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LETTERS

Identification of Tim4 as a phosphatidylserine receptor

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In programmed cell death, a large number of cells undergo apoptosis, and are engulfed by macrophages to avoid the release of noxious materials from the dying cells[1,2]. In definitive erythropoiesis, nuclei are expelled from erythroid precursor cells and are engulfed by macrophages. Phosphatidylserine is exposed on the surface of apoptotic cells[3] and on the nuclei expelled from erythroid precursor cells[4]; it works as an 'eat me' signal for phagocytes[5,6]. Phosphatidylserine is also expressed on the surface of exosomes involved in intercellular signalling[7]. Here we established a library of hamster monoclonal antibodies against mouse peritoneal macrophages, and found an antibody that strongly inhibited the phosphatidylserine-dependent engulfment of apoptotic cells. The antigen recognized by the antibody was identified by expression cloning as a type I transmembrane protein called Tim4 (T-cell immunoglobulin- and mucin-domain-containing molecule; also known as Timd4)[8]. Tim4 was expressed in Mac1$^+$ cells in various mouse tissues, including spleen, lymph nodes and fetal liver. Tim4 bound apoptotic cells by recognizing phosphatidylserine via its immunoglobulin domain. The expression of Tim4 in fibroblasts enhanced their ability to engulf apoptotic cells. When the anti-Tim4 monoclonal antibody was administered into mice, the engulfment of apoptotic cells by thymic macrophages was significantly blocked, and the mice developed autoantibodies. Among the other Tim family members, Tim1, but neither Tim2 nor Tim3, specifically bound phosphatidylserine. Tim1- or Tim4-expressing Ba/F3 B cells were bound by exosomes via phosphatidylserine, and exosomes stimulated the interaction between Tim1 and Tim4. These results indicate that Tim4 and Tim1 are phosphatidylserine receptors for the engulfment of apoptotic cells, and may also be involved in intercellular signalling in which exosomes are involved.

Caspase-activated DNase (*CAD*)-deficient cells do not undergo apoptotic DNA fragmentation, but their DNA is degraded in phagocytes after they are engulfed[9]. We used this knowledge to assay the

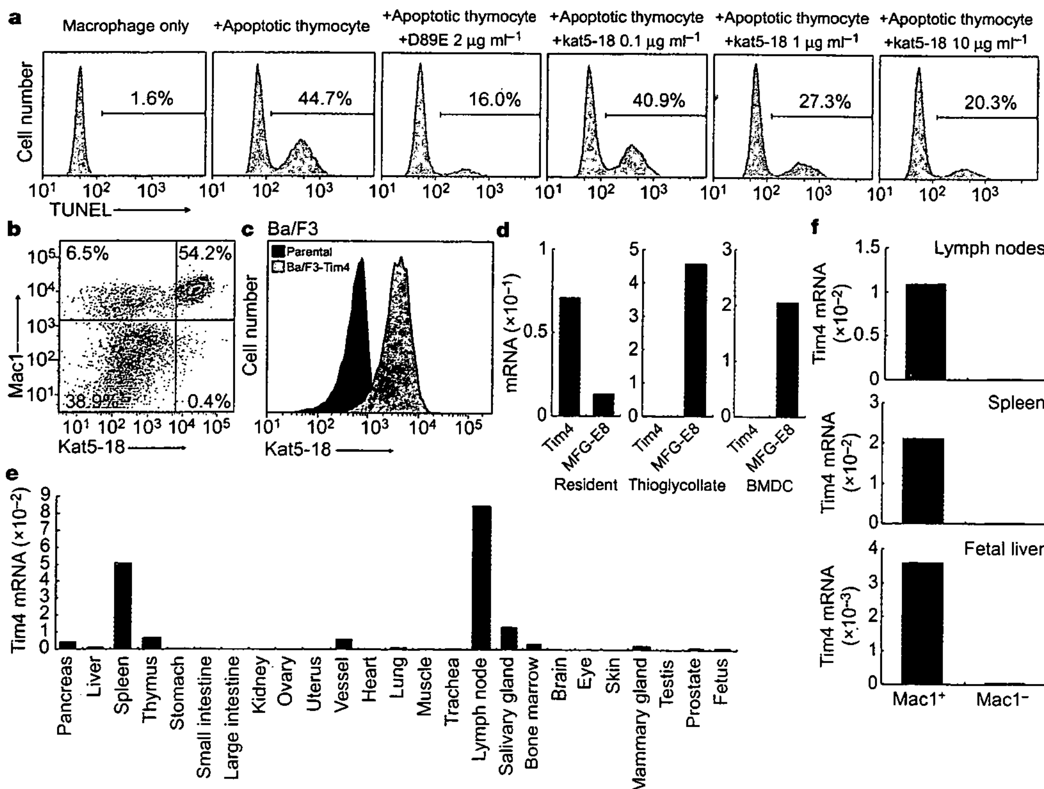

Figure 1 | Tim4 in peritoneal macrophages. a, Mouse peritoneal cells were used for phagocytosis with D89E[10] or Kat5-18, stained for TUNEL, analysed by flow cytometry, and the percentage of TUNEL$^+$ macrophages determined. Average values of two experiments are shown. **b,** Peritoneal macrophages incubated with biotin-Kat5-18, followed with FITC-streptavidin and APC-anti-Mac1. **c,** Parental and Tim4-expressing Ba/F3 analysed by flow cytometry with Kat5-18. **d,** mRNA levels of Tim4 and MFG-E8 in resident or thioglycollate-elicited peritoneal macrophages, and bone marrow-derived immature dendritic cells, determined by real-time PCR. **e,** Tim4 mRNA levels in mouse tissues, determined by real-time PCR. **f,** Real-time PCR for Tim4 mRNA in Mac1$^+$ and Mac1$^-$ cells from lymph nodes, spleen and fetal liver.

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engulfment of apoptotic cells, and identified MFG-E8 (milk fat globule EGF factor 8) that stimulates their engulfment[10]. MFG-E8 is expressed in thioglycollate-elicited peritoneal macrophages, tingible-body macrophages of lymph nodes and spleen, Langerhans cells and mammary epithelial cells[10–13]. Hu *et al.*[14] reported that naïve peritoneal macrophages engulf apoptotic cells in a phosphatidylserine (PS)-dependent manner. We confirmed this with our assay system, which uses $CAD^{-/-}$ thymocytes as prey (Fig. 1a). Peritoneal macrophages efficiently engulfed apoptotic cells, and this was inhibited by the D89E mutant of MFG-E8 that masks PS[10]. However, peritoneal macrophages expressed little MFG-E8 (see below), and this MFG-E8 deficiency did not affect the ability of these macrophages to engulf apoptotic cells (data not shown). To identify the molecules involved in this engulfment, Armenian hamsters were immunized with mouse peritoneal cells, and hybridomas were prepared. Among 1,200 hybridomas, one monoclonal antibody (Kat5-18) dose-dependently inhibited the phagocytosis of apoptotic cells by peritoneal macrophages (Fig. 1a). Most $Mac1^{+}$ peritoneal cells were stained by Kat5-18 (Fig. 1b), indicating that peritoneal macrophages expressed its antigen.

To identify the antigen for Kat5-18, we used retrovirus-mediated expression cloning[15]. A complementary DNA library was constructed with messenger RNA from mouse peritoneal cells, converted to retroviruses, and used to infect mouse Ba/F3 cells. After 2 days in culture, transformants labelled by Kat5-18 (less than 0.1%) were sorted using a cell sorter, expanded in culture, and sorted again. This cycle of sorting and expansion was repeated three times until most cells stained with Kat5-18. The sorted Kat5-18^{+} cells carried 2–3 different integrated cDNAs, and Tim4 cDNA was found in two independent experiments. When Tim4 cDNA was introduced into Ba/F3, the transformants stained strongly with Kat5-18 (Fig. 1c), confirming that Tim4 was the antigen recognized by Kat5-18. Real-time PCR indicated that Tim4 mRNA was expressed in resident, but not thioglycollate-elicited, peritoneal cells (Fig. 1d), in sharp contrast with MFG-E8, which was expressed in thioglycollate-elicited but weakly in resident peritoneal cells. A similar situation was observed with bone marrow-derived immature dendritic cells, which expressed a high level of MFG-E8, but not Tim4. Other major tissues that expressed Tim4 included the spleen, thymus, lymph nodes and salivary glands (Fig. 1e). When cells from the spleen, lymph nodes and fetal liver were sorted into $Mac1^{+}$ and $Mac1^{-}$ cells using MACS (magnetic cell sorting), Tim4 mRNA was found only in $Mac1^{+}$ cells (Fig. 1f). Flow cytometry indicated that about 20% of $Mac1^{+}$ cells in the lymph nodes expressed Tim4 (Supplementary Fig. 1).

DNase II degrades DNA of apoptotic cells in lysosomes after phagocytes engulf them[16]. To examine the role of Tim4 in the engulfment of apoptotic cells, a fibroblast cell line expressing Tim4 was established with NIH3T3 expressing DNase II[10] (NIH3T3/DNase II/Tim4) (Supplementary Fig. 2). The ability of the parental NIH3T3/DNase II to engulf apoptotic cells was weak: when incubated with apoptotic $CAD^{-/-}$ thymocytes for 120 min, only 20% of the cells engulfed them (Fig. 2a). On the other hand, more than 50% of NIH3T3/DNase II/Tim4 engulfed apoptotic cells within 60 min. This effect of Tim4 was confirmed by microscopic observation. That is, when apoptotic

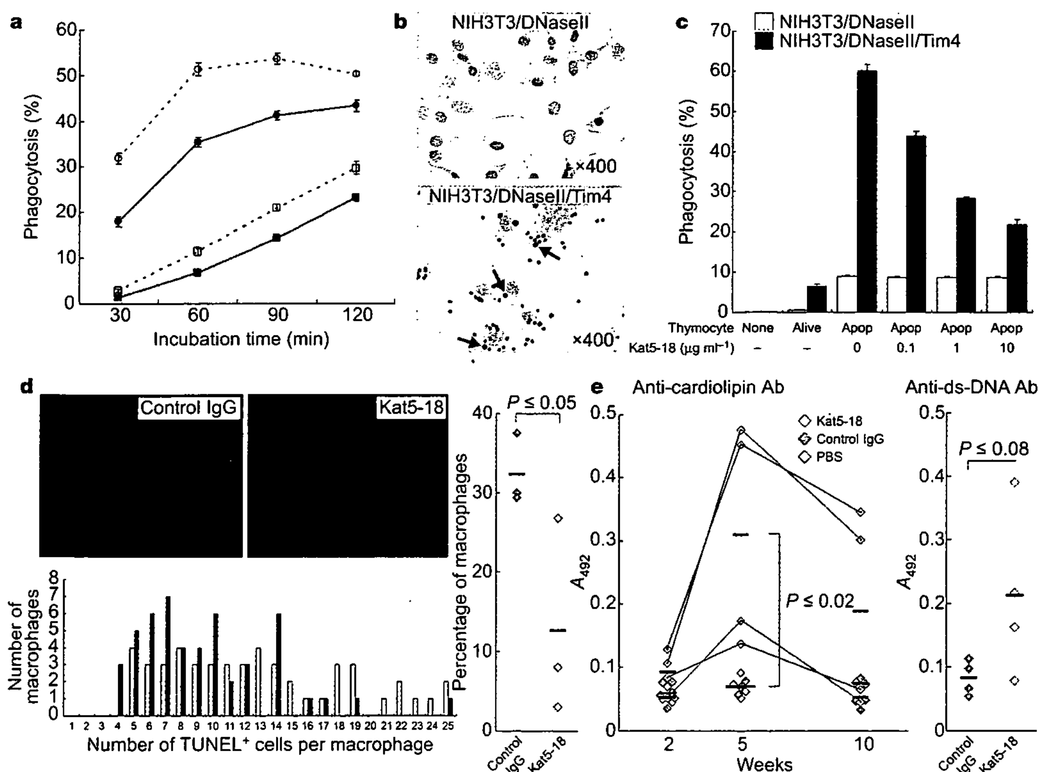

Figure 2 | Tim4-mediated engulfment of apoptotic cells. **a**, NIH3T3/DNase II (filled squares, open squares) and NIH3T3/DNase II/Tim4 (filled circles, open circles) incubated with 3.0×10^5 (filled squares, filled circles) or 6.0×10^5 (open squares, open circles) apoptotic $CAD^{-/-}$ thymocytes for the indicated periods. The percentage of $TUNEL^{+}$ macrophages was determined by flow cytometry. Average values from three experiments are shown; error bars, ±1s.e. **b**, NIH3T3/DNase II and NIH3T3/DNase II/Tim4 used for 60-min phagocytosis, stained for TUNEL, and observed by microscopy. Arrows indicate $TUNEL^{+}$ cells. **c**, NIH3T3/DNase II or NIH3T3/DNase II/Tim4 incubated with healthy or apoptotic thymocytes in the presence of Kat5-18, stained for TUNEL. The percentage of $TUNEL^{+}$ macrophages was determined by flow cytometry. Average values from three experiments are shown; error bars, ±1s.e.. **d**, $CAD^{-/-}$ mice that had received normal hamster Ab or Kat5-18 were treated with dexamethasone. Top, the thymus stained for TUNEL (green) and F4/80 (red). The number of $TUNEL^{+}$ cells determined for 50 randomly selected macrophages (bottom). Experiments were carried out three times, and the percentage of the macrophages carrying more than 15 apoptotic cells plotted (right). Mann–Whitney's U-test was used to analyse the difference, and P values are shown. **e**, Normal hamster IgG, Kat5-18 or PBS was injected into mice twice a week for 5 weeks. The serum concentration of anti-cardiolipin and anti-dsDNA was determined at 2, 5 and 10 weeks. P values determined as above are shown. A_{492}, absorbance at 492 nm.

cells were cultured with NIH3T3/DNase II, few apoptotic cells were seen attached to NIH3T3/DNase II (Fig. 2b). In contrast, many apoptotic cells associated with NIH3T3/DNase II/Tim4, and many were TUNEL-positive, indicating that these cells had been engulfed. NIH3T3/DNase II/Tim4 engulfed apoptotic cells but not healthy ones, and this engulfment was dose-dependently inhibited by Kat5-18 (Fig. 2c), confirming that Tim4 conferred the ability to engulf apoptotic cells on NIH3T3/DNase II. To confirm the function of Tim4 *in vivo*, $CAD^{-/-}$ mice were given Kat5-18, and treated with dexamethasone to induce apoptosis in the thymus. As shown in Fig. 2d, in the thymus of mice that received the control IgG, 32.3% of F4/80$^+$ macrophages carried more than 15 TUNEL$^+$ apoptotic cells. This percentage of the macrophages carrying more than 15 apoptotic cells was reduced to about 12.6% in the thymus of the mice that received Kat5-18. Inefficient engulfment of apoptotic cells often leads to autoimmune diseases[17]. In fact, the mice that received Kat5-18 developed autoantibodies (anti-cardiolipin and anti-double-stranded-DNA antibodies) within 5 weeks in their serum (Fig. 2e). Two mice strongly responded to Kat5-18 and two weakly, but the injection of normal IgG or PBS never caused the development of autoantibodies. The serum anti-cardiolipin antibody level was reduced when the injection of Kat5-18 was interrupted, confirming that this increase was due to Kat5-18.

Mouse Tim4 is a type I membrane protein, consisting of a signal sequence and extracellular, transmembrane and cytosolic regions (Fig. 3a). To examine how Tim4 enhances the engulfment of

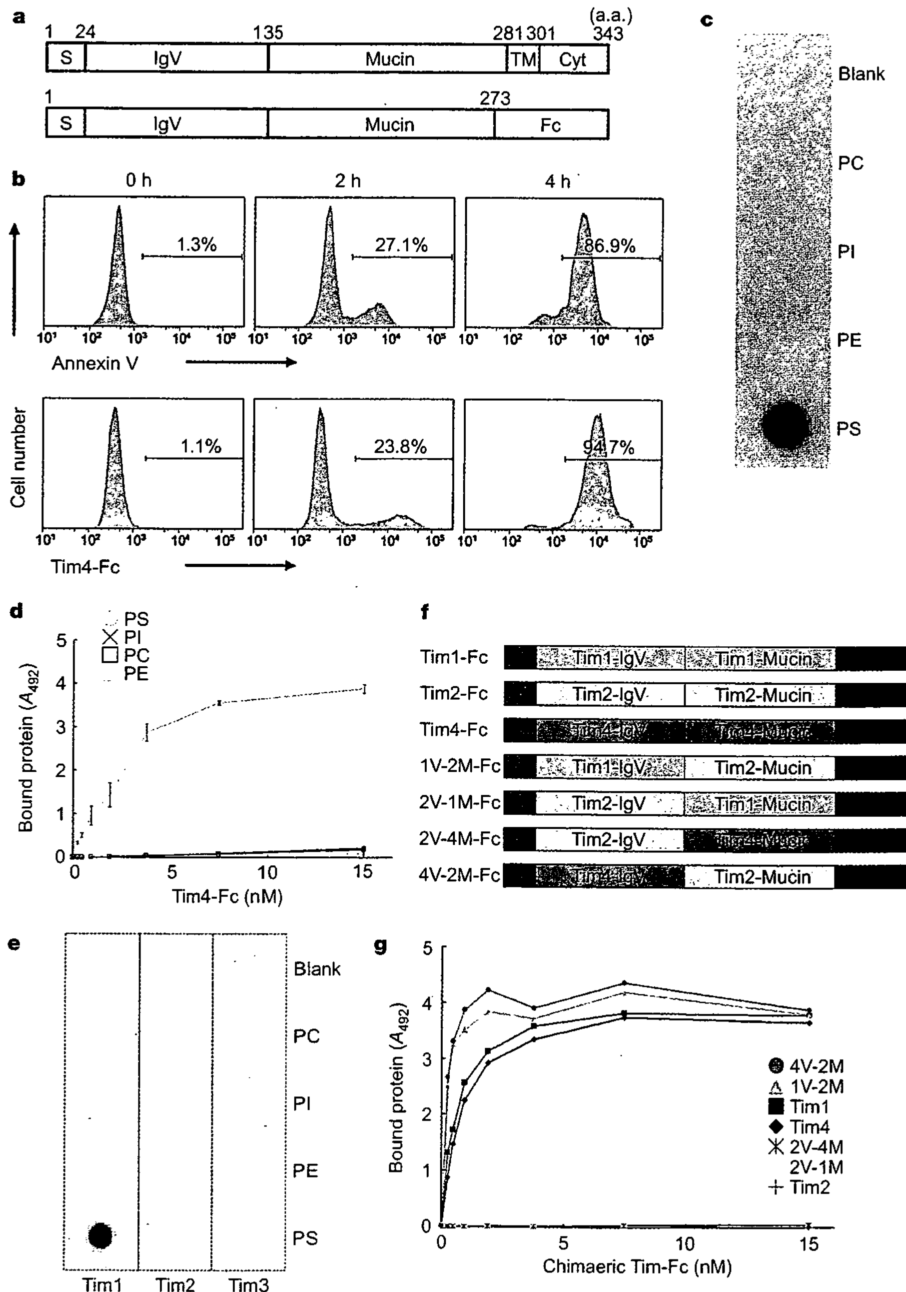

Figure 3 | Binding to phosphatidylserine. **a**, Structure of Tim4, showing signal sequence (S), IgV-like (IgV), mucin-like (Mucin), transmembrane (TM) and cytoplasmic regions (Cyt). a.a., amino acid. Tim4-Fc carries the Tim4 extracellular region and the Fc region of human IgG. **b**, Ba/F3-Fas treated with Fas ligand for the indicated periods, stained with annexin V or Tim4-Fc. **c**, A filter spotted with phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) or phosphatidylserine (PS), incubated with Tim4-Fc followed by anti-IgG. **d**, Microtitre plates coated with indicated phospholipids incubated with Tim4-Fc, followed by anti-IgG. The assay was performed in triplicate. **e**, Lipid overlay assay performed as **c** with Tim1-Fc, Tim2-Fc and Tim3-Fc. **f**, Structures of the extracellular region of Tim1, Tim2 and Tim4, and their hybrids, fused to human IgG. **g**, Microtitre plates coated with PS incubated with increasing concentrations of Tim1-Fc, Tim2-Fc, Tim4-Fc, or their hybrids.

apoptotic cells, the Tim4 extracellular region was fused to the Fc region of human IgG (Tim4-Fc). The purified Tim4-Fc showed a band at 82 kDa in SDS–PAGE under reducing conditions, and a 170 kDa band under non-reducing conditions (Supplementary Fig. 3), indicating that Tim4-Fc existed as a dimer. When Ba/F3 expressing Fas was treated with Fas ligand, they underwent apoptosis and bound annexin V (Fig. 3b), which binds to PS exposed on the surface of apoptotic cells[18]. These cells also bound Tim4-Fc, with a time course similar to their binding of annexin V. When a nitrocellulose filter spotted with various phospholipids was incubated with Tim4-Fc, Tim4-Fc bound PS, but not phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine (Fig. 3c). The affinity of Tim4-Fc for PS, determined with microtitre plates coated with phospholipids, was comparable to that of MFG-E8 ($K_d \approx 2$ nM)(Fig. 3d). These results suggested that Tim4 recognizes PS exposed on apoptotic cells as an engulfment signal. In support of this idea, the MFG-E8 mutant D89E dose-dependently inhibited the engulfment of apoptotic cells by NIH3T3/DNase II/Tim4 (Supplementary Fig. 4).

The Tim family has three members in human (TIM1, 3 and 4), and eight in mouse (Tim1–8). To examine whether other Tim family members can recognize PS, the Fc fusion proteins were prepared with mouse Tim1, Tim2 and Tim3 (Supplementary Fig. 5). Tim1-Fc, Tim2-Fc and Tim3-Fc each behaved as a dimer (Supplementary Fig. 6). The lipid overlay assay indicated that Tim1-Fc, but neither Tim2-Fc nor Tim3-Fc, specifically bound PS (Fig. 3e). Accordingly, full-length Tim1, but neither Tim2 nor Tim3, enhanced the engulfment of apoptotic cells when it was expressed in NIH3T3/DNase II cells (Supplementary Fig. 7). The extracellular region of Tim family members consists of two domains (IgG V-chain like (IgV) and mucin domains)[8]. To examine which domain of Tim1 and Tim4 is responsible for binding PS, IgV and mucin domains were swapped between Tim1 and Tim2, and between Tim4 and Tim2, and fused to the Fc region (Fig. 3f). As shown in Fig. 3g, the chimaeric molecules carrying IgV domain from Tim1 or Tim4 tightly bound PS, while no binding was observed with the chimaeric molecules carrying mucin domain from Tim1 or Tim4.

Various mammalian cells produce exosomes that expose PS on their surface[7]. The ability of Tim1 and Tim4 to bind PS suggested that exosomes bind to cells expressing Tim1 or Tim4. To examine this possibility, Ba/F3 cell lines expressing Tim1 or Tim4 (Ba/F3-Tim1 or Ba/F3-Tim4) were established. The growing Ba/F3-Tim1 or Ba/F3-Tim4 but not the parental Ba/F3 bound annexin V (Fig. 4a, and data not shown). Ba/F3-Tim1 and Ba/F3-Tim4 were then treated with biotin-labelled annexin V, followed by staining with gold-conjugated streptavidin. Observation by electron microscopy indicated that Ba/F3-Tim1 or Ba/F3-Tim4 was associated with exosome-like vesicles, and several gold particles were found on the outer leaflet of the exosomes (Fig. 4b). Such vesicles were not observed with the Ba/F3 parental cells. Meyers *et al.*[19] reported that Tim4 acts as a ligand for Tim1 expressed in Th2 cells. Accordingly, Tim1-Fc strongly bound Ba/F3-Tim4 (Fig. 4c). In addition, Tim1-Fc bound Ba/F3-Tim1, and Tim4-Fc bound Ba/F3-Tim4. The ability of Tim4 and Tim1 to bind PS suggested that these homophilic and heterophilic interactions between Tim1 and Tim4 can be stimulated by exosomes. In support of this idea, the binding of Tim4-Fc to Ba/F3-Tim4 was inhibited by annexin V (Fig. 4d). To directly confirm the effect of exosomes on association between Tim1 and Tim4, microspheres were coated with Tim4-Fc, and subjected to binding assay with Tim1-Fc. As shown in Fig. 4e, little Tim1-Fc bound to Tim4-Fc-coated beads, and exosomes from Ba/F3 dose-dependently stimulated the binding of Tim1-Fc to the beads.

Phosphatidylserine exposed on the outer leaflet of the plasma membrane is often used as a recognition signal for phagocytes to

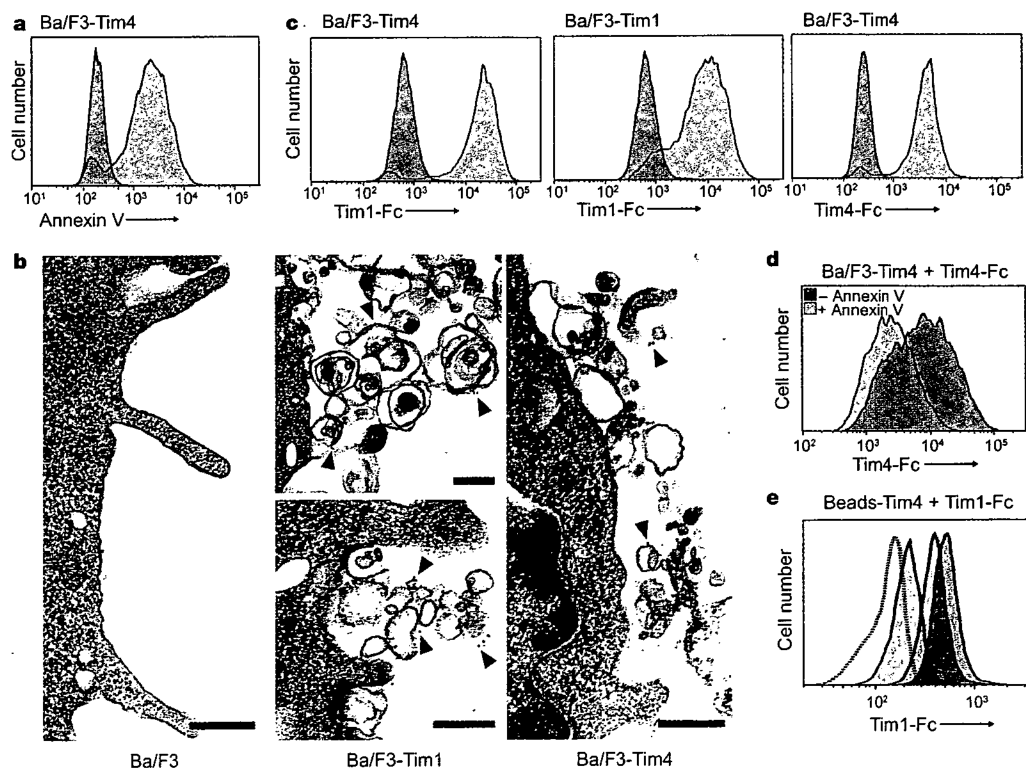

Figure 4 | Association of Tim1 and Tim4 via exosomes. **a**, Ba/F3-Tim4 incubated with PE-annexin V, and analysed by flow cytometry. Staining profile of Ba/F3 in red. **b**, Electron micrographs of Ba/F3, Ba/F3-Tim1 or Ba/F3-Tim4, incubated with biotin-annexin V and stained with streptavidin-colloidal gold. Gold particles on exosome-like vesicles indicated by arrowheads. Scale bar, 0.3 μm. **c**, Ba/F3-Tim4 or Ba/F3-Tim1 incubated with Tim1-Fc or Tim4-Fc, stained with FITC-anti-IgG, and analysed by flow cytometry. Staining profiles of Ba/F3 in red. **d**, Ba/F3-Tim4 incubated with Tim4-Fc in the absence or presence of annexin V, stained with FITC-anti-IgG. **e**, Tim4-Fc-coated latex beads incubated with Tim1-Fc in the absence (green) or presence of 10-fold (blue) or 50-fold (brown) concentrated exosomes, stained with PE-anti-Tim1, and analysed by flow cytometry. Profile without Tim1-Fc in red dotted line.

engulf apoptotic cells. Fadok *et al.* reported that phosphatidylserine receptor (PSR), a ubiquitously expressed type II-membrane protein, binds to PS on apoptotic cells and mediates their engulfment[20], and initial experiments with $PSR^{-/-}$ mice supported this notion[21,22]. However, a careful analysis of $PSR^{-/-}$ mice by Bose *et al.*[23] indicated that PSR is not involved in the engulfment of apoptotic cells, and it is unlikely to work as a receptor for PS. Here, we showed that Tim4 expressed in macrophages in various tissues mediates the engulfment of apoptotic cells by recognizing PS, indicating that Tim4 is the phosphatidylserine receptor for engulfment of apoptotic cells. Tim1 also enhanced the PS-dependent engulfment of apoptotic cells. Tim1 is expressed in kidney tubule cells post-ischaemia[24], suggesting that it may have a role in removing injured and dead cells, to help restore the morphological integrity of the kidney. It will be interesting to examine whether any other molecules work as phosphatidylserine receptors.

Some non-apoptotic T or B cells[25,26] as well as macrophages[27] have been reported to bind annexin V. Here, we showed that the cells expressing Tim1 or Tim4 associated with exosomes that carried exposed PS. Activated Th2 cells and B cells express Tim1[28,29], while the resident macrophages express Tim4, suggesting that the non-apoptotic annexin V-binding cells are those that express Tim1 or Tim4. Exosomes are secreted from various cells and act as an intercellular signalling device[7]. Tim1 and Tim4 may mediate this signalling by enhancing the uptake of exosomes. Cells expressing Tim1 or Tim4 tended to aggregate (M.M. and S.N., unpublished results). PS on activated T cells is suggested to function in cell-to-cell contact at the immunological synapse[25]. Thus, Tim1 and Tim4 may also be involved in cell-to-cell interactions via PS on exosomes. Finally, Tim family genes of human and mouse are located on a genetic interval that has linkage to a number of autoimmune diseases, such as asthma, allergy and atopy[30]. The establishment of Tim1 and Tim4 as PS receptors may contribute to the understanding of these autoimmune diseases.

METHODS SUMMARY

Production of monoclonal antibodies. Armenian hamsters (Oriental Yeast) were immunized with mouse peritoneal cells, and about 1,200 hybridomas were established by fusing cells from the popliteal and inguinal lymph nodes with $NSO^{bcl\text{-}2}$ mouse myeloma. The hybridoma supernatants were tested by a phagocytosis assay, and one hybridoma (Kat5-18) that inhibited the engulfment of apoptotic cells was identified.

Construction of cDNA library, and expression cloning. The double-stranded cDNA longer than 1.0 kb was ligated into a *Bst*XI-digested pMXs vector[15]. *Escherichia coli* DH10B was transformed by electroporation, and about 1.0×10^6 clones were produced. Plasmid DNA from the cDNA library was introduced into PLAT-E packaging cells, and the culture supernatant was used to infect Ba/F3. A subpopulation of Ba/F3 that stained intensely with Kat5-18 was selected by repeated FACS. The integrated cDNA was recovered by PCR with the vector primers from the genomic DNA of Ba/F3 transformants.

Phagocytosis assay. To assay the engulfment of apoptotic cells, $CAD^{-/-}$ thymocytes were induced to undergo apoptosis with leucine-zipper tagged Fas ligand, and co-cultured with peritoneal macrophages or NIH3T3/DNase II cells. The cells were fixed with paraformaldehyde, subjected to TUNEL staining, and analysed by flow cytometry using a FACSAria (BD Biosciences).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors

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Summary: Apoptotic cells are recognized and subsequently engulfed by macrophages and immature dendritic cells. The engulfed dead cells are transported to the lysosomes of macrophages, and their components are degraded into amino acids and nucleotides for reuse. In mammals, macrophages also engulf nuclei expelled from erythroid precursors in the final stage of definitive erythropoiesis. Failure to swiftly engulf dead cells at the germinal centers of lymphoid organs causes systemic lupus erythematosus-type autoimmune diseases. In contrast, failure to efficiently degrade the DNA of dead cells or erythroid cell nuclei activates innate immunity, causing lethal anemia in the fetus and chronic arthritis in adults.

Keywords: *apoptosis, definitive erythropoiesis, DNA degradation, phosphatidylserine, macrophages, MFG-E8*

Introduction

Every day, a large number of cells (10^8–10^9 cells) in the human body undergoes apoptosis (1, 2). In the last stage of this process, apoptotic bodies, which are fragmented cell bodies carrying cellular components, present 'eat-me' signals and are engulfed by phagocytes such as macrophages, immature dendritic cells, and microglia (3–5). Endosomes containing the engulfed dead cells fuse with lysosomes, where the dead cell components are degraded into amino acids and nucleotides. In adult humans, about 10^{11} red blood cells are produced per day. Red blood cells in mammals do not carry nuclei, and they are produced in a process called definitive erythropoiesis (6). Definitive erythropoiesis takes place in anatomical units called 'erythroblastic islands' in the fetal liver or bone marrow, where erythroblasts proliferate and differentiate in contact with the macrophages, that reside in the center of each island (7, 8). In the last stage of this process, nuclei are expelled from the erythroid precursors and are engulfed by the macrophages.

Unlike most cellular components that are degraded during apoptosis, the degradation of chromosomal DNA occurs in two steps. First, the chromosomal DNA of apoptotic cells is cell-autonomously cleaved into nucleosomal units (9) by caspase-activated DNase (CAD) (10, 11). Following engulfment by macrophages, the DNA of the dead cells is further degraded into nucleotides by DNase II, an acid DNase located in the lysosomes of macrophages (12). DNase II is also responsible for degrading nuclear DNA from erythroid precursor cells in the macrophages of bone marrow and fetal liver (13). In DNase II-deficient mice, macrophages in various tissues, particularly fetal liver and bone marrow, accumulate undigested DNA in the lysosomes (13, 14), which activate them to produce cytokines such as interferon β (IFNβ) and tumor necrosis factor α (TNFα) (12, 15). IFNβ thus produced from the macrophages in erythroblastic islands kills erythroblasts directly or indirectly, leading to lethal anemia in embryos (15). However, TNFα goes to the joints, activates synovial cells, and triggers chronic polyarthritis in adults (16).

The engulfment of apoptotic cells has been thought to prevent the release of noxious materials from dying cells, which otherwise might cause inflammation (17). However, there has been no clear evidence for this notion. We recently identified a molecule that works as a bridge between apoptotic cells and phagocytes (18). A deficiency in this molecule causes inefficient engulfment of apoptotic cells in the germinal centers of the spleen and induces systemic lupus erythematosus (SLE)-type autoimmune diseases (19, 20).

The identification of molecules involved in the engulfment and degradation of dead cells suggests that failure of this process leads to autoimmune diseases such as rheumatoid arthritis and SLE. In this review, I discuss the final stage of programmed cell death and erythropoiesis (engulfment and degradation of dead cell components as well as the erythroid nuclei) and the diseases caused by its failure.

Apoptosis

During animal development, many useless and toxic cells are generated and removed (2, 21). Cells also die in adults: that is, every cell has its own life-span, and senescent cells die and are replaced by newly generated ones. When cells are infected by bacteria or viruses, they are swiftly removed to prevent the pathogen from spreading. These physiological cell deaths, also known as programmed cell death, consist of two steps: cell death and the clearing of the corpse.

There are at least two death processes, necrosis and apoptosis, and they can be distinguished morphologically (22). In necrosis, the nuclei and mitochondria swell, the cell membranes rupture, and all the cellular contents are released. During the apoptotic process, the cell membranes convolute, and the nuclei condense and become fragmented. In the final stage of this process, the cells themselves become fragmented, and the fragments retain the cellular contents. These condensed and fragmented cells (called apoptotic bodies) are engulfed by phagocytes, and therefore the contents of the dying cells are not released. This clean apoptotic death process, regulated by gene products, is likely to account for most of the cell death that occurs during development (23). In contrast, interdigital cells, chondrocytes in bone, and intestinal epithelial cells may be removed by necrosis during development (24–27), but the molecular mechanism of necrosis is not well understood.

Most animal cells carry the machinery for apoptosis (1, 2, 28–30). During animal development, some factor(s) that triggers the death machinery is transcriptionally upregulated in certain cells and then activated at the appropriate developmental point (intrinsic cell death) (1). Cytotoxic T lymphocytes (CTLs), anti-cancer drugs, γ-irradiation, and factor deprivation also turn on the death machinery. Among these death triggers, CTLs and natural killer cells express Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), which are type II-membrane proteins of the TNF family, and trigger non-cell-autonomous apoptosis (extrinsic cell death) (31–33). Whatever the trigger, apoptotic cell death is mediated in most cases by caspases (cysteine proteases). Caspases comprise a family of 12 members in human, and they take part in an apoptotic cascade when activated. Caspases 3 and 7 are the downstream effectors of the cascade, and they specifically cleave a set of cellular enzymes and proteins (more than 300 substrates), thereby inactivating or activating them (34). The cleavage of these enzymes and proteins is responsible for the morphological and biochemical changes that occur during apoptosis and for killing the cells. There are many excellent reviews on apoptosis (1, 28, 29, 31, 32, 35–38) that describe the details of the apoptotic pathways.

Apoptotic DNA degradation

In addition to the morphological changes in the cells, apoptosis is accompanied by DNA fragmentation (9, 39, 40). At the early stage of apoptosis, chromosomal DNA is degraded into multimers of 180 bp, a nucleosomal unit. This cleavage is accomplished by CAD, also called DFF-40 (DNA fragmentation factor 40) (10, 41–43). In proliferating cells, CAD is complexed with its inhibitor, ICAD (inhibitor of CAD)

or DFF-45 (10, 11, 43). ICAD also works as a chaperone for CAD during its synthesis (44, 45), and functional CAD cannot be synthesized without ICAD (41, 46). DNA can bind to the CAD–ICAD complex, but it cannot be cleaved because the active site of CAD that is necessary for its enzymatic activity is masked by ICAD (47). Caspases 3 and 7 cleave ICAD at two positions, inactivating ICAD's ability to inhibit CAD's DNase (48). Similar to DNase I and DNase II, CAD is an endonuclease containing histidine residues in its active site (49, 50), and it cleaves double-stranded DNA with some preference for A–T-rich regions (51). CAD carries a nuclear localization signal and cleaves chromosomal DNA when the nucleosomal structure is intact. It exists as a homodimer with a scissors-like structure, and the histidine-containing active site is located in the deep cleft between the 'blades' (52). This structure prevents CAD from accessing the DNA on the nucleosomes but allows CAD to access DNA at the spacer regions between the nucleosomes. This explains why chromosomal DNA is degraded into nucleosomal units during apoptotic cell death.

There are reports claiming that residual DNA degradation (high molecular weights or nucleosomal units) takes place in ICAD-deficient cells (53, 54). However, in our hands, cells expressing caspase-resistant ICAD or cells lacking ICAD or CAD show no DNA degradation in response to any apoptotic stimuli we have tested (12, 41, 55), indicating that CAD is solely responsible for the cell-autonomous apoptotic DNA degradation. Other DNases, such as endonuclease G, DNase I, and DNase γ, have been proposed to play a role in this process (53, 56–58). However, the absence of DNA degradation in CAD-null cells and the lack of effect on apoptotic DNA degradation when the genes for *endonuclease G* or *DNase I* are deleted (59–61) indicate that these nucleases are not involved in apoptotic DNA degradation or that their role in this process is limited (62, 63).

In contrast to the lack of apoptotic DNA fragmentation observed *in vitro* in *CAD*-null cells, apoptotic DNA degradation, assessed by staining with TdT-mediated dUTP-nick end labeling (TUNEL), occurs normally *in vivo* in *CAD*-deficient mice (64). Further *in vitro* studies indicated that the DNA of apoptotic cells is degraded by DNase II, a lysosomal enzyme, after the dying cells are engulfed by macrophages (12, 65). Thus, the chromosomal DNA of apoptotic cells is degraded in two steps (12, 64, 66) (Fig. 1). First, the DNA is cleaved by CAD in the dying cells, leaving the nucleosomes intact. Next, the apoptotic cells are engulfed by macrophages and transferred into their lysosomes. Lysosomes carry not only DNase (DNase II) but also proteases, lipases, and glycosidases, and the cellular components of apoptotic cells are degraded by these enzymes into amino acids, nucleosides, fatty acids, and glycosides.

Degradation of nuclear DNA during erythropoiesis

Early in normal mouse embryogenesis, the red blood cells are produced in the yolk sac, and these cells are nucleated (67). This process is called primitive erythropoiesis. From embryonic day 12.5 (E12.5) onward, erythropoiesis takes place in the fetal liver instead of in the yolk sac. Like the erythroid cells produced in the bone marrow of the adult, those produced in the fetal liver are enucleated; this process is known as 'definitive erythropoiesis' (68). Definitive erythropoiesis takes place in anatomical units called 'erythroblastic islands' in the bone marrow and fetal liver. Each island contains a macrophage at the center, surrounded by erythroid precursor cells at different developmental stages (erythroblast, reticulocytes, and mature enucleated erythrocytes). It seems that the macrophage in the blood island supports the proliferation and differentiation of erythroid precursor cells (69, 70). Erythroid cells cell-autonomously undergo enucleation, and the expelled nuclei are engulfed by the central macrophage (Fig. 1). Like the DNA of engulfed apoptotic cells, the engulfed erythocytic nuclei are degraded in the lysosomes of the macrophages by DNase II (13).

Lethal anemia in *DNase II*-null embryos

DNase II is an endonuclease that works efficiently under acidic conditions (71). There are two subtypes of DNase II, DNase IIα (DNase II) and DNase IIβ [DNase II-like acid DNase (DLAD)] (72, 73). The expression of DLAD is limited to the lens-fiber cells of the eye (74), in particular, the fiber cells close to the organelle-free zone of the lens (75). DLAD is responsible for cell-autonomously degrading nuclear DNA during the differentiation of the fiber cells (Fig. 1). Thus, *DLAD*$^{-/-}$ mice accumulate undigested DNA in the cytoplasm of lens-fiber cells and suffer from cataract (Fig. 2).

DNase II or DNase IIα is ubiquitously expressed in various tissues, particularly in macrophages. Cell lysates prepared from the fetal livers of *DNase II*$^{-/-}$ mice do not show DNase activity under acidic conditions, indicating that lysosomes carry a single DNase, DNase IIα. The null mutation of the *DNase II* gene is embryonic lethal; *DNase II*$^{-/-}$ mice die late in embryogenesis from severe anemia (13). In these embryos, the number of primitive erythrocytes at E12.5 is normal, but the number of definitive erythrocytes at E17.5 is severely reduced (< 10% of the number in wildtype mice). This finding suggests that definitive erythropoiesis is specifically

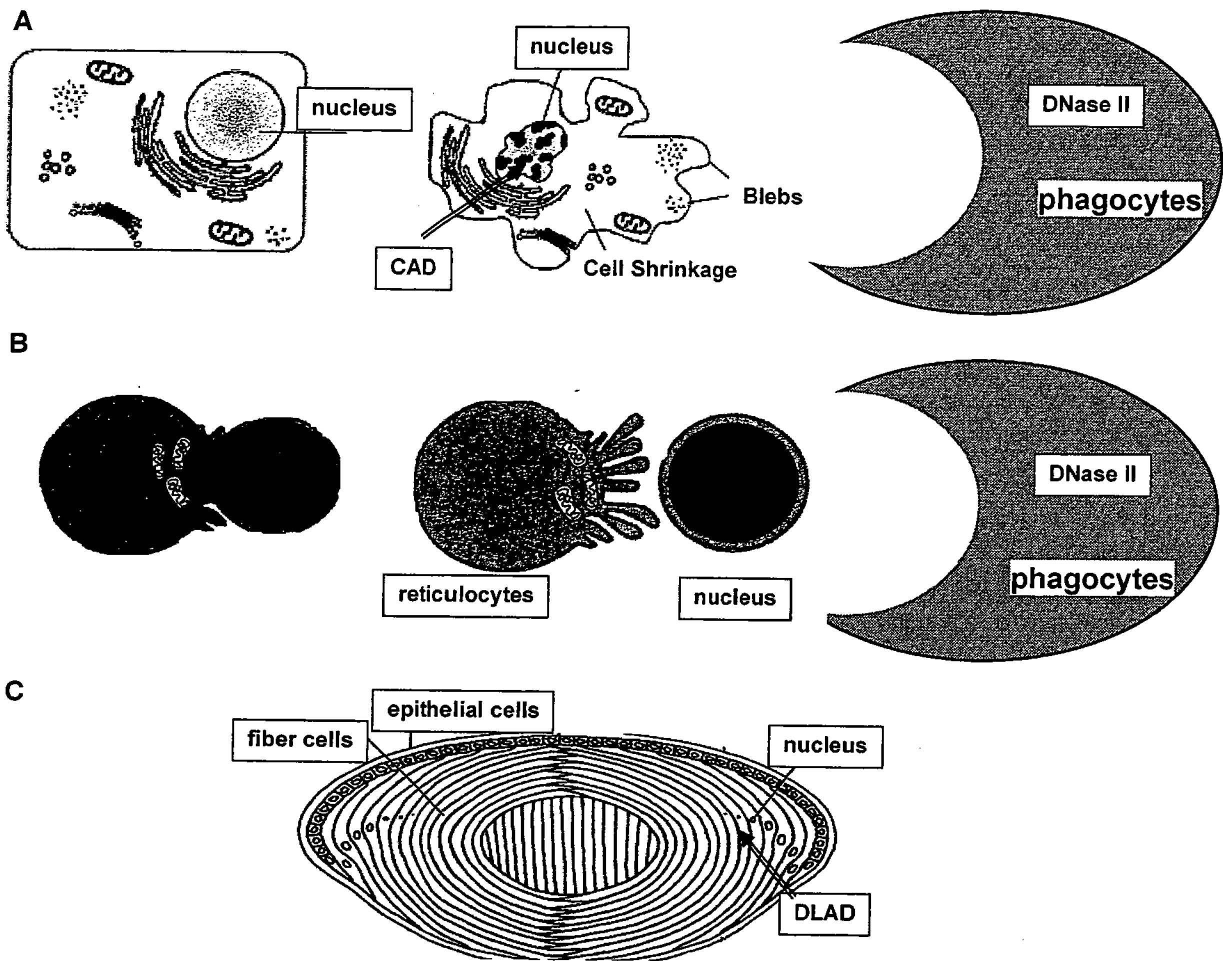

Fig. 1. DNA degradation during three developmental stages. (A) Two-step DNA degradation in programmed cell death. Cells die through apoptosis, in which the cells and nucleus are fragmented. In this process, chromosomal DNA of apoptotic cells is cleaved first by caspase-activated DNase (CAD) into nucleosomal units. Dead cells are then engulfed by phagocytes, such as macrophages and immature dendritic cells, and DNase II in their lysosomes digest DNA of the dead cells into nucleotides. (B) DNA degradation in definitive erythropoiesis. Red blood cells lack a nucleus and are produced in a process called definitive erythropoiesis, which occurs in erythroblastic islands that have a single, central macrophage. In the last stage of erythropoiesis, nuclei are extruded by the erythrocytes and engulfed by the macrophage. DNase II in the lysosomes of the macrophage digests DNA. (C) DNA degradation in lens-cell differentiation. The eye lens is composed of fiber cells that are derived from epithelial cells. At the equator, epithelial cells move toward the lens nucleus and differentiate into fiber cells. During this differentiation process, all the cell organelles, including the nucleus, mitochondria, and endoplasmic reticulum, are degraded. DNase II-like acid DNase (DLAD) in the lysosomes of fiber cells digests chromosomal DNA.

affected in the *DNase II*-null mutant, which leads to fatal anemia. When the fetal liver cells of *DNase II*$^{-/-}$ mice are transferred into an irradiated host, the *DNase* II-null precursor cells differentiate into mature enucleated erythrocytes. This finding indicates that DNase II is not necessary in the erythroid cells but in the cells that support erythropoiesis.

A large number of macrophages carrying undigested DNA are present throughout the *DNase II*$^{-/-}$ embryos (13, 14) (Fig. 2). These macrophages are found not only in tissues where many cells undergo programmed cell death, such as the thymus, brain, and kidney, but also in the fetal liver, where definitive erythropoiesis takes place, confirming that DNase II is responsible for degrading the DNA of apoptotic cells as well as the nuclear DNA from erythroid precursor cells. The gene expression profile, determined by microarray analysis, indicated that the expression levels of genes that support erythropoiesis, such as erythropoietin, stem cell factor, erythropoietin receptor, and GATA-1, are similar between the wild-type and *DNase II*$^{-/-}$ fetal livers. In contrast, a set of IFN-inducible genes are strongly activated in the fetal liver and thymus of *DNase II*$^{-/-}$-embryos (12, 15). In fact, IFNβ and IFNγ mRNAs were found in the fetal liver of *DNase II*$^{-/-}$ but not in that of wild-type mice. *In situ* hybridization showed a high level of IFNβ mRNA in macrophages carrying undigested DNA, while IFNγ mRNA was found not only in macrophages but also in other cells, such as erythroid cells, indicating that IFNβ is primarily produced by the macrophages carrying undigested DNA (15, 76).

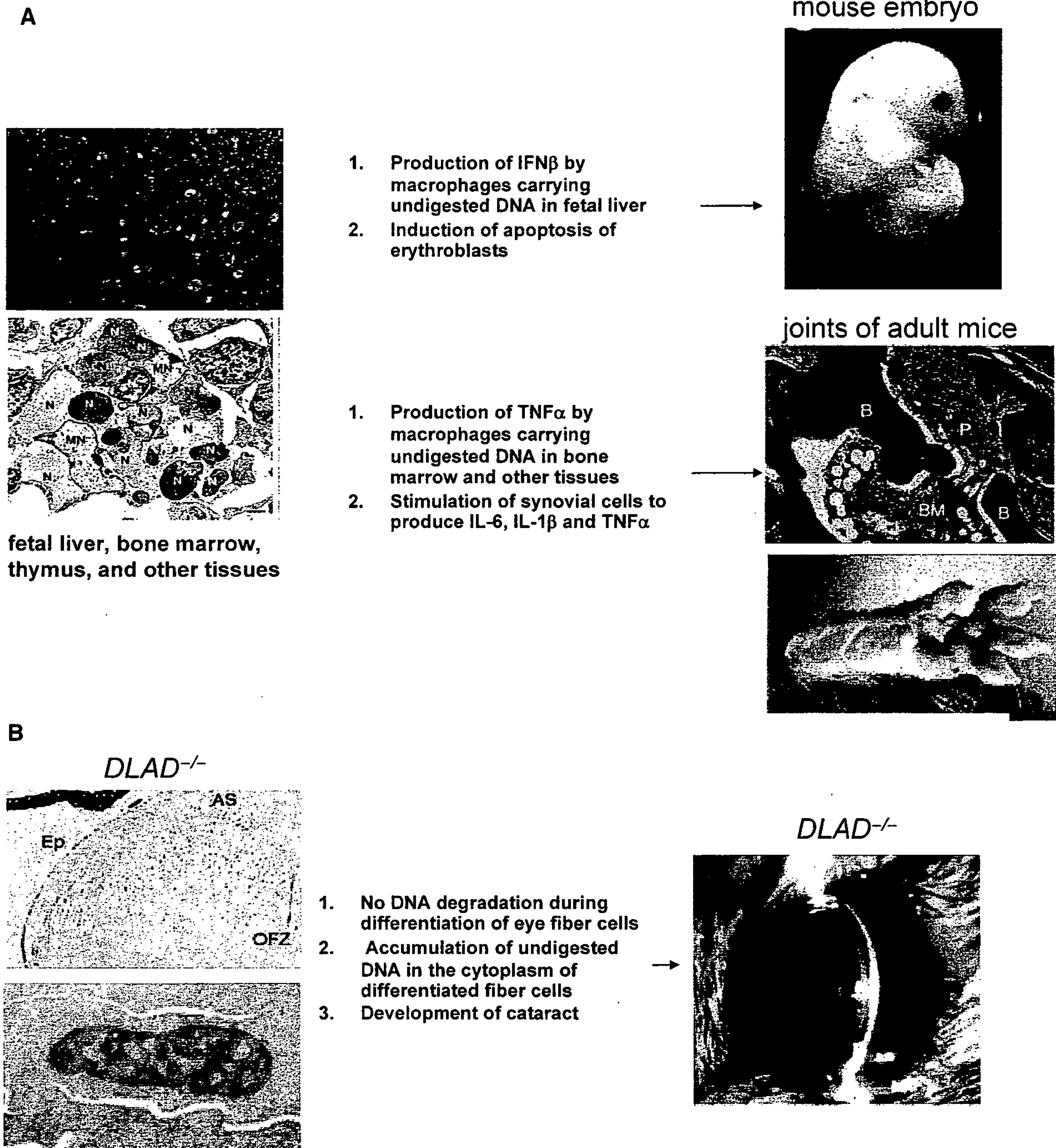

Fig. 2. Diseases caused by inefficient DNA degradation. (A) Anemia and chronic polyarthritis caused by DNase II deficiency. In *DNase II*$^{-/-}$ embryos, the macrophages in various tissues, particularly in the fetal liver, accumulate a large amount of undigested DNA in their lysosomes. These macrophages produce inflammatory cytokines such as interferon β (IFNβ) and tumor necrosis factor α (TNFα). IFNβ induces apoptosis of erythroblasts, leading to severe anemia. When the DNase II gene is deleted after birth, TNFα produced by the macrophages seems to be responsible for the activation of synovial cells in the joints, leading to chronic polyarthritis. (B) Cataract caused by the lack of DNase II-like acid DNase (DLAD). In *DLAD*$^{-/-}$ mice, chromosomal DNA is undigested during the differentiation of fiber cells in the eye lens. Thus, DNA is left in the cytoplasm of the differentiated fiber cells, where it disturbs the light path and leads to cataract formation.

Involvement of IFNβ in the lethality of *DNase II*$^{-/-}$ embryos

IFNβ is a cytokine belonging to the type I IFN family (77, 78). It protects cells from virus, but it also has strong cytotoxic effects in newborn mice (79, 80). There are two types of IFN receptors, type I (IFN-IR) and type II (IFN-IIR). The IFN-IR is responsible for mediating the signals of IFNα and IFNβ, while the IFN-IIR mediates the IFNγ signal. Mice that are doubly deficient in the genes for *DNase II* and IFN-IR (15), but not for *DNase* II and IFN-IIR (K. Kawane and S. Negata, unpublished

results), are apparently normal at birth, confirming that the IFNβ produced in the *DNase II*$^{-/-}$ mice is responsible for the lethality. Many erythroblasts in the fetal liver of *DNase II*$^{-/-}$ embryos are positive for TUNEL staining, indicating that erythroblasts undergo apoptosis in these mice (15). Because IFN-induced apoptosis depends on protein or RNA synthesis, it is likely that one or more factors induced by the elevated IFNβ indirectly activate the apoptosis program in the erythroblasts.

IFNβ is normally produced by cells infected by a virus or bacteria and is regarded as a marker gene for innate immunity (81). Toll-like receptors (TLRs) and their adapters [myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing IFNβ (TRIF)] are involved in the activation of the IFNβ gene in the innate immune reaction to bacterial or viral infection (82–84). In *DNase II*$^{-/-}$ mice, the macrophages accumulate DNA in their lysosomes and express IFNβ (15). When *DNase II*$^{-/-}$ macrophages engulf apoptotic cells *in vitro*, they also accumulate undigested DNA in their lysosomes and produce IFNβ (76), indicating that mammalian DNA that escapes degradation in lysosomes activates the innate immune system. However, a null mutation of *TLR9* or *TLR3* or of their adapters (*MyD88* and *TRIF*) had no effect on the lethality of the *DNase II*$^{-/-}$ genotype (76), suggesting that mammalian DNA activates the innate immune system in a TLR-independent manner. For bacterial components to activate the innate immune system, IRF3 (IFN-regulatory factor 3) and IRF7 are activated downstream of MyD88 or TRIF and bind to the IFN-regulatory element in the promoter region of the IFNβ gene (85, 86). The deletion of the genes for both *IRF3* and *IRF7* abolished the expression of IFNβ gene in the fetal liver of *DNase II*$^{-/-}$ embryos and rescued the lethality (Y. Okabe and S. Negata, unpublished results), indicating that an unidentified sensor for mammalian DNA is present upstream of IRF3/IRF7.

Chronic polyarthritis in *DNase II*-null mice

DNase II$^{-/-}$-*IFN-RI*$^{-/-}$ mice are born normal, but they develop polyarthritis as they age (16). When the *DNase II* gene alone is deleted after birth in an inducible conditional knockout mouse, the mice do not die but develop polyarthritis in a time-dependent manner (Fig. 2). Two to 3 months after the deletion of the *DNase II* gene, the forelimbs and hindlimbs start to swell. Similar to the development of rheumatoid arthritis in human patients, the joints affected first are at the periphery (digit), and those affected subsequently are increasingly central (foot and wrist). Eventually, all joints are affected within 8 months. The swollen joints show severe synovitis with aggressive pannus formation. Tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts are at the leading edge of the pannus, which fills the joint cavity, erodes cartilage, and often destroys the bones. Genes for inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and TNFα, are strongly activated in the affected joints. An anti-TNFα antibody and a soluble form of the TNF receptor that antagonize TNFα action have been successfully used to treat human patients with rheumatoid arthritis (87, 88). Similarly, the administration of an anti-TNFα antibody significantly improved the clinical score for the polyarthritis developed by the *DNase II*-null mice (16).

Inflammatory cytokines (IL-1β, TNFα, and IL-6) stimulate the proliferation of synovial cells in the joints, and these abnormally proliferated synovial cells form pannus and destroy the joints (89). In human rheumatoid arthritis, not only TNF antagonists but also molecules that neutralize IL-6 or IL-1β have clinically beneficial effects (90–92). Patients who respond to the TNF antagonist often respond to the IL-6 or IL-1β antagonists as well. Because the inflammatory cytokines TNFα, IL-1β, and IL-6 stimulate each other's genes (93, 94), it is likely that blocking one cytokine's action will cause the shutdown of gene expression for all the inflammatory cytokines. In this regard, it will be interesting to study whether molecules that neutralize IL-6 or IL-1β block the development of arthritis in *DNase II*$^{-/-}$ mice.

Although inflammatory cytokines are known to play an essential role in the maintenance of rheumatoid arthritis in human (95), what triggers the disease is still unknown, and the anti-TNF therapy does not cure it. Previously, Keffer *et al.* (96) showed that mice carrying human *TNFα* gene as a transgene develop polyarthritis. In the *DNase II*-null mice, the macrophages carrying undigested DNA in their lysosomes are activated as part of the innate immune reaction, and the *IFNβ* and *TNFα* genes are activated as well. In fact, *in situ* hybridization indicates that bone marrow macrophages carrying undigested DNA in *DNase II*$^{-/-}$ mice express TNFα mRNA (16). A low but significant level of TNFα is found in sera of *DNase II*$^{-/-}$ mice, at the stage before joints show any abnormality. Neutralization of TNFα by injecting anti-TNFα antibodies into young *DNase II*$^{-/-}$*IRF-IR*$^{-/-}$ mice prevented the development of polyarthritis.

Programmed cell death occurs extensively during development and then continues constitutively at a steady rate. Erythropoiesis is also strongest during development (97). It is likely that macrophages loaded with DNA from dead cells or erythroid precursors constitutively produce TNFα in *DNase II*-null mice, which triggers the development of arthritis (Fig. 2). Some patients with rheumatoid arthritis can be cured by bone marrow transplantation (98), suggesting that these patients

have a defect(s) in bone marrow-derived cells. It will be useful to learn whether these patients have defects in lysosomal DNA digestion by macrophages.

DNase II is a lysosomal enzyme, and the diseases caused by its deficiency are essentially lysosomal storage diseases, which result from defects in lysosomal function (99). Lysosomal storage diseases include more than 40 disorders with inactive or malfunctioning lysosomal enzymes, including proteases, glycosidases, and lipases. Innate immunity is activated by bacterial or viral proteins, polysaccharides, DNA, and RNA (100). As described above, mammalian DNA activates the innate immunity via a TLR-independent system. Other cellular components that escape degradation in lysosomes may also activate TNFα and IFN genes, leading to the symptoms of storage diseases. The finding that cytokines are constitutively secreted by fibroblasts derived from patients with Niemann–Pick disease type C (101), an inherited lipid storage disorder, may support this idea.

Engulfment of apoptotic cells

Although a large number of cells undergo apoptosis during mammalian development, it is difficult to detect them *in situ*, because they are quickly engulfed by professional phagocytes such as macrophages and immature dendritic cells (4, 5, 17). In animals such as *Caenorhabditis elegans*, which does not have professional phagocytes, non-professional fibroblasts or endothelial cells in the neighborhood of the apoptotic cells engulf the dead cells instead, although their efficiency of engulfment may be low (102, 103).

Macrophages engulf apoptotic cells but not healthy living cells. It is therefore thought that apoptotic cells present an eat-me signal(s) on their surface, and phagocytes recognize it. Many molecules have been proposed as the eat-me signal and its receptor. These include phosphatidylserine, intercellular adhesion molecule-3 (ICAM-3), Annexin I, and cardiolipin as the eat-me signal (17, 104, 105) and CD44, CD14, CD36, CD68, scavenger receptor, and phosphatidylserine receptor (PSR) as the receptor. However, understanding how these molecules are exposed on the cell surface of apoptotic cells and how they are involved in the cell's engulfment has been very elusive and rather confusing.

As soon as apoptotic dead cells are engulfed by phagocytes, the dead cell's components are degraded in the phagocyte's lysosomes. It is not easy, therefore, to assay the engulfment of apoptotic cells or to distinguish cells engulfed by macrophages from those that are simply attached to them. Using our knowledge that *CAD*-deficient cells undergo apoptosis without DNA fragmentation and are efficiently engulfed by macrophages (12), which efficiently cleave their DNA and produce TUNEL-positive cells (64), we established a reliable assay system for the engulfment of apoptotic cells (18). A hamster hybridoma library of monoclonal antibodies against mouse macrophages composed of more than 3000 clones was screened with this assay, and one monoclonal antibody was found to affect engulfment. Purification of the antigen recognized by the monoclonal antibody led to its identification as 'milk fat globule EGF factor VIII' (MFG-E8) (18). Mouse MFG-E8 is a secreted protein that has a signal sequence, two EGF domains, and two factor VIII-homologous domains (106, 107). MFG-E8 was first identified as a protein expressed in mammary glands and is particularly abundant when the mammary glands undergo involution (108, 109). It is also secreted from thioglycollate-elicited peritoneal macrophages but not resident peritoneal macrophages (18).

Among the many molecules proposed as eat-me signals, phosphatidylserine, a glycerophospholipid, is located inside healthy cells but exposed to the outer leaflet of the plasma membrane when the cells undergo apoptosis (110). When erythrocyte ghosts (erythrocytes whose contents have been removed) are loaded with phosphatidylserine, they are phagocytosed by macrophages (110, 111). Thus, phosphatidylserine has been a strong candidate for the eat-me signal (Fig. 3). MFG-E8 binds phosphatidylserine via its factor VIII-homologous domain and binds the $\alpha_v\beta_3$- or $\alpha_v\beta_5$-type integrin through an RGD motif in the EGF domain (18, 112–114). Because macrophages and immature dendritic cells express the $\alpha_v\beta_3$- or $\alpha_v\beta_5$ integrin, it seems likely that MFG-E8 works as a bridge between apoptotic cells and phagocytes. In fact, NIH3T3 fibroblasts, which normally do not engulf apoptotic cells, efficiently engulf apoptotic cells in the presence of MFG-E8 when they are transformed with $\alpha_v\beta_3$ integrin.

Autoimmunity caused by a defect of MFG-E8

In addition to mammary epithelial cells, MFG-E8 is expressed in thioglycollate-elicited peritoneal macrophages and granulocyte macrophage-colony-stimulating factor (GM-CSF)-induced bone marrow-derived immature dendritic cells (115). These macrophages and immature dendritic cells from *MFG-E8*-deficient mice have severe defects in engulfing apoptotic cells, indicating that these cells mainly use MFG-E8 for engulfment (19). Langerhans cells, immature dendritic cells of the skin, also express abundant MFG-E8, although its physiological role in the Langerhans cells is not clear. The secondary lymphoid

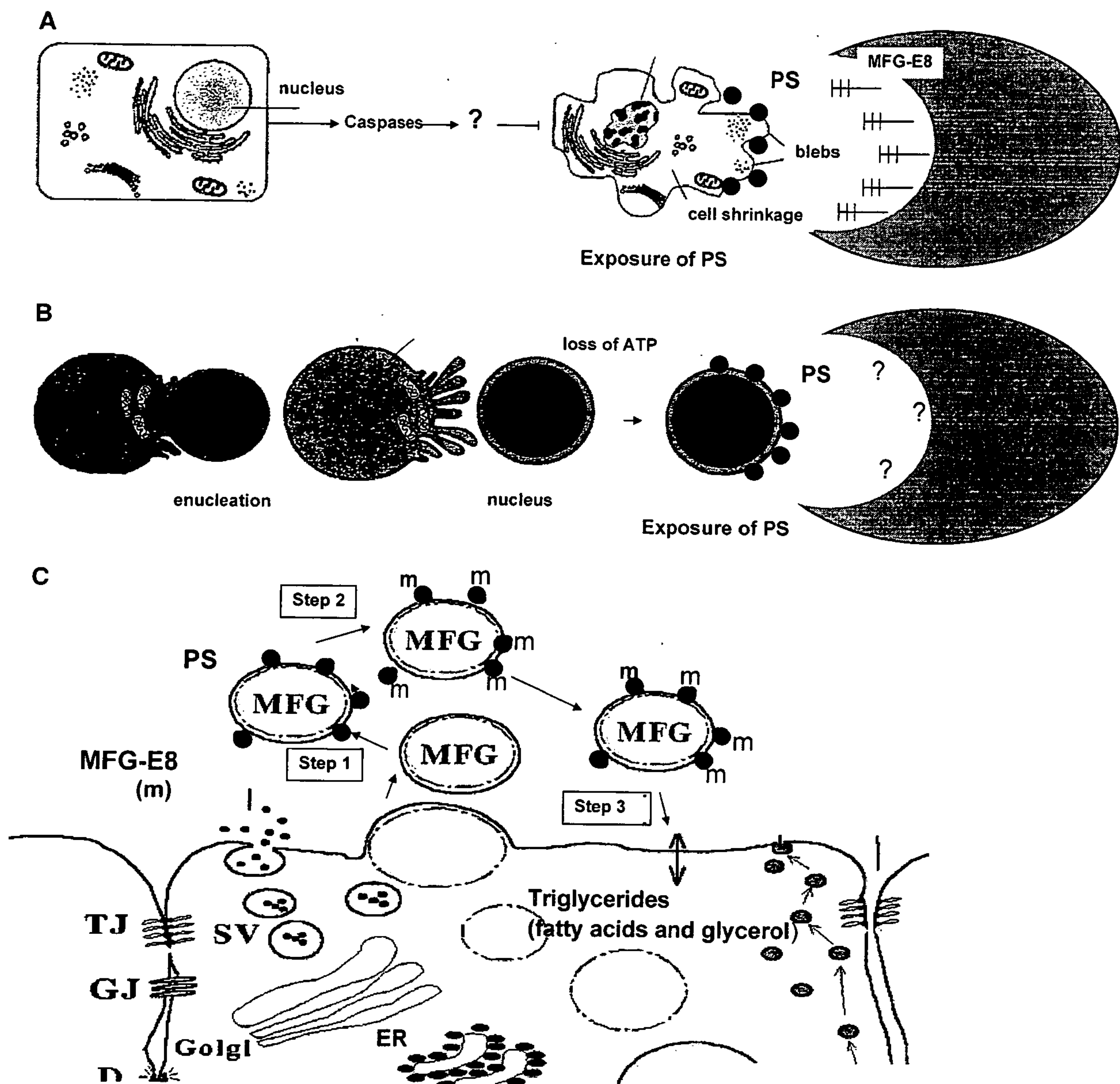

Fig. 3. Phosphatidylserine-dependent engulfment of apoptotic cells, erythroid cell nuclei, and milk fat globules (MFGs). (A) Phosphatidylserine-dependent engulfment of apoptotic cells. When cells undergo apoptosis, they expose phosphatidylserine on their surface. This process is caspase-dependent, but the detailed molecular mechanism is unknown. Macrophages use various molecules to recognize phosphatidylserine as an eat-me signal. The molecule used by tingible-body macrophage in the spleen is MFG-E8, which binds to phosphatidylserine on apoptotic cells and $\alpha_v\beta_3$ integrins on phagocytes, thus acting as a bridge between them. (B) Phosphatidylserine-dependent engulfment of nuclei expelled from erythroid precursors. When nuclei are expelled from erythroid precursor cells, they quickly lose adenosine triphosphate (ATP), because they do not have the machinery to make it. This loss of ATP inactivates the ATP-dependent translocase and activates the Ca-dependent scramblase, thus leading to the exposure of phosphatidylserine on the plasma membrane covering the nucleus. An unidentified molecule(s) on macrophages in the bone marrow or fetal liver recognizes nuclei for engulfment. (C) MFG-E8-dependent recycling of MFG. MFGs that are released from mammary epithelial cells are a kind of exosome that contains triglycerides. Because MFGs do not have an ATP-generating system, phosphatidylserine is exposed on the surface as soon as they are released from cells. MFG-E8 is also secreted by mammary epithelial cells and binds to phosphatidylserine on MFGs. When mammary glands undergo involution, MFGs left in mammary ducts are apparently re-absorbed by epithelial cells in an MFG-E8-dependent manner.

organs, the spleen and lymph nodes, contain diverse subsets of macrophages, with regard to the expression of CD68, F4/80, and MOMA-1. MFG-E8 is expressed in the tingible-body macrophages, which engulf apoptotic lymphocytes and reside at the germinal centers in the spleen and lymph nodes (19). In the germinal centers, many B lymphocytes are activated, undergo apoptosis (116), and are then engulfed. In MFG-E8-deficient mice, many TUNEL-positive apoptotic cells were

found associated with tingible-body macrophages in the germinal centers, particularly when the mice were challenged with keyhole limpet hemocyanin (KLH) to activate the B lymphocytes. These findings indicate that MFG-E8 plays an essential role in the engulfment of apoptotic cells at least in the tingible-body macrophages. However, the fact that apoptotic cells were associated with *MFG-E8*$^{-/-}$ macrophages indicates that one or more molecules other than MFG-E8 must be involved in the binding of apoptotic cells to macrophages.

MFG-E8-null mice are normal at birth. However, as they grow older, they develop splenomegaly with an enlarged white pulp and numerous germinal centers (19, 20). Many immunoglobulin G (IgG)-producing plasma cells are found in the germinal centers. Accordingly, *MFG-E8*-deficient mice develop SLE-type autoimmune diseases. At the age of 40 weeks, *MFG-E8*-deficient mice produce a large quantity of anti-double-stranded DNA and anti-nuclear antibodies (ANAs), develop glomerulonephritis from a massive deposition of Igs in the glomeruli, and suffer from proteinuria. As found in human SLE patients, the female mice exhibit more severe phenotypes than the male mice (19). When the mice were immunized with KLH, *MFG-E8*-null female mice, even young ones, quickly generated ANAs. These results confirm that if apoptotic cells, in this case B lymphocytes activated in the germinal centers, are not efficiently engulfed by macrophages, the animal will develop autoimmune disease. Recent reports on the involvement of MFG-E8 in atherosclerosis and Alzheimer disease (117, 118) also point out pathological roles of unengulfed dead cells in these diseases.

Defect in involution of mammary glands in MFG-E8-deficeint mice

Mammary epithelial cells express a high level of MFG-E8 during involution of the mammary glands (108, 109). The MFGs produced by the epithelial cells are plasma membranes covering triglycerolipids (119), and they associate with MFG-E8, which gave this molecule its name. MFGs expose phosphatidylserine because they lack an adenosine triphosphate (ATP)-generating system, and MFG-E8 binds to the MFGs through the phosphatidylserine on the plasma membrane (109) (Fig. 3). During the involution of mammary glands, epithelial cells undergo extensive apoptosis, and macrophages enter the mammary glands to engulf them (120). In addition, many of the MFGs that remain in the mammary glands are reabsorbed by epithelial cells in an MFG-E8-dependent manner. Thus, in *MFG-E8*$^{-/-}$ mice, a large number of MFGs remains in the mammary glands after the pups are weaned. This condition prevents the re-development of mammary glands for the next generation of pups, and the pups therefore starve.

Engulfment of nuclei expelled from erythroid precursors

During the differentiation of erythroid cells, nuclei are expelled from the erythroid precursors, leaving reticulocytes that further mature into red blood cells. Nuclei that are expelled from the erythroid precursor cells are engulfed by macrophages, whereas reticulocytes are not, indicating that nuclei present an 'eat nucleus' signal on their surface. As described above, apoptotic cells often use phosphatidylserine as an eat-me signal. To clarify the eat nucleus signal, a system for enucleating erythroid precursor cells was developed using erythroblasts from the spleen of phlebotomized mice (121). Nuclei expelled from erythroblasts are surrounded by a plasma membrane and are efficiently engulfed *in vitro* by macrophages from the fetal liver. This engulfment is blocked when phosphatidylserine is masked, indicating that as for apoptotic cells, phosphatidylserine is an eat-me signal for erythroid nuclei. In fact, immediately after the nuclei are disconnected from the reticulocytes, the surrounding plasma membrane exposes phosphatidylserine.

The asymmetrical distribution of phosphatidylserine on plasma membranes is thought to be maintained by an as-yet unidentified ATP-dependent aminophospholipid translocase that catalyzes the inward movement of aminophospholipids (122, 123). Another enzyme thought to contribute to the exposure of phosphatidylserine is a Ca^{2+}-dependent, ATP-independent scramblase that catalyzes both the inward and outward movements of lipids. Nuclei extruded from reticulocytes quickly lose their ATP, because they do not contain mitochondria and the glycolysis system that generates it. This inactivates the ATP-dependent aminophospholipid translocase and ATP-dependent Ca pump, which transports Ca^{2+} out of the cells against its large concentration gradient. Therefore, the Ca^{2+} concentration increases in the nuclei, and the Ca^{2+}-dependent scramblase is activated, leading to the exposure of phosphatidylserine on the nuclear plasma membrane surface (Fig. 3). Although candidate molecules for scamblases and translocases have been identified (124, 125), whether these enzymes are in fact involved in the exposure of phosphatidylserine on the plasma membranes is still controversial (126). In addition, the mechanism for exposing phosphatidylserine during apoptotic cell death remains a mystery.

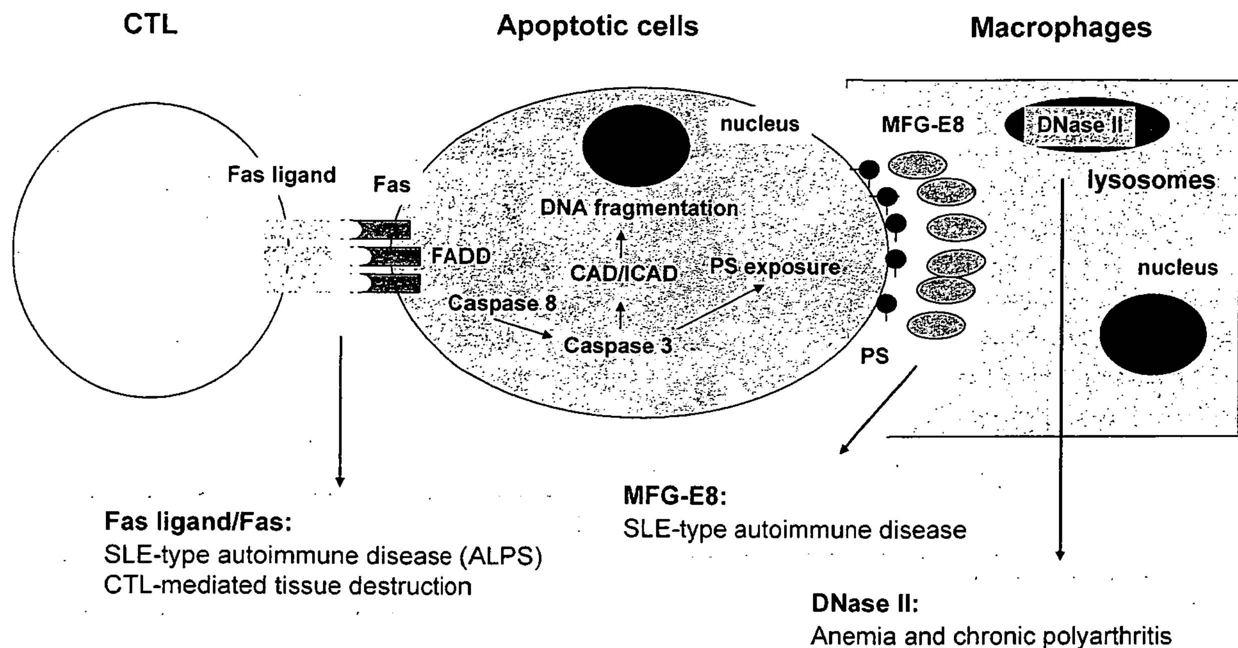

Fig. 4. Fas-mediated cell death, followed by clearance of dead cells. Fas ligand (FasL) is a type II-membrane protein expressed on cytotoxic T lymphocytes (CTLs). Binding of FasL to its receptor, Fas, activates the apoptosis program in the target cells. This program includes caspase-activated DNase (CAD)-dependent DNA fragmentation and the exposure of phosphatidylserine on the membrane surface. Macrophages engulf the apoptotic cells by recognizing phosphatidylserine and degrade all the cellular components. One molecule that recognizes phosphatidylserine is MFG-E8, which is expressed by tingible-body macrophages. DNase II in the lysosomes of macrophages degrades the DNA of apoptotic cells. Loss-of-function mutations in Fas or FasL cause an systemic lupus erythematosus (SLE)-type autoimmune disease, and exacerbation of FasL-induced apoptosis leads to tissue destruction. Inefficient engulfment of apoptotic cells because of the lack of MFG-E8 also leads to SLE-type autoimmune diseases. When DNA of apoptotic cells is left undigested in the lysosomes of macrophages because of deficiency of DNase II, the result is anemia in embryos and chronic polyarthritis in adults.

Exosomes

The engulfment of apoptotic cells, nuclei from erythroid precursor cells, and MFGs all follow the exposure of the same eat-me signal (phosphatidylserine) on the surface. Exosomes are small vesicles that are secreted by a variety of cell types (e.g. activated lymphocytes and cancer cells) (127, 128). They associate with MFG-E8 (129), are ingested by macrophages and immature dendritic cells, and seem to play a role in antigen presentation. The apoptotic bodies that are generated during apoptosis and are composed of membranous vesicles carrying cellular components are a kind of exosome.

Exosomes are implicated in activating the immune system, at least in the case of exosomes from transformed cells. The engulfment of apoptotic cells by immature dendritic cells also has an immunoregulatory role. If the dead cells are the organism's 'own healthy cells' that die during development or due to senescence, their engulfment will not activate the immune system. In contrast, if the cells undergoing apoptosis are virally infected or transformed, their engulfment may activate the immune system. The exact role played by exosomes in the immune system and whether similar mechanisms are used by different kinds of exosomes including apoptotic cells to affect immune responses remain an interesting subject for future studies.

Conclusion and perspective

All the cells in our body die sooner or later. The clearance of apoptotic cells is a normal process for recycling cellular components and maintaining homeostasis in mammals. Defects in apoptosis lead to cancer and autoimmune diseases (130–132). In particular, the autoimmune diseases caused by defects in the Fas and FasL system have been well characterized in human and mice (133–137) (Fig. 4). As described above, recent progress in understanding the clearance of apoptotic cells indicates that a defect in clearance or degradation of apoptotic cell components causes autoimmune disease, anemia, and chronic arthritis. The number of red blood cells produced per day in our bodies is about 10 times higher than the number of apoptotic cells, and an equal number of nuclei are expelled by erythroid precursor cells during red cell differentiation. A large number of exosomes seem to be produced in various processes in our bodies. Failure to engulf

and degrade these nuclei and exosomes are likely to cause problems similar to those observed in the inefficient engulfment of apoptotic cells.

MFG-E8 functions as a bridge between apoptotic cells and macrophages. However, it is expressed only in macrophages in just a few tissues. How macrophages in the thymus or microglia in the brain engulf apoptotic cells and how macrophages in the fetal liver or bone marrow engulf erythroid cell nuclei have not been elucidated. The development of specific diseases, anemia and polyarthritis, as a result of the inefficient digestion of DNA of apoptotic cells was a surprise to us. Elucidation of the detailed molecular mechanism for clearing apoptotic cells will provide more surprises and certainly contribute to the understanding of human disease.

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