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## **Two-Step Engulfment of Apoptotic Cells**

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Running title: TWO-STEP ENGULFMENT OF APOPTOTIC CELLS

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## Abstract

Apoptotic cells expose phosphatidylserine on their surface as an “eat me” signal, and macrophages respond by engulfing them. Although several molecules that specifically bind phosphatidylserine have been identified, the molecular mechanism that triggers engulfment remains elusive. Here, using a mouse pro-B cell line, Ba/F3, that grows in suspension, we reconstituted the engulfment of apoptotic cells. The parental Ba/F3 cells did not engulf apoptotic cells. Ba/F3 transformants expressing Tim4, a type I membrane protein that specifically binds phosphatidylserine efficiently bound apoptotic cells in a phosphatidylserine-dependent manner, but did not engulf them. However, Ba/F3 transformants expressing both Tim4 and the integrin  $\alpha_v\beta_3$  complex bound to and engulfed apoptotic cells in the presence of MFG-E8, a secreted protein that can bind phosphatidylserine and integrin  $\alpha_v\beta_3$ . These results indicate that the engulfment of apoptotic cells proceeds in two steps: Tim4 tethers apoptotic cells, and the integrin  $\alpha_v\beta_3$  complex mediates engulfment in coordination with MFG-E8. A similar two-step engulfment of apoptotic cells was observed with mouse resident peritoneal macrophages. Furthermore, the Tim4/integrin-mediated engulfment by the Ba/F3 cells was enhanced in cells expressing Rac1 and Rab5, suggesting that this system well reproduces the engulfment of apoptotic cells by macrophages.

## Introduction

Every day, billions of cells that are toxic, useless, and senescent die by apoptosis and are engulfed by macrophages, presumably to prevent the release of noxious materials from the dead cells (18). The system that efficiently removes apoptotic cells from the body appears to be quite elaborate, and some details of this process are unclear (25). Apoptotic cells present an “eat me” signal to macrophages, triggering their own engulfment. Among the various molecules proposed to be involved in this process, phosphatidylserine (PS) is a strong candidate for the “eat me” signal (11). PS is transferred caspase-dependently from the inner leaflet to the outer leaflet of the plasma membrane (13), and masking PS inhibits the engulfment of apoptotic cells by macrophages (2, 6, 11).

By identifying monoclonal antibodies that have positive or negative effects on the engulfment of apoptotic cells, we previously identified two molecules, Milk Fat Globule EGF Factor VIII (MFG-E8) and T-cell immunoglobulin- and mucin-domain-containing molecule (Tim4), that enhance engulfment (6, 15). MFG-E8 is a secreted protein of 75 kDa that binds to PS via its Factor VIII-homologous domain. It also binds to the integrin  $\alpha_v\beta_3$  complex in macrophages via an RGD-motif in MFG-E8's EGF domain, thus bridging apoptotic cells and macrophages. Tim4 is a type I membrane protein of 70 kDa, that binds to PS via the Immunoglobulin-like domain in its extracellular region. Its cytoplasmic region is 43 amino-acids long, and is dispensable for engulfment (22), suggesting that Tim4 itself does not transduce the signal for engulfment. Tim4, but not MFG-E8, is expressed by

resident peritoneal macrophages, and MFG-E8, but not Tim4, is expressed by thioglycollate-elicited peritoneal macrophages (15), suggesting that the two molecules function independently in the engulfment of apoptotic cells in these macrophages. On the other hand, tingible-body macrophages in the spleen express both MFG-E8 and Tim4 (7, 31), suggesting that the two molecules may co-operate in the engulfment of apoptotic cells. However, how MFG-E8 and Tim4 function in these macrophages, and how Tim4 and integrins transduce the engulfment signal have not been elucidated.

Here, we established an assay system for the engulfment of apoptotic cells using a Ba/F3 suspension culture. The parental Ba/F3 cells did not engulf apoptotic cells at all. The expression of Tim4 conferred on them the ability to recognize and bind apoptotic cells, but not to engulf them. When Tim4 and integrin  $\alpha_v\beta_3$  complex were co-expressed, the Ba/F3 transformants efficiently engulfed apoptotic cells in the presence of MFG-E8, and this efficiency was enhanced by the expression of Rac1 and Rab5. These results indicate that Tim4 and integrin  $\alpha_v\beta_3$  co-operate to mediate the engulfment of apoptotic cells, Tim4 in the tethering step and MFG-E8/integrin in the uptake step.

## MATERIALS AND METHODS

**Materials, cell lines, recombinant proteins, and antibodies.** pHrodo<sup>TM</sup> succinimidyl ester (pHrodo) was purchased from Invitrogen. CellTracker Orange (CMRA, ((9'-(4-(and 5)-chloromethyl-2-carboxyphenyl)-7'-chloro-6'-oxo-1,2,2,4-tetramethyl-1,2-dihydropyrido[2',3'-6]xanthene) was obtained from Molecular Probe. A caspase inhibitor (Q-VD-OPh, quinolyl-valyl-O-methylaspartyl-[2, 6-difluorophenoxy]-methyl ketone) was from R&D systems.

The mouse pro-B cell line, Ba/F3 was maintained in RPMI1640 supplemented with 10% fetal calf serum (FCS) and 45 units/ml mouse interleukin (IL)-3. Mouse NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented by 10% FCS.

Flag-tagged mouse recombinant MFG-E8 was produced in human 293T cells as described (6), and purified with anti-Flag-Sepharose. To prepare the leucine-zipper-tagged human Fas ligand (FasL)(27), a FasL-expression plasmid was introduced into monkey COS cells by electroporation. The COS cells were cultured in DMEM containing 1% FCS for 48 h, and FasL that was secreted into the culture supernatant was precipitated in 60%-saturated  $(\text{NH}_4)_2\text{SO}_4$ , and dialyzed against PBS. The cytotoxic activity of FasL was assayed with mouse WR19L cells expressing mouse Fas (W3 cell line) as described (29). One unit of FasL activity represented the concentration of FasL needed to produce half-maximal cytotoxicity against  $5 \times 10^4$  W3 cells. Mouse recombinant IL-3 was produced by mouse C127I cells transformed with a bovine papillomavirus expression vector for mouse IL-3 as

described (4). The biological activity of IL-3 was assayed by determining its ability to support the growth of Ba/F3 cells as described (4). One unit represented the concentration of IL-3 required for the half-maximal stimulation at 37° for 48 h of  $1 \times 10^4$  cells in 100  $\mu$ l of medium. The hamster anti-Tim4 mAb (clone Mat4) will be described elsewhere. The phycoerythrin (PE)-labeled rat anti-mouse integrin  $\alpha_v$  (clone RMV-7), PE-labeled hamster anti-mouse integrin  $\beta_3$  (clone 2C9.G2), and allophycocyanine (APC)-labeled streptavidin were from BD Pharmingen. The APC-labeled rat anti-mouse Mac1 (clone M1/70) was purchased from BioLegend.

**Mice.** The wild-type C57BL/6 mice were purchased from Japan SLC. Tim4<sup>-/-</sup> mice were established in C57BL/6 background (M. Miyanishi and S.N., unpublished), and will be described elsewhere. Mice were housed in a specific pathogen-free facility at Kyoto University Graduate School of Medicine. All animal experiments were carried out in accordance with protocols approved by the Animal Care and Use Committee at Kyoto University Graduate School of Medicine.

**Transformation.** Mouse Tim4 and integrins were expressed in NIH3T3 cells and Ba/F3 cells by retrovirus-mediated transformation. In brief, the recombinant retrovirus was produced in Plat-E cells (16) by transfecting them with the pMXs-puro or -neo retrovirus vector (10) carrying the cDNA sequence for GFP, mouse Tim4 (15), mouse Rac1 or Rab5

(19), or mouse integrin  $\alpha_v$  or integrin  $\beta_3$  (6). The retrovirus was precipitated by centrifugation at 6000 x g for 16 h, dissolved in medium containing 10% FCS, and used to infect NIH3T3 cells or Ba/F3 cells in the presence of 10  $\mu\text{g/ml}$  polybrene. The transformants were selected by culturing in the presence of 1.0  $\mu\text{g/ml}$  puromycin or 800  $\mu\text{g/ml}$  G-418. When necessary, Ba/F3 cells expressing Tim4, integrin  $\alpha_v$  or integrin  $\beta_3$  were sorted using FACS Aria (BD Biosciences).

**Labeling of apoptotic cells.** Mouse thymocytes were labeled with pHrodo as described by Miska et al. (14) for the engulfment assay or with CellTracker Orange for the binding assay. In brief, thymocytes ( $2 \times 10^7$  cells/ml) from 4- to 8-week old C57BL/6 mice were treated at 37°C for 2 h with 70 units/ml FasL, which caused about 70% of the cells to become Annexin V-positive, and less than 20% of them to be Sytox Blue-positive. The cells were washed twice with PBS, and incubated at room temperature for 30 min with 0.1  $\mu\text{g/ml}$  pHrodo. After stopping the reaction by adding 1 ml FCS, the cells were washed with PBS containing 10% FCS, suspended in the medium containing 10% FCS and used for engulfment. For labeling with CellTracker, thymocytes were incubated at 37°C for 30 min in serum-free DMEM containing 10  $\mu\text{M}$  CellTracker Orange, washed with DMEM containing 10% FCS, and incubated at 37°C for 2 h with FasL as described above.

**Engulfment of apoptotic cells by NIH3T3 cells.** To  $7.5 \times 10^4$  NIH3T3 cells in 0.5 ml



of DMEM containing 10% FCS in a 24-microtiter well,  $1 \times 10^6$  pHrodo-labeled apoptotic cells were added, and the culture was incubated at 37°C for 90 min. The cells were detached from the plate by incubating them with 0.25% trypsin and 1 mM EDTA, and collected by centrifugation. The cells were suspended in 300  $\mu$ l of 20 mM CHES-NaOH buffer (pH 9.0) containing 150 mM NaCl and 2% FCS (CHES-FACS buffer), and analyzed by flow cytometry on a FACS Aria. In some cases,  $6 \times 10^4$  NIH3T3 cells were cultured in an 8-well Lab-Tek II Chambered cover glass (Nalge Nunc), and incubated at 37°C for 90 min with  $1 \times 10^6$  pHrodo-labeled apoptotic thymocytes in 0.8 ml DMEM containing 10% FCS. After being washed with PBS, the cells were fixed at room temperature for 10 min with 1% paraformaldehyde in PBS, immersed in CHES-FACS buffer, and observed by fluorescence microscopy (BioRevo BZ-9000, Keyence).

**Engulfment of apoptotic cells with Ba/F3 cells.** The GFP-expressing Ba/F3 cells ( $1 \times 10^5$ ) and  $1-5 \times 10^6$  pHrodo-labeled apoptotic thymocytes were incubated at 37°C for 2 h in 0.6-3.0 ml RPMI 1640 containing 10% FCS. After the incubation, the cells were collected by centrifugation at 500 x g for 5 min, suspended in 300  $\mu$ l of CHES-FACS buffer, and analyzed by flow cytometry as above. For microscopic observation, the GFP-expressing Ba/F3 and pHrodo-labeled thymocytes were suspended in 100  $\mu$ l of CHES-FACS Buffer, transferred to the Lab-Tek II Chambered Cover glass, and observed by fluorescence microscopy. The numbers of GFP-positive Ba/F3 cells and pHrodo-positive thymocytes

were counted using Dynamic Cell Count software, BZ-HIC (Keyence). The phagocytosis index represents the number of the engulfed apoptotic cells per Ba/F3 cell.

**Engulfment of apoptotic cells with peritoneal macrophages.** Resident peritoneal cells were prepared from C57BL/6 mice at age of 8 weeks. The peritoneal cells ( $1 \times 10^5$ ) and  $1.0 \times 10^6$  pHrodo-labeled apoptotic thymocytes were incubated in suspension at  $37^\circ\text{C}$  for 60 min in 0.5 ml RPMI 1640 containing 10% FCS, or in PBS containing 2% FCS. After the incubation, the cells were collected by centrifugation at  $500 \times g$  for 5 min, suspended in 300  $\mu\text{l}$  of CHES-FACS buffer containing  $0.67 \mu\text{g/ml}$  APC-labeled rat anti-mouse Mac1, and analyzed by flow cytometry.

**Binding of apoptotic cells with Ba/F3 cells.** To assay the binding of apoptotic cells to Ba/F3 cells,  $1 \times 10^5$  Ba/F3-GFP-expressing cells were incubated at  $37^\circ\text{C}$  with  $5 \times 10^6$  CellTracker Orange-labeled apoptotic thymocytes in 500  $\mu\text{l}$  of PBS containing 10% FCS, and subjected to FACS analysis for GFP and CellTracker Orange. The cell population that was positive for GFP and CellTracker Orange was regarded as Ba/F3 cells to which apoptotic cells were bound. In some cases,  $1 \times 10^5$  Ba/F3 cells were incubated with  $1 \times 10^6$  CellTracker Orange-labeled apoptotic thymocytes at room temperature for 25 min in a 8-well Lab-Tek II Chamber, and then observed by fluorescence microscopy.

**Binding of apoptotic cells with resident peritoneal macrophages.** To assay the binding of apoptotic cells to the resident peritoneal macrophages,  $1 \times 10^5$  mouse peritoneal cells were incubated in suspension at room temperature with  $1 \times 10^6$  CellTracker Orange-labeled apoptotic thymocytes in 500  $\mu$ l of PBS containing 10% or 2% FCS. The APC-conjugated anti-Mac1 was added to a final concentration of 0.67  $\mu$ g/ml, and the cells were analyzed by flow cytometry. The cell population that was positive for APC and CellTracker Orange was regarded as peritoneal macrophages to which apoptotic cells were bound.

## RESULTS

**Establishment of the assay for the engulfment of apoptotic cells.** Miksa et al (14) recently developed a simple method for monitoring the engulfment of apoptotic cells by using pHrodo-labeled apoptotic cells as prey. This method permits apoptotic cells that are engulfed and transported into lysosomes to be detected by their increased light emission in the acidic lysosomal environment. We modified this method slightly. That is, since we found that the non-engulfed pHrodo-conjugated apoptotic thymocytes emit a low but significant light at neutral conditions (pH 7.4), we treated the phagocytes with basic buffer (pH 9.0) after co-incubation with apoptotic cells. Furthermore, living cells were found to incorporate pHrodo to emit light even at basic conditions. Mouse thymocytes were therefore treated with a high concentration of FasL to fully induce apoptosis in all cells in a short period (2 h) before labeling them with pHrodo. Under these conditions, about 70% of the thymocytes were Annexin V-positive, and Sytox blue-negative.

We first tested the reliability of the assay by examining the Tim4-mediated engulfment of apoptotic cells by NIH3T3 cells (15). NIH3T3 cells, which do not normally express Tim4 were stably transformed with Tim4 to generate NIH3T3/Tim4 cells (Fig. 1A) and incubated with pHrodo-labeled apoptotic thymocytes at a ratio of 1:13 (NIH3T3: thymocytes). An analysis by flow cytometry indicated that more than 60% of the NIH3T3/Tim4 cells became pHrodo-positive within 90 min of co-incubation with the apoptotic thymocytes, while most of the cells with the parental NIH3T3 remained

pHrodo-negative (Fig. 1B). Microscopic observation showed several pHrodo-positive thymocytes present inside most of the NIH3T3/Tim4 cells, with only a small percentage of NIH3T3 cells carrying a single pHrodo-positive thymocyte. In addition, many pHrodo-negative, unengulfed cells were associated with NIH3T3/Tim4 cells, but not with the parental NIH3T3 cells. These results confirmed that Tim4 strongly enhances the engulfment of apoptotic cells in NIH3T3 cells (15), and that the engulfment assay using the pHrodo-labeled apoptotic cells (14) was simple and reliable.

**Binding of apoptotic cells to Tim4-expressing Ba/F3 cells without engulfment.** In contrast to the NIH3T3 cells, when the mouse pro-B cell line Ba/F3, which grows in suspension, was transformed with Tim4 (BaF/Tim4), they hardly engulfed any apoptotic cells (data not shown). Since a small GTPase, Rac1, enhances the engulfment of apoptotic cells (see below) (19, 30), BaF/GFP and BaF/GFP/Tim4 were transformed with Rac1. As shown in Figure 2, very few apoptotic cells were engulfed by the BaF/GFP/Rac cells either. This assay showed that the expression of Tim4 rendered the cells able to engulf apoptotic cells, but even so, only 5% of the BaF/GFP/Rac/Tim4 transformants contained dead cells.

On the other hand, when the binding, rather than engulfment of CellTracker-labeled apoptotic cells was assayed, the ability of the Ba/F3 cells to bind the apoptotic cells was clearly and strongly enhanced by Tim4 expression. That is, only 1.3% of the BaF/GFP/Rac cells bound apoptotic cells within 30 min, whereas more than 50% of the

BaF/GFP/Rac/Tim4 cells bound them (Fig. 3A). Microscopic observation showed that the BaF/Tim4 cells had a tendency to aggregate (Fig. 3B), as reported previously (15). We postulated that the aggregation of the Tim4-expressing cells is due to PS-expressing exosomes (15). Apoptotic thymocytes also seemed to mediate the aggregation of BaF/GFP/Rac/Tim4 cells (Fig. 3B). The binding of apoptotic cells to BaF3/GFP/Tim4 was quick, with near-maximum binding observed within 15 min at 37°C (Fig. 3C). The D89E mutant of MFG-E8, which can mask PS (6), dose-dependently inhibited the binding of apoptotic cells to the BaF/GFP/Rac/Tim4 cells (Fig. 3D), confirming that the apoptotic cells bound to Tim4-expressing cells in a PS-dependent manner.

**Stimulation of the engulfment of apoptotic cells by integrin  $\alpha_v\beta_3$ .** The above results indicated that Tim4 recognized apoptotic cells and tethered them to phagocytes, but did not mediate their engulfment. The  $\alpha_v\beta_3$  integrin complex, which is the vitronectin receptor, is known to transduce a signal for engulfment (1, 6, 26). Macrophages express the integrin  $\alpha_v\beta_3$  complex, but Ba/F3 cells do not. We therefore expressed integrin  $\alpha_v\beta_3$  in BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells (Supplementary Figure 1). A real-time RT-PCR analysis indicated that the expression levels of Tim4 and integrin  $\alpha_v$  mRNA in the Ba/F3 transformants were comparable to those in mouse resident peritoneal macrophages (Supplementary Figure 2). As shown in Figure 4A, the expression of the integrins  $\alpha_v\beta_3$  complex alone in these Ba/F3 cells had little effect on their ability to engulf apoptotic cells.

However, the addition of MFG-E8 to the integrin  $\alpha_v\beta_3$ -expressing cells strongly enhanced the engulfment. Specifically, about 25% of the BaF/GFP/Rac/Tim4/ $\alpha\beta$  cells engulfed apoptotic cells in the presence of 0.1  $\mu\text{g/ml}$  MFG-E8. Observation by microscope showed that only a few apoptotic cells were inside Ba/F3 expressing integrins  $\alpha_v\beta_3$ , but several dead cells were found in the Ba/F3 cells expressing integrin  $\alpha_v\beta_3$  and Tim4 (Fig. 4B). Thus, the phagocytic index (the number of engulfed dead cells per phagocyte) by the BaF/Rac/Tim4 cells, increased to 0.4 with the expression of integrin  $\alpha_v\beta_3$  (Fig. 4C). The increase in the engulfment of apoptotic cells by Ba/F3 cells expressing integrin  $\alpha_v\beta_3$  was not due to a more efficient recruitment of apoptotic cells; as shown in Figure 4D, apoptotic cells did not bind to the integrin  $\alpha_v\beta_3$ -expressing Ba/F3 cells, even in the presence of MFG-E8, unless Tim4 was also expressed. These results indicated that Tim4 and integrin  $\alpha_v\beta_3$ /MFG-E8 function in different steps of the engulfment process for apoptotic cells.

To examine whether macrophages engulf apoptotic cells in two steps, we analyzed the engulfment of apoptotic cells with mouse resident peritoneal macrophages that strongly express Tim4 (15). As shown in Figure 4E, the wild-type peritoneal macrophages efficiently engulfed apoptotic cells, but this ability was lost in Tim4-deficient peritoneal macrophages, because they could not tether apoptotic cells. Binding of Tim4 to phosphatidylserine does not require  $\text{Ca}^{2+}$  (15), while the integrin signaling is known to require  $\text{Ca}^{2+}$  (28). Accordingly, apoptotic cells efficiently bound to the Tim4-expressing peritoneal macrophages in the absence or presence of  $\text{Ca}^{2+}$  (Fig. 4F). On the other hand, the

engulfment of apoptotic cells by the peritoneal macrophages did not occur in the absence of  $\text{Ca}^{2+}$ . These results indicated that the engulfment of apoptotic cells by mouse resident peritoneal macrophages proceeds in two steps,  $\text{Ca}^{2+}$ -independent tethering and  $\text{Ca}^{2+}$ -dependent uptake steps.

**Effect of small GTPases Rac1 and Rab5 on the engulfment by Ba/F3 cells.** The Rho and Rab family GTPases regulate the engulfment of apoptotic cells by macrophages and immature dendritic cells. That is, to form the phagocytic cup to engulf apoptotic cells, the integrin  $\alpha_v\beta_3$  expressed by macrophages activates Rac1 of the Rho family to induce actin polymerization (1). Rab5, a member of the Rab family, regulates the fusion of phagosomes with endosomes (3). As shown in Figure 5, the over-expression of Rac1 in BaF/Tim4/ $\alpha\beta$  cells strongly enhanced the MFG-E8-dependent engulfment of apoptotic cells, increasing the phagocytic index from 0.1 to 0.4. The addition of Rab5 expression further increased the index to 0.65. That is, less than 10% of the BaF/GFP/Tim4/ $\alpha\beta$  cells engulfed apoptotic thymocytes in the presence of MFG-E8. In contrast, about half the BaF/GFP/Tim4/ $\alpha\beta$  cells expressing Rac1 and Rab5 engulfed dead cells, and some of them contained several dead cells. These results support the idea that the engulfment of apoptotic cells in the Ba/F3 suspension culture efficiently reproduces the process of apoptotic cell engulfment by macrophages.



## DISCUSSION

The reconstitution of apoptotic cell engulfment in a system that uses defined molecules is essential for understanding the detailed molecular mechanisms involved. We and others have used fibroblasts and epithelial cell lines such as NIH3T3, LR73, and 293T cells as host cells to reconstitute engulfment, and have identified several molecules (MFG-E8/integrins, Tim4, stabilin 2, BAI1, etc.) that bind to PS (6, 15, 20, 23, 24). When they are expressed in fibroblasts, they enhance the engulfment of dead cells, suggesting that each molecule is independently involved in this process. In particular, Tim4 alone endows NIH3T3/LR73 cells with the ability to efficiently engulf apoptotic cells, yet its cytoplasmic region is not required for the engulfment of apoptotic cells, suggesting that Tim-4 works at the tethering step, and LR73 cells express one or more additional molecule that co-mediate the engulfment of apoptotic cells with Tim4 (22).

Lymphocytes that grow in suspension usually do not engulf apoptotic cells. On the other hand, immature dendritic cells established from the mouse spleen grow in suspension, and can take up mycobacteria (21). We therefore thought that a lymphocyte cell line could be a good host cell for reconstituting apoptotic cell engulfment, when coupled with pHrodo-labeled apoptotic cell engulfment assay. Here, we found that, unlike Tim4 in NIH3T3 cells, the expression of Tim4 in Ba/F3 cells was not sufficient for apoptotic cell engulfment, although apoptotic cells bound to the Tim4-expressing cells. Integrin  $\alpha_v\beta_3$  was also required for the efficient engulfment of apoptotic cells. However, the failure of

apoptotic cells to bind to Ba/F3 cells expressing integrin  $\alpha_v\beta_3$ , but not Tim4, even in the presence of MFG-E8, indicated that the role of integrin  $\alpha_v\beta_3$ /MFG-E8 is different from that of Tim4. Our experiments indicated that apoptotic cells are recruited by Tim4, and then passed to the integrin  $\alpha_v\beta_3$ /MFG-E8 complex for uptake (Figure 6). This mechanism agrees with the tether-tickle model for the engulfment of apoptotic cells, proposed by Hoffmann et al (8). A similar two-step engulfment, Tim4-dependent tethering and Ca-dependent uptake, was demonstrated by mouse resident peritoneal macrophages, indicating that the engulfment system reconstituted with the Ba/F3 cell line well represents the engulfment that naturally takes place with macrophages.

Integrins are activated by an inside-out signaling pathway or can be artificially activated with manganese (9, 17). Accordingly, when the Ba/F3 cells expressing integrin  $\alpha_v\beta_3$  were treated with 0.5 mM manganese, they engulfed apoptotic cells in the presence of MFG-E8 (S.T. R.H., and S.N., unpublished observation), suggesting that integrin  $\alpha_v\beta_3$  is activated in Ba/F3/Tim4/integrin  $\alpha_v\beta_3$  cells by inside-out signaling during the engulfment of apoptotic cells. Since Tim4 does not directly associate with the integrin complex (S.T. R.H., and S.N., unpublished observation), it is possible that there is another unidentified factor that links Tim4 with the integrin  $\alpha_v\beta_3$ /MFG-E8 system (Figure 6).

The tingible-body macrophages in the spleen express MFG-E8, and MFG-E8<sup>-/-</sup> tingible-body macrophages do not engulf apoptotic cells (7). The strong association of apoptotic cells with these macrophages supports the idea that MFG-E8 functions at the

uptake step, but not at the tethering step. Resident peritoneal macrophages express Tim4, but not MFG-E8, whereas thioglycollate-elicited peritoneal macrophages express MFG-E8, but not Tim4 (15). Yet, these resident and thioglycollate-elicited peritoneal macrophages efficiently engulf apoptotic cells, suggesting that some factor other than MFG-E8 is working in the uptake step in the resident macrophages, and some factor besides Tim4 in the tethering step in the thioglycollate-elicited macrophages. Recently, the engulfment of apoptotic cells by macrophages has been recognized to be as complicated as neurotransmission or lymphocyte activation (5, 12). A synapse-like structure consisting of many molecules called “engulfment synapse” has been proposed. The engulfment system established here, using Ba/F3 cells, will help in classifying these molecules into the tethering and uptake steps, and also in identifying new factors involved in these processes.

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## Figure Legends

**FIG. 1.** Effect of Tim4 on the engulfment of apoptotic cells by NIH3T3 cells. (A) Expression of Tim4 in mouse NIH3T3 cells. NIH3T3 cells and transformants expressing mouse Tim4 were stained with a biotinylated anti-Tim4 mAb, followed by APC-conjugated streptavidin. The staining profile was analyzed by flow cytometry using a FACS Aria. (B and C) Tim4-dependent engulfment of apoptotic cells. The pHrodo-labeled apoptotic thymocytes were incubated for 90 min with NIH3T3 cells or NIH3T3 transformants expressing Tim4, and analyzed by flow cytometry (B). In (C), the engulfment of pHrodo-labeled apoptotic cells was performed in a Lab-Tek Chamber, and observed by fluorescence microscopy.

**FIG. 2.** Effect of Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. (A) Expression of Tim4 in mouse Ba/F3 cells. The Ba/F3 cells were transformed with an expression vector for GFP and Rac1 (BaF/GFP/Rac), or with a vector for GFP, Rac1 and Tim4 (BaF/GFP/Rac/Tim4). The stable transformants were stained with a biotinylated anti-Tim4 mAb, followed by PE-conjugated streptavidin. (B and C) The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 120 min, and subjected to flow cytometry. The pHrodo profile of the GFP-positive population is shown (B). The number indicates the percentage of pHrodo-positive cells in the GFP-positive population. In (C), the BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were



incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 120 min. The cell mixture was transferred to a Lab-Tek Chamber, and observed by fluorescence microscopy.

**FIG. 3.** Tim4-dependent binding of apoptotic cells to Ba/F3 cells. (A) FACS analysis of the binding of apoptotic cells to Ba/F3 cells. Ba/F3 cells expressing GFP and Rac1 (BaF/GFP/Rac) or GFP, Rac1, and Tim4 (BaF/GFP/Rac/Tim4) were incubated at 37°C for 30 min with CellTracker Orange-labeled apoptotic thymocytes, and subjected to flow cytometry for GFP and CellTracker Orange. The number indicates the percentage of the CellTracker-positive cells in the GFP-positive population. (B) Microscopic analysis of the binding of apoptotic cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated for 25 min at room temperature with CellTracker Orange-labeled living or apoptotic thymocytes in Lab-Tek chamber, and observed by fluorescence microscopy. (C) Time course of the binding of apoptotic cells to Tim4-expressing cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with CellTracker Orange-labeled living or apoptotic thymocytes at 37°C for the indicated periods of time. The percentage of GFP-positive Ba/F3 cells that bound apoptotic cells was determined by flow cytometry as described above. The experiments were done in triplicate, and the average values are plotted (bars indicate S.D.). (D) Phosphatidylserine-dependent binding of apoptotic cells to Tim4-expressing Ba/F3 cells. The BaF/GFP/Rac and BaF/GFP/Rac/Tim4 cells were incubated with CellTracker Orange-labeled living or apoptotic thymocytes at 37°C for 30

min in the presence of the indicated amount of MFG-E8 D89E. The cell mixture was subjected to FACS analysis, and the mean fluorescence of the CellTracker-Orange in the GFP-positive cells was determined. The experiments were done in triplicate, and the average values are plotted (bars indicate S.D.).

**FIG. 4.** Effect of integrins/MFG-E8 and Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. (A) Effect of integrin  $\alpha_v\beta_3$ /MFG-E8 and Tim4 on the engulfment of apoptotic cells by Ba/F3 cells. BaF/GFP/Rac, BaF/GFP/Rac/ $\alpha\beta$ , BaF/GFP/Rac/Tim4, or BaF/GFP/Rac/Tim4/ $\alpha\beta$  cells were incubated at 37° for 120 min with pHrodo-labeled apoptotic thymocytes in the absence or presence of 0.1  $\mu$ g/ml mouse MFG-E8. The cell mixture was subjected to flow cytometry for GFP and pHrodo. The pHrodo staining profile of the GFP-positive population is shown. The experiment was performed three times, and representative data are shown. (B) Microscopic observation. BaF/GFP/Rac/ $\alpha\beta$  or BaF/GFP/Rac/Tim4/ $\alpha\beta$  cells were incubated as above with pHrodo-labeled apoptotic thymocytes in the presence of 0.1  $\mu$ g/ml mouse MFG-E8. The cells were transferred to a Lab-Tek chamber, and observed by fluorescence microscopy. (C) Enhanced engulfment of apoptotic cells by Tim4 and MFG-E8. BaF/GFP/Rac (-), BaF/GFP/Rac/ $\alpha\beta$  ( $\alpha\beta$ ), BaF/GFP/Rac/Tim4 (Tim4), or BaF/GFP/Rac/Tim4/ $\alpha\beta$  (Tim/ $\alpha\beta$ ) cells were incubated at 37°C for 120 min with pHrodo-labeled apoptotic thymocytes in the absence (-) or in the presence (+) of 0.1  $\mu$ g/ml mouse MFG-E8. The cell mixture was transferred to a Lab-Tek

chamber and observed by fluorescence microscopy. Phagocytosis index was determined for at least 3500 Ba/F3 cells in 10 fields, and is plotted. (D) No effect of integrin or MFG-E8 on the binding of apoptotic cells to Ba/F3 cells. BaF/GFP, BaF/GFP/Tim4, or BaF/GFP/ $\alpha\beta$  cells were incubated at 37°C with CellTracker Orange-labeled apoptotic thymocytes in the absence or presence of 0.1  $\mu\text{g/ml}$  mouse MFG-E8 for the indicated periods of time. The percentage of GFP-positive Ba/F3 cells that bound apoptotic cells was determined by flow cytometry. The experiments were done in triplicate, and the average values are plotted with S.D. (bars). (E) Tim4-dependent tethering and uptake of apoptotic cells by mouse resident peritoneal macrophages. In left panel, peritoneal cells from the wild-type and Tim4<sup>-/-</sup> mice were incubated with pHrodo-labeled apoptotic thymocytes. The cells were stained with APC-conjugated anti-mouse Mac1, and subjected to flow cytometry for APC and pHrodo. The engulfment was determined as the percentage of pHrodo-positive cells in the Mac1-positive population. The experiment was carried out in triplicate, and the average values were plotted with S.D. (bars). In right panel, peritoneal cells from the wild-type and Tim4<sup>-/-</sup> mice were incubated at room temperature with CellTracker-labeled apoptotic thymocytes for the indicated periods of time. After staining with APC-labeled anti-Mac1, the percentage of Mac1-positive cells that bound apoptotic cells was determined by flow cytometry. The experiments were done in triplicate, and the average values are plotted with S.D. (bars). (F) Requirement of Ca<sup>2+</sup> for the uptake but not binding of apoptotic cells to peritoneal macrophages. In left panel, peritoneal cells from the wild-type mice were

incubated at 37° for 60 min with pHrodo-labeled apoptotic thymocytes in PBS containing 2% FCS in the absence or presence of 0.4 mM Ca. The cells were stained with APC-labeled anti-Mac, and analyzed by flow cytometry as described above. The experiments were done in triplicate, and the average values are plotted with S.D. (bars). In right panel, peritoneal cells from the wild-type mice were incubated at room temperature in PBS containing 2% FCS in the absence or presence of 0.4 mM Ca with CellTracker-labeled apoptotic thymocytes for indicated periods of time, and analyzed by flow cytometry as above. The experiments were done in triplicate, and the average values are plotted with S.D. (bars).

**FIG. 5.** Effect of Rac1 and Rab5 on the engulfment of apoptotic cells by Ba/F3 cells. BaF/GFP/Tim4/ $\alpha\beta$  was transformed with Rac1 (Tim4/ $\alpha\beta$ /Rac), or Rac1 and Rab5 (Tim4/ $\alpha\beta$ /Rac/Rab), and incubated at 37°C for 120 min with pHrodo-labeled apoptotic thymocytes in the absence (open bar) or presence (filled bar) of 0.1  $\mu$ g/ml mouse MFG-E8. Ba/F3 cells were sorted by FACS, transferred to LabTek chamber, and observed by fluorescence microscopy. At bottom, the phagocytosis index for more than 4500 Ba/F3 cells in 10-12 fields is plotted.

**FIG. 6.** The tether-and-uptake model for the engulfment of apoptotic cells. In the tethering step, Tim4 catches apoptotic cells by recognizing phosphatidylserine. A putative co-receptor that associates with Tim4 then activates the integrin  $\alpha_v\beta_3$  complex to initiate

uptake, in which MFG-E8 binds to phosphatidylserine on apoptotic cells and to the activated integrin  $\alpha_v\beta_3$  complex on the phagocytes. The signal from the integrin  $\alpha_v\beta_3$  complex then activates the macrophages' internalization of apoptotic cells into lysosomes. This final step is mediated by Rac1, Rab5, and other, as-yet unidentified, molecules.

Figure 1

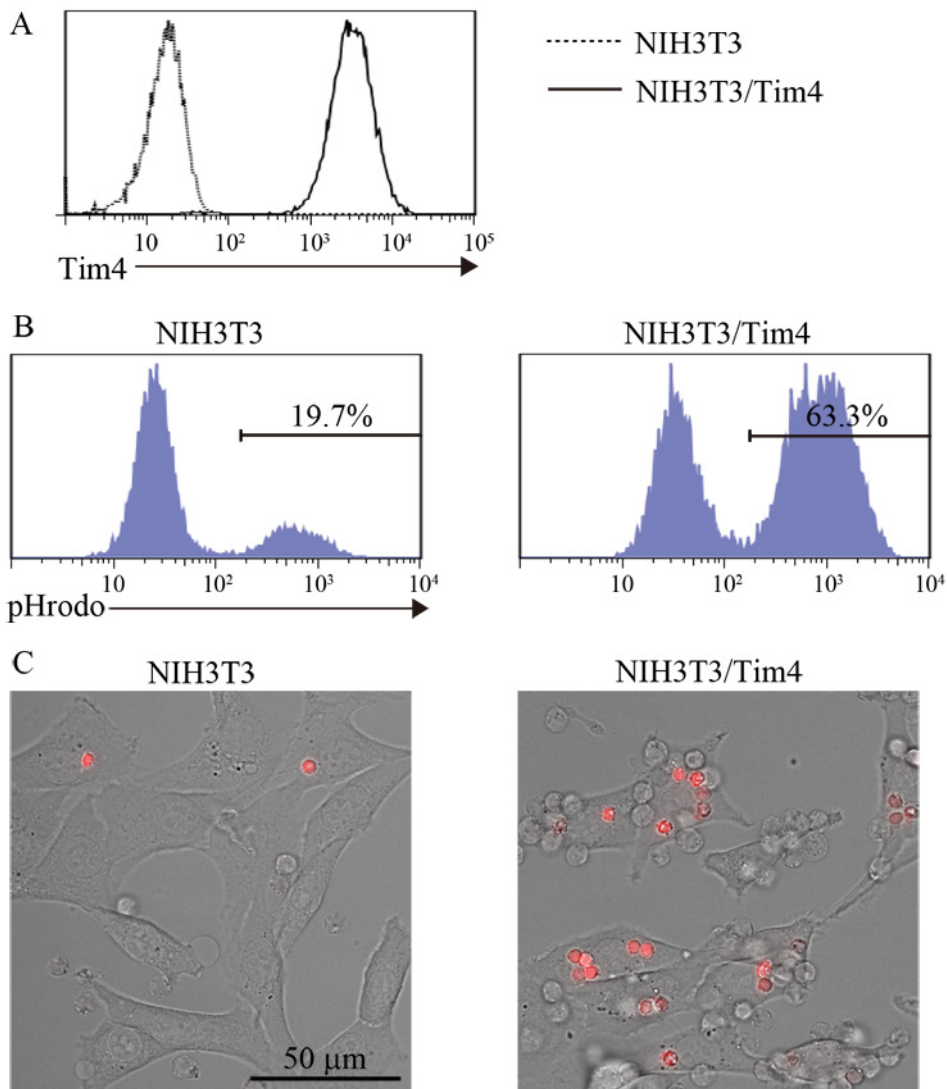


Figure 2

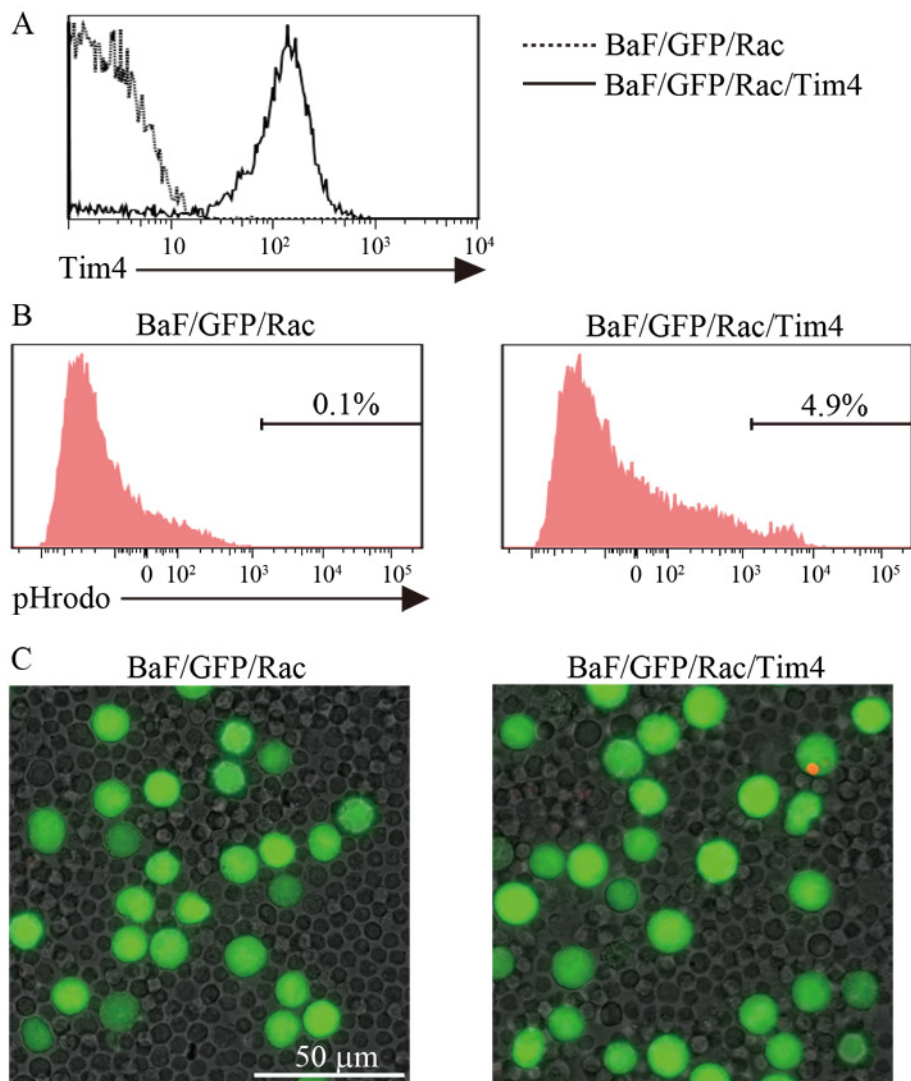


Figure 3

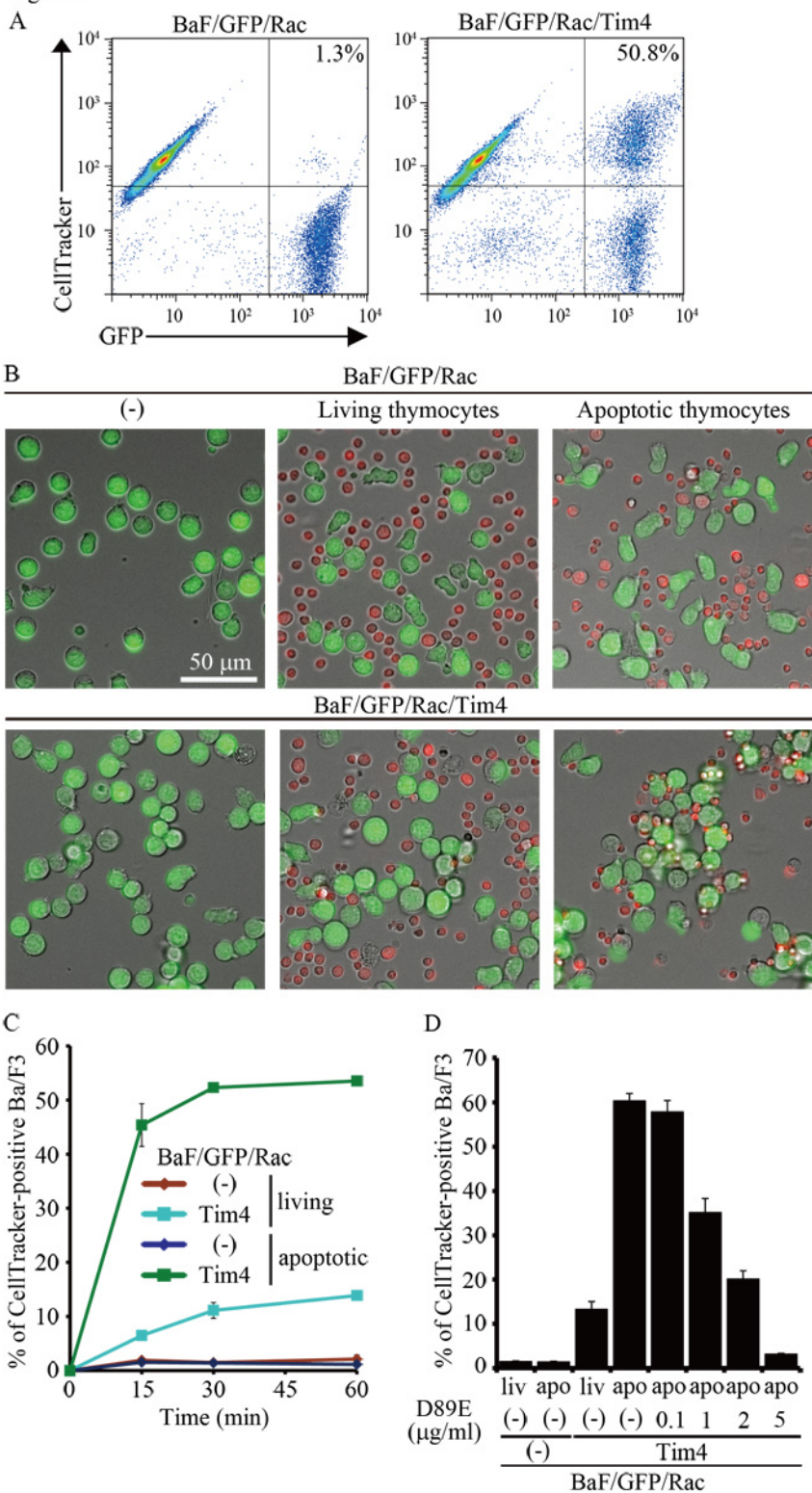




Figure 4

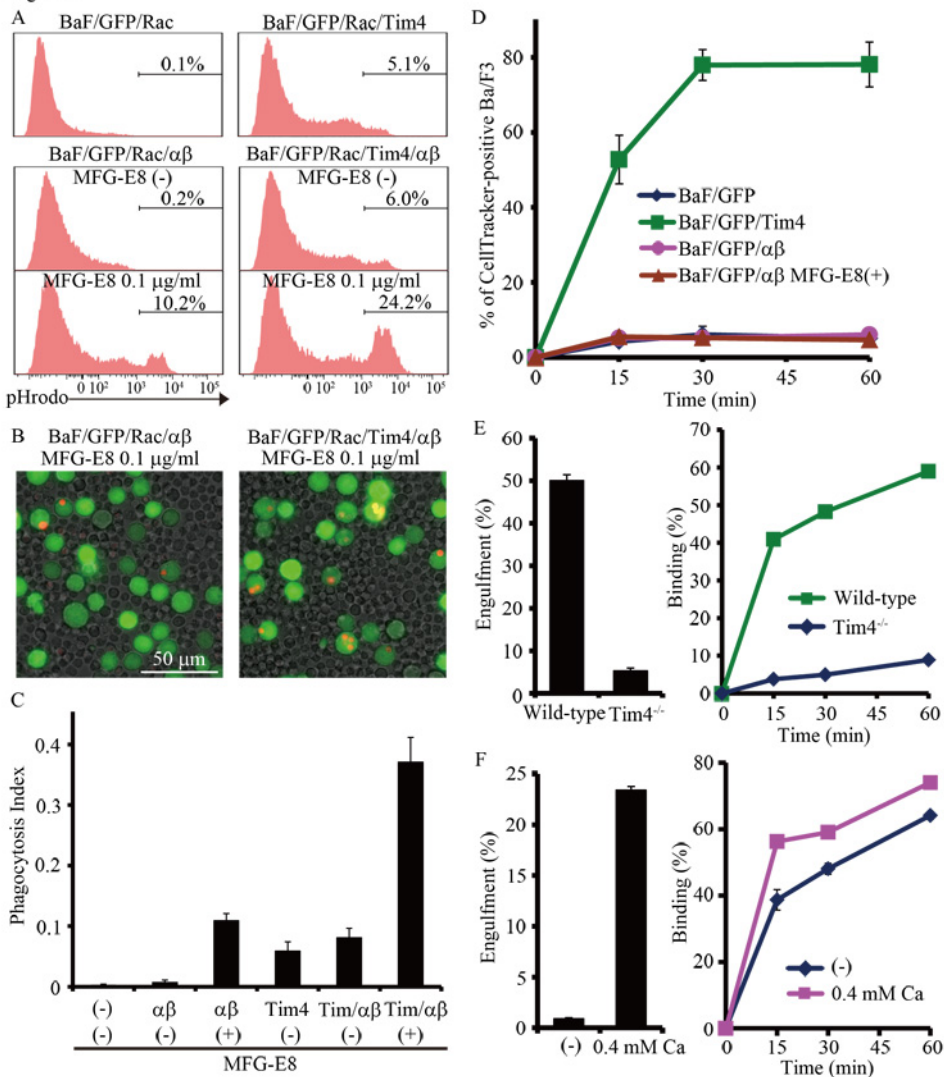


Figure 5

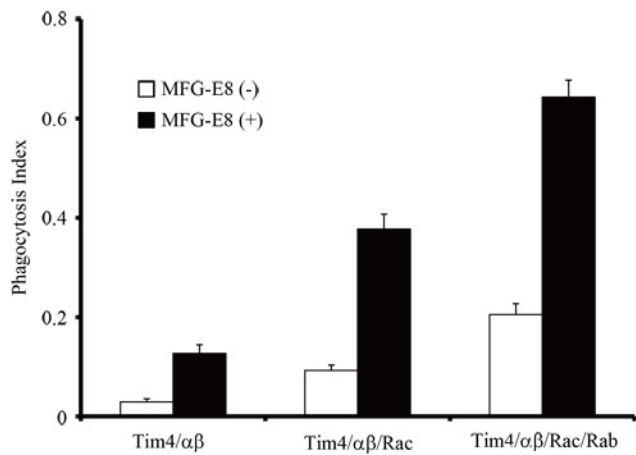
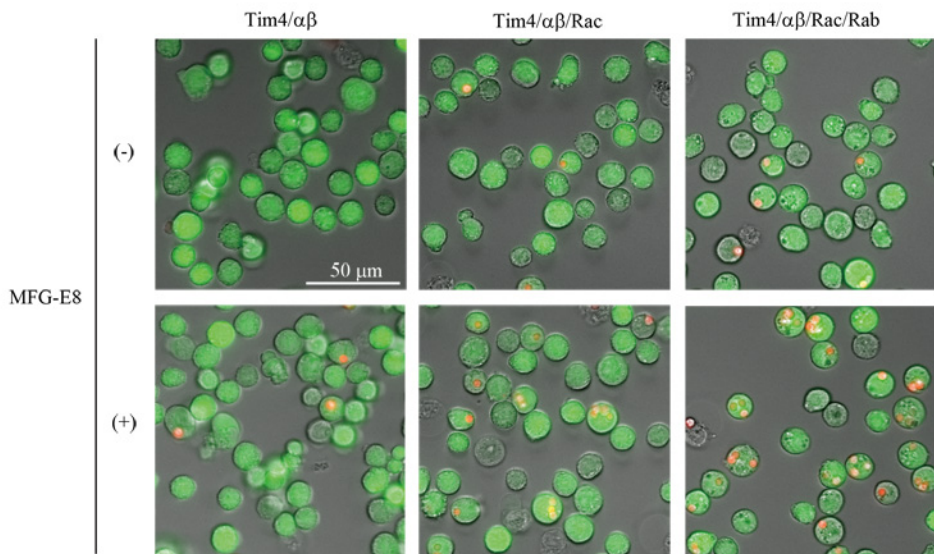
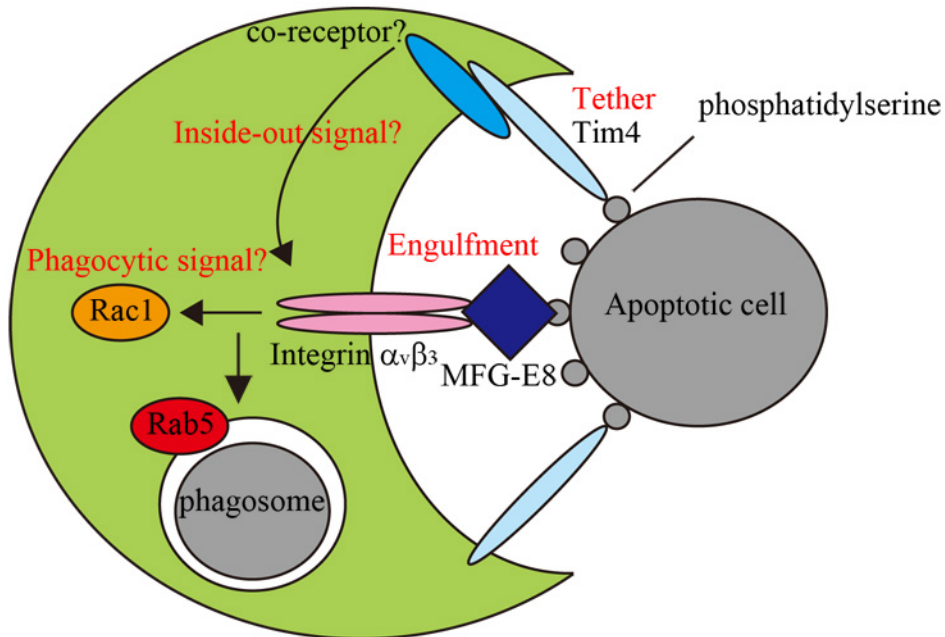
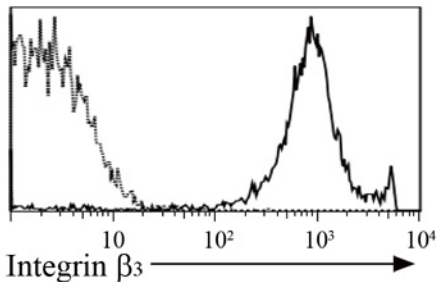
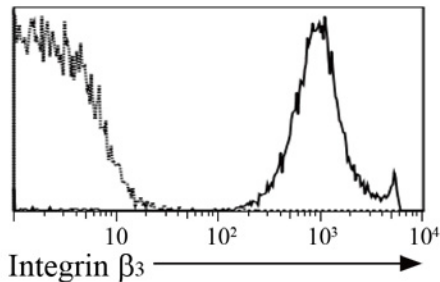
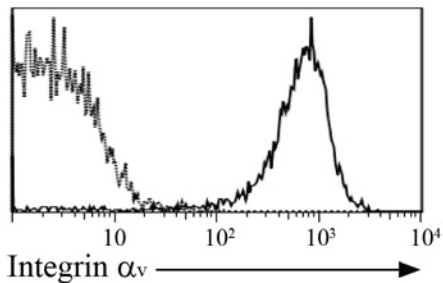
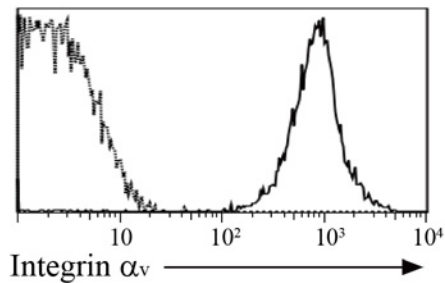


Figure 6



# Supplement Figure 1



----- BaF/GFP/Rac  
——— BaF/GFP/Rac/ $\alpha\beta$

----- BaF/GFP/Rac/Tim4  
——— BaF/GFP/Rac/Tim4/ $\alpha\beta$

Supplement Figure 2

