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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 $\alpha_v\beta_3$ 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.

Apoptosis 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 macromolecules 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

BAL1, stabilin-2, and Tim4 (5–7). Macrophages may also bind apoptotic cells indirectly via PtdSer-binding adaptor molecules such as MFG-E8, Gas6, and protein S, which are linked to phagocytic receptors such as integrin $\alpha_v\beta_3$ and MerTK (8, 9). Phagocytosis 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.

*Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; [†]Center for Innovation in Immunoregulatory Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; [‡]Department of Pathology and Biology of Diseases, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan; and [§]Department of Biochemistry and Immunology, Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan
ORCID: 0000-0003-3886-3066 (K.S.); 0000-0002-6160-2510 (M.H.).

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Address correspondence and reprint requests to Dr. Nagahiro Minato or Prof. Masakazu Hattori, Department of Immunology and Cell Biology, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan (N.M.) or Center for Innovation in Immunoregulatory Technology and Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan (M.H.). E-mail addresses: minato@imm.med.kyoto-u.ac.jp (N.M.) or hattori@ak.med.kyoto-u.ac.jp (M.H.).

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Abbreviations used in this article: ANA, anti-nuclear Ab; EGFP, enhanced GFP; f-BWFI, female New Zealand Black/White F1; FRET, fluorescence resonance energy transfer; GC, germinal center; OPN, osteopontin; PNA, peanut agglutinin; PtdSer, phosphatidylserine; SA-T, senescence-associated T; SLE, systemic lupus erythematosus; TB Mφ, tingible body macrophage.

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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 heterochromatic 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 female New Zealand Black/White F1 (f-BWFI) 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-BWFI 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 $\alpha_v\beta_3$ 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

BWFI 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

Ba/F3 mouse pro-B cells expressing both Rac1 and integrin $\alpha_v\beta_3$ (BaF/Rac/ $\alpha\beta$) were described previously (22). The cells were maintained in RPMI 1640 containing 10% FBS and 10 ng/ml mouse IL-3. Mouse NIH3T3 cells expressing integrin $\alpha_v\beta_3$ (NIH3T3/integrin) were described previously (9). The cells were grown in DMEM containing 5% FBS.

Flow cytometry

Multicolor flow cytometric analysis and cell sorting were performed with FACS Canto and FACS Aria II/III (BD Biosciences), respectively. The following Abs were used: FITC-conjugated anti-CD153 (Abcam); PE-conjugated anti-CD153 (Affymetrix) and PE-conjugated streptavidin (BD Biosciences); PerCP/Cy5.5-conjugated anti-B220 (BioLegend) and CD11b (BioLegend); PE/Cy7-conjugated anti-CD4 (TONBO) and anti-CD95 (BD Biosciences); allophycocyanin-conjugated anti-PD-1 (BioLegend) and anti-GL7 (Affymetrix); allophycocyanin/Cy7-conjugated anti-CD44 (TONBO) and anti-B220 (TONBO); and biotin-conjugated CXCR5 (BD Biosciences).

Engulfment of apoptotic cells

Engulfment of apoptotic cells was assessed, as described previously (22). In brief, thymocytes from 4- to 6-wk-old B6 mice were treated with 70 U/ml Fas ligand at 37°C for 2 h to induce apoptosis. The cells were washed with PBS and incubated with 0.1 μ g/ml pHrodo (Thermo) at room temperature for 30 min. BaF/Rac/ $\alpha\beta$ cells at 1×10^5 cells/ml were then incubated with 1×10^6 pHrodo-labeled apoptotic cells in the presence of 0.1 μ g/ml MFG-E8 at 37°C for 2 h. The cells were washed, suspended in 20 mM N-cyclohexyl-2-aminoethanesulfonic acid–NaOH buffer (pH 9) containing 150 mM NaCl and 2% FBS, and analyzed with flow cytometry. To examine the effect of OPN, 10 μ g recombinant OPN (R&D Systems) was incubated with 1 U thrombin (GE Healthcare) at 22°C for 16 h for activation (N-half

OPN), and BaF/Rac/ $\alpha\beta$ cells were preincubated with the N-half OPN at varying concentrations for 30 min.

Cell adhesion assay

A MultiSorp plate (Thermo) was coated with 3 μ g/ml PtdSer solution (Sigma-Aldrich) in methanol and air dried, followed by treatment with 1% BSA in PBS and then incubation with MFG-E8 at room temperature for 1 h. BaF/Rac/ $\alpha\beta$ cells were stained with calcein AM (BD Pharmingen) (5 mg/ml) in HBSS at 37°C for 1 h and then preincubated with N-half OPN or the cyclic peptide cRGDFV (Peptides International) (23) at room temperature for 30 min. The cells were transferred into the PtdSer-coated wells and incubated at 37°C for 30 min, followed by quantitation of adherent cells on a fluorescence microplate reader (FLEX station; Molecular Devices).

Immunostaining

Tissues were snap frozen in Optimum Cutting Temperature compound (Sakura). Frozen sections (6 μ m) were fixed serially in 95% ethanol and 100% acetone. The Abs used were anti-CD68 (BioLegend), anti-B220 (BioLegend), anti-CD3 (BD Biosciences), anti-CD4 (BioLegend), anti-PD-1 (BioLegend), anti-GFP (Thermo), and biotinylated peanut agglutinin (PNA; Vector Laboratories). TUNEL staining was performed using an in situ cell death detection kit (Roche). Immunostained sections were mounted in Mowiol (Calbiochem) and examined under a microscope (Carl Zeiss). GC areas were analyzed using the Axio Vision 4.8.2 SP1 software, as described previously (20).

Laser-capture microdissection

Frozen sections (6 μ m) of spleen were affixed to a frame membrane slide, fixed in acetone, and stained using the Histogene immunofluorescence staining kit (Thermo) with biotinylated PNA and PE-conjugated IgD (Affymetrix). After dehydration, regions of interest were dissected on an Arcturus XT system (Thermo).

Rac1GTP pull-down assay

BaF/Rac/ $\alpha\beta$ cells were incubated at 37°C for the indicated periods on plates coated with N-half OPN or MFG-E8. For blocking of integrin $\alpha_v\beta_3$, BaF/Rac/ $\alpha\beta$ cells were preincubated with cRGDFV at room temperature for 30 min 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–Sepharese-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 pPBsr2-Raichu-Rac1 and pCMV-mPBase by Lipofectamine 2000 (Thermo) and selected with 10 μ g/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 IX83 inverted microscope (Olympus, Tokyo, Japan) equipped with a UPlanSApo $\times 40$ objective lens, a CoolLED precisExcite 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; Ω Optical) were used: 440AF21 and 480AF30 for CFP, 440AF21 and 535AF25 for YFP, and 580AF20 and 636DF55 for RFP. A 455DRLP dichroic mirror (Ω Optical) was used for CFP and YFP, and an 86006bs dichroic mirror (Chroma 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 detection

NIH3T3/integrin cells (3.8×10^4 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, 2×10^7 apoptotic thymocytes were added to the sample in the presence of 0.1 μ g/ml MFG-E8, with or without 10 μ g/ml N-half OPN in FluoroBrite DMEM containing 5% FBS. Images were acquired every minute on an Olympus IX81.

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'-CCCGGTGAAAGT-GACTGATT-3' and 5'-TTCTTCAGAGGACACAGCATTC-3'; *MFG-E8*, 5'-GCTGGATAATCAGGGCAAGA-3' and 5'-TCTGAGTGCCAGGT-CAAC-3'; *Mertk*, 5'-GATGGTTCTGGCCCCACT-3' and 5'-CTGATC-TAGCTCGGTCTCTTCC-3'; *Bcl6*, 5'-TTCGCTACAAGGGCAAC-3' and 5'-CAGCGATAGGGTTTCTCACC-3'; *Cdkn2b*, 5'-ACACCTGTC-CATTGAAGAAGG-3' and 5'-CCCCAAACACACACTCAA-3'; and *Sostdc1*, 5'-AACAGACCCCTGAATCAAGC-3' and 5'-CAGCCCACTT-GAACTCGAC-3'.

Statistical analyses

Statistical analyses were performed using Student *t* test.

Results

The number of unengulfed apoptotic cells associated with TB Mφs 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 Mφs), and the average number of 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 Mφs 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 f-BWF1 mice is associated with increasing accumulation of unengulfed apoptotic cells on TB Mφs.

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 Mφs are mediated via specific adaptors and receptors (15, 24). Accordingly, we used laser-capture microdissection to examine the expression of these genes in situ in PNA⁺ GCs and IgD⁺ non-GC follicular regions of f-BWF1 and SRBC-B6 mice. PNA⁺ regions exhibited much higher *Bcl6* expression than IgD⁺ regions in both groups of

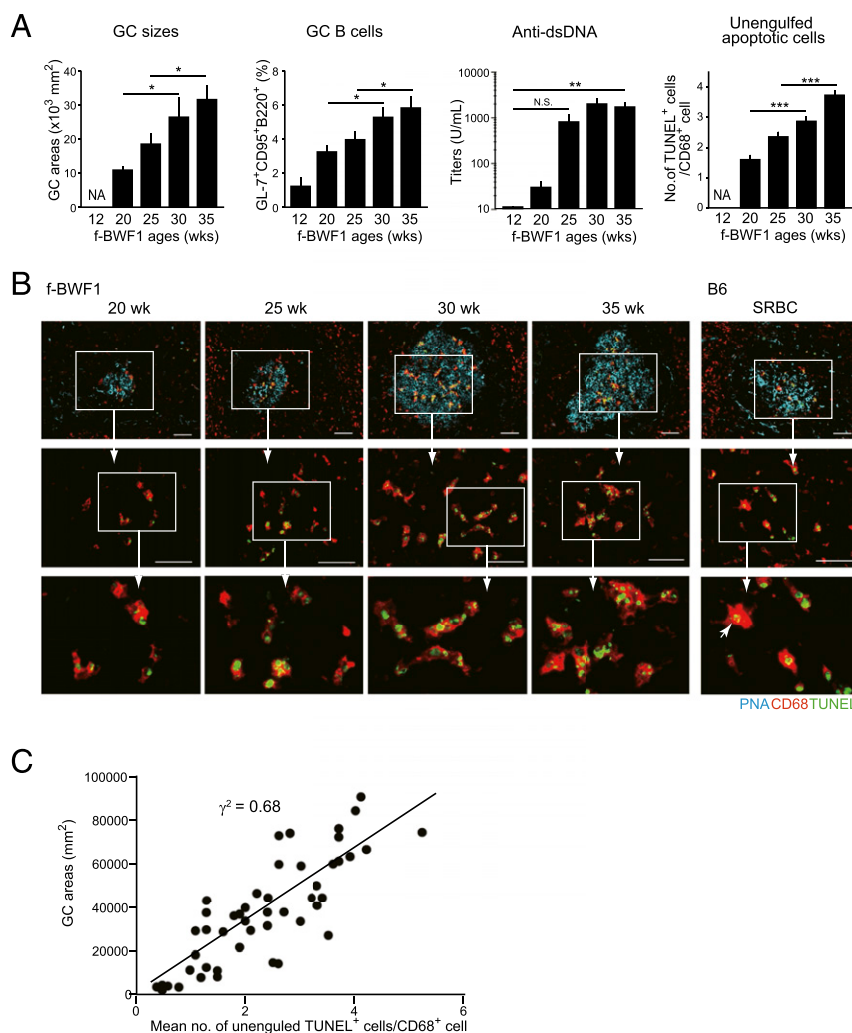
mice, verifying the dissection procedures (Fig. 2A). Expressions of the *Mfge8*, *Pros1*, and *Gas6* genes, which encode adaptor proteins, were largely similar between the two regions, except that *Gas6* was expressed at higher levels than in IgD⁺ regions; no significant difference was observed between f-BWF1 and SRBC-B6 mice (Fig. 2A). Expression of *Mertk* was also comparable in both B cell regions, whereas *Intgav* and *Intgb3* tended to be expressed at higher levels in PNA⁺ than in IgD⁺ regions. Again, no significant difference was observed between f-BWF1 and SRBC-B6 mice (Fig. 2A). PNA⁺ regions of f-BWF1 mice, but not IgD⁺ regions, exhibited remarkably elevated expression of *Spp1* (the gene encoding OPN), whereas neither region expressed *Spp1* at appreciable levels in SRBC-B6 mice (Fig. 2A). Also, *Spp1* expression in GC increased progressively with the age of f-BWF1 mice (Fig. 2A). Furthermore, the level of *Spp1* transcripts in a given GC was highly correlated with the average number of TUNEL⁺ cells per TB Mφ (Fig. 2B). To investigate the possible involvement of OPN in the increased numbers of unengulfed apoptotic cells, we treated f-BWF1 mice with anti-OPN Ab or isotype-matched control IgG twice per week for 4 wk beginning at 16 wk of age. Recipients of the anti-OPN Ab exhibited a significant decrease in the average number of apoptotic cells on TB Mφ in GCs, compared with untreated and control IgG-treated recipients (Fig. 2C). The results strongly suggested that local OPN in GCs plays a significant role in the age-related 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 α_vβ₃

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 α_vβ₃ (BaF/Rac/αβ) (22). BaF/Rac/αβ 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 BaF/Rac/αβ 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 BaF/Rac/αβ 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 BaF/Rac/αβ 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 α_vβ₃ (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).

FIGURE 1. Increase in the number of unengulfed apoptotic cells in TB Mφs in association with disease progression in f-BWF1 mice. **(A and B)** Aliquots of spleens from f-BWF1 mice at the indicated ages in weeks ($n = 6-14$ per group) were made into single-cell suspensions and analyzed for GL-7⁺ CD95⁺ B220⁺ cells (GC B cells). The remainder was frozen sectioned and multi-color immunostained with PNA, anti-CD68, and TUNEL. Sizes of PNA⁺ GC areas (μm^2) were measured in at least two sections per spleen. Spleen sections obtained from SRBC-immunized 8-wk-old B6 mice served as controls. Enlarged images of boxed regions are shown in lower columns. Representative images are shown. Scale bar, 50 μm . Arrow indicates engulfed and fragmented TUNEL⁺ cell. Numbers of unengulfed TUNEL⁺ (apoptotic) per CD68⁺ cell in PNA⁺ GC areas were determined for 50–130 CD68⁺ cells, and mean numbers and SEs are indicated. The bar indicates the value for the PNA⁺ GC area of 20-wk-old f-BWF1 mice. Anti-dsDNA Ab in plasma was measured with ELISA, and means and SEs are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(C)** Mean numbers of unengulfed apoptotic cells on TB Mφ and the mean sizes of PNA⁺ GCs to which they belonged were plotted in f-BWF1 mice at various ages from 20 to 35 wk (total $n = 49$). Correlation efficiency was determined using Pearson's correlation efficiency method. NA, not applicable.



When apoptotic cells were added to NIH/ $\alpha\beta$ /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 this was followed by the rapid dissolution of the actin cup and engulfment of apoptotic cells (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 $\alpha_v\beta_3$, 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). The GFP⁺ cells were costained with PD-1 and CD4, but not with B220 or CD68, and comprised a minor por-

portion (~17%) of follicular PD-1⁺ CD4⁺ T cells at any given time (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 in association with much larger GCs 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 Mφs.

Spp1^{high} 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

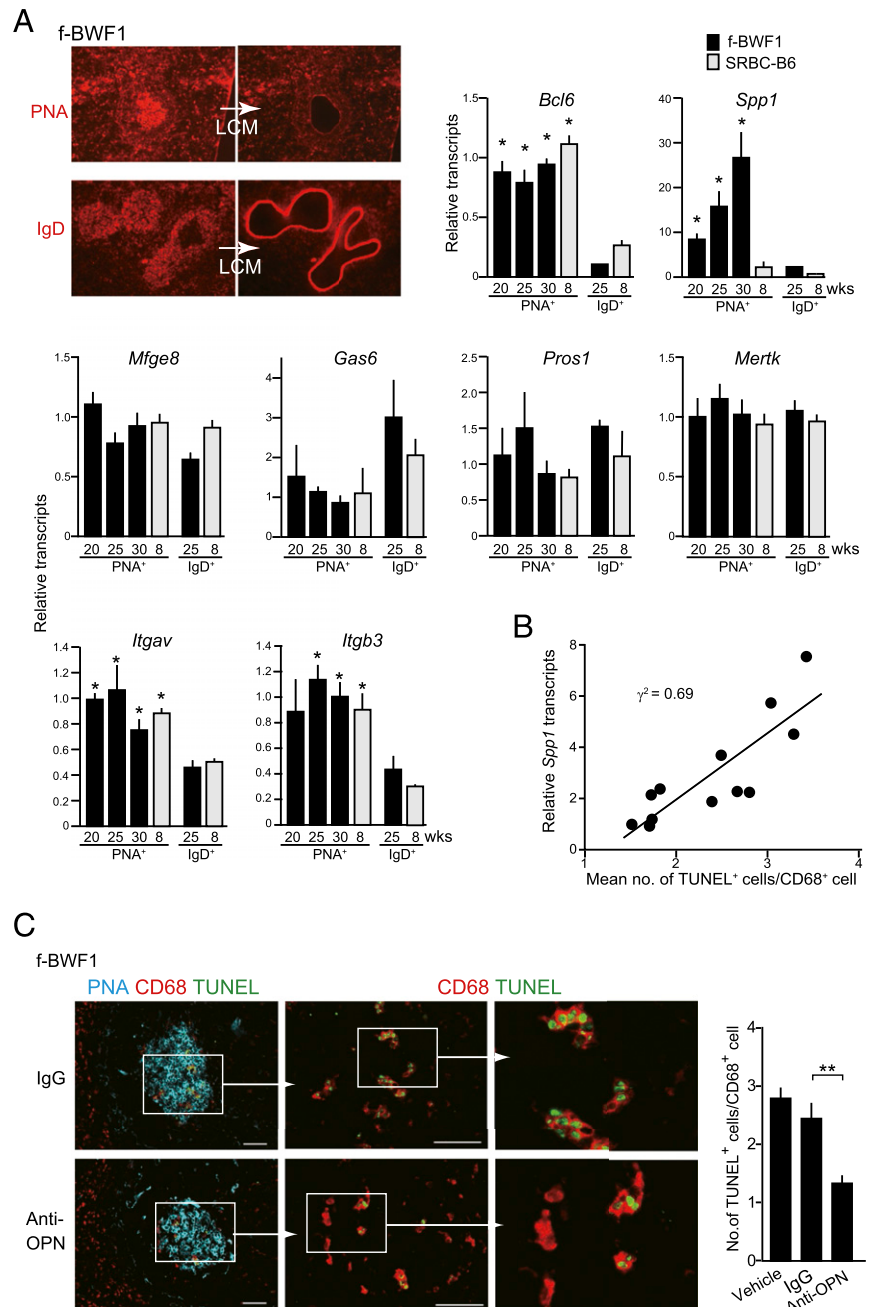


FIGURE 2. OPN in GCs plays a role in the accumulation of unengulfed apoptotic cells on TB Mφs in f-BWF1 mice. **(A)** Spleen sections were prepared from 20-, 25-, and 30-wk-old f-BWF1 mice (solid columns) and 8-wk-old B6 mice immunized with SRBC (gray columns) and stained with PNA or anti-IgD. PNA⁺ and IgD⁺ regions were gouged out by laser-capture microdissection (LCM); RNAs were extracted, and expression of the indicated transcripts was assessed with quantitative RT-PCR. Means and SEs of three to five samples are shown. Statistical analysis was performed to evaluate the difference between PNA⁺ and IgD⁺ regions in each group of mice. * $p < 0.05$. **(B)** The levels of *Spp1* transcripts and mean numbers of unengulfed apoptotic cells on a CD68⁺ cell in PNA⁺ regions of f-BWF1 mice ($n = 3$ –5, per group) at the ages of 20–35 wk were assessed in serial sections and plotted. Each symbol represents a PNA⁺ region. Correlation efficiency was determined by the Pearson correlation efficiency method. **(C)** f-BWF1 mice at 16 wk of age were treated with anti-OPN Ab or normal rat IgG (500 μ g/mouse) twice per week for 4 wk, and spleen sections were multicolor immunostained with PNA, anti-CD68, and TUNEL. Enlarged images of boxed regions are shown. Scale bar, 50 μ m. Numbers of unengulfed TUNEL⁺ cells per CD68⁺ cell in PNA⁺ GCs were counted. Means and SEs for 50–130 CD68⁺ cells in five mice from each group are indicated. ** $p < 0.01$.

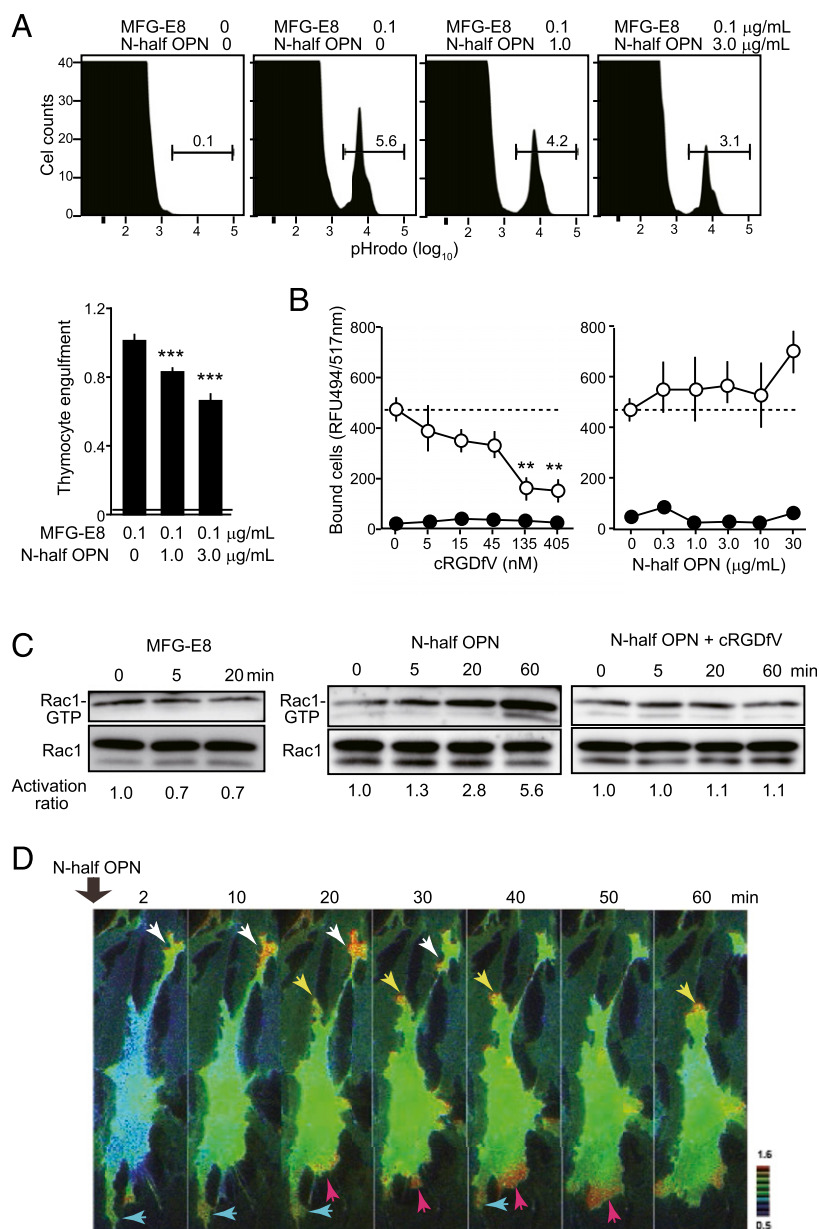
involvement of CD153⁺ SA-T cells and OPN in TLR7-mediated GC development and ANA production. We confirmed that continuous administration of the TLR7 ligand R848 induced GC reactions and significant production of anti-histone Ab in B6 mice, whereas poly(I:C) (TLR3 ligand) or LPS (TLR4 ligand) had little 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⁺ PD-1⁺ CD4⁺ 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 Mφs 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

FIGURE 3. OPN induces diffuse and prolonged Rac1 activation in phagocytes and inhibits MFG-E8-mediated phagocytosis of apoptotic cells. **(A)** BaF/Rac/αβ cells were cultured with pHrodo-labeled Fas ligand-treated thymocytes in the presence of MFG-E8 at 37°C for 2 h with or without the indicated concentrations of N-half OPN. pHrodo⁺ cells engulfed inside the GFP⁺ BaF/Rac/αβ cells were assessed by flow cytometry. The bar indicates the value in the absence of MFG-E8. Representative FACS profiles and relative thymocyte engulfment with means and SEs of normalized phagocytosis are indicated; profiles were obtained in triplicate. ****p* < 0.001. **(B)** Calcein AM-labeled BaF/Rac/αβ cells were incubated with the indicated concentrations of RGD-binding peptide (cRGDFV) or N-half OPN at 37°C for 30 min in microplates coated with BSA (solid circles) or PtdSer (open circles). MFG-E8 (0.1 μg/ml) was included in all culture wells. The numbers of cells adhered to the wells were assessed using a fluorescent microplate reader. Means and SEs of triplicate cultures are indicated. ***p* < 0.01. **(C)** BaF/Rac/αβ cells were starved from IL-3 for 6 h, followed by addition of 10 μg/ml MFG-E8 or incubation for indicated periods of time in N-half OPN-coated plates in the presence or absence of cRGDFV. The cells were recovered and subjected to GTP-Rac1 pull-down assay using PAK-1 PBD, followed by immunoblotting with anti-Rac1 Ab. Relative intensities of Rac1 activation (Rac1GTP/total Rac1) are indicated. Representative images from three independent experiments with similar results are shown. **(D)** N-half OPN (10 μg/ml) was added to the culture of NIH/αβ/Raichu-Rac1 cells with CFP and FRET, and differential interference contrast images were obtained every 2 min. Representative YFP/CFP ratio images obtained at the indicated time points are shown. Colored arrowheads indicate Rac1 activation at the distinct regions.



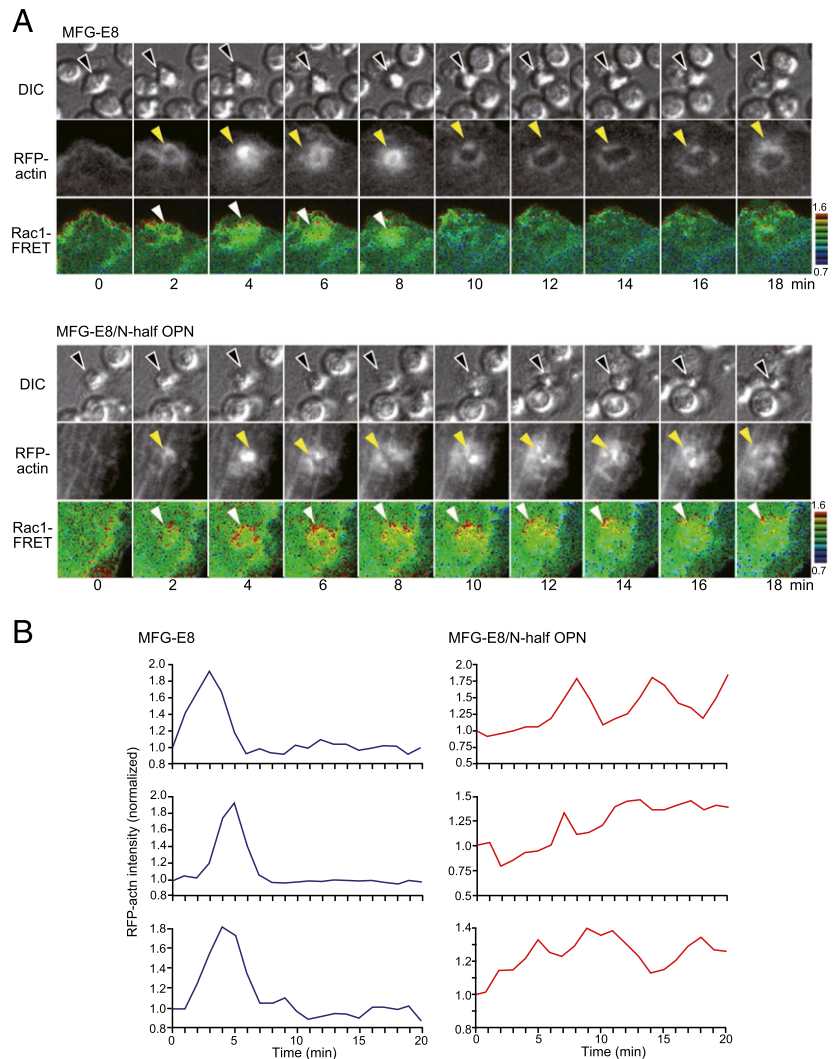
with spontaneous development of GCs (29). In mouse models of SLE, nucleic acid-sensing TLRs such as TLR7 and TLR9 in B cells play important roles in the GC reaction of autoreactive B cells, either positively or negatively (27, 30, 31). Specific stimulation of autoreactive B cells via the BCR is also 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 Mφs, apparently without being engulfed, and the average number of unengulfed apoptotic cells on TB Mφs 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 *Mertk*, 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 Mφ-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 Mφs 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.

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/αβ/Raichu-Rac1 cells transfected with RFP actin in the presence of 0.1 μg/ml MFG-E8 with or without 10 μg/ml N-half OPN, and 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 indicate engulfed cells. 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.



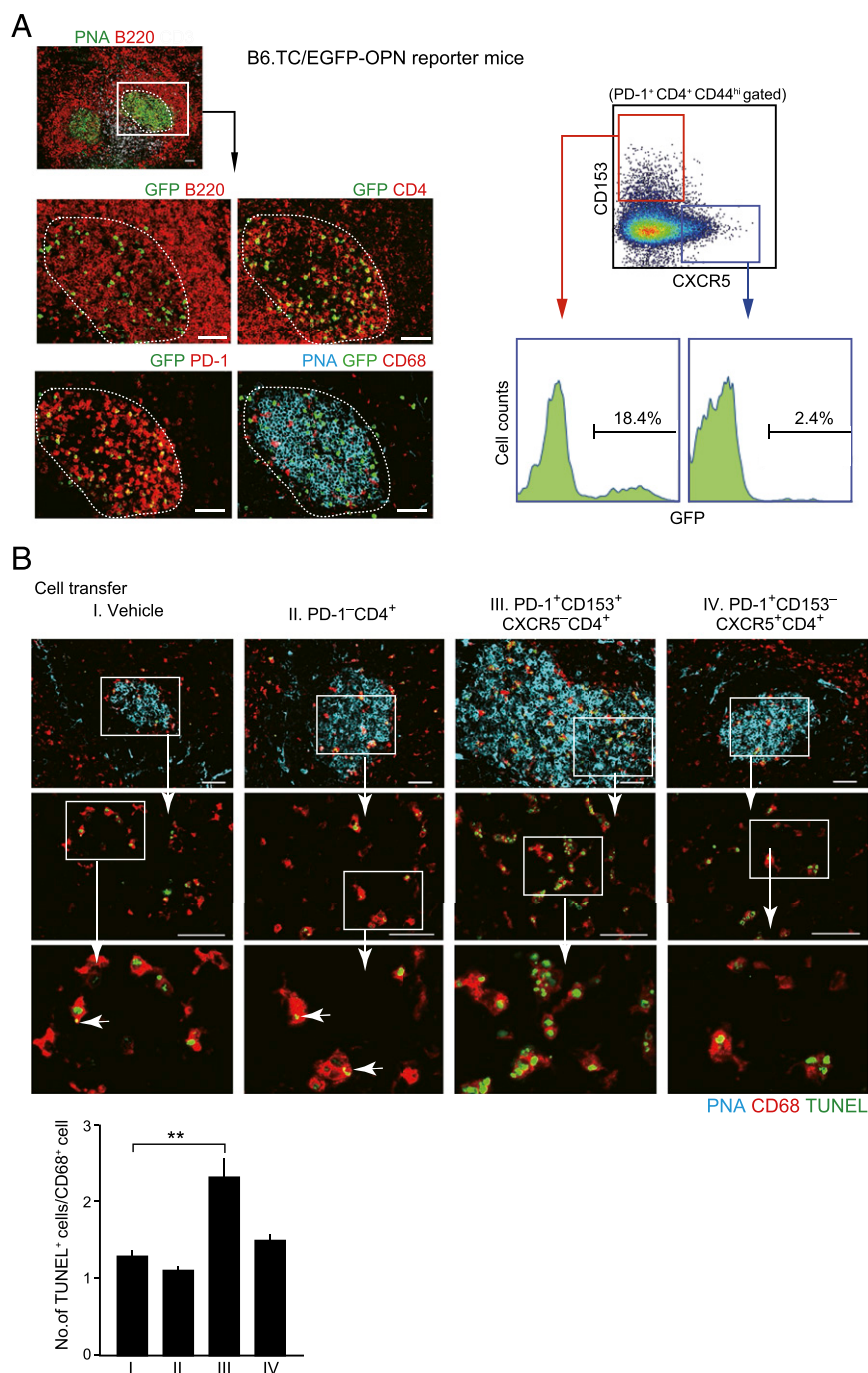
Importantly, however, N-half OPN did not affect specific binding of MFG-E8 to PtdSer or MFG-E8-mediated binding of apoptotic cells to integrin $\alpha_v\beta_3$ -expressing BaF cells. This was rather unexpected because it is reported that both OPN and MFG-E8 bind to integrin $\alpha_v\beta_3$ with comparable 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 Mφs in vivo, in f-BWFL 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 MFG-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 Mφs are expected to eventually rupture via secondary necrosis (11), thereby releasing intracellular contents into the GC micro-environment.

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 5. CD153⁺ SA-T cells show *Spp1* activation in GCs of f-BWFL mice and mediate apoptotic cell accumulation on TB Mφs. **(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⁺ CD4^{high} CD4⁺ cells. The proportions of GFP⁺ cells are indicated (lower). **(B)** Subfractions of CD4⁺ T cells, PD-1[−] (II), PD-1⁺ CD153[−] CXCR5[−] CD44^{high} CD4⁺ (III), and PD-1⁺ CD153[−] CXCR5⁺ CD44^{high} CD4⁺ (IV), were sorted from 37-wk-old f-BWFL mice and injected in temperature-sensitive biogel into the spleens of 6-wk-old f-BWFL 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 Ab. 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. ** $p < 0.01$.



Mφs in GCs when transferred in vivo in young f-BWFL 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-BWFL 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 spontaneous GC reaction and ANA production (27, 28, 37, 38). TLR7 ligand induced a persistent increase in CD153⁺ CXCR5[−] SA-T cells with remarkably high *Spp1* expression in B6 mice, suggesting that the development of CD153⁺ SA-T cells is efficiently induced by TLR7-driven autoreactive B cells.

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

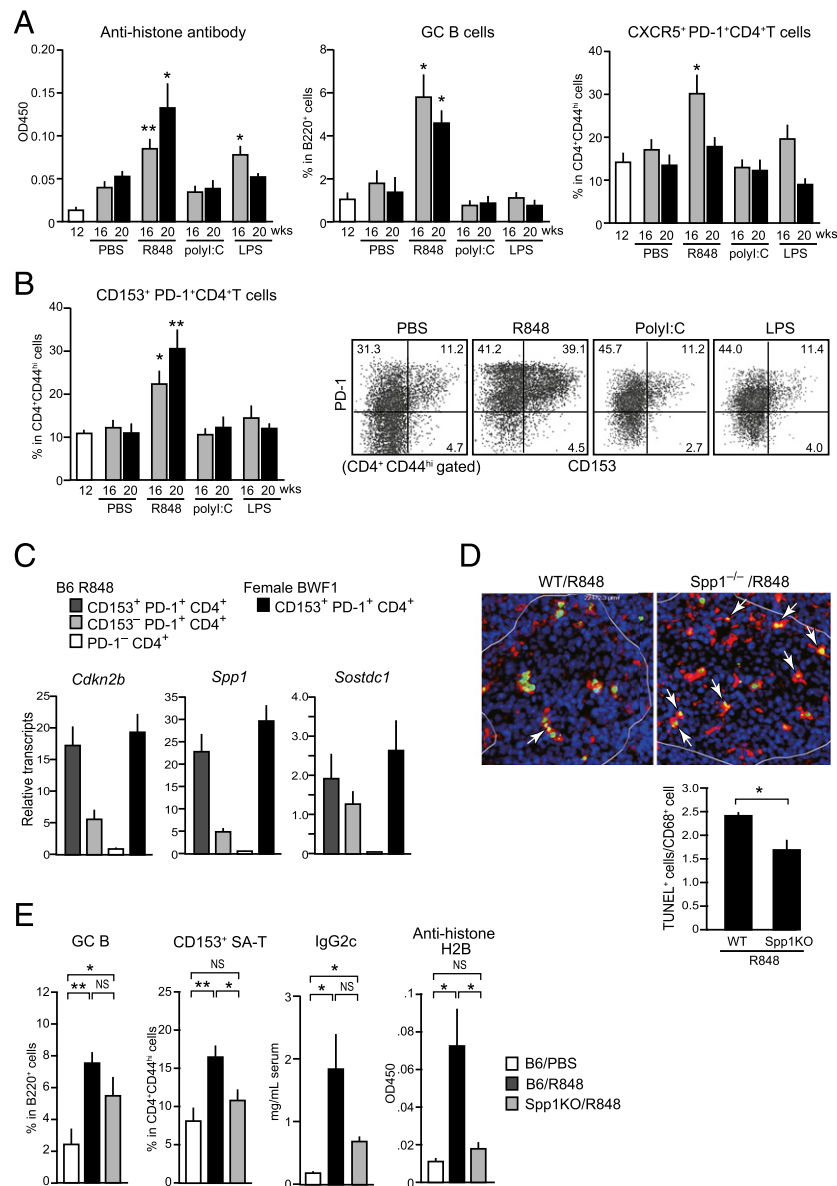


FIGURE 6. *Spp1*^{high} CD153⁺ SA-T cells are induced by TLR7 ligand administration in vivo and are involved in TLR7-driven ANA production via OPN production. (**A** and **B**) Female B6 mice at the age of 12 wk were administered R848 (2.5 μ g/mouse), poly(I:C) (5 μ g/mouse), LPS (1 μ g/mouse), or saline three times per week, and the mice were sacrificed at the age of 16 wk (gray columns) or 20 wk (solid columns). Anti-histone Ab in plasma was assessed with ELISA. The splenocytes were analyzed for the proportions of GL-7⁺ CD95⁺ B220⁺ (GC B) cells, CXCR5⁺ PD-1⁺ CD44^{high} CD4⁺ (T follicular helper) cells, and CD153⁺ PD-1⁺ CD44^{high} CD4⁺ (SA-T) cells. Mean proportions and SEs from five mice are indicated. Representative profile of PD-1 and CD153 expression in CD44^{high} CD4⁺ cell gate in each group is also shown. Statistical analysis was performed to evaluate the differences in mice receiving PBS and TLR ligands. **p* < 0.05, ***p* < 0.01. (**C**) CD44^{high} CD4⁺ T cell subfractions, CD153⁺ PD-1⁺, CD153⁺ PD-1⁺, and PD-1⁺, were sorted from R848-treated f-B6 mice at the age of 20 wk. CD153⁺ PD-1⁺ CD44^{high} CD4⁺ T cells were isolated from 40-wk-old f-BWF1 mice. RNAs were extracted, and expression of the indicated genes was assessed with quantitative RT-PCR. Means and SEs of triplicate determination are shown. (**D** and **E**) Female wild-type or *Spp1*^{-/-} B6 mice at the age of 12 wk were administered R848 (5 or 7.5 μ g/mouse) three times per week. Two weeks later (R848 7.5 μ g/mouse), the spleen sections were multicolor immunostained with PNA, TUNEL, and anti-CD68 Ab. Dotted lines indicate PNA⁺ regions, and arrows show engulfed apoptotic cells (merged yellow and fragmented). Numbers of unengulfed TUNEL⁺ cells (green) per CD68⁺ cell were counted for 50 CD68⁺ cells in each group, and the mean numbers and SEs are also indicated. **p* < 0.05. Four weeks later (R848 5 μ g/mouse), spleen cells were subjected to flow cytometry to determine the proportions of GL-7⁺ CD95⁺ B220⁺ (GC B) and CD153⁺ PD-1⁺ CD44^{high} CD4⁺ (SA-T) cells. IgG2c and anti-histone Ab in plasma were assessed with ELISA. Means and SEs of five mice are indicated. **p* < 0.05, ***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 ϕ 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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Disclosures

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