

# Masking of Phosphatidylserine Inhibits Apoptotic Cell Engulfment and Induces Autoantibody Production in Mice

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## Abstract

Apoptotic cells are rapidly phagocytosed by professional phagocytes, such as macrophages and dendritic cells. This process prevents the release of potentially noxious or immunogenic intracellular materials from dying cells, and is thought to play a critical role for the maintenance of normal functions in surrounding tissues. Milk fat globule-EGF-factor 8 (MFG-E8), secreted by activated macrophages and immature dendritic cells, links apoptotic cells and phagocytes, and promotes phagocytosis of apoptotic cells. Here, we report that an MFG-E8 mutant, designated as D89E, carrying a point mutation in an RGD motif, inhibited not only the phagocytosis of apoptotic cells by a wide variety of phagocytes, but also inhibited the enhanced production of IL-10 by thioglycollate-elicited peritoneal macrophages phagocytosing apoptotic cells. When intravenously injected into mice, the D89E protein induced the production of autoantibodies including antiphospholipids antibodies and antinuclear antibodies. The production of autoantibodies was enhanced by the coinjection of syngeneic apoptotic thymocytes. After the induction of autoantibody production by D89E, the treated mice showed a long-term elevation of the titer for autoantibodies, and developed IgG deposition in the glomeruli. These results indicated that the impairment of apoptotic cell phagocytosis led to autoantibody production.

Key words: apoptosis • phagocytosis • MFG-E8 • phosphatidylserine • autoimmunity

## Introduction

Unnecessary or potentially harmful cells are eliminated by programmed cell death or apoptosis (1, 2). When cells receive apoptotic stimuli, endogenous caspases—a family of cysteine proteases—are autonomously activated. These active caspases cleave their target molecules, including cytoskeletal and structure proteins, and cell cycle regulators (3, 4). This, proteolysis makes the targets either active or inactive, which lead to morphological and biochemical changes that characterize apoptotic cell death. Apoptotic cells are rapidly engulfed by phagocytes, such as macrophages and dendritic cells, or neighboring cells. The clearance of dead cells prevents the release of potentially toxic or immunogenic intracellular materials from dead cell corpses. There-

fore, even tissues with high cell turnover rates are free from inflammation. Soon after engulfment, the components of apoptotic cell corpses are degraded in the lysosome of phagocytes. Chromosomal DNA degradation of apoptotic cells is performed not only cell autonomously by the action of CAD, but also by the lysosomal DNase of phagocytes (5). DNase II-deficient mice showed dead cell-derived DNA accumulation in thymic macrophages and impairment of thymic development in association with the induction of the interferon genes (6), indicating that phagocytosis of apoptotic cells and subsequent degradation of apoptotic cell materials are essential for organ development and homeostasis.

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Abbreviations used in this paper: ANA, antinuclear antibody; BMDM, bone marrow-derived macrophage; CL, cardiolipin; ds, double stranded; MFG-E8, milk fat globule-EGF-factor 8; PS, phosphatidylserine.

To engulf apoptotic cells, phagocytes must recognize ligands found on apoptotic cells that are absent on living cells (7–9). In mammals, the recognition and engulfment of apoptotic cells by phagocytes is an intricate process, and a number of molecules on phagocytes were found to be involved in this phenomenon (10–12). According to the two-step model (13), apoptotic cells are first tethered to phagocytes through the interaction between putative ligands and receptors, and then are engulfed by phagocytes through recognition of phosphatidylserine (PS) exposed on apoptotic cells. Some receptor molecules, including phosphatidylserine receptor (14), LDL receptor (15), and scavenger receptors (16), directly recognize PS on apoptotic cells. In addition, soluble proteins that bind to PS on apoptotic cells for phagocytosis were also reported (17). However, how these receptors are involved in the recognition and engulfment of apoptotic cells has not been fully understood.

We have previously shown that milk fat globule-EGF-factor 8 (MFG-E8) is involved in apoptotic cell phagocytosis by phagocytes (18). MFG-E8 was originally identified as a component of milk fat globules (19–21), and is also produced and secreted by activated macrophages. MFG-E8 specifically binds to PS exposed on apoptotic cells via COOH-terminal factor VIII homologous domains. When MFG-E8 is engaged by apoptotic cells, it binds to  $\alpha_v\beta_3$  integrin expressed in phagocytes via a NH<sub>2</sub>-terminal EGF-like domain, and promotes the phagocytosis of apoptotic cells.

In this paper, we show that an MFG-E8 mutant protein, carrying a mutation in the RGD sequence, inhibited the phagocytosis of apoptotic cells by a wide variety of phagocytes through the masking of PS on apoptotic cells. To reveal the consequence of failure for apoptotic cell engulfment *in vivo*, D89E protein was intravenously injected into mice. The mice developed sustained autoantibody production and deposition of immunoglobulin in the glomeruli of the kidney. The production of autoantibodies was enhanced by the coinjection of apoptotic cells. These results suggested that apoptotic cell engulfment is required for the maintenance of self-tolerance.

## Materials and Methods

**Preparation of Recombinant MFG-E8.** Recombinant MFG-E8-L and its mutant proteins were prepared as described previously (18). In brief, the expression plasmid of wild-type MFG-E8-L, D89E carrying RGE sequence instead of RGD in the second EGF-like domain, or E1E2PT lacking the C1C2 domains were introduced into human 293T cells by the calcium phosphate precipitation method. The culture medium was replaced with DMEM/2% FCS 16 h after transfection, and the transfected cells were cultured for another 48 h. The recombinant proteins secreted into the medium were purified using anti-FLAG M2 affinity gel (Sigma-Aldrich), or anti-MFG-E8 antibody (clone 2422)-conjugated protein A-Sepharose 4FF beads (Amersham Biosciences). To examine the purity of the proteins, they were subjected to SDS-PAGE and Western blotting. For SDS-PAGE, proteins were separated by electrophoresis on a 10/20% polyacrylamide gradient gel and stained with Coomassie brilliant blue. For Western blotting, proteins were separated by electrophoresis,

transferred to PVDF membranes, and detected with anti-FLAG antibody. We confirmed that the recombinant proteins alone did not stimulate the production of inflammatory cytokines including TNF $\alpha$  and IL-1 $\beta$  in macrophages.

**In Vitro Phagocytosis Assay and Measurement of Cytokines Production.** For the preparation of bone marrow-derived macrophages (BMDMs), bone marrow cells were obtained by flushing the femurs from 8–10-wk-old C57BL/6 mice. The cells were treated with RBC lysis buffer (17 mM Tris-HCl, pH 7.5, 144 mM ammonium chloride, and 0.5% FCS) for 1 min at room temperature. Next, the cells were suspended in  $\alpha$ MEM/10% FCS medium, and were plated at a density of  $10^6$  cells/ml in the presence of recombinant mouse M-CSF (a gift from A. Kudo, Tokyo Institute of Technology, Kanagawa, Japan). Cells were harvested on day 3, diluted 1:10 with the medium, and cultured for another 3 d at 37°C. On day 6, the cells were used for phagocytosis assay.  $2.5 \times 10^5$  BMDMs were seeded in a 48-well cell culture cluster (Corning Inc.) and cultured overnight at 37°C. Cells were preincubated with or without D89E (1, 2, and 4  $\mu$ g/ml) or E1E2PT protein (4  $\mu$ g/ml) for 30 min. For the preparation of apoptotic cells, thymocytes from 4 to 8-wk-old CAD-deficient mice (6) were incubated with 10  $\mu$ M dexamethasone at 37°C for 4 h.  $2.5 \times 10^6$  of apoptotic thymocytes were added to BMDM cultures, and phagocytosis was allowed to proceed for 2 h. Cells not being engulfed were removed by washing with PBS, and the BMDMs were detached with 1 mM EDTA/PBS. Afterwards, the cells were stained with PE-conjugated anti-mouse CD11b, followed by TUNEL staining as described previously (18).

Resident peritoneal macrophages were suspended in DMEM/10% FCS and  $10^5$  cells were seeded in a 96-well cell culture cluster. The cells were incubated 3 h at 37°C and used for the phagocytosis assay.

For cytokine assay,  $10^5$  cells of thioglycollate-elicited peritoneal macrophages from C57BL/6 were seeded in a 96-well cell culture cluster. The macrophages were preincubated with or without D89E or E1E2PT protein for 30 min. Then,  $2 \times 10^6$  cells of apoptotic thymocytes induced by UV irradiation were added to the macrophages for phagocytosis. After 2-h incubation, the macrophages were washed twice, and were stimulated with or without 1  $\mu$ g/ml LPS for 20 h. For the measurement of TGF- $\beta$ , the culture medium was changed to AIM-V (Invitrogen) 1 h after stimulation with or without LPS, and the cells were incubated for 20 h. Cytokine concentrations in the culture supernatants were measured using ELISA kits for IL-10, TNF $\alpha$  (BD Biosciences), and TGF- $\beta$  (R&D Systems) according to manufacturer's protocols.

**Injection of Recombinant Proteins and Apoptotic Cells.** The purified recombinant proteins were diluted with PBS containing 2.5% normal mouse serum obtained from C57BL/6 mice, and 300  $\mu$ l of the solution was intravenously injected into 8-wk-old C57BL/6 female mice through the tail vein. In the case of apoptotic thymocyte injection, thymi from 4 to 6-wk-old C57BL/6 mice were removed and squeezed between glass slides. Thymocytes were then filtrated through a nylon mesh, and suspended in serum-free RPMI 1640 medium. The cells were irradiated with 40 J/m<sup>2</sup> UV light to induce apoptosis and were cultured in RPMI 1640 medium containing 1% normal mouse serum at 37°C for 20 h. Next, the cells were washed twice with PBS containing 1 mg/ml of mouse serum albumin and suspended in PBS containing 2.5% normal mouse serum. Recombinant MFG-E8 was added to apoptotic thymocytes 30 min before injection, and the cells were intravenously injected into mice. Immunizations were performed weekly for a total of four to six injections.

**Detection of Autoantibodies** The serum levels of anticardiolipin antibody and anti-PS antibody were detected by ELISA. 96-well ELISA plates (Immulon 1B microtiter plate; ThermoLabsystems) were coated with 10  $\mu\text{g/ml}$  cardiolipin (CL) in methanol or 10  $\mu\text{g/ml}$  L- $\alpha$ -phosphatidyl-L-serine, dioleoyl (Sigma-Aldrich) in ethanol. After blocking with 10% FCS, mice sera diluted 50 times with PBS were added and incubated for 1 h at room temperature. The mouse antibodies bound to the plate were detected using goat anti-mouse Ig conjugated to HRP (ICN Biomedicals) at a dilution of 1:2,000. The peroxidase activity was detected using *o*-phenylenediamine in the peroxidase detection kit (Sumitomo) as a substrate. The color reaction was read at 492 nm using a microplate reader (Titertek Instruments).

Antinuclear antibody (ANA) was detected by indirect immunofluorescence and ELISA. For immunofluorescence, serum samples were diluted 50 times with PBS, and were added on glass slides coated with Hep-2 cells (MBL). The slides were incubated at 37°C in a humid chamber for 30 min. The antibodies bound to the slides were detected by Cy3-conjugated F(ab')<sub>2</sub> of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) diluted 100 times with PBS/10% normal goat serum. Slides were observed by fluorescence microscopy (model IX-70; Olympus).

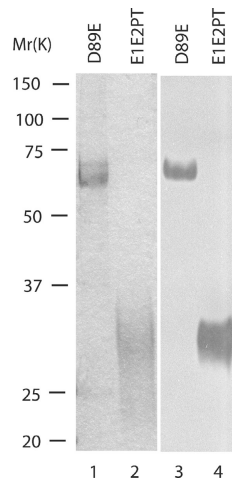
ANAs were also detected by using an ANA detection kit (MBL). Mouse sera diluted 100 times with the reaction buffer of the kit were added to a microcup coated with a mixture of human nuclear antigens (three types of RNP epitope [70k, RNP-A, and RNP-C], native Sm, native SS-A, recombinant SS-B, recombinant Scl-70, cenp-B, recombinant Jo-1, and  $\gamma$  phage DNA antigen) and incubated for 1 h at room temperature. The antibodies bound to the plate were detected using goat anti-mouse Ig's conjugated to HRP (ICN Biomedicals) at a dilution of 1:1,000. The peroxidase activity was detected using TMB as a substrate. The color reaction was read at 450 nm using a microplate reader (Titertek Instruments).

Anti-double stranded (ds) DNA antibody was detected by using anti-dsDNA detection kit (MBL). Mouse sera diluted 100 times with the reaction buffer of the kit were added to a microcup coated with human dsDNA and incubated 1 h at room temperature. The antibodies bound to the microplate were detected using goat anti-mouse Ig's conjugated to HRP (ICN Biomedicals) at a dilution of 1:1,000. The peroxidase activity was detected as described in the previous paragraph. The color reaction was read at 450 nm using a microplate reader (Titertek Instruments).

**Immunohistochemistry** 20–36-wk-old mouse kidneys were fixed with 4% paraformaldehyde/4% sucrose in 0.1 M phosphate buffer, pH 7.2, and embedded in paraffin. 4- $\mu\text{m}$ -thick sections were prepared and mounted on silanized slide glasses. For immunohistochemistry, sections were incubated for 60 min at room temperature in PBS containing 0.1% Triton X-100 and 10% normal goat serum and were stained for 60 min at room temperature with Cy3-conjugated F(ab')<sub>2</sub> of goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) used at a dilution of 1:100. The sections were washed three times with PBS containing 0.1% Triton X-100 and observed by fluorescence microscopy.

## Results

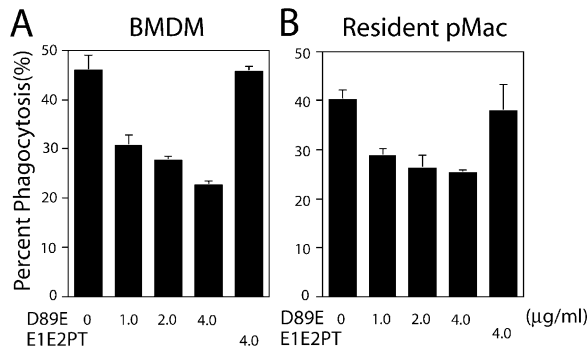
**Preparation of MFG-E8 Mutant Proteins.** MFG-E8 is produced and secreted by macrophages and works as a bridging molecule between apoptotic cells and macrophages to promote phagocytosis of apoptotic cells. This bridging function depends on an RGD sequence located in



**Figure 1.** Preparation of recombinant MFG-E8. The expression plasmids of D89E carrying the RGE sequence instead of RGD in the second EGF-like domain of mouse MFG-E8, or E1E2PT lacking the C1C2 domains were introduced into human 293T cells. Recombinant proteins in the supernatants were purified using anti-FLAG M2 affinity gel. D89E (lanes 1 and 3) or E1E2PT (lanes 2 and 4) were separated by SDS-PAGE on a 10/20% polyacrylamide gradient gel and stained with CBB (lanes 1 and 2), or transferred to a PVDF membrane and detected with anti-FLAG antibody (lanes 3 and 4). Positions of molecular mass standard proteins are indicated in kilodaltons on the left.

the second EGF domain, and the mutant MFG-E8, designated as D89E, carrying a point mutation in an RGD motif, inhibited the phagocytosis of apoptotic cells by thioglycollate-elicited peritoneal macrophages (18). To examine the effects of the D89E mutant protein in vivo, we produced the mutant D89E protein in 293T cells, and then purified it by affinity chromatography. The purified D89E protein showed a single band of 67 kD in SDS-PAGE that was recognized by an anti-FLAG antibody (Fig. 1). No other proteins were detected in SDS-PAGE, indicating a high purity of the protein. We also produced and purified another mutant (E1E2PT) lacking the C1 and C2 domains, showing a band of 30 kD in SDS-PAGE.

**The D89E Mutant Protein Inhibits Phagocytosis of Apoptotic Cells by Phagocytes.** We have shown that D89E inhibits the phagocytosis of apoptotic cells by thioglycollate-elicited mouse peritoneal macrophages (18), which produce a large amount of MFG-E8. We have also confirmed that D89E inhibits the phagocytosis of apoptotic cells, but not phagocytosis of microspheres by BAM3 cells, a macrophage line that produces Del-1, which is homologous to MFG-E8 (22, 23; unpublished data). In these cases, D89E works as a dominant negative form of MFG-E8, or competes with Del-1. Here, we examined whether D89E inhibits the phagocytosis of apoptotic cells by macrophages that produced neither MFG-E8 nor Del-1. BMDMs and peritoneal resident macrophages were pretreated with D89E protein, and then cocultured with apoptotic thymocytes for phagocytosis. As shown in Fig. 2, D89E inhibited phagocytosis by both kinds of macrophages in a dose-dependent manner. On the other hand, the E1E2PT protein, which cannot bind to PS exposed on apoptotic cells because it lacks the C1 and C2 domains, did not affect the phagocytosis of apoptotic thymocytes. These results indicated that BMDMs and peritoneal resident macrophages recognize PS on apoptotic cells for phagocytosis, and that the D89E protein can inhibit apoptotic cell engulfment by a wide variety of phagocytes by masking the PS on dead cells. We have previously shown that a high concentration of wild-type recombinant MFG-E8-L does not promote phagocytosis of

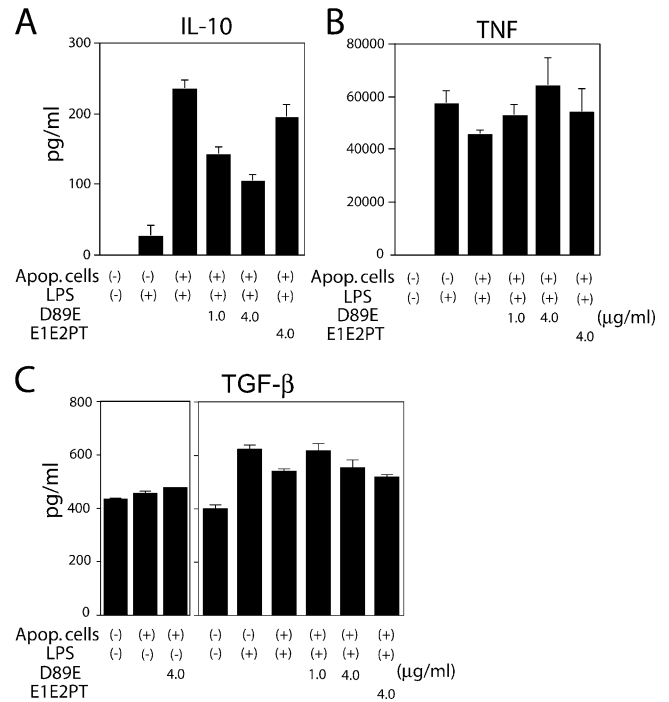


**Figure 2.** The D89E inhibits phagocytosis of apoptotic cells by macrophages. (A) Mouse bone marrow-derived macrophages (BMDM) or (B) resident peritoneal macrophages (Resident pMac) were pretreated for 30 min with indicated concentrations of D89E or E1E2PT and were cocultured with dexamethasone-treated apoptotic thymocytes from CAD-deficient mice. The cells were stained for Mac-1 and TUNEL, analyzed by flow cytometry, and the percentage of TUNEL-positive cells in the Mac-1-positive population was determined. The assay was performed in triplicate, and mean values are shown with SD. These results are representative of three independent experiments.

apoptotic cells, but rather inhibits this phenomenon (18). We also tested the effect of wild-type recombinant MFG-E8-L on phagocytosis of apoptotic cells by BMDMs and peritoneal resident macrophages, and found that 4.0 μg/ml of wild-type MFG-E8 had the inhibitory effects to the same extent as the D89E protein (unpublished data).

Phagocytosis of apoptotic cells has immunosuppressive effects on phagocytes. That is, macrophages phagocytosing apoptotic cells produce higher levels of the antiinflammatory cytokines IL-10 and TGF-β (24, 25). We next examined whether the D89E protein has inhibitory effects on the immunosuppressant associated with phagocytosis of apoptotic cells. Thioglycollate-elicited peritoneal macrophages were cocultured with thymocytes irradiated with UV for phagocytosis. Then, the cells were stimulated with LPS for 20 h and IL-10, TGF-β, and TNFα levels in the supernatant was measured by ELISA. IL-10 production (Fig. 3) by LPS in the macrophages engulfing apoptotic cells was 10-fold higher than that in the cells not engulfing apoptotic cells. D89E dose dependently inhibited the augmentation of IL-10 production associated with apoptotic cell engulfment, whereas E1E2PT did not have any effects. In contrast to IL-10, apoptotic cell engulfment did not change either TGF-β or TNF production in the macrophages, and D89E had little effect on these cytokine productions.

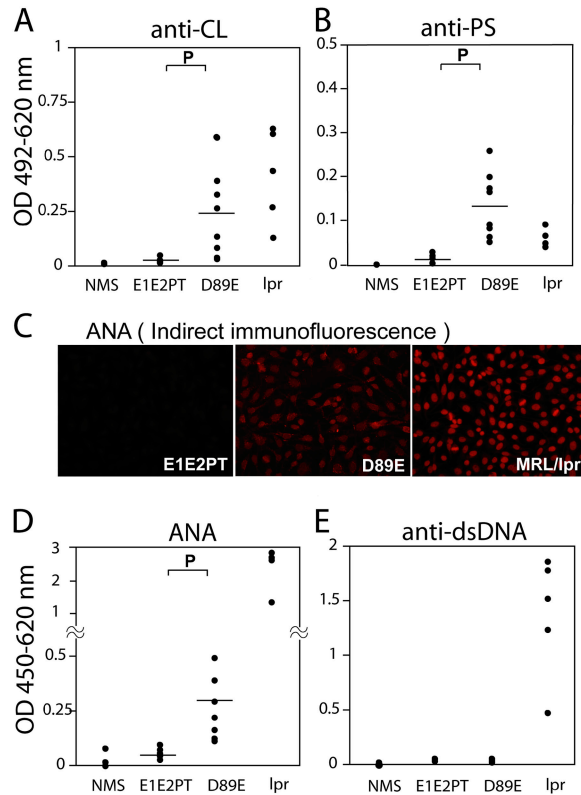
**Intravenous Injection of D89E Induces Autoantibody Production in Mice.** The impairment of apoptotic cell phagocytosis, or the exposure of a large number of apoptotic cells causes autoantibody production in mice (26–28). To examine the effects on autoantibody production of the recombinant MFG-E8 protein in vivo, we intravenously administered the recombinant protein into normal mice, and autoantibodies in sera were measured with an ELISA assay. described in the previous section, both wild-type MFG-E8 and D89E proteins have the inhibitory effects on apoptotic cell phagocytosis at a high concentration. However, be-



**Figure 3.** D89E inhibits the enhanced production of IL-10 in macrophages that engulf apoptotic cells. Thioglycollate-elicited mouse peritoneal macrophages were cocultured with or without UV-irradiated thymocytes for 2 h in the absence or presence of indicated concentrations of the D89E or E1E2PT protein. After washing twice, the macrophages were treated with or without 1 μg/ml of LPS for 20 h. The concentrations of IL-10 (A), TNFα (B), and TGF-β (C) were determined by ELISA. The assay was performed in triplicate, and mean values are shown with SD. These results are representative of three independent experiments.

cause the D89E protein was more effective than the wild-type protein at a low concentration (unpublished data), we used the D89E protein for in vivo study. All mice injected with the D89E protein developed anti-CL antibody after four injections (Fig. 4 A), whereas none of the mice injected with the E1E2PT protein was anti-CL antibody positive. The titer of antibody against CL did not decrease by D89E addition to the assay (unpublished data), indicating that the antibodies to CL in the sera were not cross-reactive to the D89E protein. Injection of the D89E protein without FLAG tag also induced anti-CL antibody production (unpublished data), indicating that this autoantibody production was not due to immunization of FLAG peptide fused to the D89E protein. Anti-CL antibodies often cross-react with PS, which is another anionic phospholipid. As shown in Fig. 4 B, the sera from mice injected with the D89E protein contained anti-PS antibody, whereas the E1E2PT protein did not induce anti-PS antibody production. We also examined ANA, and anti-dsDNA antibody in the sera. ANA was initially detected by indirect immunofluorescence on Hep-2 cells. All mice injected with the D89E protein were ANA positive, whereas none of the mice injected with the E1E2PT protein developed ANA (Fig. 4 C). Most ANA-positive sera showed either homogeneous or speckled patterns, or a combination of both.



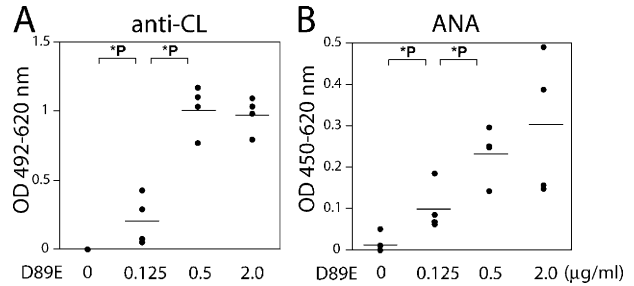


**Figure 4.** Intravenous injection of D89E induces autoantibody production in mice. Either D89E or E1E2PT (0.6  $\mu\text{g}$ ) were diluted in 300  $\mu\text{l}$  PBS containing 2.5% normal syngeneic mouse serum (concentration, 2.0  $\mu\text{g}/\text{ml}$ ), and injected into C57BL/6 mice weekly for the total of four (A and B) or six (C–E) injections. Blood samples were obtained 1 wk after the last injection, and serum levels of anti-CL (A), anti-PS (B), and anti-dsDNA (E) were evaluated by ELISA as described in Materials and Methods. ANAs were detected by indirect immunofluorescence on Hep-2 cells (C) and ELISA (D). Serum levels of autoantibodies in MRL/MpJ-lpr/lpr mice (16–40 wk old) are shown as a control. Horizontal bars represent the means. Statistical significance was determined by a one-tailed Mann-Whitney test. P indicates a difference with a  $P < 0.01$ .

We also measured ANA with a plate coated with several kinds of nuclear proteins that are potential antigens against autoantibodies. Injection of D89E (Fig. 4 D), but not E1E2PT, into mice induced ANA production. On the other hand, anti-dsDNA antibody was not detected in the mice injected with the D89E protein (Fig. 4 E).

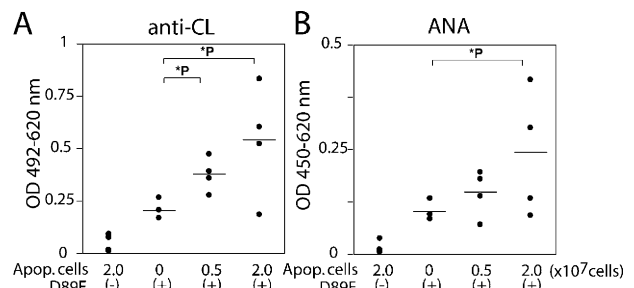
We next administered different amounts of D89E protein into mice. 0.125–2.0  $\mu\text{g}/\text{ml}$  of D89E solution was prepared in PBS/2.5% syngeneic mouse serum and was injected into mice six times at a weekly interval. Injection of the D89E protein induced anti-CL antibody and ANA in a dose-dependent manner (Fig. 5).

**Coinjection of Apoptotic Cells Augments Autoantibody Production Induced by D89E.** Intravenous injection of a large number of apoptotic thymocytes induced autoantibody production, especially anti-CL antibody (28). We examined whether D89E-induced autoantibody production was augmented by coinjection of apoptotic thymocytes. Injection of heterologous  $\beta$ 2-glycoprotein I ( $\beta$ 2GPI), which

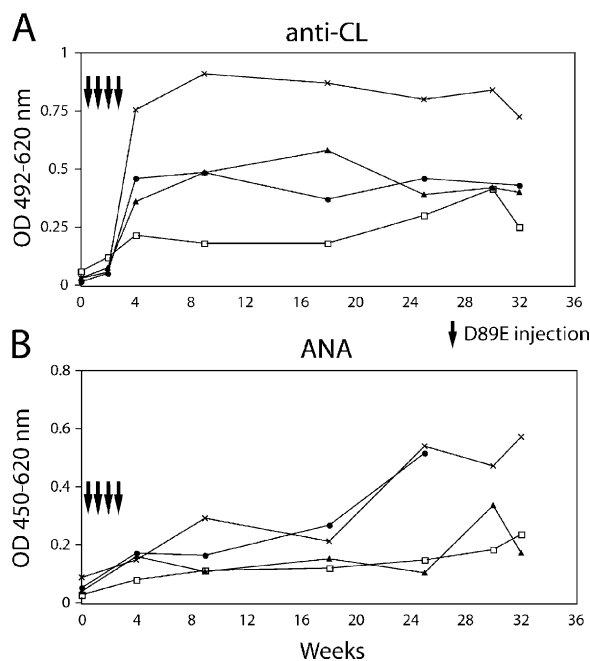


**Figure 5.** Dose-dependent effects of D89E on the development of autoantibodies. The D89E protein was diluted in PBS containing 2.5% normal syngeneic mouse serum at a concentration of 0.125, 0.5, or 2.0  $\mu\text{g}/\text{ml}$ , and 300  $\mu\text{l}$  of the solution was injected intravenously into C57BL/6 mice. Mice were given six injections at weekly intervals and bled a week after the last immunization. Serum levels of anti-CL (A) and ANA (B) were measured by ELISA. \*P indicates a difference with a  $P < 0.05$ .

can bind to anionic phospholipid, induces antiphospholipid autoantibodies (29, 30). To avoid the contact of apoptotic thymocytes to  $\beta$ 2GPI in FCS, thymocytes from C57BL/6 mice were suspended in DMEM/1% syngeneic mouse serum and irradiated with 40  $\text{J}/\text{m}^2$  UV light to induce apoptosis. After incubation for 20 h, TUNEL staining and Annexin V staining showed that >90% of thymocytes underwent apoptosis (unpublished data). The cells were intravenously injected with or without the D89E protein into syngeneic mice. As shown in Fig. 6, the injection of apoptotic thymocytes alone induced anti-CL antibody production, although the titer of the antibody was far lower compared with those in the mice injected with the D89E protein. When the mice were coinjected with apoptotic thymocytes and the D89E protein, they showed a higher titer of anti-CL antibody and ANA than those in mice injected with the D89E protein alone. However, coinjection of E1E2PT and apoptotic thymocytes did not cause enhanced production of autoantibodies (unpublished data). These results suggested that the D89E protein injection



**Figure 6.** Coinjection of apoptotic cells augments autoantibody production induced by D89E. Thymocytes were prepared from C57BL/6 mice, cultured in RPMI 1640 containing 1% syngeneic mouse serum, and irradiated with 40  $\text{J}/\text{m}^2$  UV light. After incubation for 20 h to induce apoptosis, the indicated number of cells was intravenously injected with or without 0.6  $\mu\text{g}$  of D89E protein into syngeneic mice. Serum levels of anti-CL (A) and ANA (B) were measured by ELISA after four or six weekly injections, respectively. Horizontal bars represent the means. \*P indicates a difference with a  $P < 0.05$ .

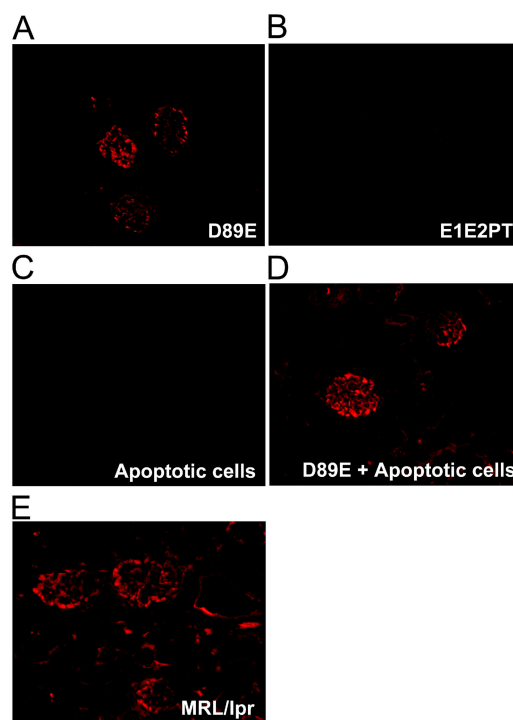


**Figure 7.** Sustained elevation of autoantibodies in mice injected with D89E. Titers of autoantibodies were followed over 32 wk after immunization. C57BL/6 mice were injected intravenously with 300  $\mu$ l of 2.0  $\mu$ g/ml D89E. Injections were administered weekly for a total of four immunizations (arrows). Mice were bled 2, 4, 9, 18, 25, 30, and 32 wk after the first immunization. Anti-CL (A) and ANA (B) were measured by ELISA as described in Materials and Methods.

caused autoantibody production by the inhibition of phagocytosis of apoptotic cells in vivo.

**Sustained Elevation of Autoantibodies in Mice Injected with D89E Protein.** We followed the titer of autoantibodies in mice injected with the D89E protein over a long period. The titer of anti-CL antibody (Fig. 7) was rapidly increased after four injections of D89E, and it was not decreased even after injections were stopped. We confirmed that the titer of anti-CL antibody and ANA was sustained at least until week 32, suggesting that autoantibody production was continuously stimulated by endogenous autoantigens in the mice once self-tolerance was broken by the injection of D89E protein.

**Immunoglobulin Deposition in the Glomeruli of the Mice Injected with D89E Protein.** Autoantibody production often causes deposition of Ig complexes in the glomeruli of kidney and glomerulonephritis. We performed immunohistochemical staining of the kidney sections from the mice injected with D89E. Fig. 8 shows that deposition of IgG was detected in the glomeruli of kidneys from mice injected with the D89E protein, whereas no deposition was observed in the kidneys from mice injected with E1E2PT. We followed Ig deposition in injected mice 28 wk after the initial injection of the D89E protein, and all of the mice showed deposition in glomeruli at this point. Mice injected with the D89E protein and apoptotic thymocytes also showed immunoglobulin deposition in the glomeruli, whereas the deposition was barely detectable in mice injected with apoptotic cells alone. We also stained the kid-



**Figure 8.** Immunohistochemical detection of IgG deposition in the glomeruli of kidneys in mice injected with D89E. C57BL/6 mice were injected intravenously with D89E or E1E2PT with or without apoptotic thymocytes six times at weekly intervals. Kidneys obtained 17 wk after the initial injection were fixed with 4% paraformaldehyde and embedded in paraffin. The sections were stained with Cy3-conjugated F(ab')<sub>2</sub> of goat anti-mouse IgG and were observed with fluorescence microscopy. 25 wk-old MRL/lpr mice (E) were used as a positive control. (A) 0.5  $\mu$ g/ml D89E, (B) 2.0  $\mu$ g/ml E1E2PT, (C)  $2.0 \times 10^7$  UV-irradiated thymocytes, and (D) 2.0  $\mu$ g/ml D89E with  $2.0 \times 10^7$  of UV-irradiated thymocytes.

ney sections of injected mice with an anti-mouse C3 antibody. However, C3 could not be detected in any sections from mice injected with the D89E protein or D89E and apoptotic cells (unpublished data).

## Discussion

Apoptotic cells are swiftly engulfed by professional phagocytes, such as macrophages and dendritic cells. We have previously shown that MFG-E8, produced by activated macrophages, links between apoptotic cells and phagocytes to promote the engulfment of dying cells, and that the mutant protein D89E inhibits phagocytosis (18, 22). Here, we showed that D89E induced autoantibody production when intravenously injected into normal mice. For this effect, D89E requires its binding domains to PS exposed on apoptotic cells.

Many molecules have been proposed to be involved in the phagocytosis of apoptotic cells (10, 12, 31). Some of these molecules were demonstrated to be responsible for engulfment of apoptotic cells in vivo. Mer-deficient mice showed impaired phagocytosis of apoptotic immature T cells in the thymus (26). The clearance of apoptotic cells

injected into the mouse peritoneum was delayed in mice with homozygous C1q deficiency, which is a component of the complement classical pathway (32). Both Mer- and C1q-deficient mice spontaneously showed autoantibody production, and developed glomerulonephritis (27, 33). Moreover, recently, we have established MFG-E8-deficient mice, which showed impaired phagocytosis of apoptotic B cells in the germinal center of the spleen and suffered from autoimmune diseases with ANA and anti-DNA antibodies production (34). Judging from these results, it is most likely that the administration of D89E led to autoantibody production in mice by inhibiting phagocytosis of apoptotic cells in certain organs. The enhanced production of autoantibodies by the coinjection of apoptotic cells with D89E supports this model. A small amount of D89E (<1 µg per mouse) injection was sufficient to induce autoantibodies production, suggesting that disturbing the clearance of even a small number of apoptotic cells can cause autoantibodies production if occurring repeatedly.

In spite of increasing evidences that the impairment of apoptotic cell clearance causes autoantibody production, the underlying mechanisms remain elusive, and several possibilities may arise. Liu et al. (35) reported that when apoptotic cells loaded with an exogenous protein (e.g., ovalbumin) were injected into mice, immunotolerance for the protein was induced in the animal. Injected apoptotic cells were phagocytosed by CD8<sup>+</sup> dendritic cells in the spleen, which in turn presented cell-associated proteins to induce tolerance. These findings suggest that peripheral immune tolerance may be maintained by consistent presentation of self antigens by dendritic cells and macrophages that phagocytose dying self cells during physiological tissue turn over, and that the inhibition of the phagocytosis may cause the breakdown of self-tolerance. Phagocytosis of apoptotic cells has immunosuppressive effects on phagocytes, and the D89E protein inhibits the enhanced production of IL-10 in LPS-stimulated macrophages with apoptotic cells (Fig. 3). Thus, it is possible that D89E induces inflammation in association with apoptotic cell death and subsequent phagocytosis of cell corpses in vivo, and consequently, leads to the breakdown of self-tolerance. Alternatively, the clearance of dead cells prevents the release of potentially toxic or immunogenic intracellular materials from dead cell corpse. A recent paper by Shi et al. (36) described that uric acid, released from injured cells, stimulates dendritic cell maturation, and acts as an endogenous adjuvant. Heat shock protein 70 can also modulate immune responses by promoting functional maturation of dendritic cells (37). Impaired phagocytosis of apoptotic cells results in secondary necrosis of dead cells, and the released materials from the cell corpses may induce abnormal immune responses including stimulation of self-reactive T cells. Nevertheless, impairment of apoptotic cell phagocytosis can cause the breakdown of self-tolerance, and further study to reveal the precise mechanisms of this phenomenon should be required for a deeper understanding of autoimmune diseases.

MFG-E8 is expressed in a limited kind of macrophage. In physiological conditions, only tingible body macro-

phages in the spleen and lymph nodes produced MFG-E8, and the protein could not be detected in the mouse serum at a 1 ng/ml order (unpublished data). We have previously shown that there is an optimal concentration of MFG-E8 for promoting apoptotic cell phagocytosis, and that this effect changes toward inhibition above this concentration. In fact, large amounts of wild-type recombinant MFG-E8 also induced autoantibody production when injected into mice (data not shown). These results strongly suggest that the expression level of MFG-E8 is strictly regulated in the macrophages in vivo, and that the excess amount of MFG-E8 may cause production of autoantibodies in pathological conditions. A large amount of MFG-E8 is produced in thioglycollate-elicited peritoneal macrophages, whereas resident peritoneal macrophages shows no expression, suggesting that large amounts of MFG-E8 may be produced in the macrophages in response to inflammation. In another case, the induction of MFG-E8 gene expression was found to occur in the mammary gland during lactation (38, 39). It will be of considerable interest to examine the correlation between MFG-E8 production and the levels of autoantibodies in patients with autoimmune diseases.

MFG-E8 has two C domains that share homology with the discoidin family, including the lipid-binding C domain of blood coagulation factor V and factor VIII. These coagulation factors bind to phospholipids including PS on platelets via the C domain to proceed the coagulation cascade. Shi and Gilbert (40) reported that purified MFG-E8 inhibited blood coagulation by competing with coagulation factors for membrane-binding sites, suggesting that MFG-E8 could serve as an anticoagulant drug. However, the present paper may caution against MFG-E8 usage as a therapeutic drug.

In summary, the D89E protein has inhibitory effects on the phagocytosis of apoptotic cells by a wide variety of phagocytes, and induces autoantibodies production when injected into mice. These results imply a relationship between apoptotic cell phagocytosis and autoimmune disease. Further studies of the mechanisms of phagocytosis of dead cells will be required to reveal the pathophysiology of human autoimmune diseases.

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