mouse models of lupus; these autoantibodies potentially affect macrophage phagocytosis of ACs (7).

Capture of AC debris by B cells and their intrafollicular migration

The functional activities of the MZMs were evaluated by the ability to clear exogenous CFSE-labeled apoptotic thymocytes (Fig. 2A). Compared with B6 mice, the apoptotic thymocytes persisted for an extended period of time in 2-mo-old BXD2 mice. Thus, the functional defects in the MZMs precede the loss of these cells and represent one of the earliest autoimmune defects detected in the BXD2 strain.

However, the failure to clear Ags alone is not sufficient to induce an autoimmune response in BXD2 mice, because large GCs are found inside the follicles. Cyster and colleagues (15) showed that B cells can shuttle between the MZ and FO areas, thereby facilitating Ag transport. We established previously that, in BXD2 mice, there is the expansion in number of MZ-P B cells that is associated with the reduction in the MZ B cells and the heightened Ag delivery by MZ-P B cells (17, 18).

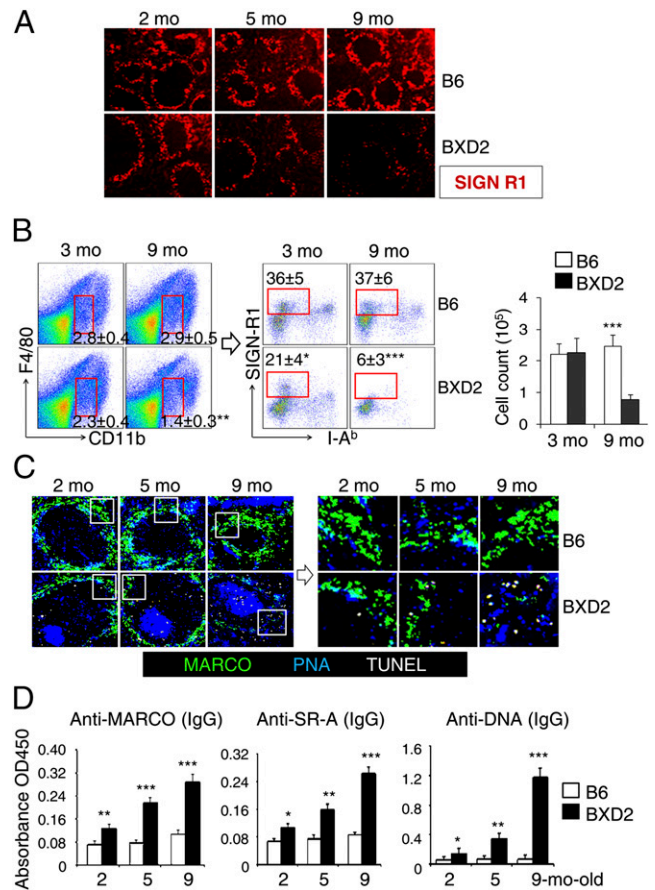


FIGURE 1. Age-related decrease in MZMs in BXD2 spleens. (A–C) Naive B6 and BXD2 mice were sacrificed at the indicated age. (A) Confocal imaging showing the decline of MZMs in the spleen in BXD2 mice. Staining was carried out using anti-SIGN-R1 (eBioscience). Original magnification \times 50. (B) FACS analysis showing the frequency of CD11b^{int}F4/80^{lo}SIGN-R1⁺I-Ab^{neg} MZMs (rectangle gated cells) in the spleen of B6 and BXD2 mice (left and middle panels). MZM cell count was calculated by multiplying the percentage of MZMs by the total spleen cell count obtained from each mouse (right panel). (C) Confocal imaging showing the anatomic location of ACs (TUNEL⁺) relative to the location of MZMs (MARCO⁺) and GCs (PNA⁺) in the representative follicle (left panel). Staining was carried out using an anti-MARCO Ab (AbD Serotec), peanut agglutinin (PNA, Vector Laboratories) and an In Situ Apoptosis Detection Kit (EMD Millipore). High-power view of the boxed areas (right panel). Original magnification \times 200. (D) ELISA analysis of serum titers of the indicated autoantibodies in the indicated strains. All data are mean \pm SEM ($n = 3$ –4/group) for two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, B6 mice versus BXD2 mice of the same age.

To determine whether BXD2 B cells can capture and deliver AC Ags in MZM-deficient conditions, we tracked CFSE-labeled apoptotic thymocytes in mice that were treated with 150 μ g of CL. This dose was used because it selectively induced uptake of liposomes by MZMs (Supplemental Fig. 1A). In mice administered CL, there was a time-dependent capture of ACs by MZ B and MZ-P B cells but not FO B cells (Fig. 2B, Supplemental Fig. 1B). The numbers of CFSE⁺ cells, which were located predominantly in the MZ, peaked at 5–15 min, and the majority was processed at 60 min (Fig. 2C, left panel). This may be associated with the higher uptake of CFSE by MZ B cells compared with MZ-P B cells during these early time points (Fig. 2B, Supplemental Fig. 1B). Within 5–30 min, there was a rapid migration of MZ-P B cells to the periphery of the follicle and into the MZ (Fig. 2C, right panel, Supplemental Fig. 1C, 1D). By 1 h after

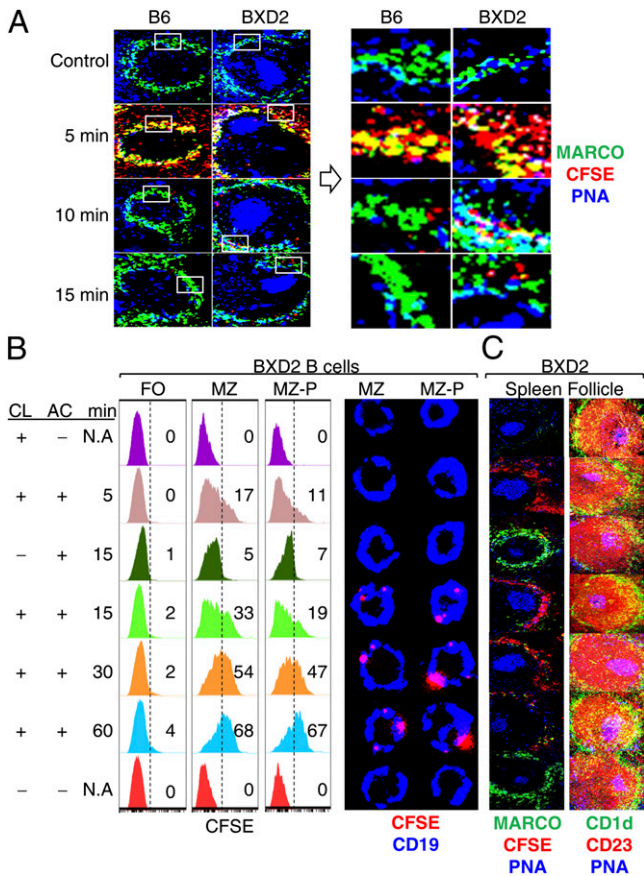


FIGURE 2. Defective clearance of apoptotic debris is associated with increased uptake of ACs by MZ/FO shuttling MZ-P B cells in the spleens of BXD2 mice. **(A)** Confocal imaging showing the kinetics of clearance of administered CFSE-labeled apoptotic thymocytes (pseudocolor red) in the MZ area in the spleen of 2-mo-old mice (*left panel*). High-power view of the indicated (box) areas (*right panel*). Original magnification $\times 200$. **(B and C)** BXD2 mice (2 mo) were treated with either CL- or PBS-liposome 4 h prior to CFSE-labeled apoptotic thymocyte administration. Following AC administration, mice were sacrificed at the indicated time points. **(B)** FACS analysis of subpopulations of B cells that were positive for AC Ags (CFSE⁺) (*left panel*). FO, MZ, and MZ-P B cells were gated as described (17, 18). Confocal imaging assessment of the binding of FACS-sorted MZ or MZ-P B cells with CFSE⁺ (pseudocolor red) apoptotic debris (*right panel*). A representative cell from the indicated subset is shown. Original magnification $\times 1000$. **(C)** Confocal imaging showing a representative spleen follicle from each group to reveal the clearance of the administered CFSE⁺ apoptotic thymocytes (pseudocolor red) in the spleen (*left panel*) and the anatomic location of FO (red: CD1d⁺CD23⁺), MZ (green: CD1d⁺CD23⁻), MZ-P (yellow: CD1d⁺CD23⁺), and GC (blue: PNA⁺) in each group (*right panel*). Original magnification $\times 200$. ($n = 2-3$ mice/group for three independent experiments).

administration of the ACs, the majority of MZ-P B cells, but not MZ B cells, exhibited massive inward migration into the follicles. At this time point, the majority of both the MZ-P and MZ B cells carried the apoptotic debris (Fig. 2B, 2C, Supplemental Fig. 1B).

Apoptotic Ags capturing MZ-P B cells stimulate CD4⁺ T cells

To determine whether MZMs are mechanistically required to suppress the T cell response to ACs and associated Ags, we transferred CFSE-labeled OT-II TCR Tg T cells into BXD2 and B6 mice and assessed the response of V β 5⁺ T cells to apoptotic thymocytes derived from mOVA Tg mice. Significantly higher numbers of OT-II T cells entered the cell cycle

in the spleens of the 2- and 9-mo-old BXD2 mice than the spleens of age-matched B6 mice postapoptotic mOVA⁺ thymocyte transfer (Fig. 3A, *right panel*). The percentages of CFSE-labeled OT-II T cells that entered the cell cycle in mice administered CL were similar to that observed in 9-mo-old BXD2 mice (Fig. 3A, *right panel*), which is consistent with the dramatic loss of MZMs observed in the older BXD2 mice (Fig. 1A, 1B).

We showed previously that the T cell costimulatory function of MZ-P B cells is superior to other B cells in BXD2 mice (17, 18). Therefore, we cocultured CFSE-labeled OT-II CD4⁺ T cells with F4/80⁺ RPMs or subpopulations of B cells sorted

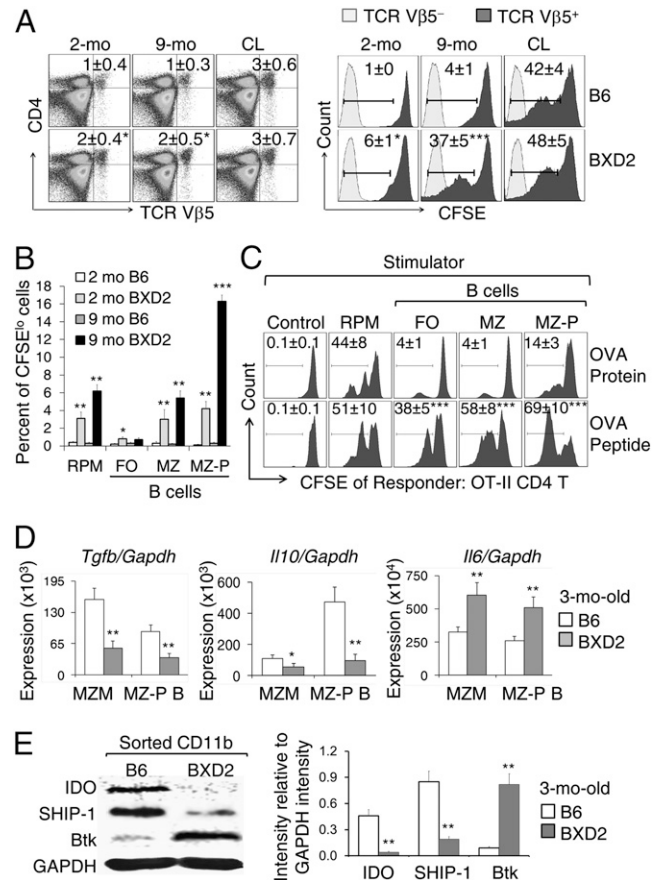


FIGURE 3. Stimulation of CD4 T cells by MZ-P B cells carrying AC Ags. **(A)** Flow cytometry analysis of the percentage of the transferred BXD2 OT-II CD4 T cells (TCR V β 5) in total spleen cells in the indicated recipient groups (*left panels*). Flow cytometry analysis of the in vivo OVA Ag-specific proliferative response of the transferred OT-II TCR CD4 T cells, indicated by attenuation of CFSE intensity (*right panels*). Control mice (2 mo old) were administered CL. **(B)** Bar graph represents the in vitro proliferative response of OT-II TCR CD4 T cells (CFSE^{lo}) 72 h after coculture with the indicated cell populations, which were FACS sorted from the spleens of mice 30 min postadministration of apoptotic mOVA⁺ thymocytes. **(C)** Flow cytometry analysis of the in vitro OT-II T cell–proliferative response (CFSE^{lo}) to either OVA protein or the OVA₃₂₃₋₃₃₉ peptide processed or presented by the indicated populations of cells that were FACS sorted from naive 2-mo-old BXD2 mice. CFSE-labeled OT-II T cells alone were used as control. **(D)** RT-PCR analysis of the expression of the indicated gene in sorted MZMs and MZ-P B cells derived from the spleen of naive mice. **(E)** Western blot analysis of the expression of the indicated protein in sorted CD11b cells from naive mice (*left panel*). ImageJ analysis of the intensity of each protein after normalization to the intensity of GAPDH ($n = 3-4$ /group) (*right panel*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus the same population of cells from B6 mice in (A), (B), (D), and (E) or from OVA protein–stimulated cells in (C).

from the spleens of BXD2 mice that had been administered mOVA thymocytes. These results show that both MZ and MZ-P B cells can present Ags derived from ACs, and this Ag presentation and stimulation of T cells are higher in BXD2 mice compared with B6 mice (Fig. 3B, Supplemental Fig. 2). Although the ability of MZ-P B cells to process intact OVA proteins for stimulation of CD4⁺ T cells was lower than that of RPMs, the ability of MZ-P B cells to present OVA peptide for stimulation of T cells was superior to that of all other tested B cell populations from BXD2 spleens (Fig. 3C). This suggests that MZ-P B cells that have migrated into the FO region may either directly process or capture processed AC Ags, thereby stimulating CD4⁺ T cells.

Under normal conditions, phagocytic cells that ingest ACs produce tolerogenic signals, including TGF- β and IL-10, which maintain tolerance to self-Ags (19). Both MZMs and MZ-P B cells from 3-mo-old BXD2 mice exhibited lower expression of genes encoding tolerogenic cytokines, including TGF- β and IL-10, and higher expression of the gene encoding the immunogenic cytokine IL-6 compared with those from B6 MZMs and MZ-P B cells (Fig. 3D). IDO was recently shown to be an important tolerogenic signal inducer in MZMs of B6 mice following AC administration (6). However, IDO was detectable in MZMs of unmanipulated younger MRL-Fas^{lpr/lpr} mice, and its inhibition with D-1-methyl-tryptophan promoted autoimmunity (6), suggesting that low IDO can enhance, but is not the primary cause of, autoimmunity in MRL-Fas^{lpr/lpr} mice. In contrast, to our knowledge, the current study demonstrated for the first time that CD11b⁺ cells from a mouse model of spontaneous autoimmunity expressed significantly lower levels of IDO compared with those from normal B6 mice (Fig. 3E). Also, local high levels of IL-6 in the MZ area might contribute to an immunogenic signal for AC Ags, because IL-6 degrades IDO (20). The TGF- β -IDO-ITIMs stimulate SHIP-1 (*Inpp5d*), and the TGF- β -IDO-SHP axis was recently identified as the major long-term tolerogenic signal in DCs (21). There also were dramatically lower levels of SHIP-1, together with higher levels of its counteracting molecule, Btk (9), in the CD11b⁺ cells from the spleens of BXD2 mice (Fig. 3E). These findings are consistent with the observations that loss of the expression of SHIP-1 in myeloid cells of Lyz.Cre *Ship1*^{-/-} mice was associated with disrupted MZM integrity and enhanced splenomegaly and that deficiency of Btk in Lyz.Cre *Ship1*^{-/-} mice reversed these phenotypes (9).

In summary, we showed previously that the development of the high titers of autoantibodies in BXD2 mice is dependent on abnormalities in two compartments in the spleen: enhanced production of type I IFNs in the MZ induces FO translocation of Ag-bearing MZ-P B cells, which costimulate T cells (17, 18), and enhanced IL-17 production in the GCs that upregulates regulator of G-protein signaling proteins, thereby enhancing T-B cell interactions (12, 22). We now have observed that these abnormalities are preceded by the development of defects in the MZ barrier in BXD2 mice. Collectively, the present results suggest a novel mechanism of autoimmunity in which "leaks" in the FO exclusion of self-apoptotic Ags, together with enhanced activity of carrier B cells, subverts otherwise protected adaptive immune processes in the follicles. McHeyzer-Williams et al. (23) elegantly described the importance of B cells as initial Ag-transporting cells in adaptive immunity. The present study directly demonstrates that, in

a situation in which MZM apoptotic debris ingestion and tolerogenic functions are defective, FO translocating immunogenic B cells can capture and deliver the autoantigens into the follicles, thus providing the previously unknown bridging mechanism between defective clearance of apoptotic debris and the development of autoantibodies. Strategies that restore and retolerize MZM function may represent a novel approach to attenuation of autoimmune disease.

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Disclosures

The authors have no financial conflicts of interest.

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