The Journal of Immunology

Expression of Developmental Endothelial Locus-1 in a Subset of Macrophages for Engulfment of Apoptotic Cells

This information is current as of October 25, 2021.

Rikinari Hanayama, Masato Tanaka, Keiko Miwa and Shigekazu Nagata

---

References	This article **cites 49 articles**, 16 of which you can access for free at: http://www.jimmunol.org/content/172/6/3876.full#ref-list-1

Why *The JI*? Submit online.

- **Rapid Reviews! 30 days*** from submission to initial decision

- **No Triage!** Every submission reviewed by practicing scientists

- **Fast Publication!** 4 weeks from acceptance to publication

*average

Subscription	Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription
Permissions	Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

Expression of Developmental Endothelial Locus-1 in a Subset of Macrophages for Engulfment of Apoptotic Cells[1]

Rikinari Hanayama,* Masato Tanaka,*† Keiko Miwa,*‡ and Shigekazu Nagata[2]*‡§

A major function of macrophages is to engulf apoptotic cells to prevent them from releasing noxious materials as they die. Milk fat globule-EGF-factor 8 (MFG-E8) is a glycoprotein secreted by activated macrophages that works as a bridge between apoptotic cells and phagocytes by specifically recognizing phosphatidylserine exposed on apoptotic cells. In this study, we found that developmental endothelial locus-1 (Del-1), originally identified as an embryonic endothelial cell protein that binds $\alpha_v\beta_3$ integrin, is structurally and functionally homologous to MFG-E8. That is, both consist of a signal sequence, two epidermal growth factor domains and two factor VIII-homologous domains (C1 and C2). Del-1 bound to the apoptotic cells by recognizing phosphatidylserine via the factor VIII-homologous domains with an affinity similar to that of MFG-E8. The phagocytic activity of NIH 3T3 cells against apoptotic cells was enhanced by Del-1 through an interaction between the epidermal growth factor domain in Del-1 and $\alpha_v\beta_3$ integrin expressed in the NIH 3T3 cells. Screening of primary macrophages and macrophage cell lines for the expression of MFG-E8 and Del-1 indicated that MFG-E8 and Del-1 are expressed in different sets of macrophages. These results suggest the existence of macrophage subsets that use MFG-E8 or Del-1 differently to engulf apoptotic cells. *The Journal of Immunology,* **2004, 172: 3876–3882.**

A poptosis removes toxic or useless cells in metazoans (1, 2). It is involved in organ development, tissue remodeling, and tissue turn over. Apoptosis is induced by intrinsic and extrinsic stimuli (3, 4) and is mediated by a family of caspases that cleave a set of cellular substrates and by a specific DNase (caspase-activated DNase) that degrades the chromosomal DNA (5–7).

Apoptotic cells are swiftly removed by professional phagocytes (macrophages and immature dendritic cells) or amateur phagocytes such as fibroblasts and epithelial cells (8, 9). The engulfment of apoptotic cells is believed to benefit animals by preventing the inflammation and tissue damage that can be caused by intracellular materials being released from dying cells. Thus, unless the burden of apoptotic cells exceeds the capacity of the macrophages, most of the apoptotic cells generated during animal development or tissue turnover are engulfed by macrophages (10).

Macrophages engulf apoptotic cells but not living cells, indicating that apoptotic cells present an "eat me" signal(s) that can be recognized by macrophages (11). Among various molecules proposed as an "eat me" signal, phosphatidylserine is the most likely candidate (12, 13). Except for myoblasts, which are fusion-competent, the extracellular leaflet of the plasma membrane of living cells is devoid of phosphatidylserine (14). When apoptosis is induced in cells, phosphatidylserine is translocated from the inner leaflet to the outer leaflet of the plasma membranes (12, 15). This exposure of phosphatidylserine is thought to be mediated by the concomitant activation of the plasma membrane phospholipid scramblase and inhibition of an ATP-dependent aminophospholipid translocase (16, 17). A number of macrophage receptors have been suggested to recognize apoptotic cells, and several of these molecules including CD36, CD68, and class B scavenger receptor type I, were reported to directly bind phosphatidylserine (18–20).

By screening a library of mAbs against the cell surface proteins of mouse macrophages, we recently identified a factor that can link apoptotic cells to macrophages (21). The factor, called milk fat globule epidermal growth factor-8 (MFG-E8)[3] in mice and lactadherin in humans, is comprised of two epidermal growth factor (EGF) domains and two factor VIII-homologous domains (discoidin domain), and is expressed in thioglycolate-elicited peritoneal macrophages. In this report, we found that Del-1, originally identified as a matrix protein expressed in the endothelial cells of mouse embryos (22), is highly homologous to MFG-E8 in its structure and function. The recombinant Del-1 specifically bound to phosphatidylserine through its factor VIII-homologous domains, and to $\alpha_v\beta_3$ integrins via an Arg-Gly-Asp motif in the second EGF domain. Del-1 was as potent as MFG-E8 in enhancing the engulfment of apoptotic cells by NIH 3T3 cells. Del-1 was expressed in macrophages, but the type of macrophages expressing Del-1 was different from that expressing MFG-E8. That is, thioglycolate-elicited macrophages expressed MFG-E8, but not Del-1. Fetal liver and thymic macrophages expressed Del-1 but not MFG-E8. These results suggested that different macrophages use MFG-E8 or Del-1 to engulf apoptotic cells.

*Department of Genetics, Osaka University Medical School, Osaka, Japan; †Laboratory for Innate Cellular Immunity, RIKEN Research Center for Allergy and Immunology, Yokohama, Kanagawa, Japan; ‡Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Osaka, Japan; and §Laboratory of Genetics, Integrated Biology Laboratories, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

[3] Abbreviations used in this paper: MFG-E8, milk fat globule epidermal growth factor 8; EGF, epidermal growth factor; Del-1, developmental endothelial locus-1; wt, wild type, MFG-E8L, MFG-E8 long; MFG-E8S, MFG-E8 short.

Materials and Methods

Mice, Armenian hamsters, and cell lines

The transgenic mice (ICAD-Sdm) expressing a caspase-resistant form of inhibitor of caspase-activated DNase-short form were described previously (23). C57BL/6 mice and Armenian hamsters were purchased from Japan SLC (Shizuoka, Japan) and Oriental Yeast (Tokyo, Japan), respectively. Macrophages were prepared from the thymus and liver of mouse embryos as described previously (24). Bone marrow-derived macrophages were obtained by culturing bone marrow cells for 7 days in the presence of M-CSF. Mouse NIH 3T3 transformants expressing integrin $\alpha_v\beta_3$ were described previously (21). The NIH 3T3 cells were further transformed with DNase II to enhance the TUNEL reactivity.

Production of rDel-1 and rMFG-E8

A DNA fragment for the full-length coding sequence of mouse Del-1 was prepared by RT-PCR with RNA from E15.5 mouse embryo using primers whose sequences have been published (22). After joining a FLAG epitope at the 3′ end, the Del-1 cDNA was inserted into the pEF-BOS-EX vector (25). The expression plasmids for Del-1 mutants carrying a series of point mutations were generated by means of recombinant PCR. The primers used were: 5′ GAAGCCTATCGAGGAGAAACATTCATAGGC-3′ for D98E, 5′-TATCCCTACTATGCTGCCCTTAATAAGAAG-3′ for R195A, 5′-AATCAAAGCTCAGTATGTAGCCCCTCTACCC-3′ for R295A, and 5′-CGAAGGCATTGTACTTTAGCCATGGAACTT-3′ for R308A. The expression plasmids were introduced into human 293T cells by the calcium phosphate precipitation method, and Del-1 secreted into the medium was purified using ANTI-FLAG M2 affinity gel (Sigma-Aldrich, St. Louis, MO). The production of mouse MFG-E8 long (MFG-E8L) was described previously (21).

Preparation of mAbs

mAbs were prepared by immunizing Armenian hamsters with rDel-1. In brief, the purified Del-1 (100 μg) was injected s.c. into hamsters twice in a 2-wk interval. A final booster was performed by injecting 40 μg of Del-1 into the footpads. Three days later, the hamsters were sacrificed, and lymphocytes were harvested from the lymph nodes and fused with P3U1 mouse myeloma cells. Hybridomas were tested by ELISA for binding activity to Del-1, and positive clones were screened further to identify clones that were suitable for Western blotting (clone 8-29) and immunoprecipitation (clone 4-9). After they were subjected to limiting dilution, the hybridomas were cultured in GIT medium (Nihon Seiyaku, Tokyo, Japan), and the mAb was purified by protein A-Sepharose 4FF beads (Amersham Biosciences, Piscataway, NJ). The biotinylation of mAb and chemical cross-linking of mAb to protein A-Sepharose were performed according to standard protocols (26, 27).

Immunoprecipitation and Western blotting

Macrophages (1×10^7 cells) from various sources were cultured in AIM V medium (Invitrogen, Carlsbad, CA) for 48 h. The culture medium was collected, and adjusted to 1% Triton X-100 as described (28). Del-1 was then immunoprecipitated with 10 μl of anti-Del-1 mAb (clone 4-9)-protein A-Sepharose, and eluted from the beads with 30 μl of triethylamine (pH 11.7) containing 0.1% Triton X-100. An aliquot (20 μl) of the eluate was mixed 1:1 with 2× sample buffer without a reducing agent, separated by 10% PAGE, and the proteins were transferred to a nitrocellulose filter. The filter was subjected to Western blotting with biotinylated anti-Del-1 mAb (clone 8-29) and peroxidase-conjugated streptavidin (Roche, Indianapolis, IN). Proteins recognized by the Ab were visualized using a chemiluminescence reaction (Western Lightning Chemiluminescence Reagent; PerkinElmer, Wellesley, MA).

### Protein lipid overlay assay and solid-phase ELISA

L-α-phosphatidyl-L-serine, L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, and L-α-phosphatidylinositol were purchased from Sigma-Aldrich. The protein lipid overlay assay was performed as described (29). In brief, each phospholipid (500 pmol) was spotted onto Hybond-C extra membrane (Amersham Biosciences, Piscataway, NJ) and allowed to dry at room temperature. After blocking nonspecific binding sites by incubating the membranes in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 2 mg/ml BSA, it was incubated for 2 h at room temperature with 10 nM FLAG-tagged MFG-E8 or Del-1. The membrane was washed with TBST, and the bound proteins were detected with peroxidase-conjugated ANTI-FLAG mAb (M2; Sigma-Aldrich), followed by a chemiluminescence reaction.

The solid phase ELISA for Del-1 and MFG-E8 binding to phospholipids was conducted as previously described (21). In brief, phospholipid (100 μl, 3 μg/ml) in methanol was added to 96-well Immulon 1B microtiter plates (Thermo Environmental Instruments, Franklin, MA) and air-dried. Nonspecific binding sites were blocked by treating the wells with 10 mg/ml BSA in PBS. Del-1 and MFG-E8 were diluted to the appropriate concentrations with PBS containing 0.05% Tween 20 and 10 mg/ml BSA, added to the wells, and incubated at room temperature for 1 h. After the wells were washed with PBS containing 0.05% Tween 20, the bound proteins were quantified by ELISA using biotinylated ANTI-FLAG mAb and peroxidase-conjugated streptavidin. The peroxidase activity was detected using a peroxidase-detecting kit (Sumitomo Chemical, Tokyo, Japan), and quantified by measuring the absorbance at 450 nm with a microELISA reader.

In vitro phagocytosis assay

Phagocytosis of apoptotic cells was performed as described previously (21). In brief, NIH 3T3 cells or their transformants (2×10^4 cells) were

FIGURE 1. Comparison of the amino acid sequences of murine Del-1 and MFG-E8. The structures of Del-1 and MFG-E8 are shown schematically at the *top*, and their amino acid sequences are aligned to give maximal homology by introducing several gaps (−). Both Del-1 and MFG-E8 carry from the N terminus, a signal sequence (S), two EGF-like domains (E1 and E2) and two factor VIII-homologous domains (C1 and C2). The third EGF domain (E3) found in Del-1 is not found in MFG-E8, and the corresponding region in MFG-E8 is rich in proline and threonine (PT). An Arg-Gly-Asp motif in the second EGF domain is marked with a double underline.
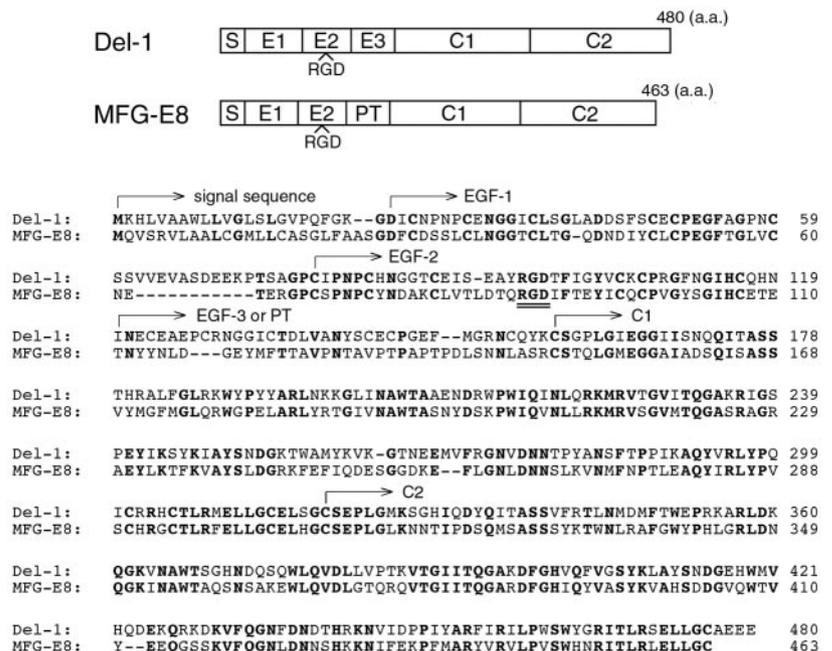
cultured in 8-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) that had been coated with 0.1% gelatin. Thymocytes from 4- to 8-wk-old ICAD-Sdm mice were treated with 10 μM dexamethasone to induce apoptosis. The apoptotic thymocytes were added to the well, and phagocytosis was allowed to proceed for 2 h. After fixation with 1% paraformaldehyde, the adherent cells were subjected to the TUNEL reaction using an Apoptag kit (Intergen, Purchase, NY) and DAB-black (Zymed Laboratories, South San Francisco, CA) and observed by light microscopy. The TUNEL-positive thymocytes in a total of 150 NIH 3T3 cells were counted, and the number of engulfed apoptotic thymocytes per NIH 3T3 cell (phagocytosis index) was determined.

Results

Binding of Del-1 to apoptotic cells

Del-1 is a protein of 480 amino acids, and consists of a signal sequence, three EGF-like domains including one with an Arg-Gly-Asp motif, and two factor VIII-homologous (discoidin I-like) domains (C1 and C2)(Fig. 1). This structure is similar to that of MFG-E8, except that the third EGF domain in Del-1 is not found in MFG-E8. There are two forms for MFG-E8 (MFG-E8 short (MFG-E8S) and MFG-E8L for MFG-E8 short and long forms, respectively) that are generated by alternative splicing (30). In MFG-E8S, there is no domain corresponding to the third EGF domain of Del-1, but in MFG-E8L the third EGF repeat is replaced by a proline-threonine-rich (P/T-rich) region (Fig. 1). The overall identity and similarity between Del-1 and MFG-E8L are 48% and 54%, respectively, at the amino acid sequence level.

We previously showed that MFG-E8, and in particular MFG-E8L, binds to apoptotic cells by recognizing phosphatidylserine (21). To examine whether Del-1 also binds to apoptotic cells, rDel-1 was produced in human 293T cells and purified. The Del-1 did not bind to freshly isolated thymocytes (Fig. 2A). In contrast, when thymocytes were treated for 6 h with dexamethasone, ~70% of them underwent apoptosis, as judged by TUNEL staining, and Del-1 specifically bound to these apoptotic cells (Fig. 2A). Annexin V binds to apoptotic cells by recognizing phosphatidylserine (31). Del-1 inhibited the binding of Annexin V to the apoptotic cells in a dose-dependent manner, and its inhibition was complete at a concentration of 1.0 μg/ml (Fig. 2B). The binding of MFG-E8L to apoptotic cells was also inhibited by Del-1. These results suggested that Del-1 binds to apoptotic cells by recognizing phosphatidylserine.

Binding of Del-1 to phosphatidylserine

The possibility that Del-1 binds directly to phosphatidylserine was examined using the protein lipid overlay assay (29). When a nitrocellulose filter spotted with various phospholipids was incubated with MFG-E8L or Del-1, both MFG-E8L and Del-1 specifically bound to phosphatidylserine (Fig. 2C). In contrast, neither of MFG-E8L nor Del-1 bound to phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine. The affinities of MFG-E8L and Del-1 to phosphatidylserine, determined with microtiter plates coated with phosphatidylserine, were comparable (Fig. 2D). Considering that the affinity of MFG-E8S to phosphatidylserine is ~10 times less than that of MFG-E8L (21), it seems that the third EGF domain in Del-1 may have a similar function as the P/T-rich domain of MFG-E8L in the binding of phosphatidylserine.

The C1 and C2 domains of Del-1 and MFG-E8 belong to the discoidin-1 family (32), which includes the C1 and C2 domains of factor V and factor VIII. Factors V and VIII are phosphatidylserine-binding cofactors for the factor IX serine protease in blood coagulation (33). The tertiary structure of the C2 domains of factor V and VIII has been determined by x-ray analysis (34, 35). They have a jelly-roll β-barrel motif consisting of 19 β-strands including 8 core β-strands. The amino acid sequences of the C1 and C2 domains of
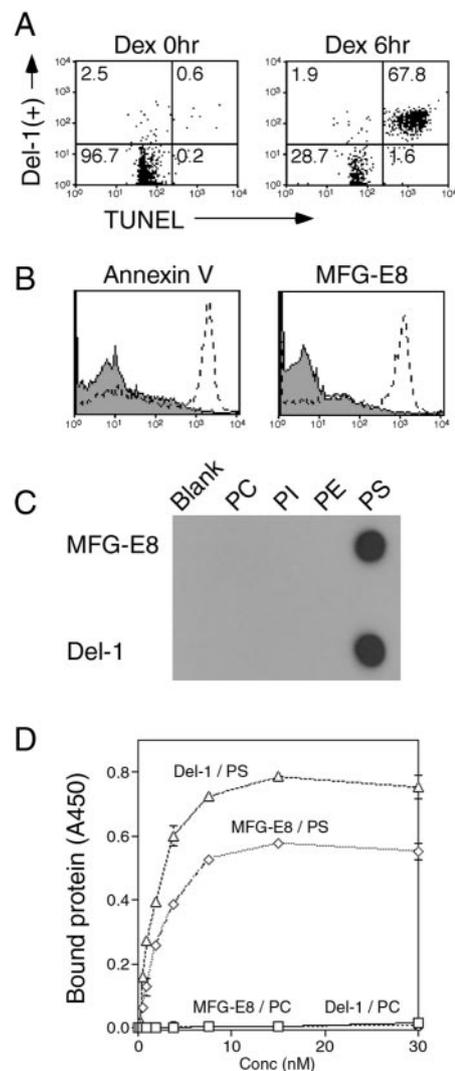

FIGURE 2. Binding of Del-1 to phosphatidylserine exposed on apoptotic cells. *A,* Binding of Del-1 to apoptotic cells. Freshly prepared wt thymocytes (1 × 10⁵ cells) (*left panel*) or thymocytes treated with dexamethasone for 6 h (*right panel*) were incubated for 30 min with 0.1 μg/ml Del-1, followed by staining with biotinylated anti-Del-1 mAb (clone 8-29) and PE-conjugated streptavidin. After fixation with 1% paraformaldehyde, the cells were subjected to TUNEL staining with FITC-labeled dUTP, and analyzed by FACS. *B,* Effect of Del-1 on the binding of annexin V or MFG-E8 to apoptotic thymocytes. Apoptotic thymocytes were stained in the presence of 1.0 μg/ml Del-1 with 10 μg/ml PE-conjugated annexin V (*left panel*) or with 0.1 μg/ml MFG-E8 followed by the biotinylated anti-MFG-E8 mAb (clone 2422) and PE-conjugated streptavidin (*right panel*), and analyzed by FACS. The annexin V- and MFG-E8-staining profiles without Del-1 are shown by dotted lines. *C,* Specific binding of Del-1 and MFG-E8 to phosphatidylserine. A nitrocellulose filter was spotted with phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), or phosphatidylserine (PS) and incubated at room temperature for 2 h with 10 nM FLAG-tagged MFG-E8 or Del-1. Proteins that bound to the phospholipids were detected by an ANTI-FLAG mAb as described in *Materials and Methods*. *D,* Dose-dependent binding of Del-1 or MFG-E8 to phosphatidylserine. Microtiter plates coated with PS or PC were incubated with increasing concentrations of Del-1 or MFG-E8, and proteins that bound to the wells were quantified by ELISA using ANTI-FLAG mAb. The absorbance at 450 nm is shown. Assays were done in triplicate, and the average values are plotted with SD.

Del-1 and MFG-E8 are well conserved with those of factors V and VIII, particularly for the 8 β-strand core region (Fig. 3, *A* and *B*). Among the conserved amino acids, the arginine residues at amino
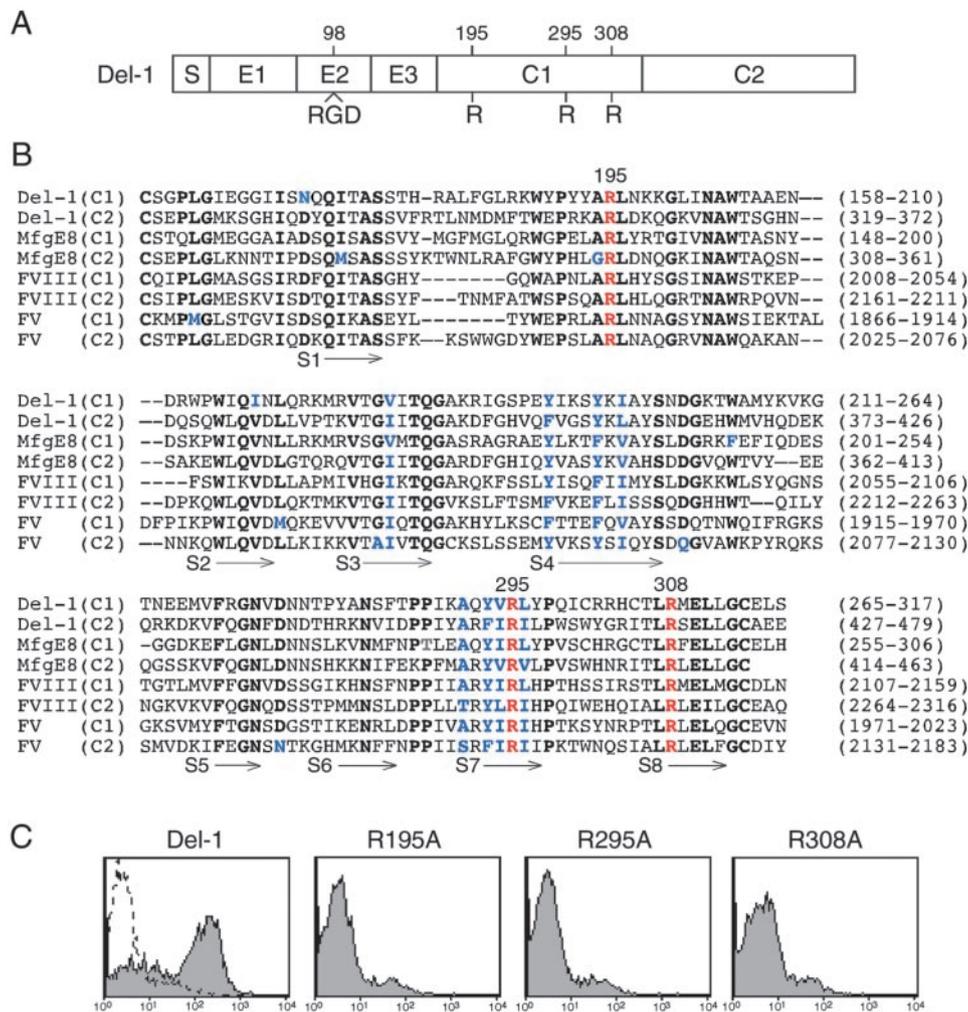
FIGURE 3. Binding of Del-1 mutants to apoptotic cells. *A*, Schematic representation of Del-1. In D98E, the aspartic acid at amino acid position 98 of the RGD motif was replaced by glutamic acid. In R195A, R295A, and R308A, the arginine at amino acid position 195, 295, or 308 in the C1 domain was replaced with alanine. All Del-1 derivatives were tagged with a FLAG epitope at the C terminus. *B*, Alignment of the amino acid sequences of the C1 and C2 domains of Del-1, MFG-E8, factor VIII, and factor V. Amino acid residues that are identical or regarded as favored substitutions at any given aligned position among the 8 sequences are shown in bold black or blue type, respectively. The core eight β-strands determined by the x-ray analysis for factor V and factor VIII (34, 35) are indicated by arrows. The three conserved arginine residues at amino acid positions 195, 295, and 308 of Del-1 are shown in red. *C*, Binding of Del-1 mutants to apoptotic cells. Thymocytes were treated with dexamethasone for 6 h, and incubated with 0.1 μg/ml the indicated wt or mutant Del-1. Del-1 that bound to the thymocytes was detected by FACS using the FITC-labeled ANTI-FLAG mAb. As a control, apoptotic thymocytes were stained with FITC-labeled ANTI-FLAG mAb alone, and its staining profile is indicated by the dashed line in the *first panel*.

acid positions 195, 295, and 308 are essential for the function of factors V and VIII (36). To confirm that the tertiary structure of the C1 and C2 domains of Del-1 is similar to that of factors V and VIII, these arginine residues were replaced by alanine. As shown in Fig. 3*C*, none of the Del-1 mutants could bind to apoptotic cells, suggesting that these arginine residues are involved in the binding of phosphatidyl-serine or in stabilizing the protein fold.

Enhancement of phagocytosis of apoptotic cells by Del-1

We previously showed that MFG-E8 stimulates NIH 3T3 cells to engulf apoptotic cells. To examine whether Del-1 has a similar activity, the phagocytosis assay with NIH 3T3 cells was conducted using thymocytes from the ICAD-Sdm mice as prey (21). In this assay system, DNA of the apoptotic thymocytes becomes TUNEL-positive only after the thymocytes are phagocytosed. When freshly prepared thymocytes from ICAD-Sdm mice were cocultured with NIH 3T3 cells, no thymocytes were engulfed by the NIH 3T3 cells in the presence or absence of Del-1, as judged by the TUNEL-staining of the NIH 3T3 cells (data not shown). In contrast, when

apoptotic thymocytes were cocultured with NIH 3T3 cells (3T3/wt), a significant percentage of NIH 3T3 cells engulfed the apoptotic thymocytes, and this percentage was enhanced by Del-1. That is, the phagocytosis index (the average number of apoptotic cells per NIH 3T3 cell) was 0.18 in the absence of Del-1 and increased to 1.06 following the addition of Del-1 (Fig. 4*A*). This effect of Del-1 on the engulfment of apoptotic cells was pronounced when the NIH 3T3 cells expressing $\alpha_v\beta_3$ integrin were used as phagocytes (Fig. 4*A*), and many of these NIH 3T3 cells contained >8 apoptotic cells (Fig. 4*B*). The ability of Del-1 to stimulate the engulfment of apoptotic cells was comparable to that of MFG-E8. The Del-1 mutants (R195A, R295A, and R308A) that could not bind apoptotic cells did not enhance the engulfment, confirming the specific effect of Del-1 on the engulfment of apoptotic cells. When the aspartic acid at aa 98 in the RGD motif was mutated to glutamic acid, the mutant Del-1 (D98E) bound the apoptotic cells (data not shown) but did not stimulate their engulfment by NIH 3T3 cells (Fig. 4). These results indicated that, as
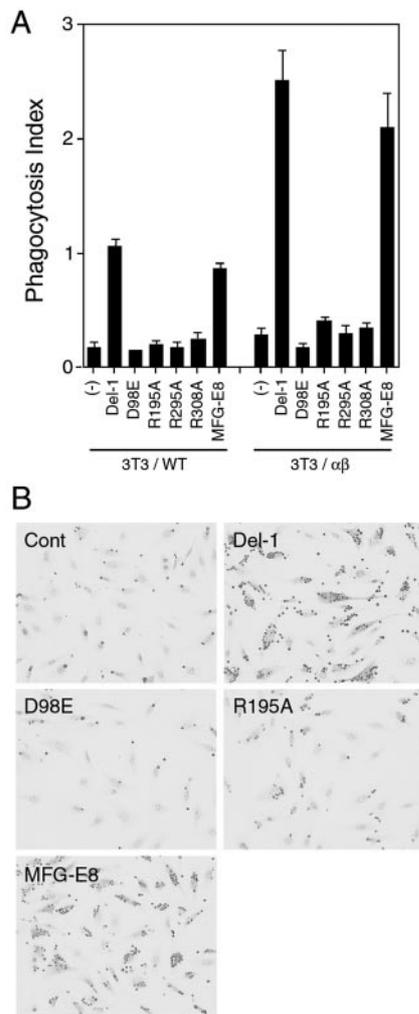
FIGURE 4. Del-1-dependent engulfment of apoptotic cells by NIH 3T3 cells. *A*, Effect of Del-1 on the engulfment of apoptotic cells. NIH 3T3 cells (2×10^4 cells) transformed with the cDNA for DNase II (3T3/wt) or with cDNA for DNase II and $\alpha_v\beta_3$ integrin (3T3/$\alpha\beta$) were grown in chamber slides, and incubated for 2 h with the apoptotic thymocytes (1×10^6 cells) from ICAD-Sdm mice in the absence (−) or presence of 0.3 µg/ml Del-1, its mutants D98E, R195A, R295A, and R308A, or MFG-E8L. The cells were fixed with 1% paraformaldehyde, subjected to the TUNEL reaction, and the phagocytosis index was determined as described in *Materials and Methods*. The experiments were performed three times in duplicate and the average number is shown with SD. *B*, Engulfment of apoptotic cells by NIH 3T3 cells. Engulfment of apoptotic cells was performed as described above with NIH 3T3 cells transformed with DNase II and $\alpha_v\beta_3$ integrin in the absence (cont) or presence of 0.3 µg/ml Del-1, its D98E or R195A mutant, or MFG-E8. Original magnification, ×200.

with MFG-E8, the RGD motif in the second EGF domain of Del-1 is essential for the binding of Del-1 to the integrin-expressing cells.

Expression of Del-1 in macrophages that do not express MFG-E8

The above results indicated that Del-1 and MFG-E8 have a similar structure and function. However, the expression profile of Del-1 was strikingly different from that of MFG-E8. As shown in Fig. 5*A*, Del-1 mRNA was expressed in fetal thymic and fetal liver macrophages, and weakly in bone marrow-derived macrophages. These macrophages did not express MFG-E8. The macrophage cell lines BAM3 and J774A.1 expressed Del-1, but not MFG-E8. In contrast, MFG-E8 mRNA was detected in thioglycolate-elicited peritoneal macrophages and the P388D1 macrophage cell line,
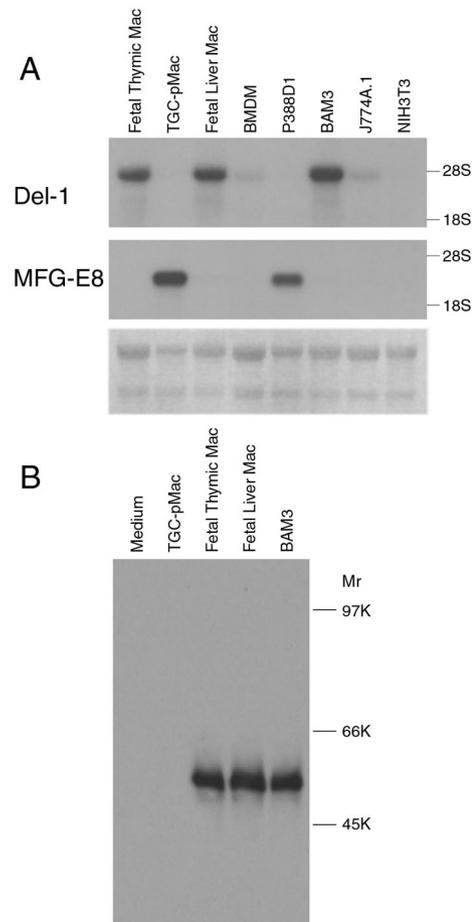

FIGURE 5. Expression of Del-1 and MFG-E8 in macrophages. *A*, Northern hybridization. Total RNA (7.5 µg per lane) was prepared from indicated macrophages of different sources and cell lines, separated by electrophoresis on a 1.5% agarose gel and analyzed by Northern hybridization using ^{32}P-labeled murine Del-1 cDNA (*upper panel*) or MFG-E8 cDNA (*middle panel*). In the *lower panel*, the filter was stained with 0.05% (w/v) methylene blue. *B*, Secretion of Del-1 from macrophages. Thioglycolate-elicited peritoneal macrophages (TGC-pMac), fetal thymic macrophages, fetal liver macrophages, and BAM3 cells were cultured for 48 h. The culture medium was adjusted to 1% Triton X-100, and subjected to immunoprecipitation followed by Western blotting. In the *first lane*, the control medium was subjected to the same procedure.

which did not express Del-1. Neither Del-1 nor MFG-E8 was expressed in thymocytes, the NIH 3T3 fibroblast cell line, or in lymphoid or myeloid cell lines (Fig. 5*A*, and data not shown). MFG-E8 is secreted from macrophages as a component of exosomes (28)(K. Miyasaka, R. Hanayama, S. Nagata, unpublished results). Similarly, when conditioned medium from fetal thymic macrophages, fetal liver macrophages, or the BAM3 cell line was spun, no Del-1 protein was detected in the supernatant (data not shown). In contrast, when the conditioned medium of these macrophages was treated with 1.0% Triton X-100 before centrifugation, a large amount of M_r 53K Del-1 protein was detected in the supernatants (Fig. 5*B*), suggesting that Del-1 is also produced by macrophages as a component of exosomes.

Discussion

Del-1 was originally identified by an enhancer trap event in one transgenic mouse line as a protein that is transiently expressed in endothelial cells during early mouse embryogenesis (22). Del-1

inhibits the formation of the vascular-like structure from the endothelial-like yolk sac cells (22). It also stimulates angiogenesis in the in ovo chick chorioallantoic membrane assay (37). These activities of Del-1 are mediated by its binding to $\alpha_v\beta_3$ integrin expressed on endothelial cells. In this report, we showed that Del-1 is structurally and functionally homologous to MFG-E8L, which works as a bridge between apoptotic cells and phagocytes. That is, Del-1 specifically bound apoptotic cells by recognizing phosphatidylserine via its C1 and C2 domain. Del-1 that was engaged by phosphatidylserine on microtiter plates recruited NIH 3T3 cell expressing $\alpha_v\beta_3$ integrin via the RGD motif (data not shown). Del-1 also stimulated the engulfment of apoptotic cells by NIH 3T3 fibroblasts. It is possible that the functions of Del-1 in the engulfment of apoptotic cells and angiogenesis in the fetus are independent and that Del-1 has dual functions. However, the vascular remodeling in normal developmental, as well as pathological, neovascularization is often associated with endothelial cell apoptosis (38, 39). Del-1 expressed in the epithelial cells in the fetus may have a role in the engulfment of the dying endothelial cells, thus enhancing neovascularization.

We previously observed weak but significant binding of MFG-E8 to phosphatidylethanolamine (21). Kagen et al. (15) recently analyzed the phospholipids in human cell lines that were exposed during Fas-induced apoptosis, and reported that although the exposure of phosphatidylserine is strictly regulated by the apoptotic process, a significant amount of phosphatidylethanolamine is exposed even in living cells. Therefore, we re-evaluated the binding of MFG-E8 and Del-1 to phospholipids, and found that the addition of 0.05% Tween 20 to the assay mixture completely inhibited the binding of MFG-E8 or Del-1 to phosphatidylethanolamine. In contrast, this concentration of Tween 20 had no effect on the binding of MFG-E8 or Del-1 to phosphatidylserine. MFG-E8 did not bind phosphatidylcholine or phosphatidylinositol even in the absence of the detergent. Therefore, we have no clear explanation for the binding of Del-1 or MFG-E8 to phosphatidylethanolamine in the absence of detergent. The C1 and C2 domains of factors V and VIII in the discoidin family bind phosphatidylserine but not other phospholipids (40, 41). In this study, we showed that the arginine residues conserved among the discoidin family members are essential for the binding of Del-1 to apoptotic cells, and thus phosphatidylserine. It is therefore reasonable to conclude that Del-1 and MFG-E8 preferentially bind to phosphatidylserine.

Lymphocytes have been progressively subdivided into a large number of subtypes with distinct functions. Mouse mononuclear phagocytes are defined as blood mononuclear cells that express CD11b and F4/80, and the heterogeneity of macrophages and dendritic cells has been recognized (42–45). Very recently, Geissmann et al. (46) reported the existence of monocyte subsets that have different surface markers. According to them, the $CX_3CR1^{low}Gr1^+$ subset of monocytes is short-lived and represents the immediate circulating precursors for dendritic cells and CD11c-myeloid cells in inflammatory conditions (inflammatory subset). The second subset of monocytes ($CX_3CR1^{high}Gr1^-$) persists longer in tissues and serves as a precursor for resident myeloid cells in noninflamed tissues (resident subset). Del-1 was expressed in macrophages in the fetal liver, fetal thymus, and bone marrow, whereas MFG-E8 was expressed in thioglycolate-elicited macrophages, suggesting that Del-1 and MFG-E8 are expressed in resident and inflammatory subsets of macrophages, respectively. It is likely that these resident and inflammatory subsets of macrophages correspond to "unactivated and activated" macrophage subsets defined by Pradhan et al. (45). If the differential expression of Del-1 and MFG-E8 in the resident and inflammatory macro-

phages can be confirmed, Del-1 and MFG-E8 will be good surface markers for distinguishing these two subsets of monocytes in the blood, and macrophages and dendritic cells in tissues. In this regard, it is noteworthy that the 5′ promoter sequences of Del-1 and MFG-E8 differ significantly compared with their conserved coding sequence. The murine and human Del-1 gene promoters contain binding sites for NF-AT, δ-EF1, and Tal1 transcription factors that are necessary for the development of blood cells (47–49) while binding sites for the NF-κB and CREB transcription factors can be found on the promoters of murine and human MFG-E8. Studies using the Del-1 and MFG-E8 gene promoter in a transgenic and/or knockin mouse strategy may help characterize the functions of subsets of mononuclear phagocytes.

In this study, we described that Del-1 as well as MFG-E8 bind phosphatidylserine exposed on apoptotic cells with high affinities, and can work as a bridge between apoptotic cells and phagocytes. We recently established a mouse line that is deficient in MFG-E8.[4] The ability of thioglycolate-elicited peritoneal macrophages to engulf apoptotic cells was significantly reduced in MFG-E8-null mice, suggesting a nonredundant role of MFG-E8 in thioglycolate-elicited peritoneal macrophages for engulfment of apoptotic cells. To determine whether Del-1 also plays a nonredundant role in the engulfment of apoptotic cells in fetal macrophages, it will be necessary to establish mice deficient in the Del-1 gene.

Acknowledgments

References

1. Jacobson, M. D., M. Weil, and M. C. Raff. 1997. Programmed cell death in animal development. *Cell 88:347.*
2. Vaux, D. L., and S. J. Korsmeyer. 1999. Cell death in development. *Cell 96:245.*
3. Hengartner, M. O. 2000. The biochemistry of apoptosis. *Nature 407:770.*
4. Nagata, S. 1997. Apoptosis by death factor. *Cell 88:355.*
5. Thornberry, N. A., and Y. Lazebnik. 1998. Caspases: enemies within. *Science 281:1312.*
6. Fischer, U., R. U. Janicke, and K. Schulze-Osthoff. 2003. Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ. 10:76.*
7. Nagata, S., H. Nagase, K. Kawane, N. Mukae, and H. Fukuyama. 2003. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ. 10:108.*
8. Henson, P. M., D. L. Bratton, and V. A. Fadok. 2001. Apoptotic cell removal. *Curr. Biol. 11:R795.*
9. Savill, J., and V. Fadok. 2000. Corpse clearance defines the meaning of cell death. *Nature 407:784.*
10. Surh, C. D., and J. Sprent. 1994. T-cell apoptosis detected in situ during positive and negative selection in the thymus. *Nature 372:100.*
11. Platt, N., R. P. da Silva, and S. Gordon. 1998. Recognizing death: the phagocytosis of apoptotic cells. *Trends Cell Biol. 8:365.*
12. Fadok, V. A., D. L. Bratton, S. C. Frasch, M. L. Warner, and P. M. Henson. 1998. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ. 5:551.*
13. Fadok, V. A., D. R. Voelker, P. A. Campbell, J. J. Cohen, D. L. Bratton, and P. M. Henson. 1992. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J. Immunol. 148:2207.*
14. Zachowski, A. 1993. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem. J. 294:1.*
15. Kagan, V. E., B. Gleiss, Y. Y. Tyurina, V. A. Tyurin, C. Elenstrom-Magnusson, S. X. Liu, F. B. Serinkan, A. Arroyo, J. Chandra, S. Orrenius, and B. Fadeel. 2002. A role for oxidative stress in apoptosis: oxidation and externalization of phosphatidylserine is required for macrophage clearance of cells undergoing Fas-mediated apoptosis. *J. Immunol. 169:487.*
16. Verhoven, B., R. A. Schlegel, and P. Williamson. 1995. Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J. Exp. Med. 182:1597.*
17. Bratton, D. L., V. A. Fadok, D. A. Richter, J. M. Kailey, L. A. Guthrie, and P. M. Henson. 1997. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J. Biol. Chem. 272:26159.*
18. Ramprasad, M. P., W. Fischer, J. L. Witztum, G. R. Sambrano, O. Quehenberger, and D. Steinberg. 1995. The 94- to 97-kDa mouse macrophage membrane protein that recognizes oxidized low density lipoprotein and phosphatidylserine-rich liposomes is identical to macrosialin, the mouse homologue of human CD68. *Proc. Natl. Acad. Sci. USA 92:9580.*

19. Fadok, V. A., M. L. Warner, D. L. Bratton, and P. M. Henson. 1998. CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor ($\alpha_v\beta_3$). *J. Immunol. 161:6250.*

20. Kawasaki, Y., A. Nakagawa, K. Nagaosa, A. Shiratsuchi, and Y. Nakanishi. 2002. Phosphatidylserine binding of class B scavenger receptor type I, a phagocytosis receptor of testicular sertoli cells. *J. Biol. Chem. 277:27559.*

21. Hanayama, R., M. Tanaka, K. Miwa, A. Shinohara, A. Iwamatsu, and S. Nagata. 2002. Identification of a factor that links apoptotic cells to phagocytes. *Nature 417:182.*

22. Hidai, C., T. Zupancic, K. Penta, A. Mikhail, M. Kawana, E. E. Quertermous, Y. Aoka, M. Fukagawa, Y. Matsui, D. Platika, et al. 1998. Cloning and characterization of developmental endothelial locus-1: an embryonic endothelial cell protein that binds the $\alpha_v\beta_3$ integrin receptor. *Genes Dev. 12:21.*

23. McIlroy, D., M. Tanaka, H. Sakahira, H. Fukuyama, M. Suzuki, K. Yamamura, Y. Ohsawa, Y. Uchiyama, and S. Nagata. 2000. An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev. 14:549.*

24. Kawane, K., H. Fukuyama, H. Yoshida, H. Nagase, Y. Ohsawa, Y. Uchiyama, T. Iida, K. Okada, and S. Nagata. 2003. Impaired thymic development in mouse embryos deficient in apoptotic DNA degradation. *Nat. Immun. 4:138.*

25. Murai, K., H. Murakami, and S. Nagata. 1998. Myeloid-specific transcriptional activation by murine myeloid zinc finger protein-2. *Proc. Natl. Acad. Sci. USA 95:3461.*

26. Bayer, E. A., and M. Wilchek. 1980. The use of the avidin-biotin complex as a tool in molecular biology. *Methods Biochem. Anal. 26:1.*

27. Gersten, D. M., and J. J. Marchalonis. 1978. A rapid, novel method for the solid-phase derivatization of IgG antibodies for immune-affinity chromatography. *J. Immunol. Methods 24:305.*

28. Oshima, K., N. Aoki, T. Kato, K. Kitajima, and T. Matsuda. 2002. Secretion of a peripheral membrane protein, MFG-E8, as a complex with membrane vesicles. *Eur. J. Biochem. 269:1209.*

29. Dowler, S., G. Kular, and D. R. Alessi. 2002. Protein lipid overlay assay. *Sci. STKE 2002:PL6.*

30. Oshima, K., N. Aoki, M. Negi, M. Kishi, K. Kitajima, and T. Matsuda. 1999. Lactation-dependent expression of an mRNA splice variant with an exon for a multiply *O*-glycosylated domain of mouse milk fat globule glycoprotein MFG-E8. *Biochem. Biophys. Res. Commun. 254:522.*

31. Vermes, I., C. Haanen, H. Steffens-Nakken, and C. Reutelingsperger. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods 184:39.*

32. Baumgartner, S., K. Hofmann, R. Chiquet-Ehrismann, and P. Bucher. 1998. The discoidin domain family revisited: new members from prokaryotes and a homology-based fold prediction. *Protein Sci. 7:1626.*

33. Davie, E. W., K. Fujikawa, and W. Kisiel. 1991. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry 30:10363.*

34. Macedo-Ribeiro, S., W. Bode, R. Huber, M. A. Quinn-Allen, S. W. Kim, T. L. Ortel, G. P. Bourenkov, H. D. Bartunik, M. T. Stubbs, W. H. Kane, and P. Fuentes-Prior. 1999. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. *Nature 402:434.*

35. Pratt, K. P., B. W. Shen, K. Takeshima, E. W. Davie, K. Fujikawa, and B. L. Stoddard. 1999. Structure of the C2 domain of human factor VIII at 1.5 Å resolution. *Nature 402:439.*

36. Kim, S. W., M. A. Quinn-Allen, J. T. Camp, S. Macedo-Ribeiro, P. Fuentes-Prior, W. Bode, and W. H. Kane. 2000. Identification of functionally important amino acid residues within the C2-domain of human factor V using alanine-scanning mutagenesis. *Biochemistry 39:1951.*

37. Penta, K., J. A. Varner, L. Liaw, C. Hidai, R. Schatzman, and T. Quertermous. 1999. Del1 induces integrin signaling and angiogenesis by ligation of $\alpha_v\beta_3$. *J. Biol. Chem. 274:11101.*

38. Ishida, S., K. Yamashiro, T. Usui, Y. Kaji, Y. Ogura, T. Hida, Y. Honda, Y. Oguchi, and A. P. Adamis. 2003. Leukocytes mediate retinal vascular remodeling during development and vaso-obliteration in disease. *Nat. Med. 9:781.*

39. Alon, T., I. Hemo, A. Itin, J. Pe'er, J. Stone, and E. Keshet. 1995. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. *Nat. Med. 1:1024.*

40. Gilbert, G. E., and D. Drinkwater. 1993. Specific membrane binding of factor VIII is mediated by *O*-phospho-L-serine, a moiety of phosphatidylserine. *Biochemistry 32:9577.*

41. Comfurius, P., E. F. Smeets, G. M. Willems, E. M. Bevers, and R. F. Zwaal. 1994. Assembly of the prothrombinase complex on lipid vesicles depends on the stereochemical configuration of the polar headgroup of phosphatidylserine. *Biochemistry 33:10319.*

42. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol. 3:23.*

43. Wiktor-Jedrzejczak, W., and S. Gordon. 1996. Cytokine regulation of the macrophage ($M\phi$) system studied using the colony stimulating factor-1-deficient *op/op* mouse. *Physiol. Rev. 76:927.*

44. Fadok, V. A., J. S. Savill, C. Haslett, D. L. Bratton, D. E. Doherty, P. A. Campbell, and P. M. Henson. 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J. Immunol. 149:4029.*

45. Pradhan, D., S. Krahling, P. Williamson, and R. A. Schlegel. 1997. Multiple systems for recognition of apoptotic lymphocytes by macrophages. *Mol. Biol. Cell. 8:767.*

46. Geissmann, F., S. Jung, and D. R. Littman. 2003. Blood monocytes consists of two different principal subsets with distinct migratory properties. *Immunity 19:71.*

47. Peng, S. L., A. J. Gerth, A. M. Ranger, and L. H. Glimcher. 2001. NFATc1 and NFATc2 together control both T and B cell activation and differentiation. *Immunity 14:13.*

48. Higashi, Y., H. Moribe, T. Takagi, R. Sekido, K. Kawakami, H. Kikutani, and H. Kondoh. 1997. Impairment of T cell development in δEF1 mutant mice. *J. Exp. Med. 185:1467.*

49. Shivdasani, R. A., E. L. Mayer, and S. H. Orkin. 1995. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature 373:432.*