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Osteopontin in Spontaneous Germinal Centers Inhibits Apoptotic Cell Engulfment and Promotes Anti-Nuclear Antibody Production in Lupus-Prone Mice

Keiko Sakamoto,* Yuji Fukushima,† Koyu Ito,‡ Michiyuki Matsuda,§ Shigekazu Nagata,§ Nagahiro Minato,* and Masakazu Hattori†

Disposal of apoptotic cells is important for tissue homeostasis. Defects in this process in immune tissues may lead to breakdown of self-tolerance against intracellular molecules, including nuclear components. Development of diverse anti-nuclear Abs (ANAs) is a hallmark of lupus, which may arise, in part, due to impaired apoptotic cell clearance. In this work, we demonstrate that spontaneous germinal centers (GCs) in lupus-prone mice contain significantly elevated levels of unengulfed apoptotic cells, which are otherwise swiftly engulfed by tingible body macrophages. We indicate that osteopontin (OPN) secreted by CD153+ senescence-associated T cells, which selectively accumulate in the GCs of lupus-prone mice, interferes with phagocytosis of apoptotic cells specifically captured via MFG-E8. OPN induced diffuse and prolonged Rac1 activation in phagocytes via integrin αβ3 and inhibited the dissolution of phagocytic actin cup, causing defective apoptotic cell engulfment. In wild-type B6 mice, administration of TLR7 ligand also caused spontaneous GC reactions with increasing unengulfed apoptotic cells and ANA production, whereas B6 mice deficient for Spp1 encoding OPN showed less apoptotic cells and developed significantly reduced ANAs in response to TLR7 ligand. Our results suggest that OPN secreted by follicular CD153+ senescence-associated T cells in GCs promotes a continuous supply of intracellular autoantigens via apoptotic cells, thus playing a key role in the progression of the autoreactive GC reaction and leading to pathogenic autoantibody production in lupus-prone mice. The Journal of Immunology, 2016, 197: 000–000.

A poptosis occurs under physiological conditions in most tissues, and swift clearance of apoptotic cells is important for maintaining tissue homeostasis (1). Apoptotic cells are removed via phagocytosis by tissue-resident macrophages or sequestered by macrocomplements such as complement components (2, 3). Phagocytes recognize apoptotic cells via specific cues, including cell surface expression of phosphatidylserine (PtdSer), which in healthy cells is restricted to the inner leaflet of the plasma membrane (4). Macrophages specifically bind and engulf apoptotic cells through phagocytic receptors that recognize PtdSer, including

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Pathogenesis of bound apoptotic cells is regulated by Rho family GTPases, including Rac1, which induce focal and finely tuned actin reorganization at the binding sites. This cytoskeletal rearrangement leads to engulfment of apoptotic cells, followed by their rapid degradation in lysosomes (10). Unengulfed apoptotic cells eventually spontaneously rupture, leading to the release of their cellular contents (including nuclear components) into the microenvironment (11). Thus, unengulfed apoptotic cells are a potential source of nuclear Ags, against which immune self-tolerance may operate inefficiently (3, 12).

Apoptotic cell death occurs at remarkably high rates in germinal centers (GCs) of lymphoid tissues. In GCs, Ag-reactive B cells undergo robust clonal proliferation associated with somatic hypermutation in the variable regions of BCR genes (13). Although B cell progeny that have acquired higher Ag affinity survive, the majority of progeny with lower affinity die by apoptosis (14). Nonetheless, due to their swift phagocytosis by professional phagocytes called tingible body macrophages (TB Mφs), apoptotic cells are rarely seen in GCs during immune responses. TB Mφs express abundant amounts of MFG-E8, which serve as an adaptor molecule specific for apoptotic cell binding; MFG-E8 deficiency results in the development of spontaneous GCs and anti-nuclear Ab (ANA) production reminiscent of systemic lupus erythematosus (SLE) (15). SLE, a multiorgan disease preferentially affecting females, is characterized by the development of a wide variety of autoantibodies including ANAs, thus leading to immune complex-mediated inflammation in vital organs (16–19). Although accumulating evidence suggests that apoptotic cell clearance plays a crucial role in preventing systemic autoimmunity (1), the exact mechanism linking apoptotic cell clearance in GCs and development of lupus disease remains elusive.
We previously showed that a unique PD-1⁺ memory phenotype CD4⁺ T cell population bearing the follicular T cell nature plays an important role in spontaneous GC reactions in lupus-prone mice (20). We termed these T cells senescence-associated T (SA-T) cells because their abundance increased with age in normal mice and exhibited multiple features of cell senescence, including defective proliferation capacity, elevated expression of senescence-related genes, abundant heterochromatin nuclear foci, and preferential secretion of proinflammatory cytokines such as secreted phosphoprotein 1 (Spp1), also called osteopontin (OPN); these features were especially prominent in a CD153⁺ subset among them (20, 21). Notably, CD153⁺ SA-T cells robustly accumulate prematurely in spontaneous GCs of lupus-prone New Zealand Black/White F1 (f-BWF1) mice (20). The CD153⁺ SA-T cells are apparently autoreactive because they are specifically activated to produce OPN in response to autologous GC B cells of f-BWF1 mice in a TCR/MHC-II–dependent manner, suggesting that autoreactive B cells may function as effective APCs for these T cells (20).

In this study, we show that OPN induces prolonged Rac1 activation via integrin αβ3 in phagocytes and thereby inhibits the engulfment process of bound apoptotic cells by phagocytes. We propose that OPN secretion by CD153⁺ SA-T cells in lupus-prone mice plays a key role in the progression of autoreactive GC reactions by supporting a continuous supply of autoantigens from unengulfed apoptotic cells.

Materials and Methods

Mice

BWF1 and C57BL/6N (B6) mice were purchased from Japan SLC (Shizuoka, Japan). B6.TC (B6.NZMSle1/Sle2/Sle3) mice and Spp1–/– mice were purchased from The Jackson Laboratory. Generation of enhanced GFP (EGFP)-OPN reporter mice was described previously (20). B6.TC/EGFP-OPN reporter mice were generated by backcrossing EGFP-OPN reporter mice into B6.TC genetic background. All mice were maintained under specific pathogen-free conditions at the Center for Experimental Animals of Kyoto University, and the animal experiments were performed in accordance with institutional guidelines.

Cell and cultures

Bax−/− or Bax−/+, Bax−/−, and Bax−/− were briefly, thymocytes from 4- to 6-wk-old B6 mice were treated with 70 U/ml recombinant OPN (R&D Systems) was incubated at 37°C for the indicated periods on plates coated with N-half OPN or MFG-E8. For blocking of integrin αβ3, BaF/Rac/β cells were incubated with cRGDfV at room temperature for 2 h before incubation with N-half OPN. Cells were lysed with lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM MgCl₂, 1% Nonidet P-40, and 5% glycerol) containing protease inhibitor mixture (Complete EDTA-free tablets; Roche), and the resultant lysates were incubated with PAK-1 PBD protein (Merck Millipore) for 45 min on ice, followed by incubation with glutathione–Sepharose-4B beads for 30 min on ice. The beads were washed three times with lysis buffer and immunoblotted with anti-Rac1 Ab (Merck Millipore).

Fluorescence resonance energy transfer image analysis

NIH3T3/integrin cells were cotransfected with pPBbsr2-Raichu-Rac1 and pCMV-mPBase by Lipofectamine 2000 (Thermo) and selected with 10 μg/ml blastocidin. The cells were plated on 35-mm glass-bottom dishes and cultured in low-background DMEM (FluoroBrite DMEM; Thermo) containing 5% FBS. Cells were imaged on an IX81 inverted microscope (Olympus, Tokyo, Japan) equipped with a UPlanSapo ×40 objective lens, a CoolLED preciscExcite LED illumination system (Molecular Devices), and a cooled charge-coupled device camera (DOC-Cam HR-M, Molecular Devices). The following filter sets (excitation and emission filters; μ Optical) were used: 440AF21 and 480AF30 for CFP, 440AF21 and 535AF25 for YFP, and 580AF20 and 636DF55 for RFP. A 455DLRP dichroic mirror (μ Optical) was used for CFP and YFP, and an 80006sbs dichroic mirror (Chrome Technology) was used when RFP fluorescence was monitored with fluorescence resonance energy transfer (FRET) probes. At each time point, three to four images were acquired with the following exposure times at binning 4 × 4: CFP, 300 ms; YFP, 300 ms; RFP, 400 ms; and differential interference contrast, 50 ms. Ratio images of YFP/CFP reflecting FRET efficiency were generated with MetaMorph software (Molecular Devices) and displayed in eight-color mode (red to blue) in the intensity-modulated display mode.

Phagocytic cup capture

NIH3T3/integrin cells (3.8 × 10⁶ cells total) with Raichu-Rac1 were transfected with CellLight Actin-RFP, BacMam 2.0 (Thermo), on poly-L-lysine-coated 35-mm glass-bottom dishes. Twenty hours later, 2 × 10⁶ apoptotic thymocytes were added to the sample in the presence of 0.1 μg/ml MFG-E8, with or without 10 μg/ml N-half OPN in FluoroBrite DMEM containing 5% FBS. Images were acquired every minute on an Olympus IX81.

INHIBITION OF APOPTOTIC CELL ENGULFMENT BY OSTEOPONTIN
Cell transfer
Sorted T cell subpopulations from 37-wk-old f-BWF1 mice were suspended in temperature-sensitive biogel (Mebiol Gel; Mebiol) at 4°C and directly injected into the spleens of anesthetized 6-wk-old f-BWF1 mice.

Ab administration
Anti-OPN mAb, 35B6, was provided by T. Uede (Hokkaido University, Sapporo, Japan). The 35B6 or isotype-matched mouse IgG (R&D Systems) was administered, as described previously (20).

TLR ligand administration
Twelve-week-old female B6 mice or female Spp1−/− mice were injected i.p. with 2.5, 5, or 7.5 μg R848 (InvivoGen) three times per week for 1–2 mo. Twelve-week-old B6 mice were injected i.p. with 5 μg poly(I:C) (InvivoGen) or 1 μg LPS (Escherichia coli O26; B6; Sigma-Aldrich) three times per week for 1 mo.

Quantitative PCR
Quantitative PCR was performed as described previously (20). Primer sequences for genes were as follows: Spp1, 5′-CCCGTGAAGTGACGTGAT-3′ and 5′-TTCTCTAGGAGGACACACAGTC-3′; MFG-E8, 5′-GCTGATGATCGAGAGGAAA-3′ and 5′-TCTGTTCGCCAGGTCAAC-3′; Merk, 5′-GTAGTTGTCTGGGCCCCACT-3′ and 5′-CTGAGTC-TAGCTGCTGGTCCTTC-3′; Bcl6, 5′-TTCTGCTGACCAAGGGCAAC-3′ and 5′-CAGGATAGGGTTGTTCTACAC-3′; Cdkn2b, 5′-ACAACCTGTCATTTGAAGAAGG-3′ and 5′-CCTCCCACACACACACTAA-3′; and Sostdc1, 5′-AAGCATCACCTGATACAGG-3′ and 5′-CAGGCCCACCTGAAC-3′.

Statistical analyses
Statistical analyses were performed using Student t test.

Results
The number of unengulfed apoptotic cells associated with TB M̃ ̃bs increases over the course of disease progression in f-BWF1 mice
Histologically, overt GCs became spontaneously detectable in the spleens of lupus-prone f-BWF1 mice starting at 20 wk of age, and the numbers of CD95+ GL-7+ GC B cells as well as the mean sizes of GCs increased steadily thereafter, followed by a robust increase in the level of plasma anti-dsDNA Ab (Fig. 1A). We first investigated apoptotic cells in spleens of f-BWF1 mice at various ages by immunostaining. TUNEL+ cells were mostly detected in GC regions in association with CD68+ cells (TB M̃ ̃bs) by immunostaining. TUNEL+ cells were mostly detected in GC regions in association with CD68+ cells. The majority of TUNEL+ cells (green colored) were not fragmented, indicating that the apoptotic cells were outside TB M̃ ̃bs and had not undergone phagocytosis (Fig. 1B). The GCs actively induced in B6 mice by immunization with SRBC-B6 contained significantly fewer TUNEL+ cells associated with CD68+ cells than those in f-BWF1 mice at all ages older than 25 wk (Fig. 1A, 1B). Furthermore, regardless of the ages of f-BWF1 mice, the average number of unengulfed TUNEL+ cells per CD68+ cell progressively increased as disease progressed until 35 wk of age (Fig. 1A, 1B). The majority of TUNEL+ cells (green colored) were not fragmented, indicating that the apoptotic cells were outside TB M̃ ̃bs and had not undergone phagocytosis (Fig. 1B). The GCs actively induced in B6 mice by immunization with SRBC-B6 contained significantly fewer TUNEL+ cells associated with CD68+ cells than those in f-BWF1 mice at all ages older than 25 wk (Fig. 1A, 1B). The majority of TUNEL+ cells (green colored) were not fragmented, indicating that the apoptotic cells were outside TB M̃ ̃bs and had not undergone phagocytosis (Fig. 1B). The GCs actively induced in B6 mice by immunization with SRBC-B6 contained significantly fewer TUNEL+ cells associated with CD68+ cells than those in f-BWF1 mice at all ages older than 25 wk (Fig. 1A, 1B). Furthermore, regardless of the ages of f-BWF1 mice, the average number of unengulfed TUNEL+ cells per CD68+ cell in a given GC was highly correlated with GC size (Fig. 1C). The results suggest that progressive expansion of spontaneous GCs in GCs plays a role in the accumulation of unengulfed apoptotic cells on TB M̃ ̃bs.

OPN in GCs plays a role in the accumulation of unengulfed apoptotic cells in f-BWF1 mice
Binding and phagocytosis of apoptotic cells by TB M̃ ̃bs is associated with increasing accumulation of unengulfed apoptotic cells in f-BWF1 mice.

OPN inhibits MFG-E8−mediated phagocytosis of apoptotic cells by inducing sustained Rac1 activation via integrin αvβ3
Next, because MFG-E8 plays a nonredundant role in GCs (9), we directly examined the effects of OPN on the binding and phagocytosis of apoptotic cells mediated by MFG-E8. To this end, we used Ba/F3 cells expressing Rac1 and integrin αvβ3 (Ba/F3/Rac/αvβ3) (22). Ba/F3/Rac/αvβ3 cells were incubated with Fas ligand−treated thymocytes labeled with pHrodo to allow flow cytometric detection of apoptotic cell phagocytosis. The addition of MFG-E8 (0.1 μg/ml) caused significant engulfment of apoptotic cells, although the efficiency was rather low, most likely due to the lack of Tim4 expression in Ba/F3/Rac/αvβ3 cells (Fig. 3A). Nonetheless, the MFG-E8−mediated engulfment of apoptotic cells was significantly inhibited in the presence of an active form of OPN, N-half OPN, generated by the treatment with thrombin at 1 μg/ml or more (Fig. 3A). However, N-half OPN had no effect on MFG-E8−mediated binding of Ba/F3/Rac/αvβ3 cells to PtdSer, although the binding was inhibited in the presence of integrin inhibitor cRGDfV (Fig. 3B). These results suggested that OPN selectively inhibits the engulfment of apoptotic cells without affecting their binding to phagocytes.

Apoptotic cell engulfment requires transient activation of Rac1 at binding sites (25). We found that N-half OPN induced Rac1 activation in Ba/F3/Rac/αvβ3 cells in the absence of apoptotic cells or MFG-E8, which persisted for at least 60 min (Fig. 3C). OPN−induced Rac1 activation was completely inhibited in the presence of cRGDfV, suggesting that OPN directly activates Rac1 via integrin αvβ3 (Fig. 3C). In the absence of apoptotic cells, MFG-E8 caused no detectable Rac1 activation (Fig. 3C). To determine whether such unusually persistent Rac1 activation occurs in individual OPN-stimulated cells, we visualized Rac1 activation using NIH3T3/integrin cells expressing the Raichu-Rac1 FRET biosensor (NIH/αβ/Raichu-Rac1). After addition of N-half OPN, a NIH/αβ/Raichu-Rac1 cell exhibited strong and prolonged Rac1 activation lasting for 20 min or more at multiple sites of lamellipodia, where portals for apoptotic cell engulfment exist; thus, a single cell exhibited overall Rac1 activation for at least 60 min in the presence of OPN (Fig. 3D).
When apoptotic cells were added to NIH/αβ/RaiChu-Rac1 cells in the presence of MFG-E8, Rac1 activation occurred at sites of apoptotic cell binding called portals. There, a thick actin-based ring structure surrounding the apoptotic cell, termed the phagocytic cup, was formed. Within 10 min, local Rac1 activation dissipated, and the apoptotic cells remained unengulfed (Fig. 4A, upper). In the presence of N-half OPN, however, strong Rac1 activation and rather disorganized phagocytic cups persisted at the apoptotic cell binding sites for >20 min, and the apoptotic cells remained unengulfed (Fig. 4A, lower, Supplemental Video 1). The failure of phagocytic cup resolution was observed frequently in phagocytic portals in the presence of N-half OPN (Fig. 4B). Together, these observations strongly suggest that OPN inhibits the engulfment of apoptotic cells by inducing potent and sustained Rac1 activation via integrin αvβ3, thereby interfering with resolution of the phagocytic cup.

**CD153+ SA-T cells are the main source of OPN causing accumulation of apoptotic cells in GCs of f-BWF1 mice**

To identify the cells responsible for the production of OPN in the GCs of lupus-prone mice, we established EGFP-OPN reporter mice in the B6.TC background, which harbors Sle1, Sle2, and Sle3 loci and develops lupus disease (26). Immunostaining analysis revealed that GFP+ cells were present almost exclusively in the splenic PNA+ GC regions of B6.TC/EGFP-OPN reporter mice (Fig. 5A, left). Flow cytometric analysis indicated that GFP expression was mostly confined to a CD153+, but barely CXCR5+, cell population among PD-1+ CD4+ T cells (Fig. 5A, right). To confirm functional involvement of the follicular CD153+ SA-T cells in apoptotic cell clearance in GCs, we performed cell transfer analysis. We sorted subfractions of CD4+ T cells, PD-1+, PD-1+ CD153+ CXCR5−, and PD-1+ CD153+ CXCR5+, from 35-wk-old f-BWF1 mice and injected them directly into spleens of 8-wk-old f-BWF1 mice in temperature-sensitive biogel. Three weeks after cell transplantation, only the recipients of PD-1+ CD153+ CXCR5− CD4+ cells showed significantly more accumulation of TUNEL+ cells on CD68+ cells compared with control mice (Fig. 5B). These results suggest that CD153+ SA-T cells are activated for Spp1 expression in situ in GCs of lupus-prone mice and are responsible for the inhibition of apoptotic cell phagocytosis by TB Møs.

Spp1<sup>physb</sup> CD153+ SA-T cells are increased by TLR7 ligand administration in vivo and are involved in ANA production

Accumulating evidence indicates that signaling of TLR7, which recognizes ssRNA, is essential for spontaneous GC development and overt ANA production, in which B cells play a primary role (27). Notably, overexpression of TLR7 in B cells results in development of a broad range of ANAs, including those against non-RNA–associated autoantigens (27, 28). Because the development of CD153+ SA-T cells depends on B cells (20), we investigated the
involvement of CD153+ SA-T cells and OPN in TLR7-mediated GC development and ANA production. We confirmed that continuous administration of the TLR7 ligand R848 induced GC reactions and significant production of anti-histone Ab in B6 mice, whereas poly(I:C) (TLR3 ligand) or LPS (TLR4 ligand) had little effect (Fig. 6A). Although the R848 recipients exhibited a transient increase in the number of regular CXCR5+ PD-1+ CD4+ T follicular helper cells (Fig. 6A), these mice also exhibited even more sustained and progressive increase in CD153+ PD-1+ CD4+ T cells (Fig. 6B). We confirmed that the pattern of gene expression in CD153+ PD-1+ CD4+ T cells was similar to that of CD153+ SA-T cells in f-BWF1 mice, including prominent upregulation of Cdkn2b, Spp1, and Sostdc1; CD153+ SA-T cells (including CXCR5+ T follicular helper cells) exhibited only marginal expression of Cdkn2b and Spp1 (Fig. 6C). To examine the involvement of OPN in R848-induced ANA production, we next used Spp1−/− mice. R848-treated Spp1−/− mice exhibited significantly less accumulation of unengulfed apoptotic cells on TB Ms in GCs than R848-treated wild-type B6 mice (Fig. 6D). Consistent with the results, the development of anti-histone H2B Ab was nearly completely suppressed in the R848-treated Spp1−/− mice, although slight decrease in total IgG2c was statistically insignificant (Fig. 6E). Notably, CD153+ SA-T cells in R848-treated Spp1−/− mice were also decreased to the levels in untreated B6 mice, whereas total GC B cells were reduced only marginally (Fig. 6E), suggesting that OPN was also involved in the increase in CD153+ SA-T cells via R848, either directly or indirectly. In either case, these results indicate that OPN derived from CD153+ SA-T cells plays a crucial role in promoting endogenous GC reaction and overt ANA production by TLR7-driven B cells.

**Discussion**

Production of autoantibodies against nucleic acids and other related nuclear components is a hallmark of SLE and is associated
with spontaneous development of GCs (29). In mouse models of SLE, nucleic acid–sensing TLRs such as TLR7 and TLR9 in B cells play important roles in the GC reaction of autoreactive B cells, either positively or negatively (27, 30, 31). Specific stimulation of autoreactive B cells via the BCR is also crucial for the sustained expansion of GCs, leading to the development of class-switched, high-affinity pathogenic autoantibodies (32). Accumulating evidence suggests that defective clearance of apoptotic cells, which are potential sources of intracellular autoantigens, may underlie lupus disease (2, 12, 33), although the exact mechanism remains elusive.

In this study, we confirmed that lupus-prone f-BWF1 mice exhibit a progressive increase in the apoptotic cells in GCs with age. The majority of apoptotic cells were associated with TB Møs, apparently without being engulfed, and the average number of unengulfed apoptotic cells on TB Møs was well correlated with the size of a given spontaneous GC. It is reported that defects in genes involved in specific phagocytosis of apoptotic cells, such as Mfge8 and Merit, result in spontaneous GC development and ANA production, reminiscent of lupus-prone mice (15, 24). However, expression of these genes in f-BWF1 mice was essentially comparable to that in B6 mice. Instead, we found that expression of Spp1 was remarkably elevated specifically in spontaneous GC regions of f-BWF1 mice as the disease progressed, but not in SRBC-induced GCs in B6 mice. Moreover, the extent of Spp1 expression closely paralleled the increase in TB Mø–associated unengulfed apoptotic cells in individual GCs. Furthermore, administration of anti-OPN Ab in vivo in f-BWF1 mice significantly decreased the accumulation of unengulfed apoptotic cells on TB Møs in GCs, consistent with the reduced GC expansion and ameliorated ANA production and nephritis by the treatment with anti-OPN Ab (20). Notably, neither increase in Spp1 expression nor apoptotic cell accumulation was observed in GCs induced by SRBC immunization in young f-BWF1 mice (K. Sakamoto and M. Hattori, unpublished observations). These results suggested that the remarkably elevated Spp1 expression in GCs is specifically linked to the impaired phagocytosis of apoptotic cells in the spontaneous GC reaction of lupus-prone mice.

We found that an active form of OPN (N-half OPN) significantly inhibits the MFG-E8–mediated apoptotic cell phagocytosis in vitro.
Importantly, however, N-half OPN did not affect specific binding of MFG-E8 to PtdSer or MFG-E8–mediated binding of apoptotic cells to integrin $\alpha v\beta 3$-expressing BaF cells. This was rather unexpected because it is reported that both OPN and MFG-E8 bind to integrin $\alpha v\beta 3$ with comparable affinity, at least in cell-free conditions (34). However, MFG-E8 does not bind to integrin $\alpha v\beta 3$-expressing cells in the absence of PtdSer, which markedly increases its adherence to $\alpha v\beta 3$-expressing cells (9). Also, the binding of apoptotic cells with MFG-E8 apparently occurs at limited numbers of portals on the phagocyte surface, rather than randomly (25). Furthermore, OPN induces Rac1 activation via integrin $\alpha v\beta 3$, but free MFG-E8 does not. Thus, binding of apoptotic cell/MFG-E8 to integrin $\alpha v\beta 3$ on phagocytes may occur in a context distinct from that of OPN, avoiding simple competitive inhibition of integrin $\alpha v\beta 3$ binding. In any case, these results strongly suggested that OPN specifically affects the engulfment process of apoptotic cells specifically bound to phagocytes, and it is consistent with the accumulation of unengulfed apoptotic cells, mostly on top of TB Mfs in vivo, in f-BWF1 mice. The engulfment of apoptotic cells occurs at the selective lamellipodial sites of phagocytes, where a ring of F-actin is formed surrounding the apoptotic cell. This structure, called the phagocytic cup, is associated with local Rac1 activation and integrin recruitment to form the engulfment synapse. As soon as the apoptotic cell sinks into the cup, Rac1 is inactivated, causing breakdown of the phagocytic cup and apoptotic cell engulfment; the process is quite rapid and is complete within several minutes (25, 35, 36). Thus, the dynamic actin reorganization involved in engulfment is controlled by finely tuned Rac1 activation, followed by inactivation at binding sites. Consequently, either defective Rac1 activation or impaired inactivation of Rac1-GTP results in the failure of apoptotic cell engulfment (10, 25). FRET imaging of Rac1 activity at the single-cell level revealed that N-half OPN induces potent and prolonged Rac1 activation, which is distributed diffusely at multiple sites of lamellipodial regions of phagocytes. This effect is mediated via integrin $\alpha v\beta 3$ in phagocytes, but is independent of either MGF-E8 or apoptotic cells. Prolonged Rac1 activation in the presence of N-half OPN did not affect the phagocytic cup formation, but it remarkably delayed the dissolution of phagocytic cups and impaired the completion of apoptotic cell engulfment in much the same way as phagocytes transduced with a constitutively active Rac1 mutant gene. The apoptotic cells left unengulfed on TB Mfs are expected to eventually rupture via secondary necrosis (11), thereby releasing intracellular contents into the GC microenvironment.

The results of this study unambiguously show that follicular CD153$^+$ SA-T cells are responsible for OPN production in spontaneous GCs of lupus-prone mice. Thus, in EGFP-$Spp1$ reporter mice in the lupus-prone genetic background, CD153$^+$ CXCR5$^+$ PD-1$^+$ CD4$^+$ T cells in GCs preferentially exhibited significant activation of $Spp1$. Furthermore, only CD153$^+$ SA-T cells induced conspicuous accumulation of unengulfed apoptotic cells on TB

**FIGURE 4.** OPN inhibits apoptotic cell phagocytosis by interfering with dissolution of the phagocytic cup due to prolonged Rac1 activation. (A) Fas ligand–treated thymocytes were added to the culture of NIH/$\alpha v\beta 3$Raichu–Rac1 cells transfected with RFP actin in the presence of 0.1 $\mu$g/ml MFG-E8 with or without 10 $\mu$g/ml N-half OPN, and differential interference contrast images were obtained every 1 min. Images of differential interference contrast, RFP (F-actin), and FRET/CFP (Rac1 activation) at the indicated time points (minutes) are indicated. Solid arrowheads indicate apoptotic cells bound to phagocytes. Actins forming phagocytic cups are indicated with yellow arrowheads, and FRET signals are shown with white arrowheads. Black arrowheads indicate engulfed cells. (B) Intensities of RFP images at apoptotic cell binding sites in the absence (blue lines) or presence (red lines) of N-half OPN were quantitated at the indicated time points. Results from three independent regions are indicated.
Mφs in GCs when transferred in vivo in young f-BWF1 mice. A major question, then, is how CD153+ SA-T cells are induced and activated in spontaneous GCs. We previously reported that the generation of CD153+ SA-T cells in vivo depends on the presence of B cells, and that these cells from f-BWF1 mice are induced to secrete OPN in response to autologous GC B cells in vitro in a TCR/MHC-II–dependent manner, suggesting that GC B cells function as efficient APCs for CD153+ SA-T cells (20). In contrast, TLR7-driven activation of potentially autoreactive B cells plays an essential role in GC reactions and ANA production (27, 28, 37). Thus, it seems unlikely that TLR ligands simply function as RNA-related autoantigens; rather, diverse endogenous nuclear Ags must be involved in the process. Our results indicated that R848-treated Spp1−/− B6 mice exhibited a significant decrease in the proportion of unengulfed apoptotic cells in GCs compared with wild-type B6 mice, and this effect was associated with almost complete suppression of anti-histone Ab. The results are consistent with the notion that nuclear Ags derived from unengulfed apoptotic cells in GCs make a major contribution to the sustained expansion of TLR7-driven autoreactive B cells via BCR stimulation. Interestingly, the R848-induced increase in CD153+ SA-T cells was also suppressed in

Excessive TLR7 signaling in B cells results in the production of a wide repertoire of ANAs, including anti-dsDNA, anti-histone, and anti-nucleosome Abs, in addition to an acidic nuclear protein (Smith) Ag/ribonucleoprotein Ab (27, 28, 37). Thus, it seems unlikely that TLR ligands simply function as RNA-related autoantigens; rather, diverse endogenous nuclear Ags must be involved in the process. Our results indicated that R848-treated Spp1−/− B6 mice exhibited a significant decrease in the proportion of unengulfed apoptotic cells in GCs compared with wild-type B6 mice, and this effect was associated with almost complete suppression of anti-histone Ab. The results are consistent with the notion that nuclear Ags derived from unengulfed apoptotic cells in GCs make a major contribution to the sustained expansion of TLR7-driven autoreactive B cells via BCR stimulation. Interestingly, the R848-induced increase in CD153+ SA-T cells was also suppressed in

**FIGURE 5.** CD153+ SA-T cells show Spp1 activation in GCs of f-BWF1 mice and mediate apoptotic cell accumulation in MCs. (A) Spleen sections from 17-wk-old B6.TC/EGFP-OPN reporter mice were multicolor immunostained with PNA and the indicated Abs (left). Enlarged images of the boxed region are indicated. Dotted region corresponds to the PNA* region in the box. Scale bar, 50 μm. Representative images from three mice are shown. Aliquots of spleen cells were analyzed for the expression of GFP in CD153+ (red box) and CXCR5+ (blue box) populations of PD-1+ CD44high CD4+ cells. The proportions of GFP+ cells are indicated (lower). (B) Subfractions of CD4+ T cells, PD-1− (I), PD-1+ CD153− CXCR5− CD44high CD4+ (II), and PD-1+ CD153− CXCR5− CD44high CD4+ (III), and PD-1+ CD153− CXCR5+ CD44high CD4+ (IV), were sorted from 37-wk-old f-BWF1 mice and injected in temperature-sensitive biogel into the spleens of 6-wk-old f-BWF1 mice at 2 × 10^6 cells per mouse. Control mice received biogel containing no cells (I). Three weeks after transplantation, spleen sections were multicolor immunostained with PNA, TUNEL, and anti-CD68 Abs. Enlarged images of the boxed region are shown. Scale bar, 50 μm. Arrows indicate fragmented apoptotic cells engulfed in CD68+ cells. Representative images from five mice of each group are shown. The numbers of unengulfed TUNEL+ cells per CD68+ cell in GCs were counted for 50–100 CD68+ cells in each group of mice; means and SEs are indicated. **p < 0.01.
Spp1$^{+/−}$ mice. It is possible that OPN might directly act on CD153$^+$ SA-T cells to promote their survival. It is reported that OPN directly promotes the survival of pathogenic CD4$^+$ T cells in mice in an experimental allergic encephalitis model (39). Alternatively, and more likely, the diminished expansion of autoreactive B cells due to limited autoantigens from apoptotic cells resulted in the reduced APC activity required for CD153$^+$ SA-T cell activation. These possibilities are certainly not exclusive of each other.

Overall, our results indicate that CD153$^+$ SA-T cells that produce abundant OPN play an important role in the progression of endogenous autoreactive GC reaction leading to ANA production. OPN derived from CD153$^+$ SA-T cells in GCs inhibits apoptotic cell engulfment by TB M$^+$s and promotes the continuous supply of a variety of nuclear autoantigens to potentially autoreactive B cells, which in turn activate CD153$^+$ SA-T cells as APCs, although the actual Ags are yet to be identified. Because apoptosis...
of GC B cells increases as GCs expand, such processes may comprise an amplification circuit that promotes sustained autoantibody production. Notably, CD153+ SA-T cells also produce significant IFN-γ (20), which may contribute to class switching and somatic hypermutation of autoreactive B cells, thus allowing affinity maturation as well as epitope spreading of autoantibodies. Consistent with female predominance in human SLE, the CD153+ SA-T cells increase much more dramatically in female rather than male BWF1 mice (20), but the reason remains to be investigated. Nonetheless, because such an amplification circuit is apparently unique to the endogenous autoreactive GC reaction, OPN and CD153+ SA-T cells may provide suitable targets for controlling systemic autoimmune diseases, including SLE.

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