Opposite Effects of Rho Family GTPases on Engagement of Apoptotic Cells by Macrophages

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The efficient engulfment of apoptotic cells by professional or nonprofessional phagocytes is critical to maintain mammalian homeostasis. To identify molecules involved in the engulfment of apoptotic cells, we established a retrovirus-based expression cloning system coupled with the engulfment assay. By screening a cDNA library of a mouse macrophage cell line, we identified two small GTPase family members (RhoG and Rab5) that enhanced the engulfment of apoptotic cells. By examining other small GTPase family members, we found that Rac1 enhanced the engulfment of apoptotic cells, whereas RhoA inhibited the process. Accordingly, the expression of a dominant-negative form of RhoG or Rac1 in primary macrophage cultures severely reduced the ability of the macrophages to engulf apoptotic cells, and a dominant-negative form of RhoA enhanced the process. These results indicated that the efficient engulfment of apoptotic cells requires the concerted action of small GTPase family members. We demonstrated previously that NIH3T3 cells expressing the αβ integrin efficiently engulf apoptotic cells in the presence of milk fat globule epidermal growth factor 8 via a phosphatidyserine-dependent mechanism. The dominant-negative form of RhoG or Rac1 inhibited this process, which suggested RhoG and Rac1 are also involved in the integrin-mediated engulfment.

Apoptosis is a cell-autonomous process that eliminates harmful or useless cells in metazoans (1, 2). It contributes to development, tissue remodeling, and the resolution of inflammation. The apoptotic program is triggered by a variety of stimuli, including anti-cancer drugs and death factors, and is mediated by a family of caspases (3, 4). Caspases cleave a set of cellular proteins such as cytoskeletal and structural proteins, which results in the morphological changes that characterize apoptotic cell death (5). The activation of caspases is also responsible for the extensive degradation of chromosomal DNA, another hallmark of apoptosis, which is mediated by a specific DNase (caspase-activated DNase, CAD) (6).

Apoptotic cells are rapidly engulfed by professional phagocytes (macrophages and immature dendritic cells) or less efficiently by non-professional phagocytes such as fibroblasts and epithelial cells (7, 8). This prompt engulfment seems to prevent the release of potentially noxious or immunogenic intracellular contents from dying cells, thereby preserving the integrity and function of the surrounding tissues. Phagocytes engulf apoptotic cells but not healthy cells, indicating that the apoptotic cells display an “eat me” signal(s) on the cell surface, and the phagocytes directly or indirectly recognize this signal. Among the variety of molecules that have been proposed as an eat me signal, phosphatidylinerine (PS) is the best candidate (9). In living cells, PS is confined to the inner leaflet of the plasma membrane, but it is quickly exposed to the cell surface when cells undergo apoptosis. We showed previously that a factor called milk fat globule epidermal growth factor 8 (MFG-E8) or developmental endothelial locus-1 (Del-1) binds to apoptotic cells by specifically recognizing PS and passes them to phagocytes (10, 11). MFG-E8 and Del-1 are expressed only in a limited set of phagocytes such as tingible body macrophages in the spleen, thioylcollate-elicited peritoneal macrophages, and Langerhans cells in the skin (12, 13). Macrophages in bone marrow and resident peritoneal macrophages do not express MFG-E8 or Del-1, suggesting that a molecule(s) other than MFG-E8 or Del-1 is involved in the engulfment of apoptotic cells in the macrophage of these tissues.

The recognition of apoptotic cells seems to activate a cascade of intracellular molecules in phagocytes, leading to a rearrangement of the cytoskeleton that permits the efficient engulfment of apoptotic cells. Genetic analysis of programmed cell death in Caenorhabditis elegans has identified a set of genes that are involved in removal of cell corpses (14). Of these genes, ced-2, ced-5, ced-10, and ced-12 are on the same signaling pathway and encode homologs of mammalian CrkII, DOCK180, Rac1, and Elmo (15–17); these mammalian proteins are also thought to be involved in the engulfment of apoptotic cells (18–21). However, how these proteins and other related proteins regulate the engulfment of apoptotic cells remains very elusive.

To identify the phagocyte receptors and their downstream molecules involved in the engulfment of apoptotic cells, we developed a functional screening strategy using a retrovirus cDNA library. With this assay, we isolated cDNA clones encoding Rab5 and RhoG that enhanced the engulfment of apoptotic cells. By expressing the wild-type or dominant-negative forms of Rho family GTPases, we showed that Rac1/RhoG and RhoA had opposite effects on the engulfment of apoptotic cells.

**EXPERIMENTAL PROCEDURES**

**Mice, Cell Lines, and Recombinant Proteins—**C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). Mice deficient in CAD bovine serum; PBS, phosphate-buffered saline; CMFDA, 5-chloromethylfluorescein diacetate; FACS, fluorescence-activated cell sorter.
were described previously (22). The mouse NIH3T3 cell line and a transformant cell line (NIH3T3/integrin/DNase II), expressing α,β3 integrin and DNase II (10), and the macrophage cell line BAM3 (23) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (FBS; Invitrogen). The FLAG-tagged recombinant mouse MFG-E8 was prepared as described previously (10).

Construction of the cDNA Library—Total RNA was prepared from BAM3 cells using Isogen (Nippon Gene, Saitama, Japan), and poly(A) RNA was purified with an mRNA purification kit (Amersham Biosciences). Double-stranded cDNA was synthesized with random hexamers as primers using a cDNA synthesis kit (Invitrogen) and size-fractionated by electrophoresis through an agarose gel. DNA fragments larger than 500 bp were recovered from the gel and ligated into a BstXI-digested pMX-puro vector (24) with a BstXI adaptor. Escherichia coli DH10B cells (Electromax DH10B; Invitrogen) were transformed by electroporation using a Gene Pulser (Bio-Rad). A total of 3.5 × 10⁶ bacterial colonies were plated on agar at a density of about 50 colonies per well in 24-well microtiter plates. Bacteria were cultured for each group, and plasmid DNA was prepared using QIAprep (Qiagen, Tokyo, Japan).

Screening the Library—Plasmid DNA from each group was introduced by lipofection using FuGENE 6 (Roche Applied Science) into 3 × 10⁴ PLAT-E packaging cells (25) grown in 96-well microtiter plates. Two days after transfection, the culture supernatant (100 μl) was adjusted to 10 μg/ml Polybrene (Sigma), and used to infect 5 × 10⁴ NIH3T3 cells in 96-well plates. After a 24-h culture, the medium was replaced with fresh medium, and the cells were cultured for 3 more days.

As prey for engulfment, thymocytes from 4- to 8-week-old C57BL/6 mice were labeled with 1 μM CMFDA (Molecular Probes, Eugene, OR) by incubating them at 37°C for 30 min in serum-free Dulbecco’s modified Eagle’s medium, and treating them at 37°C for 4.5 h with 10 μM dexamethasone to induce apoptosis. The CMFDA-labeled thymocytes (1 × 10⁶ cells/well) were added to the NIH3T3 cells (1 × 10⁶ cells/well) in a 96-well plate and incubated for 1 h at 37°C. The NIH3T3 cells were thoroughly washed with PBS, suspended in PBS containing 2% FBS and 0.02% sodium azide, and analyzed by flow cytometry using a FACS-Calibur (BD Biosciences).

Construction of Expression Plasmids—The full-length coding sequences for mouse Rac1, Cdc42, and RhoA were prepared by reverse transcription-PCR with RNA from BAM3 cells, and inserted into pGEM-T-EASY (Promega, Madison, WI). The authenticity of the cDNAs was verified by DNA sequencing. The cDNAs for the dominant-negative forms of Rac1 (Rac1 T17N), Cdc42 (Cdc42 T12N), RhoA (RhoA T19N), RhoG (RhoG F37A), and Rab5 (Rab5 S34N) were prepared by PCR-mediated mutagenesis (26) and inserted into pMX-puro retrovirus vector.

Transformation of Primary Macrophages—Bone marrow-derived macrophages were transformed by retrovirus-mediated transfection. In brief, bone marrow cells were collected from 10-week-old female C57BL/6 mice and treated with red blood cell lysis buffer (17 mM Tris-HCl, pH 7.5, 144 mM NH₄Cl, 0.5% FBS). The remaining cells were seeded at 1 × 10⁶ cells/ml in RPMI 1640 containing 10% FBS, cultured for 2 days in the presence of 10 units/ml macrophage colony-stimulating factor (M-CSF), and subjected to retrovirus infection as described above. 24 h after transfection, puromycin was added to the medium at a final concentration of 2.5 μg/ml to select the stably transfected M-CSF was prepared from the conditioned medium of mouse L929 cells transformed with a human M-CSF expression plasmid (27).

In Vitro Phagocytosis Assay with TUNEL Staining—The engulfment of apoptotic cells was also assayed by TUNEL staining with Cad-deficient thymocytes essentially as described previously (10). In brief, thymocytes from Cad-deficient mice (22) were induced to undergo apoptosis by treatment with 10 μM dexamethasone for 4 h; they were then co-cultured for 2 h with 6 × 10⁴ NIH3T3/integrin/DNase II cells per well or 1 × 10⁴ bone marrow-derived macrophages per well in 24-well plates. After the incubation, the cells were washed with PBS, fixed with 1% paraformaldehyde, and subjected to TUNEL staining with fluorescein isothiocyanate-labeled dUTP (Roche Applied Science). In some cases, engulfment of apoptotic cells and TUNEL staining were performed in 8-well Lab-TekII chamber slides (Nalgene Nunc International, Rochester, NY) and evaluated by observing the cells under a microscope.

RESULTS

Identification of cDNAs that Enhance Engulfment of Apoptotic Cells—To identify molecules involved in the engulfment of apoptotic cells by macrophages, we used a retrovirus-mediated expression cloning system (Fig. 1A). In brief, double-stranded cDNA was synthesized using mRNA from a BAM3 mouse macrophage cell line that efficiently engulfs apoptotic thymocytes (28). A cDNA library (700 pools of about 50 clones each) was prepared with pMX retrovirus vector. Plasmid DNA from each pool was introduced into PLAT-E packaging cells, and the retrovirus produced by the packaging cells was used to infect mouse NIH3T3 cells. At 72 h post-infection, NIH3T3 cells were incubated at 37°C for 2 h with CMFDA-labeled apoptotic thymocytes, washed thoroughly, and analyzed by flow cytometry. Thirteen to 15% of untransfected NIH3T3 cells were CMFDA-positive under these assay conditions, confirming that NIH3T3 cells can engulf apoptotic cells weakly. Transfection of NIH3T3 cells with the empty retrovirus vector had little effect on the frequency of CMFDA-positive cells (Fig. 1B). In contrast, NIH3T3 cells transfected with retrovirus prepared from 9 of 700 cDNA pools from the library showed a significantly greater CMFDA-positive fraction (18–20%). The positive single cDNA clones were then identified by the sibling procedure for each of the nine pools. Fig. 1B shows representative results from clones 46–4–36 and 88–5–46, which reproducibly increased the percentage of CMFDA-positive fractions in NIH3T3 cells to 34 and 28%, respectively.

Expression of Rho Family GTPases in Macrophages—The DNA sequence analysis of the nine positive cDNA clones indicated that four of them carried the full-length coding sequence for Src homology 2 domain-bearing protein-tyrosine phosphatase substrate-1 (SHPS-1); one (46–4–36) had the full-length sequence for SHPS-1; one (46–4–36) had the full-length sequence for Rab5, and one (88–5–46) had the full-length sequence for RabG. SHPS-1 is a transmembrane protein (29) expressed in macrophages. We showed previously that SHPS-1 enhances the engulfment of apoptotic cells during the tethering step (28). Rab5 is a member of the Rab subfamily of small GTPases and is involved in intracellular trafficking, in particular the endosome pathway (30). RabG is a member of the Rab subfamily of small GTPases (31). Some Rab family members (Rac1, RhoA, and Cdc42) have been shown to play a role in FcR- and complement-mediated phagocytosis by macrophages (32, 33). To investigate the involvement of Rho family GTPases in the engulfment of apoptotic cells by macrophages, we determined the expression levels of Rac1, Cdc42, RhoA, Rab5, and RabB in bone marrow-derived macrophages and the NIH3T3 fibroblast cell line by real-time PCR. As shown in Fig. 2, Rac1 was expressed at a similar level in the macrophages and NIH3T3 cells. The expression level of RabG was about three times higher in the macrophages than in NIH3T3 cells, although the expression level of Rab5 was about twice as high in the NIH3T3 cells than in the macrophages.
Enhancing or Inhibitory Effects of Rho Family GTPases on the Engulfment of Apoptotic Cells by NIH3T3 Cells

To confirm the effect of Rab5 and RhoG, as well as other Rho family GTPases, on the engulfment of apoptotic cells, retrovirus expression vectors for wild-type Rac1, Cdc42, RhoA, RhoG, and Rab5 and their dominant-negative forms (Rac1 T17N, Cdc42 T17N, RhoG F37A, RhoA T19N, and Rab5 S34N) were prepared. Infection of NIH3T3 cells with the retrovirus led to the expression of the respective small GTPases at a similar level, as judged by real time PCR (data not shown). The ability of the transformants to engulf apoptotic cells was then assayed using apoptotic CAD-deficient thymocytes as prey. In this assay, the chromosomal DNA of apoptotic cells is degraded in the lysosomes of macrophages only after the apoptotic cells are engulfed. Thus, the engulfment of apoptotic cells could be quantified by TUNEL staining (10). We assayed the engulfment of apoptotic cells under two different conditions, i.e. a higher or lower ratio of apoptotic cells to NIH3T3 cells. As shown in Fig. 3A, when apoptotic CAD-deficient thymocytes were incubated for 90 min with NIH3T3 cells at 10:1, about 12% of the NIH3T3 cells engulfed the apoptotic cells. NIH3T3 cells overexpressing wild-type Rac1, RhoG, or Rab5 showed an enhanced ability to engulf apoptotic cells, although wild-type RhoA inhibited the amount of engulfment. When the NIH3T3 cells were incubated for a longer period of time (120 min) with a higher ratio of apoptotic cells to NIH3T3 cells (20:1), about 28% of the NIH3T3 cells engulfed apoptotic thymocytes (Fig. 3B). This engulfment was strongly inhibited by expressing the dominant-negative forms of Rac1 or Rab5 and was moderately inhibited by the dominant-negative forms of Cdc42 and RhoG.

Opposite Effects of Rho Family GTPases on Apoptotic Cells by Macrophages—The effect of these Rho family GTPases in the engulfment of apoptotic cells was then examined using bone marrow-derived macrophages as phagocytes. Primary macrophages are known to be difficult to transfect. However, we found that these primary macrophages could be infected at high efficiency during their growing phase. Namely, adherent cells from mouse bone marrow were grown in the presence of M-CSF and infected on day 3 by retrovirus carrying the wild-type or dominant-negative forms of Rho family GTPases. The cells were further cultured in the presence of M-CSF for 5 days, at which point more than...
99% of the cells were Mac-1⁺, indicating they had a mature phenotype. The efficiency of the infection, assayed by transfecting the macrophages with a retrovirus carrying enhanced green fluorescent protein cDNA and analyzing them by flow cytometry, was more than 90%. The real time PCR indicated that the mRNA for each exogenous GTPase was 10–100 times more abundant than the endogenous mRNA. The engulfment of apoptotic cells was again assayed using a higher (20:1) or lower (10:1) ratio of apoptotic cells to macrophages. As shown in Fig. 4A, when the bone marrow-derived macrophages were incubated with the lower ratio of apoptotic cells, about 10% of the Mac-1⁺ cells engulfed the apoptotic cells. The overexpression of wild-type Rac1 or Rab5 enhanced the macrophages’ engulfment of apoptotic cells, although the overexpression of RhoA inhibited this process. When the macrophages were cultured with the higher ratio of apoptotic cells, about 33% of the macrophages engulfed the apoptotic thymocytes. This engulfment was inhibited by the expression of the dominant-negative form of Rac1, Rab5, or the dominant-negative form of RhoA and were incubated for a short time with the low ratio of apoptotic thymocytes. The number of apoptotic thymocytes engulfed per macrophage (phagocytosis index) of the vector-transfected macrophages was about 0.5 under this condition. This number increased between 1.2 and 1.7 in macrophages overexpressing Rac1, Rab5, or the dominant-negative RhoA and some transformed macrophages carried more than 10 apoptotic cells.

Involvement of Rac1 and RhoG in the MFG-E8-mediated Engulfment of Apoptotic Cells—MFG-E8, a glycoprotein secreted from macrophages, binds to apoptotic cells by recognizing phosphatidylserine and passes them to phagocytes expressing αvβ3 integrins (10). To examine whether this system also utilizes Rho family GTPases for the engulfment of apoptotic cells, dominant-negative Rac1 and RhoG and Rac1 were introduced into NIH3T3 cells expressing αvβ3 integrins. As shown in Fig. 5, the αvβ3 integrin-expressing NIH3T3 cells efficiently engulfed apoptotic cells in the presence of MFG-E8, and their phagocytosis index in the presence of MFG-E8 was 8 times higher than that observed in its absence. This MFG-E8-mediated engulfment of apoptotic cells was strongly inhibited by expressing the dominant-negative form of RhoG or Rac1, suggesting that this
Involvement of RhoG and Rac1 in the MFG-E8-dependent engulfment of apoptotic cells. NIH3T3 cells expressing α/β3 integrin were infected by retrovirus carrying the empty vector or by retrovirus carrying the dominant-negative form of RhoG (F37A) or Rac1 (T17N). The infected cells (5 × 10^4 cells) were co-cultured at 37°C for 90 min with 4 × 10^5 Cad−/− apoptotic thymocytes, fixed by paraformaldehyde, stained for TUNEL, and analyzed by flow cytometry. The percentage of TUNEL-positive macrophages is shown as the percentage of phagocytosis. The experiment was performed at least three times, and the average number is shown with S.D. The probabilities of statistical differences between the cells infected with the virus carrying the cDNA for wild-type GTPases were determined using Student’s t test (p < 0.01).

Opposite Effects of Rho GTPases on Apoptotic Cell Engulfment

Effect of the small GTPase family on the ability of bone marrow-derived macrophages to engulf apoptotic cells. A, effect of wild-type (WT) GTPases. Bone marrow-derived macrophages were infected with the empty retrovirus vector or the virus carrying the cDNA for wild-type Rac1, Cdc42, RhoA, RhoG, or Rab5. The infected cells (5 × 10^4 cells) were co-cultured at 37°C for 90 min with 4 × 10^5 Cad−/− apoptotic thymocytes, fixed by paraformaldehyde, stained for TUNEL, and analyzed by flow cytometry. The percentage of TUNEL-positive macrophages is shown as the percentage of phagocytosis. The experiment was performed at least three times, and the average number is shown with S.D. The probabilities of statistical differences between the cells infected with the empty retrovirus vector and the cells infected with the virus carrying the cDNA for wild-type GTPases were determined using Student’s t test (p < 0.01). B, effect of the dominant-negative forms of GTPases. Bone marrow-derived macrophages were transformed with the empty retrovirus or retrovirus carrying the dominant-negative forms of Rac1, Cdc42, RhoA, and RhoG. The infected cells (5 × 10^4 cells) were co-cultured at 37°C for 2 h with 1 × 10^6 Cad−/− apoptotic thymocytes, and the engulfment was analyzed as described above. The assay was performed at least three times, and the average numbers are shown with S.D. The probabilities of statistical differences between the cells infected with the empty vector and the cells infected with the virus carrying the cDNA for dominant-negative forms of GTPases were determined using Student’s t test (p < 0.01).

C, effect of the GTPase on the engulfing ability of individual macrophages. Bone marrow-derived macrophages were infected with the empty retrovirus or virus carrying the cDNA for the wild-type Rac1, RhoG, Rab5, or the dominant-negative form of RhoA (T19N). The infected cells (4 × 10^4 cells) were incubated at 37°C for 2 h with 1 × 10^6 Cad−/− apoptotic thymocytes in chamber slides, stained for TUNEL, and observed by light microscopy. Magnification, ×400. In the panel at the bottom right corner, the phagocytosis index (the number of apoptotic cells per macrophage) was determined as described under “Experimental Procedures” and is shown as an average of three independent experiments. The probabilities of statistical difference between the cells infected with the empty retrovirus vector and the cells infected with the virus carrying the cDNA for Rac1, RhoG, Rab5, or the dominant-negative form of RhoA were determined using Student’s t test (p < 0.01).
engulfment system faithfully reflects the mechanism of engulfment used by macrophages.

**DISCUSSION**

The engulfment of apoptotic cells by phagocytes is initiated when specific receptors on the phagocytes recognize their ligand(s) on apoptotic cells. This causes the recruitment of diverse signaling molecules and enzymes to the receptor, resulting in reorganization of the actin cytoskeletons of the phagocytes and internalization of the apoptotic cells (32). The internalized dead cells are then carried to lysosomes and degraded into their amino acids and nucleotides for reuse. Here, to examine the molecular mechanism of the engulfment of apoptotic cells, we established a retrovirus-vector-based expression cloning system. We used this system to introduce the cDNA library into NIH3T3 cells, because NIH3T3 cells could be transformed by this method at an efficiency of 100%. We also found that primary bone marrow-derived macrophages, which are professional phagocytes that efficiently engulf apoptotic cells, could be efficiently transformed using this retrovirus vector.

Negatively charged microbeads are often used as prey for phagocytes (34). However, to ensure the engulfment of apoptotic cells, we used dexamethasone-treated mouse thymocytes, which are a rather homogeneous population of apoptotic cells that can be efficiently engulfed by macrophages and fibroblasts (10). The repeated isolation by this cloning procedure of SHPS-1, a cell-surface protein involved in the tethering step of apoptotic cells (28), indicated that this screening procedure worked efficiently.

Among the newly identified genes, RhoG and Rab5 strongly enhanced the engulfment of apoptotic cells by NIH3T3 cells. We confirmed their critical role in the engulfment of apoptotic cells by expressing their wild-type or dominant-negative form in primary macrophages. RhoG is a member of the Rho GTPase family, which consists of at least 20 members that are divided into the Rho-like (represented by RhoA), Rnd-like, Cdc42-like, Rac-like (represented by Rac1 and RhoG), and RhoBTB (31) subfamilies. Two distinct mechanisms controlled by different Rho GTPases have been proposed for type I phagocytosis, mediated by the immunoglobulin receptor, and the type II phagocytosis, mediated by complement (33). In type I phagocytosis, Cdc42 and Rac regulate phagocytosis; in type II phagocytosis, Rho plays this role. Here, the engulfment of apoptotic cells by macrophages was severely inhibited by the dominant-negative form of Rac1, but dominant-negative Cdc42 had little effect, although dominant-negative RhoA rather enhanced the engulfment. These results indicate that the molecular mechanism used to engulf apoptotic cells may differ from both the type I and type II phagocytoses proposed previously. The complement system has been suggested as a mechanism for the engulfment of apoptotic cells by macrophages (35). However, given that macrophages use Rho GTPases that are different from the ones used by the complement system, the complement system may not play a major role in the engulfment of apoptotic cells. In contrast, we found that the MFG-E8-supported engulfment of apoptotic cells by αβ3 integrin-expressing NIH3T3 cells was mediated by RhoG and Rac1, just like the engulfment by bone-marrow derived macrophages, suggesting that this αβ3 integrin-mediated system is a good model for the engulfment of apoptotic cells by macrophages.

The Dock180 (CED-5)-ELMO (CED-12) complex was shown to work as the guanine nucleotide exchange factor for Rac1 (CED-10) to induce cytoskeletal reorganization during engulfment (16, 20, 34). Recently, Ravichandran and co-workers (21) further showed that RhoG and TRIO (UNC-73) both function upstream of the Dock-ELMO signaling pathway. In accord with this model, the RhoG-stimulated engulfment of apoptotic cells by macrophages was completely inhibited by dominant-negative Rac1, although dominant-negative RhoG had no effect on the Rac1-stimulated engulfment of apoptotic cells. MFG-E8 stimulates the engulfment of apoptotic cells through integrin αβ3 expressed on phagocytes (10), and an integrin is known to activate the CrkII-Dock180-Rac1 signaling (19), suggesting that the binding of MFG-E8-coated apoptotic cells to macrophages activates Rac via the RhoG-CrkII-Dock180 pathway.

As found previously (36), we showed the engulfment of apoptotic cells was negatively regulated by RhoA. In the directed cell movement of neutrophils, Rac and RhoA are activated at the front and rear of the cells, respectively, and regulate the polarity of the cell during chemotaxis (37). Similarly, the coordinated activation of Rac and RhoA is likely to be required for the efficient engulfment of apoptotic cells by macrophages. It will be interesting to study the spatiotemporal activation of Rac and RhoA in macrophages during the engulfment of apoptotic cells. Finally, the engulfment of apoptotic cells does not cause inflammation (38). Rather, it inhibits inflammation by producing anti-inflammatory cytokines such as transforming growth factor-β and platelet-activating factor. The Rho family of GTPases can activate gene expression through NF-κB and/or STAT-3 (39, 40). Whether RhoG, Rac, and RhoA, which positively and negatively regulate the engulfment of apoptotic cells, are involved in the expression of genes that are specifically expressed in macrophages in the process of engulfing apoptotic cells remains to be studied.

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