Lupus-like autoimmune disease caused by a lack of Xkr8, a caspase-dependent phospholipid scramblase

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Apoptotic cells expose phosphatidylserine (PtdSer) on their cell surface and are recognized by macrophages for clearance. Xkr8 is a scramblase that exposes PtdSer in a caspase-dependent manner. Here, we found that among the three Xkr members with caspase-dependent scramblase activity, mouse hematopoietic cells express only Xkr8. The PtdSer exposure of apoptotic thymocytes, splenocytes, and neutrophils was strongly reduced when Xkr8 was absent. While wild-type apoptotic lymphocytes and neutrophils were efficiently engulfed in vitro by phagocytes expressing Tim4 and MerTK, Xkr8-deficient apoptotic cells were hardly engulfed by these phagocytes. Accordingly, the number of apoptotic thymocytes in the thymus and neutrophils in the peritoneal cavity of the zymosan-treated mice was significantly increased in Xkr8-deficient mice. The percentage of CD62L<sup>+</sup> senescent neutrophils was increased in the spleen of Xkr8-null mice, especially after the treatment with granulocyte colony-stimulating factor. Xkr8-null mice on an MRL background showed high levels of autoantibodies, splenomegaly with high levels of effector CD4<sup>+</sup> T cells, and glomerulonephritis development with immune-complex deposition at glomeruli. These results indicate that the Xkr8-mediated PtdSer exposure in apoptotic lymphocytes and aged neutrophils is essential for their clearance, and its defect activates the immune system, leading to lupus-like autoimmune disease.

efferocytosis | macrophages | neutrophils | scramblase | senescence

Figure S1

During animal development, many unnecessary or toxic cells die (1). In animals after birth, large numbers of senescent cells, and cells infected by viruses and bacteria, die or are killed by cytotoxic cells. Among several programmed cell death processes (apoptosis, necroptosis, and pyroptosis), apoptosis is important in the physiological cell death during animal development. In apoptosis, various molecules activate a cascade of caspases, the last of which is caspase 3, and cleaves more than 400 cellular substrates, manifesting the hallmarks of apoptosis such as DNA fragmentation, membrane blebbing, and cell shrinkage (2). The apoptotic pathway is used to block necroptosis, and its defect causes abnormal cell accumulation or tissue necrosis, depending on the step at which the apoptosis is blocked (3).

Apoptotic cells are swiftly recognized and engulfed by macrophages before their plasma membrane is ruptured, to prevent the release of intracellular materials that may activate the immune system (4–6). Macrophages engulf apoptotic cells but not living cells, which led to the proposal that apoptotic cells expose an “eat me” signal (7). The process of apoptotic-cell engulfment is called “efferocytosis,” and phosphatidylserine (PtdSer) is the strongest candidate for the eat me signal (5, 8). PtdSer is an abundant phospholipid in the plasma membrane, where it is confined to the inner leaflet by the action of flippases that translocate PtdSer and phosphatidylethanolamine from the outer leaflet (9, 10). We recently identified two P4-type ATPases (ATP11A and ATP11C) that are ubiquitously expressed in the plasma membrane as the flipase (11, 12). These flipases contain caspase-recognition sites in the middle of the molecule and are inactivated by caspase during apoptosis. This caspase-mediated flipase inactivation is necessary but not sufficient for the apoptotic PtdSer exposure (5).

To quickly expose PtdSer on the surface of apoptotic cells, a scramblase that nonspecifically and bidirectionally translocates phospholipids between the inner and outer membrane leaflets has been postulated (9). We previously identified three Xkr members (Xkr4, Xkr8, and Xkr9) as scramblases that are activated in apoptotic cells. These molecules are membrane proteins with 10 transmembrane regions, and carry a caspase-recognition site in the C-terminal region (13, 14). The cleavage of Xkr8 by caspase 3 induces a conformational change that turns on its scramblase activity (15).

In this study, we found that mouse hematopoietic cells express only Xkr8 among the three Xkr members with caspase-dependent scramblase activity. The PtdSer exposure of apoptotic thymocytes, splenocytes, and neutrophils was strongly delayed by Xkr8 deficiency. Accordingly, the efferocytosis of these cells was retarded. We also found that the percentage of senescent CD62L<sup>+</sup> neutrophils in the spleen increased in Xkr8<sup>−/−</sup> mice. The Xkr8-null mice developed autoantibodies [anti-DNA and antinuclear antibodies (ANA)], and suffered from glomerulonephritis. These results indicate that the Xkr8-mediated PtdSer exposure in apoptotic lymphocytes and senescent neutrophils, followed by their engulfment, is a critical step in apoptosis that prevents the release of noxious materials from dying or senescent cells.

Results

Specific Expression of Xkr8 in Mouse Hematopoietic Cells. We previously reported that mouse thymus and spleen express Xkr8, Xkr4, and Xkr9 (13, 14). Here we found by real-time RT-PCR that a substantial level of Xkr4 as well as Xkr9 mRNA was present in the thymus and spleen (Fig. S1). In contrast, cells in the bone marrow (13, 14) and mononuclear leukocytes in the

Significance

Every day in our bodies, billions of neutrophils become senescent and are cleared by macrophages. This number exceeds the number of cells that undergo apoptosis. Xkr8 is a member of the Xkr membrane-protein family and has caspase-dependent phospholipid scramblase activity. Here, we show that not only apoptotic thymocytes and splenocytes, but also senescent neutrophils require Xkr8 for swift clearance in vivo. Xkr8-deficient female mice on the MRL background carried high levels of autoantibodies, accumulated effector CD4<sup>+</sup> T cells, and developed glomerulonephritis. These results indicate that in addition to unengulfed apoptotic cells, uncleared senescent neutrophils contribute to the development of autoimmune diseases.

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To examine the requirement of primary mouse neutrophils/macrophages, confirming that the apoptotic cells were quickly engulfed by macrophages (Fig. 1A and Fig. S2). More than 90% TUNEL-positive cells were associated with CD68+ macrophages, confirming that the apoptotic cells were quickly engulfed by macrophages (18). The thymus of Xkr8-null mice contained twice as many TUNEL-positive cells (1.5%), and the percentage of the TUNEL-positive cells that were not associated with macrophages significantly increased, suggesting that the efferocytosis of Xkr8+/− thymocytes was retarded.

Treating mice with dexamethasone causes massive apoptosis of thymocytes that are cleared by macrophages in the thymus (18). At 12 h after dexamethasone treatment, 60% more TUNEL-positive cells were observed in the thymic cortex of Xkr8−/− mice than in that of wild-type mice (Fig. 3B). Since the sensitivity to dexamethasone-induced apoptosis was similar between the wild-type and Xkr8−/− blood expressed only Xkr8. Since the thymus and spleen consist of not only lymphocytes but also stromal cells, we suspected that the Xkr4 or Xkr9 mRNA was expressed in stromal cells, but not in lymphocytes. In fact, thymocytes and lymphocytes expressed a high level of Xkr8 mRNA, but neither Xkr4 nor Xkr9 mRNA (Fig. S1).

**Xkr8-Dependent Apoptotic PtdSer Exposure and Cell Engulfment by Phagocytes.** To examine the requirement of primary mouse hematopoietic cells for Xkr8 to expose PtdSer during apoptosis, thymocytes and neutrophils were treated with Fas ligand (FasL), while splenocytes were treated with ABT737, a BH3 mimetic that activates the mitochondrial apoptosis pathway (16). As shown in Fig. 1A, the wild-type CD4+CD8+ thymocytes quickly exposed PtdSer upon FasL treatment (within 75 min), while the FasL-induced PtdSer exposure was severely retarded in Xkr8−/− thymocytes. Similar Xkr8-dependent PtdSer exposure was observed in ABT737-treated splenic CD4+ T cells (Fig. 1B) and FasL-treated blood Gr1+ neutrophils (Fig. 1C).

PtdSer-exposing apoptotic cells are engulfed by phagocytes in a PtdSer-dependent manner (5). We recently showed that resident peritoneal macrophages and Kupffer cells engulf apoptotic cells via a PtdSer-receptor Tim4 and a TAM family receptor (17). The ability of these macrophages to perform efferocytosis can be recapitulated using an NIH 3T3-derivative expressing Tim4 and MerTK (TKO-Tim4/Mer) (17). To examine the effect of the Xkr8-null mutation on efferocytosis, thymocytes, CD3+ splenocytes, and blood neutrophils were labeled with pHrodo, treated with an apoptosis inducer, and added to TKO-Tim4/Mer cells at a ratio of 1:2–1:5 (phagocytes:prey). As shown in Fig. 2, the wild-type apoptotic cells were engulfed within 45–60 min, and many pHrodo-positive dead cells were found inside the phagocytes. On the other hand, 65–94% fewer pHrodo-positive dead cells were found in the phagocytes when Xkr8−/− cells were used as prey. These results indicated that the Xkr8-mediated PtdSer exposure is essential for efficient efferocytosis.

**Effect of Xkr8 on the Clearance of Apoptotic Thymocytes in Vivo.** In the thymus, many T cells are eliminated by apoptosis (18). TUNEL staining (19) of the thymus of 5-wk-old wild-type mice for apoptotic cells revealed that about 0.6% of the thymocytes scattered in the cortex were TUNEL positive (Fig. 3A and Fig. S2). More than 90% TUNEL-positive cells were associated with CD68+ macrophages, confirming that the apoptotic cells were quickly engulfed by macrophages (18). The thymus of Xkr8-null mice contained twice as many TUNEL-positive cells (1.5%), and the percentage of the TUNEL-positive cells that were not associated with macrophages significantly increased, suggesting that the efferocytosis of Xkr8+/− thymocytes was retarded.
of the Gr1+/ neutrophils for CD62L revealed two populations, and CD62Llo but not CD62Lhi cells contained caspase 3/7-positive cells (Fig. 4B). The CD62Llo population increased by about 21% in the Xkr8−/− mice. The percentage of the caspase 3/7-positive cells in CD62Llo neutrophils also increased from 5.5 to 7.6%, supporting the idea that the senescent neutrophils underwent apoptosis and were engulfed by macrophages in a Xkr8-dependent manner. To confirm the requirement of Xkr8 in the clearance of CD62Llo senescent neutrophils, mice were treated with granulocyte colony-stimulating factor (G-CSF) once a day for 3 d. This treatment increased the number of blood neutrophils from 1 × 10⁶/mL to 5 × 10⁶/mL in both the wild-type and Xkr8−/− mice. Ten hours after the last injection of G-CSF, the percentage of neutrophils in the spleen was higher by 70% in the Xkr8−/− mice than in the wild-type mice. This increase in the number of neutrophils in the spleen of Xkr8−/− mice was accompanied by an increase in the CD62Llo neutrophils from 42% to 65% (Fig. 4B).

Elevation of Autoantibodies and Glomerulonephritis in Xkr8−/− Mice. The deficiency in clearing apoptotic cells and senescent neutrophils suggested that the Xkr8−/− mice might develop a lupus-like phenotype, but the C57BL/6-Xkr8−/− mice did not develop autoimmunity. Since the MRL mouse strain has an exaggerated response to low-affinity antigens for developing autoimmunity compared with C57BL/6 mice (24), the Xkr8−/− locus was transferred to the MRL strain using speed congenics. After dexamethasone treatment, as found with the C57BL/6 strain, the MRL mice with the Xkr8-null mutation had a defect in clearing apoptotic thymocytes (Fig. S4). Like MRL-lpr mice, which lack Fas, the female MRL-Xkr8−/− mice developed splenomegaly in an age-dependent manner. The size of the spleen of 50-wk-old Xkr8-deficient mice was approximately twice that of the wild-type MRL mice (Fig. S4). About 78% of the CD4+ T cells in the spleen of MRL-Xkr8−/− mice appeared to be activated with a CD44hiCD26lo cell-surface phenotype, which was much higher than that found in the wild-type MRL mice (27% of CD4+ T cells) (Fig. 5B). This high percentage of activated T cells in the MRL-Xkr8−/− mice was comparable to or higher than that observed in MRL-lpr mice (25). The levels of ANAs and anti-dsDNA antibodies in the serum of 40-wk-old female MRL-Xkr8−/− mice were two to three times those in the wild-type littermate serum (Fig. 5C), indicating that B cells against autoantigens were activated by the Xkr8 deficiency. Circulating autoantibodies are known to cause immune-complex deposition in the glomeruli of the kidney. Notably, the glomeruli in 50-wk-old female MRL-Xkr8−/− mice showed substantial IgG deposition (Fig. 5D). This deposition was accompanied by the enlargement and hypercellularity of the glomeruli in about 60% of these mice.

Discussion

The XKR family has nine and eight members in humans and mice, respectively (26), three of which (Xk4, Xkr8, and Xkr9) have caspase-dependent scramblase activity (14). We found that although both Xk4 and Xkr8 mRNAs were present in the mouse spleen and thymus, the thymocytes and splenocytes expressed only Xkr8. Xkr8 is also expressed in embryonal fibroblasts and hepatocytes (13), indicating that Xkr8 is broadly expressed in various cell types, while Xk4 and Xkr9 are expressed in a cell type-specific manner. The genes of XKR family members are scattered on various chromosomes, and the promoter sequences of Xkr4, Xkr8, and Xkr9 have no apparent similarity. Like the human Xkr8 gene (13), the promoter region of the mouse Xkr8 gene is rich in CpG motifs and has several Sp1-binding sites, which is a characteristic of housekeeping genes. Cells in the brain and intestine express Xkr4 or Xkr9 in addition to the ubiquitous Xkr8. Whether different cell populations in these tissues use Xkr4, Xkr8, or Xkr9 for their caspase-dependent scramblase activity remains to be investigated.

**Fig. 3.** Xkr8-null mutation decreases the clearance of apoptotic thymocytes in vivo. (A) Effect of the Xkr8-null mutation on TUNEL-positive thymocytes. Cryosections of the thymic cortex from 5-wk-old wild-type (WT) and Xkr8−/− C57BL/6 mice (n = 3 for each) were stained for TUNEL and DAPI, counterstained with DAPI, and observed by fluorescence microscopy. In Right panels, the area of TUNEL-positive spots was quantified in five fields and expressed as a ratio to the DAPI-positive area. Bar indicates the average value. The TUNEL-positive spots that were not associated with CD8+ area were also quantified and expressed as percentage of the TUNEL-positive area. (B) Six- to 8-wk-old wild-type or Xkr8−/− C57BL/6 (n = 5 for each) were i.p. injected with dexamethasone. Twelve hours later, cryosections of the thymic cortex were stained for TUNEL and DAPI. At Right, the area of TUNEL-positive spots was quantified and expressed as above. *P < 0.05, **P < 0.01, ***P < 0.001; two-sided Student’s t test. (Scale bar, 50 μm.)

**Fig. 4.** Elevation of autoantibodies and glomerulonephritis in Xkr8−/− mice. (A) The in vivo effect of the Xkr8-mediated PtdSer exposure in the clearance of apoptotic neutrophils was examined by zymosan-induced peritonitis, in which a large number of neutrophils are infiltrated into the peritoneal cavity and undergo apoptosis (20, 21). These apoptotic neutrophils are cleared by the macrophages that were subsequently recruited into the peritoneal cavity. As shown in Fig. 4A, at 18 h after the injection of zymosan, the number of neutrophils in the cavity was 3.6 × 10⁶ and 7.2 × 10⁶ cells in the wild-type and Xkr8−/− mice, respectively, indicating that the apoptotic neutrophils generated during peritonitis were not efficiently cleared without Xkr8.

We then examined the effect of Xkr8 on the clearance of senescent neutrophils. Since senescent neutrophils down-regulate CD62L (L-selectin), they can be recognized as a CD62Llo population (22). The number of CD62LloGr1+ cells in the blood was similar between wild-type and Xkr8−/− mice at the age of 7 wk. Senescent neutrophils are cleared in the liver, spleen, and bone marrow (23). The number of Gr1+ neutrophils in the spleen of Xkr8−/− mice was comparable to that in wild-type spleen. Staining thymocytes (Fig. S3), these results suggested that the clearance of dexamethasone-induced apoptotic thymocytes was delayed in the absence of Xkr8.

**Effect of Xkr8 on the Clearance of Neutrophils in Vivo.** The in vivo effect of the Xkr8-mediated PtdSer exposure in the clearance of apoptotic neutrophils was examined by zymosan-induced peritonitis, in which a large number of neutrophils are infiltrated into the peritoneal cavity and undergo apoptosis (20, 21). These apoptotic neutrophils are cleared by the macrophages that were subsequently recruited into the peritoneal cavity. As shown in Fig. 4A, at 18 h after the injection of zymosan, the number of neutrophils in the cavity was 3.6 × 10⁶ and 7.2 × 10⁶ cells in the wild-type and Xkr8−/− mice, respectively, indicating that the apoptotic neutrophils generated during peritonitis were not efficiently cleared without Xkr8.

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Masking PtdSer inhibits the efferocytosis of apoptotic thymocytes by peritoneal macrophages (27), and the loss of a PtdSer receptor (Tim4), PtdSer-binding proteins (Gas6 and Protein S), or TAM receptors (MerTK, Axl, and Tyro3) blocks efferocytosis (17, 28), together indicating that PtdSer exposure is indispensable for efferocytosis. During apoptosis, caspase cleaves and inactivates ATP11A and ATP11C, which normally flip PtdSer from the outer to inner leaflet of the plasma membrane (11, 12). Xkr8’s scramblase activity is required for the PtdSer exposure and the efficient efferocytosis of apoptotic thymocytes, lymphocytes, and neutrophils, confirming that the PtdSer exposure by flippase inactivation alone is too slow for this process. Since the PtdSer exposed by Xkr8 scramblase can return to the inner layer by the flippase action of P4-ATPase, it is likely that both caspase-mediated activation of Xkr8 scramblase and inactivation of flippases are required for efferocytosis (26). How other molecules that have been proposed as “eat me” or “do not eat me” signals, including ICAM3, calreticulin, annexin, and CD47 (5, 29, 30) function in this process remains to be clarified.

Neutrophils have a short half life of about 12 h (31) and are programmed to undergo apoptosis (32). Although the trigger for neutrophil apoptosis in the aging process has not been elucidated, Bcl2-transgenic mice (33) and Noxa/Bim- or Bax/Bak double-deficient mice have twice the normal number of neutrophils in the circulation, indicating that the neutrophils’ spontaneous apoptosis is mediated by the intrinsic mitochondrial death pathway (34, 35). We found that the number of CD62Llo senescent neutrophils increased in the spleen of Xkr8-deficient mice, and some of them carried active caspase 3/7. These results indicate that, like apoptosis-induced neutrophils, caspase 3 is activated in senescent neutrophils and cleaves Xkr8 to elicit PtdSer exposure. It is likely that macrophages in the spleen, liver, and bone marrow engulf the senescent neutrophils in a PtdSer-dependent manner. In contrast to the increased number of senescent neutrophils in the spleen of Xkr8-deficient mice, the number in the blood was comparable to that in wild-type mice, which differs from mice carrying the deregulated Bcl-2 gene (34, 35). This observation may indicate that the Xkr8-mediated PtdSer exposure does not play a role in the migration of senescent neutrophils into the spleen. A caspase-dependent process is involved in recruiting macrophages to apoptotic cells (29); therefore, a similar caspase-dependent process may be involved in causing senescent neutrophils to approach macrophages in the spleen, liver, or bone marrow. In any case, Xkr8+/− mice, in particular after G-CSF treatment, accumulated a large amount of CD62Llo neutrophils.

This cell population may be useful for studies aimed at defining the so-far poorly characterized senescent neutrophils.

Similar to the knockout mouse of other efferocytosis-related genes (MFG-E8, Tim4, and MerTK) (36–38), Xkr8-deficient mice on the MRL background showed high levels of anti-DNA antibodies and ANAs in the serum, activated effector T cells in the spleen, and IgG deposits in the glomeruli. It is likely that unengulfed apoptotic cells undergo secondary necrosis, releasing cellular contents including chromatin. Chromatin released from dying cells is a strong immunogen (39), but is normally cleaved by DNase1L3, a DNase I-like serum DNase (40). The MRL mouse strain carries a missense mutation in DNase1L3 that significantly reduces its DNase activity against chromatin (41). Thus, the chromatin released from unengulfed apoptotic cells due to inefficient efferocytosis may not be fully digested in this mouse strain. To confirm this possibility, it will be necessary to establish efferocytosis mutant mice on a DNase1L3-deficient C57BL/6 background. In any case, the treatment of patients with systemic lupus erythematosus (SLE) with G-CSF often causes severe flares (42). It is tempting to speculate that patients with SLE carry a defect in the efferocytosis system, and that overloading them with an increased number of apoptotic neutrophils worsens the disease.

The similar nephritis phenotype of mice deficient in Xkr8, MFG-E8, Tim4, and MerTK (36–38, 43) confirmed that these molecules are involved in the same process, PtdSer-dependent efferocytosis. MFG-E8 and Tim4 are expressed in different and limited subsets of macrophages (17, 44), while MerTK is expressed in almost all macrophages (44). Xkr8 is involved in the PtdSer exposure on apoptotic cells including senescent neutrophils. However, it is apparently not involved in the PtdSer exposure on pyrocytes (nuclei expelled from erythroblasts) (45) or aged red blood cells (46), in which caspase(s) is apparently not activated. Pyrocytes and aged red blood cells are still engulfed by macrophages in a PtdSer-dependent manner, in which MerTK and/or Tim4 may be involved. Thus, the severity of the SLE phenotype may differ among these knockout mice. In addition to lupus, the MFG-E8 deficiency causes dermatitis (47), and a worsened recovery after myocardial infarction due to the inefficient engulfment of apoptotic cells (48). It will be interesting to study whether the Xkr8 deficiency causes similar phenotypes in the skin and heart, in particular in humans with the autoimmune disease.

Materials and Methods

Mice. C57BL/6J mice were purchased from JapanCLEA or Japan SLC. MRL/MpJf1−/− mice were from Japan SLC. The Xkr8−/− mice on the C57BL/6 background are described in ref. 13. The MRL-Xkr8−/− mice were generated by backcrossing C57BL/6-Xkr8−/− mice to MRL/MpJf1−/− mice using speed congenics (49). Genotyping with primers for a mouse marker set (49) indicated...
that the backcrossed mice had the MRL background at 98%. All mouse studies were approved by the Animal Care and Use Committee of the Research Institute of Microbial Diseases, Osaka University. All animals were maintained under specific pathogen-free conditions.

**Cell Lines, Recombinant Proteins, and Antibodies.** The Axl−/−Tyro3−/−Gas6−/−NIH 3T3 cells expressing mouse Tim4 and MerTK (TKO-Tim4/Mer) (17) were maintained in DMEM containing 10% FCS. Human Fasl was produced as described (50) and was partially purified by (NH₄)₂SO₄ precipitation at 60% saturation. Human G-CSF was provided by Chugai Pharmaceutical Co. Rat anti-mouse CD68 (clone FA-11) was from Bio-Rad. PE- or PE/Cy7-labeled anti-mGr1 (clone RB6-8C5), PE-anti-mCD3 (clone 145–2C11), FITC- or Brilliant Violet 510-labeled anti-mCD4 (clone RM4-5), PE/Cy7- or PerCP/Cy5.5-labeled anti-mCD8 (clone 53-67.7), APC-anti-mCD62L (clone MEL-14), and PE/Cy7-anti-mCD44 (clone IM7) mAbs were from BioLegend. Alexa Fluor 488-goat anti-mouse IgG and Alexa Fluor 594-goat anti-rat IgG were from Invitrogen. Horseradish peroxidase (HRP)-rabbit anti-mouse Ig and alkaline phosphatase (AP)-rabbit anti-mouse Ig were from Dako.

**Real-Time RT-PCR.** RNA was reverse-transcribed using a High-Capacity RNA-to-cDNA kit (Applied Biosystems). Quantitative PCR was performed using the LightCycler 480 SYBR Green Master Mix (Roche Diagnostics) on a LightCycler 480 system. Plasmid DNA carrying the Xkr4 cDNA was linearized by digesting with restriction enzymes and used as a reference. Primers used for PCR were: GAPDH, 5′-AACAGACCTCTTTGATGGAC-3′ and 5′-TCCAGATACATTGGACGAAG-3′; Xkr4, 5′-GCGAGTGACCGTCTACACT-3′ and 5′-CCCGACGACGACGATCTC-3′; Xkr4, 5′-GGGAAGCGTGGCCCAATCTGA-3′ and 5′-TGGGCGCAGATGTCTGGAGAAGA-3′.

**Flow Cytometry and Apoptotic PtdSer Exposure.** Cell suspensions were prepared from mouse spleen and thymus by squishing the tissue between glass slides. The cell suspensions were treated with RBC lysis solution (ACK Lysis Buffer; Thermo Fisher Scientific), filtered through nylon strainers, incubated with antibodies, and analyzed by flow cytometry (FACS Canto; BD Biosciences). In some cases, cells were incubated at 37 °C for 30 min with 1 μM CellEvent Caspase-3/7 Green (Thermo Fisher Scientific) before the flow cytometry. For TUNEL staining, cells were fixed at room temperature for 15 min in 2% paraformaldehyde (PFA), and permeabilized by incubating for 5 min in 0.1% Triton X-100. To prepare CD3+ splenocytes and Gr1+ blood neutrophils, splenocytes or blood leukocytes were obtained from 9-wk-old mice, stained with 0.5–1.0 μg/mL PE-anti-mouse CD3 or anti-Gr1 mAb, and sorted using a FACS Aria II. The sorted CD3+ splenocytes and Gr1+ neutrophils (about 9% of the blood leukocytes) were used for the efferocytosis assay.

To examine the apoptotic PtdSer exposure, thymocytes, splenocytes, or blood neutrophils were incubated at 37 °C for 0.5–3 h with 100 units/mL FasL or 10 μM ABT737 (Symantec). After washing with annexin V staining buffer [10 mM HEPES-NaOH buffer (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂], the cells were stained on ice for 15 min with 1 μg/mL antibody for CD3, CD4, CD8, or Gr1, and 1,500-fold diluted Cy5-annexin V (Biovision) in annexin V staining buffer, and analyzed by flow cytometry using a FACS Canto.

**Efferocytosis.** Efferocytosis was carried out essentially as described previously in refs. 17 and 51. In brief, thymocytes and neutrophils were labeled with 0.1 μg/mL pHrodo Red, succinimidyl ester (Thermo Fisher Scientific), washed with PBS containing 10% FCS, and incubated at 37 °C with 100 units/mL FasL for 30 min (for thymocytes) or 1 h (for neutrophils) in DMEM containing 10% FCS. CD3+ splenocytes were similarly labeled with pHrodo, and incubated at 37 °C for 30 min with 10 μM ABT737 in DMEM containing 10% FCS. The pHrodo-labeled apoptotic cells were washed with DMEM containing 10% FCS, and used as prey. For efferocytosis, 1.5 × 10⁴ TKO-Tim4/Mer cells were cultured at 37 °C with the pHrodo-labeled prey in 0.3 mL of DMEM containing 10% FCS. After incubation, the cells were washed with PBS, and observed by confocal microscopy (FV1000, Olympus) in Fluororite DMEM (Thermo Fisher Scientific) containing 10% FCS.

**Treatment with Dexamethasone, G-CSF, or Zymosan.** Dexamethasone (Wako Pure Chemical) was i.p. administered to 6–to 9-wk-old mice at a dose of 8 mg/kg. The thymuses were harvested 12 h after the injection for histological analysis. Human G-CSF was s.c. injected into mice at a dose of 2.5 μg per mouse, and the spleens were harvested for flow cytometry analysis with PE/Cy7-labeled anti-Gr1 and APC-labeled anti-CD62L. Zymosan-induced peritonitis was performed essentially as described (52). In brief, mice at 6–8 wk of age were injected i.p. with zymosan A particles (250 μg/kg, 0.5 mL saline; Sigma-Aldrich). Cells in the peritoneal lavage fluid were harvested with 5 mL PBS containing 2% FCS at 18 h after the injection, counted, and subjected to the flow cytometry analysis for Gr1+ neutrophils as described above.

**Histochemochemical Analysis.** For periodic acid Schiff (PAS) staining, tissues were fixed at 4 °C overnight with 4% PFA in PBS. Embedded in paraffin, sectioned at 3 μm, and stained at room temperature for 30 min with Schiff's reagent (Merck). To detect the immune-complex deposition in glomeruli, fresh kidneys were embedded in optimal cutting temperature (OCT) compound
Measurements of Autoantibodies in Serum. The serum level of anti-dsDNA antibodies and ANAs were quantitated essentially as described (27). In brief, linearized plasmid DNA (5 μg/ml) was immobilized to NucleoLink plates (Nunc) by treatment with 10 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Alrich) in 10 mM 1-methylimidazole (Sigma-Alrich) at 50 °C for 5 h. Anti-dsDNA with 5% saline-sodium citrate (SSC) (150 mM NaCl, 15 mM sodium citrate, pH 7.0) containing 0.25% SDS, nonspecific binding sites on the DNA-coated plates were blocked by incubation with 1% BSA in PBS (blocking buffer) at 4 °C overnight. Mouse sera were diluted 100 times with the blocking buffer, and 100-μl aliquots were applied to the DNA-coated plates and incubated for 1 h at room temperature. After washing with PBS containing 0.1% Tween 20, the bound antibodies were detected using AP-conjugated rabbit anti-mouse IgG and the BluePhos Microwell Phosphatase Substrate system, and quantified by measuring the absorbance at 620 nm. The ANA level was determined using a Mesacup EIA system [Medical Biological Laboratories (MBL) for human ANA, except that the HRP-conjugated rabbit anti-mouse IgG was replaced with HRP-conjugated rabbit anti-mouse IgG.

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