

Tim4- and MerTK- mediated engulfment of apoptotic cells by mouse resident peritoneal macrophages

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Summary

Apoptotic cells are swiftly engulfed by macrophages to prevent the release of noxious materials from dying cells. Apoptotic cells expose phosphatidylserine (PtdSer) on their surface, and macrophages engulf them by recognizing PtdSer using specific receptors and opsonins. Here, we found that mouse resident peritoneal macrophages expressing Tim4 and MerTK are highly efficient at engulfing apoptotic cells. Neutralizing antibodies against either Tim4 or MerTK inhibited the macrophage engulfment of apoptotic cells. *Tim4*-null macrophages exhibited reduced binding and engulfment of apoptotic cells, whereas *MerTK*-null macrophages retained the ability to bind apoptotic cells, but failed to engulf them. The incubation of wild-type peritoneal macrophages with apoptotic cells induced the rapid tyrosine phosphorylation of MerTK, which was not observed with *Tim4*-null macrophages. When mouse Ba/F3 cells were transformed with Tim4, apoptotic cells bound to the transformants, but were not engulfed. Transformation of Ba/F3 cells with MerTK had no effect on the binding or engulfment of apoptotic cells; however, Tim4/MerTK-transformants exhibited strong engulfment activity. Taken together, these results indicate that the engulfment of apoptotic cells by resident peritoneal macrophages proceeds in two steps: binding to Tim4, a PtdSer-receptor, followed by MerTK-mediated cell engulfment.

Introduction

Millions of harmful, useless, or senescent cells in our body undergo apoptosis every minute, and are swiftly engulfed by macrophages for clearance (1, 2). Engulfment is required to prevent apoptotic cells from undergoing secondary necrosis and releasing noxious materials. Inefficient engulfment of apoptotic cells can activate the immune system and contribute to the development of systemic lupus erythematosus (SLE)-type autoimmune diseases (3). The engulfment of apoptotic cells is also important for digesting dead cell components into their building units for recycling and energy (4).

For apoptotic cells to be recognized by phagocytes, they expose phosphatidylserine (PtdSer) on their surface (5, 6). We recently showed that a membrane protein (Xkr8) containing six transmembrane domains is activated by caspase, and plays an indispensable role in apoptotic PtdSer- exposure (7). Several molecules are reported to recognize PtdSer on apoptotic cells (1, 2). Milk-Fat Globule Epidermal Growth factor 8 (MFG-E8) is a soluble protein secreted from a subset of macrophages. It contains an EGF domain that binds to the integrin- $\alpha_v\beta_3$ complex on macrophages, and a discoidin domain that binds to PtdSer on apoptotic cells, thus serving as a bridging molecule between apoptotic cells and macrophages. Protein S and Gas6 (Growth Arrest-Specific 6) also function as bridges by binding to receptors on macrophages and PtdSer on apoptotic cells (8). The receptors for Protein S and Gas6 are type I membrane proteins of the TAM family (Tyro 3, Axl, and MerTK), which have intracellular tyrosine kinase domains (9). Type I membrane proteins such as Tim (T-cell immunoglobulin and mucin domain containing) family proteins, stabilins, and BAI1 also directly bind PtdSer and enhance the engulfment of apoptotic cells

by phagocytes (10-12). In addition, many other molecules such as RAGE (receptor for advanced glycation end products) (13), Complement C1q (14), and Calreticulin (15) have been shown to be involved in the engulfment of apoptotic cells. Whether these molecules are redundant or have specific roles in the process remains to be determined.

Here we report that mouse resident peritoneal macrophages (rpMacs) that express both Tim4 and MerTK are highly capable of engulfing apoptotic cells. *Tim4* or *MerTK*-null mutations prevented rpMacs-mediated apoptotic cell engulfment. *Tim4*- but not *MerTK*-null macrophages lost their ability to tether apoptotic cells. The apoptotic cell-induced tyrosine-phosphorylation of MerTK, observed in wild-type macrophages, was abolished in *Tim4*-null macrophages. Tim4-transformed Ba/F3 cells were found to bind apoptotic cells, but their apoptotic cell engulfment activity was weak. In contrast, transformants expressing both Tim4 and MerTK efficiently engulfed apoptotic cells. These results indicate that mouse rpMacs require two PtdSer-dependent systems for efficient engulfment of apoptotic cells: the Tim4-mediated tethering of apoptotic cells, and Protein S/MerTK-mediated cell engulfment, and strongly confirm the “tether/tickle” hypothesis proposed by Henson’s group (16, 17).

MATERIALS AND METHODS

Mice, cell lines, recombinant proteins, antibodies, and reagents. C57BL/6J mice and *MerTK*^{-/-} mice (18) were purchased from Japan SLC and the Jackson Laboratory, respectively. *Tim4*^{-/-} mice were described previously (19). All mouse studies were approved by the ethics review committee for animal experimentation of the Graduate School of Medicine, Kyoto University.

Mouse IL (interleukin)-3 dependent Ba/F3 cells were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) and 45 units/ml mouse IL-3 as described (20). Human leucine-zipper-tagged Fas ligand (FasL) was produced by COS7 cells as described (21), and concentrated by precipitation in 60%-saturated (NH₄)₂SO₄, followed by dialysis against PBS. Hamster anti-mouse Tim4 monoclonal antibodies (mAbs) (clone Kat5-18) were described previously (10). Biotin-conjugated goat anti-mouse MerTK was from R & D Systems. Allophycocyanin (APC)- or PerCP-CyTM 5.5-labeled rat anti-mouse Mac1 mAb (CD11b, clone M1/70) was purchased from BioLegend and BD PharMingen, respectively. Mouse anti-phosphotyrosine mAb (clone 4G10) and mouse anti-Flag mAb (clone M2) were from Merck Millipore and Sigma-Aldrich, respectively. APC-conjugated goat anti-hamster IgG was purchased from Jackson Laboratory.

CellTrackerTM Orange {CMRA; 9'-(4[and5]-chloromethyl-2-carboxyphenyl)-7'-chloro-6'-oxo-1,2,2,4-tetramethyl-1,2-dihydropyrido[2',3'-6]xanthene} and pHrodo Red succinimidyl ester (pHrodo) were purchased from Life Technology. Alexa488-conjugated streptavidin was from Molecular Probes. Human Protein S was purchased from Enzyme Research Laboratories.

Transformation of Ba/F3 cells. Mouse retroviral vectors, pMXpuro (22) and pENV-IRES-puro (23), were provided by Dr. T. Kitamura (Institute of Medical Science, University of Tokyo). Lentiviral expression vectors (CSII-EF, pCAG-HIVgp, pENV-IRES-puro, and pRSV-Rev) were from Dr. H. Miyoshi, Riken Resource Center. pMX-Tim4 was previously described (10). Mouse MerTK cDNA (GenBank: NM_008587.1) was isolated by RT-PCR from kidney, flag-tagged at the C-terminus, and inserted into the CSII-EF vector.

Mouse MerTK was expressed in Ba/F3 cells by using the lentiviral vector system. In brief, human HEK293T cells were co-transfected with the CSII-EF vector expressing a Flag-tagged MerTK cDNA, pCAG-HIVgp, pRSV-Rev, and pENV-IRES-puro. After culturing for 48 h, viruses in the cell supernatant were concentrated by centrifugation at 6,000 x g for 16 h at 4°C, and used to spin-infect Ba/F3 cells as described (7). Transformants expressing MerTK were sorted with a FACSAria™ II. To establish Ba/F3 transformants expressing Tim4, the Tim4 cDNA was placed downstream of the human EF-1 α promoter of pNEF-BOS-EX, which carries an SV40 promoter-driven neomycin-resistance gene in pEF-BOS-EX (24). The construct was then introduced into Ba/F3 cells by electroporation using a Super Electroporator NEPA21 type II (Nepa Gene Co.), and the cells were cultured for 3 days. The Tim4-expressing cells were sorted with FACSAria™ II, and cultured in the presence of 800 μ g/ml geneticin (Gibco) at 0.3 cells/well in 96-well microtiter plates. Clones expressing high levels of Tim4 were expanded for further analysis.

Flow cytometry. Cells were incubated on ice for 30 min with 1 $\mu\text{g/ml}$ hamster anti-mouse Tim4 (clone Kat5-18) (10) and 1 $\mu\text{g/ml}$ biotinylated anti-MerTK Ab in 50 μl of PBS containing 2% FCS, followed by incubation with 1000-times diluted Alexa488- conjugated streptavidin, 1 $\mu\text{g/ml}$ PerCP-CyTM 5.5-labeled rat anti-mouse Mac1, and 1000 times diluted APC-labeled anti-hamster IgG. The cells were then stained with 0.5 μM SYTOX Blue (Life Technologies) to exclude dead cells, and analyzed by flow cytometry with a FACSCantoTM II (BD Biosciences).

Engulfment of apoptotic cells. Engulfment of apoptotic cells was assayed with pHrodo-labeled prey (20, 25). In brief, thymocytes from 4- to 8-week-old C57BL/6J mice were treated with 100 units/ml FasL in DMEM containing 10% FCS for 1.5-2 h at 37°C to induce apoptosis, washed with PBS, and incubated with 0.1 $\mu\text{g/ml}$ pHrodo for 30 min at room temperature. After stopping the reaction with 1 ml FCS, the cells were washed with PBS containing 10% FCS, and used as prey. At this stage, Annexin V⁺PI⁺ cell population was usually less than 30%. In some cases, thymocytes were incubated with FasL in serum-free DMEM, labeled with pHrodo as above, and washed with PBS containing 0.5% BSA and 0.25% globulin.

To prepare peritoneal macrophages, peritoneal cells (5×10^5) from wild-type and mutant C57BL/6 mice at 8-14 weeks of age were incubated in 12-well plates at 37°C for 2 h in DMEM containing 10% FCS, and washed with PBS to remove non-adherent cells. The adherent cells were incubated at 37°C with 2×10^6 pHrodo-labeled apoptotic thymocytes in

1 ml of DMEM containing 10% FCS, washed with PBS, and then treated at 37°C with 0.25% trypsin in PBS containing 1 mM EDTA. Cells were collected by centrifugation at 500 × g for 5 min, suspended in 300-500 µl of CHES-FACS buffer [20 mM CHES buffer (pH 9.0) containing 150 mM NaCl and 2% FCS] supplemented with 0.2-0.4 µg/ml APC-labeled rat anti-mouse Mac1, and analyzed by flow cytometry with a FACSCanto™ II.

For the Ba/F3 engulfment of apoptotic cells (20), 1×10^5 Ba/F3 cells and 1×10^6 pHrodo-labeled apoptotic thymocytes were co-incubated at 37°C in 0.6 ml of RPMI1640 containing 10% FCS and 45 units/ml mouse IL-3. The cells were collected by centrifugation at 500 × g for 5 min, suspended in 500 µl of CHES-FACS buffer, and analyzed by flow cytometry as described above. For microscopic observation, Ba/F3 cells co-incubated with pHrodo-labeled thymocytes were suspended in 500 µl of CHES-FACS buffer, transferred to Lab-Tek II chambered cover glasses (ThermoFisher Scientific), and examined by fluorescence microscopy (BioRevo BZ-9000, Keyence).

Binding of apoptotic cells to phagocytes. The binding of apoptotic cells to phagocytes was assayed by using the CellTracker Orange-labeled cells as described (20). In brief, approximately 1×10^6 thymocytes were labeled by incubation in 5 ml of serum-free DMEM containing 10 µM CellTracker Orange at 37°C for 30 min, and then incubated with FasL in DMEM containing 10% FCS at 37°C for 2 h to induce apoptosis. Peritoneal cells or Ba/F3 cells (1×10^5) were then co-incubated in suspension with the CellTracker Orange-labeled apoptotic cells in PBS supplemented with 10% FCS, and then stained with

500 nM SYTOX Blue, and analyzed by FACSCanto™ II. For peritoneal macrophages, the cells were stained with APC-conjugated anti-Mac-1.

Immunoprecipitation and western blotting. Resident peritoneal cells ($3-6 \times 10^6$ cells) on 3.5-cm plates were incubated at 37°C with $1.5-3 \times 10^7$ apoptotic thymocytes in 1 ml of DMEM containing 10% FCS, washed with cold PBS to remove apoptotic cells, and lysed by incubation at 4°C for 30 min in 1.5 ml of lysis buffer [25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton-X100, 5% glycerol, 2 mM Na_2VO_4 , and a cocktail of protease inhibitors (cOmplete, Mini, EDTA-free, Roche)]. The lysates were centrifuged at 15000 rpm for 10 min at 4°C, and the supernatants were used for immunoprecipitation.

Dynabeads® Protein G (10 μl) (Life Technologies) was conjugated with 2.5 μg of goat anti-mouse MerTK Ab according to the supplier's instruction. The macrophage cell lysates prepared from 6×10^6 cells were incubated overnight at 4°C with anti-MerTK-conjugated protein G-beads in 1.5 ml lysis buffer. The beads were collected, washed with lysis buffer, and suspended in 30 μl of SDS-sample buffer [63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.1% BPB, 2% β -mercaptoethanol]. Proteins were eluted from the beads by heating the samples at 95°C for 5 min, and 15 μl aliquots were separated by electrophoresis on 7.5% polyacrylamide gels. After transferring the proteins to PVDF membranes, the membranes were incubated at room temperature for 1 h in TBS-T [25 mM Tris-HCl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20] containing 5% BSA or skin milk to block non-specific binding site. The membranes were then incubated at 4°C overnight with 1000-fold diluted HRP-conjugated anti-phosphotyrosine mAb (4G10) or 0.5 $\mu\text{g}/\text{ml}$

biotinylated anti-MerTK Ab in TBS-T containing 5% BSA or skin milk, followed by incubation with 1000-fold diluted HRP-conjugated streptavidin. Proteins recognized by the antibody were visualized by a chemiluminescence reaction (Renaissance; NEN life Science Products).

RESULTS

Tim4 and MerTK expression in resident peritoneal macrophages. We previously reported that mouse rpMacs express Tim4, and that Tim4 is required for these macrophages to engulf apoptotic cells (19). In addition, Seitz et al. (26) showed that peritoneal macrophages require MerTK to engulf apoptotic cells. To examine whether the same rpMacs population expresses Tim4 and MerTK, peritoneal cells were stained for Mac1, Tim4 and MerTK. Approximately, 40-48% of the cells in the peritoneal cavity strongly expressed Mac1 and F4/80, among which 36% were Tim4⁻MerTK⁺ and 61% were Tim4⁺MerTK⁺, indicating that rpMacs consist of two populations, Tim4⁻ and MerTK-double positive cells, and MerTK-single positive cells (Fig. 1A, and Fig. S1). The ability of each macrophage population to engulf apoptotic cells was then examined. Mouse thymocytes were treated with FasL to induce approximately 70% of the cells to undergo apoptosis (as determined by Annexin V staining), and then labeled with pHrodo and used as prey for rpMacs. The majority of the pHrodo-positive macrophages, or the macrophages engulfing apoptotic cells, were Tim4⁺MerTK⁺ cells, and very few Tim4⁻MerTK⁺ macrophages (Fig. 1B). Neutralizing monoclonal antibodies against either Tim4 or MerTK strongly inhibited the uptake of apoptotic cells by the rpMacs (Fig. 1C). Taken together, these results suggest that of the two distinct rpMac populations, Tim4⁺MerTK⁺ macrophages are the population that engulfs apoptotic cells. These findings further implicate the involvement of both Tim4 and MerTK in the process.

Requirement of Tim4 and MerTK for the rpMac engulfment of apoptotic thymocytes.

To confirm the requirement of Tim4 and MerTK for the rpMac engulfment of apoptotic

cells, macrophages were prepared from *Tim4*^{-/-} or *MerTK*^{-/-} mice. In the *Tim4*^{-/-} mice, the *Neo* gene replaces part of exons 1 and 2, encoding Tim4's extracellular region (19). In these mice, all the Mac1⁺ rpMacs were found to express MerTK, but not Tim4 (Fig. 2A). *MerTK*^{-/-} mice were previously established by deleting part of the MerTK cytoplasmic region (18), yet its extracellular region was not detected in *MerTK*^{-/-} macrophages as reported earlier (27), most likely due to the nonsense-mediated mRNA decay. As expected, the peritoneal cells of *MerTK*^{-/-} mice were divided into two populations, Tim4⁺Mac1⁺ cells and Tim4⁻Mac1⁺ cells. When rpMacs from either *Tim4*^{-/-} or *MerTK*^{-/-} mice were tested for their ability to engulf apoptotic cells, their engulfment activities were found to be severely reduced (Fig. 2B and Fig. S4), confirming that neither Tim4 nor MerTK alone is sufficient for apoptotic cell engulfment by these macrophages.

Tim4 is a PtdSer receptor that tethers apoptotic cells (10), while MerTK is a receptor for the bridging proteins, Gas6 or Protein S, that binds PtdSer (9, 28). Since Protein S is abundant (about 300 nM or 25 µg/ml) in the serum (29) used for the engulfment assay, the macrophages expressing MerTK can bind apoptotic cells *via* Protein S. To examine whether the *MerTK*^{-/-} and *Tim4*^{-/-} macrophages bind apoptotic cells, the macrophages were incubated with FasL-treated mouse thymocytes that were labeled with CellTracker™ Orange. As shown in Fig. 2C, approximately 50-60% of the Mac1⁺ rpMacs from wild-type and *MerTK*^{-/-} mice were associated with CellTracker-labeled thymocytes, while there was minimal association of *Tim4*^{-/-} macrophages with labeled apoptotic cells. Similar results were obtained in a serum-free condition (Fig. S3), suggesting that Tim4 is required to tether apoptotic cells to rpMacs, and that the MerTK-Protein S interaction is not sufficient for

recruiting apoptotic cells. In fact, of the two populations of wild-type rpMacs ($\text{Tim4}^+\text{MerTK}^+$ and $\text{Tim4}^-\text{MerTK}^+$), the Tim4-positive population was found to strongly associate with the CellTracker-labeled cells (Fig. 2D).

Tim4-dependent tyrosine-phosphorylation of MerTK. MerTK is a type I membrane protein with a cytoplasmic tyrosine kinase domain (9). Todt *et al.* (30) previously reported that when mouse rpMacs are exposed to apoptotic cells, MerTK is phosphorylated at tyrosine residues. We ascertained that if Tim4 was involved in the binding of apoptotic cells, then Tim4 expression might affect the tyrosine phosphorylation of MerTK. To examine this possibility, rpMacs were incubated with apoptotic thymocytes in the presence or absence of an anti-Tim4 neutralizing antibody. MerTK was immunoprecipitated with anti-MerTK antibodies, and western blotted with an anti-phosphotyrosine mAb. As shown in Fig. 3A, rpMac incubation with apoptotic cells resulted in strong phosphorylation of MerTK, which was significantly inhibited by the presence of the anti-Tim4 Ab.

To further confirm the role of Tim4 in activating MerTK, the wild-type and *Tim4*^{-/-} rpMacs were incubated with apoptotic cells. MerTK in the wild-type macrophages was strongly tyrosine-phosphorylated after 10 min of incubation with apoptotic cells (Fig. 3B). This phosphorylation was transient and had decreased after 30 min of incubation. The *Tim4*^{-/-} rpMacs did not exhibit clear tyrosine phosphorylation of MerTK after incubation with apoptotic cells for 10 min, and only weak phosphorylation was detected at 30 min. The tyrosine kinase activity of MerTK can also be activated by cross-linking MerTK with anti-MerTK Ab (30). Accordingly, when peritoneal macrophages were treated with goat

anti-mouse MerTK, followed by rabbit anti-goat antibodies, MerTK was tyrosine-phosphorylated. However, cross-linking-induced tyrosine-phosphorylation of MerTK was not affected by the *Tim4*-null mutation (Fig. 3C), suggesting that MerTK is functional without Tim4, and Tim4 indirectly affects the activation of MerTK during engulfment of apoptotic cells. Gas6 and/or Protein S are known to bridge apoptotic cells to receptors on macrophages (28, 31). If Tim4 was sufficient for directly tethering apoptotic cells to rpMacs, bridging molecules might not be necessary for the MerTK-mediated engulfment of apoptotic cells. However, the engulfment of apoptotic cells by mouse rpMacs required FCS (Fig. 4). Furthermore, Protein S promoted the engulfment of apoptotic cells in serum-free medium resulting in a bell-shaped response with the maximum response at 1.0 $\mu\text{g/ml}$. Since FCS contains approximately 25 $\mu\text{g/ml}$ Protein S (29), the serum requirement for the engulfment of apoptotic cells may be at least partially due to Protein S present in the serum.

Reconstitution of apoptotic cell engulfment with the mouse Ba/F3 pro-B cell line. The above results indicated that both Tim4 and MerTK are indispensable for the engulfment of apoptotic cells by mouse rpMacs. We recently developed an engulfment system using the mouse B cell line, Ba/F3, that grew in suspension, and showed that engulfment proceeds *via* two distinct steps, tethering and engulfment (20). To study the roles of Tim4 and MerTK, Ba/F3 cells, which expressed neither Tim4 nor MerTK endogenously, were transformed with mouse Tim4, mouse MerTK, or both (Fig. 5A). As shown in Fig. 5B, the CellTracker-labeled apoptotic cells bound strongly to Tim4-expressing Ba/F3 (BaF3-Tim4

and BaF3-Tim4/MerTK), but not to the parental Ba/F3 or the MerTK-transformants, in agreement with the results obtained with rpMacs. When the engulfment of apoptotic cells was assayed with pHrodo-labeled thymocytes, both the MerTK- and Tim4-transformants exhibited limited engulfment activity. In contrast, Ba/F3 transformants that co-expressed Tim4 and MerTK exhibited strongly enhanced engulfment activity (Fig. 5C). Microscopic observation of the Ba/F3 cells incubated with apoptotic cells indicated that Tim4-expressing cells rarely contained a pHrodo-positive cell after a 90 min-incubation with apoptotic cells, while approximately half of the Ba/F3 transformants expressing both Tim4 and MerTK contained one or more pHrodo-positive cells, confirming that Tim4 and MerTK cooperate to engulf apoptotic cells.

DISCUSSION

Macrophages and immature dendritic cells that efficiently engulf apoptotic cells by recognizing PtdSer exposed on the apoptotic cells are highly heterogeneous (33, 34). Many soluble and transmembrane proteins have been shown to mediate the PtdSer-dependent engulfment of apoptotic cells (35); however, the mechanism(s) by which these molecules function in different macrophage populations has not been thoroughly investigated. Here we found that the efficient engulfment of apoptotic cells by mouse rpMacs requires the expression of Tim4 and MerTK. Tim4 is a type I membrane protein that functions as a receptor for apoptotic cells by directly binding to the PtdSer on apoptotic cells (10). Accordingly, apoptotic cells strongly bound to Tim4-expressing Ba/F3 cells and wild-type rpMacs. Tim4 deficiency completely blocked the binding of apoptotic cells to the rpMacs, but the null mutation of MerTK had no effect on this process. These results agree with previous observations that peritoneal macrophages from *MerTK*^{-/-} mice still bind apoptotic cells (36), and suggest that Tim4 functions to tether apoptotic cells to the phagocytic rpMac.

MerTK does not directly recognize apoptotic cells, and Protein S or Gas6, ligands for MerTK that bind PtdSer, are proposed to serve as bridges between apoptotic cells and macrophages expressing MerTK (9). Yet, MerTK-expressing Ba/F3 cells or *Tim4*^{-/-} peritoneal macrophages that express MerTK did not bind apoptotic cells in the presence of FCS, which contains Protein S. The affinity of Protein S for PtdSer (K_d of approximately 28 nM) (37) is one tenth that of Tim4 for PtdSer (K_d of 2 nM), and its affinity for MerTK is also quite low (38), which may explain the limited binding of apoptotic cells to

MerTK-expressing cells in the absence of Tim4. An example of a well-characterized two-receptor system is the TNF receptor system, in which the signal-transducing TNF-R1 exhibits a low affinity for TNF α , while the non-signal-transducing TNF-R2 exhibits a high affinity for TNF α . A ligand-passing model was proposed (39), in which TNF α first binds to TNF-R2, and is then delivered to TNF-R1 to initiate signal transduction. Similar to the TNF receptor system, we propose that in rpMacs, Tim4 recruits apoptotic cells to increase the local concentration of apoptotic cells at the cell surface. The recruited apoptotic cells are then passed to MerTK for engulfment, *via* Protein S. In migration of T cells in the endothelium system, T cells first bind to endothelial cells at their focal region where the high affinity LFA-1 is localized. This causes clustering of intermediate affinity LFA-1 at leading edge, allowing this region to bind to endothelium cells for cell's rolling (40). Similarly, binding of apoptotic cells to macrophages via Tim4 may cause clustering PtdSer, which allows the dead cells to bind to MerTK *via* Protein S.

The TAM family kinases have been shown to physically interact with other receptors; Axl interacts with IFN-RI (Interferon receptor, type I) in dendritic cells to regulate the IFN response (41), and integrin- α_5 interacts with MerTK to promote the engulfment of apoptotic cells in HEK293T cells (32). On the other hand, the immunoprecipitation of MerTK, followed by western blotting with an anti-Tim4 mAb showed no clear evidence of an association between MerTK and Tim4 (C.N., K.S, and S.N., unpublished observation). However, the co-incubation of peritoneal macrophages with apoptotic cells consistently reduced the MerTK protein level in a Tim4-dependent manner, indicating that apoptotic cells are internalized together with Tim4 and MerTK (Fig. 3). Additional studies will be

required to understand the molecular mechanism underlying Tim4's enhancement of the MerTK-mediated cell engulfment.

Thioglycollate-elicited peritoneal macrophages (thio-pMacs) also express MerTK, and require MerTK for the efficient engulfment of apoptotic cells (26, 42)(Fig. S2).

Interestingly, thio-pMacs do not express Tim4, or any other Tim family members (Tim-1 and Tim-3) that bind PtdSer (10). In contrast to *MerTK*^{-/-} rpMacs, *MerTK*^{-/-} thio-pMacs did not bind apoptotic cells (data not shown), suggesting that these macrophages may not require a MerTK-independent tethering step to engulf apoptotic cells. Thio-pMacs express MFG-E8 (43, 44), and integrin- $\alpha_v\beta_3$ or integrin- $\alpha_v\beta_5$, both of which bind MFG-E8 and synergize with MerTK to enhance apoptotic cell engulfment (32). On the other hand, MFG-E8 did not enhance the apoptotic cell uptake by rpMacs, and the expression of integrin- $\alpha_v\beta_3$ in the Tim4- and MerTK-expressing Ba/F3 cells had no effect on their ability to engulf apoptotic cells (data not shown). These results suggest that the Tim4-MerTK and integrin-MerTK systems are independent and function differently in different cell types. It is possible that the synergistic effects of integrin- $\alpha_v\beta_3$ (or integrin- $\alpha_v\beta_5$) and MerTK may mitigate the requirement of a separate tethering step in thio-pMacs.

Tim4 and MerTK are co-expressed not only in rpMacs, but also in splenic tingible-body macrophages, and thymic macrophages. Requirement of MerTK for the engulfment of apoptotic cells by tingible-body macrophages and thymic macrophages has been reported (36, 45). Whether Tim4 or other Tim family members are required for the engulfment of apoptotic cells in these and other macrophage populations remains to be determined. In this regards, the engulfment system using Ba/F3 cells may well be suitable to examine the

contribution of the proposed molecules in engulfment of apoptotic cells. Finally, *MerTK*^{-/-} mice develop an SLE-type autoimmune disease (46), and it was recently shown that the *Tim4* and *MFG-E8*-null mutations synergistically affect the development of autoimmunity in mice (19). It will be interesting to determine whether the *Tim4*-null mutation enhances the autoimmunity that develops in *MerTK*^{-/-} mice.

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FIGURE LEGENDS

Figure 1. Tim4 and MerTK in mouse resident peritoneal macrophages. (A) Expression of Tim4 and MerTK in mouse resident peritoneal macrophages (rpMacs). Peritoneal cells from C57BL/6J mice were stained with PerCP-Cy5.5-conjugated anti-mouse Mac1 mAb, biotinylated goat anti-mouse MerTK Ab, and hamster anti-Tim4 mAb, followed by staining with Alexa488-conjugated streptavidin and APC-conjugated anti-hamster IgG. The samples were then analyzed by flow cytometry. The staining profiles for Mac1, and for Tim4 and MerTK in Mac1-positive cells are shown. The Mac1⁺ cells enclosed in left panel are analyzed in right. Numbers indicate the percentage of Mac1⁺ cells in left panel, and that of Tim4⁻MerTK⁺ and Tim4⁺MerTK⁺ cells in right panel. (B) Engulfment of apoptotic cells by rpMacs. Resident peritoneal macrophages (5×10^5) were incubated with 2×10^6 pHrodo-labeled apoptotic thymocytes at 37°C for 60 min, stained with PerCP-Cy5.5-conjugated anti-mouse Mac1 mAb, and hamster anti-Tim4 mAb, followed by staining with APC-conjugated anti-hamster IgG, and analyzed by flow cytometry. Representative FACS profiles for the pHrodo-positive cells in Tim4⁺MerTK⁺ and Tim4⁻MerTK⁺ populations in the Mac1⁺ cells are shown. The number indicates the percentage (phagocytosis) of the pHrodo-positive cells in each population. The experiments were performed in triplicate, and the average values with S.D. (bars) are plotted. (C) Effect of Tim4 and MerTK neutralizing antibodies on rpMac engulfment of apoptotic cells. Resident peritoneal cells (5×10^5) were incubated with pHrodo-labeled apoptotic thymocytes (2×10^6) in the presence or absence of 10 µg/ml goat normal IgG, hamster anti-mouse Tim4 mAb, or goat anti-mouse MerTK Ab at 37°C for the indicated time. The

percentage of pHrodo-positive macrophages was determined by flow cytometry, and plotted.

Figure 2. Requirement of MerTK and Tim4 for apoptotic cell engulfment by rpMacs. (A) Expression of Tim4 and MerTK in wild-type and knockout rpMacs. Cells in the peritoneal cavity of wild-type, *Tim4*^{-/-}, or *MerTK*^{-/-} mice were incubated with PerCP-Cy5.5-conjugated anti-mouse Mac1 mAb, biotinylated goat anti-MerTK Ab, and hamster anti-Tim4 mAb, followed by staining with Alexa488-conjugated streptavidin and APC-conjugated anti-hamster IgG. The stained cells were then analyzed by flow cytometry. The expression profiles of MerTK and Tim4 in the Mac-1-positive population are shown. The experiments were carried out independently with 6 mice, and average percentage for MerTK⁻Tim4⁻, MerTK⁺Tim4⁻, MerTK⁻Tim4⁺, and MerTK⁺Tim4⁺ cell population are indicated with S.D. (B) Engulfment of apoptotic cells by rpMacs. Cells in the peritoneal cavity of wild-type, *Tim4*^{-/-}, or *MerTK*^{-/-} mice were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 30 or 60 min, and analyzed by flow cytometry. The percentage of pHrodo-positive cells in the Mac1-positive population was determined by flow cytometry. The experiments were performed three times, and the average values are plotted with the S.D. (bars). Representative FACS profiles of pHrodo-positive cells in Mac1⁺ cells, obtained by incubation for 60 min, are shown in right. (C) Binding of apoptotic thymocytes to rpMacs. CellTracker-labeled apoptotic thymocytes were incubated with peritoneal cells from wild-type, *Tim4*^{-/-}, or *MerTK*^{-/-} mice at 37°C for 30 min and analyzed by flow cytometry. The number indicates the percentage of CellTracker-positive cells. The experiments were

done in triplicate, and the average values with the S.D. (bars) are plotted. At right, a representative CellTracker-staining profile of the Mac1-positive cells is shown. (D) Binding of apoptotic cells by Tim4⁺MerTK⁺ rpMacs. Resident peritoneal cells (1×10^5) were incubated at 37°C for 30 min with CellTracker-labeled apoptotic thymocytes (1×10^6), stained with PerCP-Cy5.5-conjugated anti-mouse Mac1 mAb, and hamster anti-Tim4 mAb, followed by staining with APC-conjugated anti-hamster IgG, and analyzed by flow cytometry. The number indicates the percentage of the CellTracker-positive cells in each population. The experiments were performed in triplicate, and the average values with S.D. (bars) are plotted. Representative FACS profiles for CellTracker-positive cells for each population are shown at right.

Figure 3. Involvement of Tim4 in apoptotic cell-induced tyrosine phosphorylation of MerTK. (A) Effect of the anti-Tim4 neutralizing antibody on the apoptotic cell-induced phosphorylation of MerTK. Resident peritoneal cells (6×10^6) were incubated for 30 min with 3×10^7 apoptotic thymocytes in the absence or presence of 10 µg/ml hamster control IgG, or an anti-Tim4 mAb. The rpMac cell lysates were subjected to immunoprecipitation with an anti-MerTK Ab, separated by SDS-PAGE, and analyzed by western blotting using an anti-phosphotyrosine mAb (4G10) (upper panel) or anti-MerTK Ab (lower panel). (B) Requirement of Tim4 expression for the apoptosis cell-induced phosphorylation of MerTK. Resident peritoneal cells (6×10^6) from wild-type or *Tim4*^{-/-} mice were incubated with 3×10^7 apoptotic thymocytes at 37°C for 10 or 30 min. The cell lysates were immunoprecipitated with an anti-MerTK Ab and analyzed by western blotting with an

anti-phosphotyrosine mAb (upper panel) or anti-Mer Ab (lower panel). (C) Cross-linking induced MerTK phosphorylation. Resident peritoneal cells (3×10^6) from wild-type or *Tim4*^{-/-} mice were incubated with 30 $\mu\text{g/ml}$ goat anti-Mer Ab or goat control IgG at 37°C for 30 min, washed with PBS, and incubated with 30 $\mu\text{g/ml}$ anti-goat IgG at 37°C for 10 min. The cell lysates were immunoprecipitated with anti-Mer Ab and analyzed by western blotting with anti-phosphotyrosine mAb (upper panel) or anti-Mer Ab (lower panel).

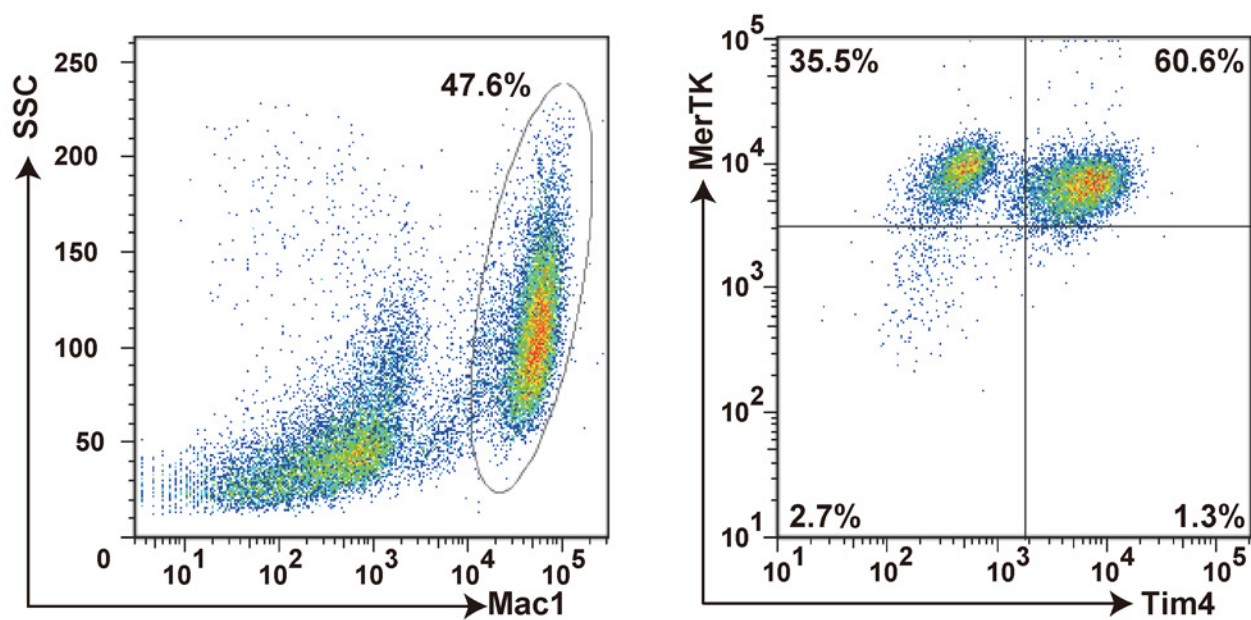
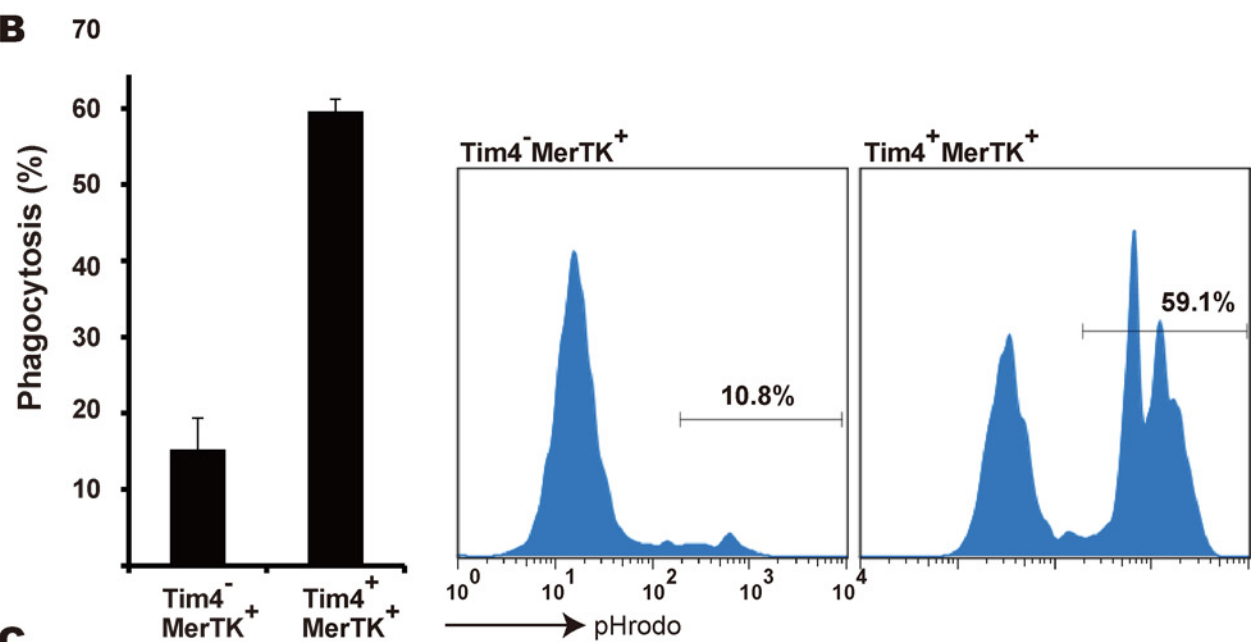
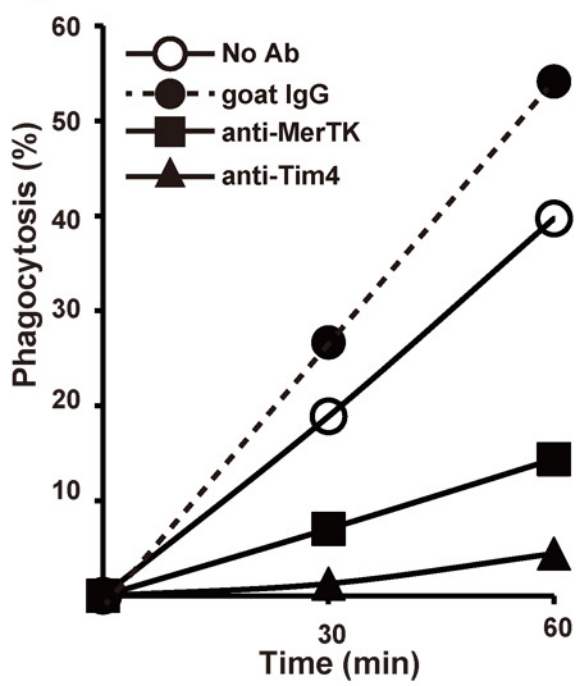
Figure 4. Requirement of Protein S for the engulfment of apoptotic cells by rpMacs.

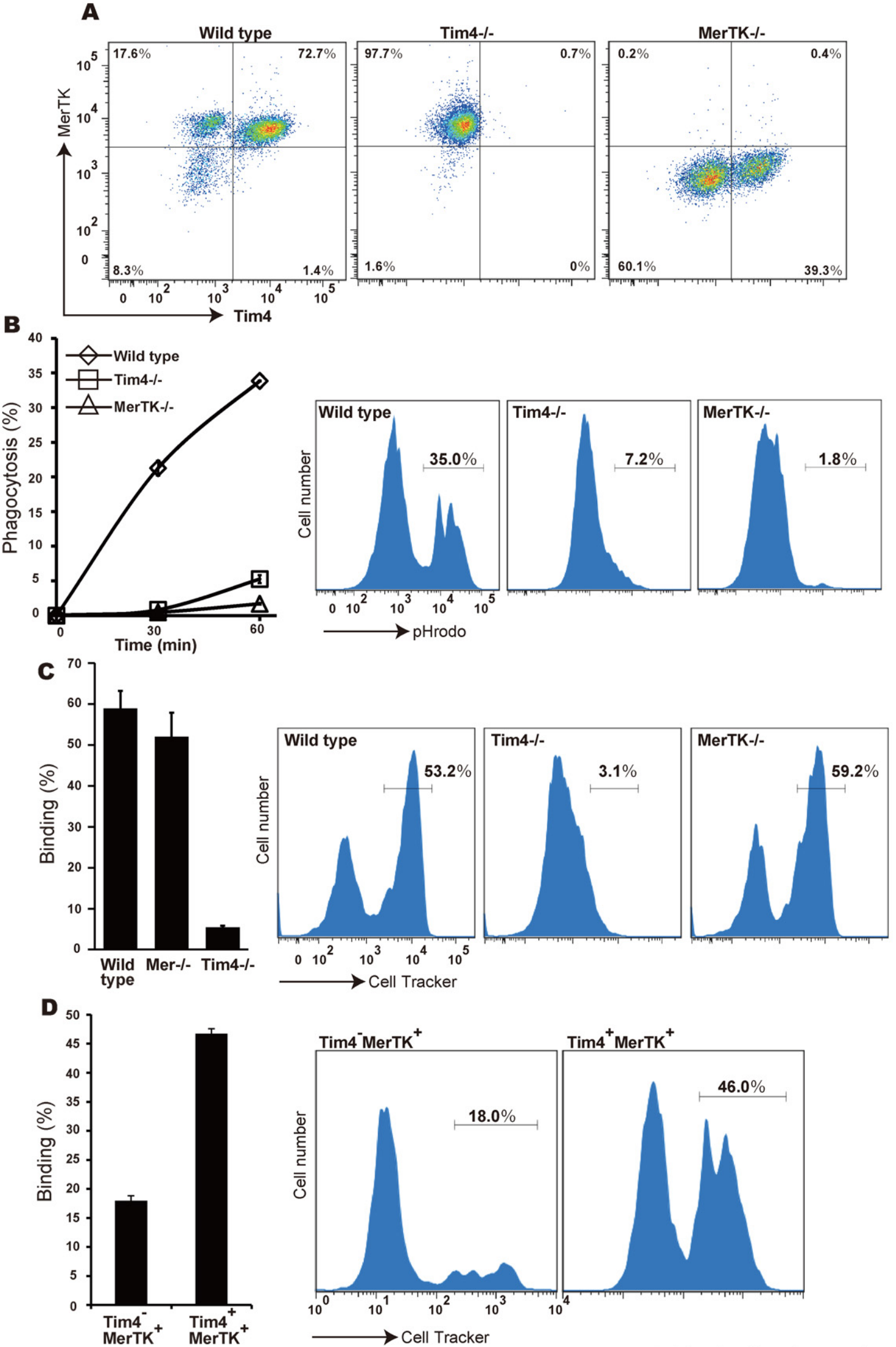
Resident peritoneal cells (5×10^5) were incubated with 2×10^6 pHrodo-labeled apoptotic thymocytes in 10% FCS, or serum-free DMEM containing the indicated concentration of protein S at 37°C for 60 min, and then subjected to flow cytometry. The pHrodo-staining profiles of the Mac1-positive population are shown in right with numbers indicating the percentage of pHrodo-positive macrophages. The experiments were performed three times, and the average percentage of the pHrodo-positive macrophages is plotted with S.D. (bars).

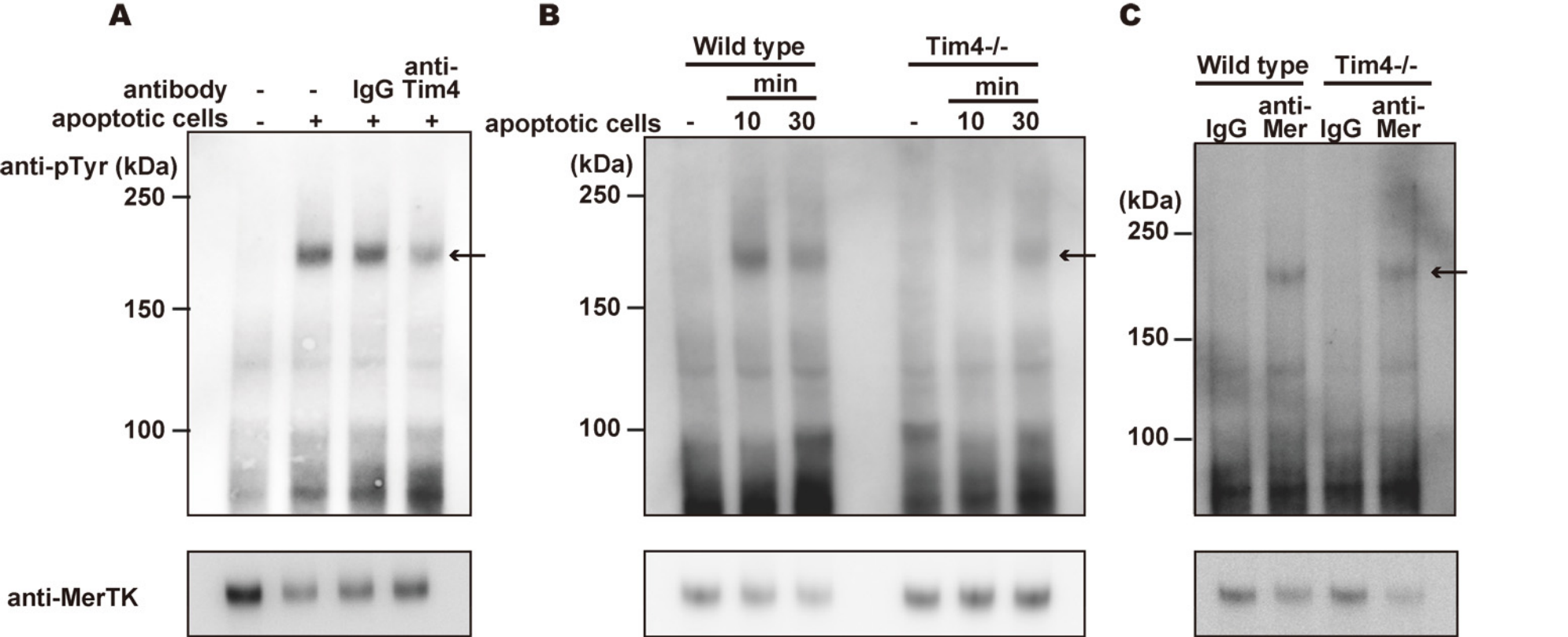
Figure 5. Reconstitution of Tim4- and MerTK- mediated phagocytosis with mouse Ba/F3 cells.

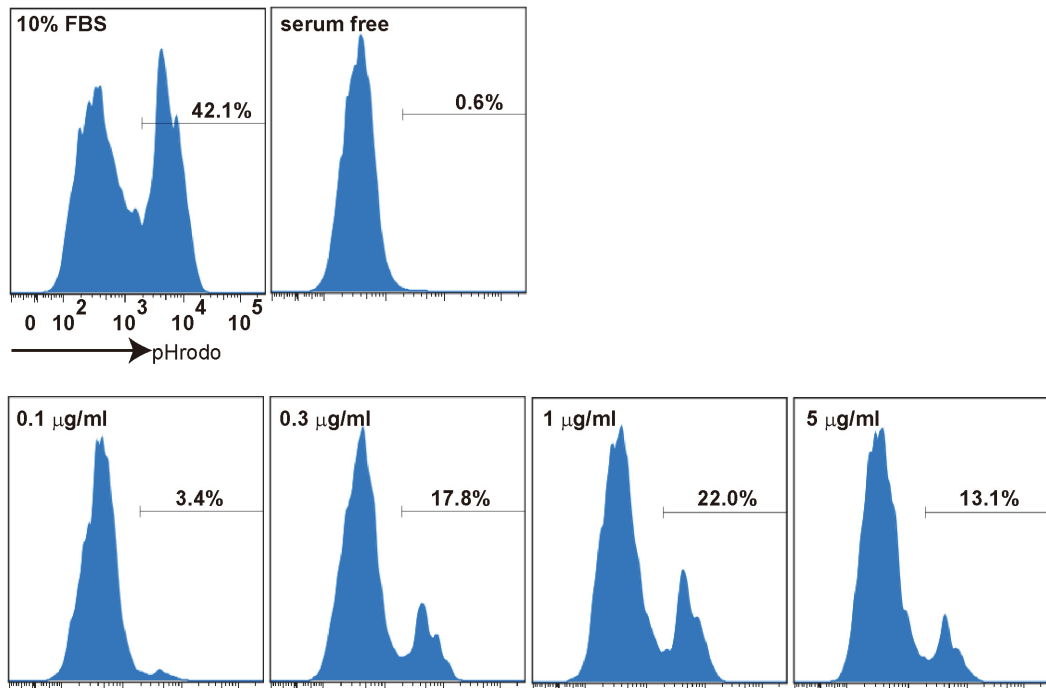
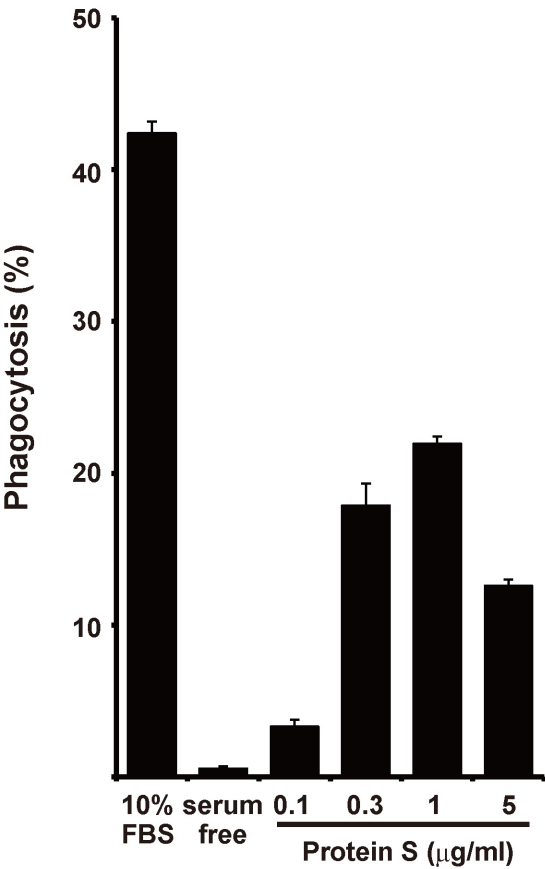
(A) Expression of Tim4 and MerTK in Ba/F3 transformants. Ba/F3 stable transformants expressing MerTK, Tim4, or both were stained with a biotinylated anti-MerTK Ab and hamster anti-Tim4 mAb, followed by Alexa488-conjugated streptavidin and APC-conjugated anti-hamster IgG. The cells were analyzed by flow cytometry. (B) Binding of apoptotic thymocytes to Ba/F3 transformants. Ba/F3 transformants (1×10^5 cells) expressing Tim4, MerTK, or Tim4 and MerTK were incubated

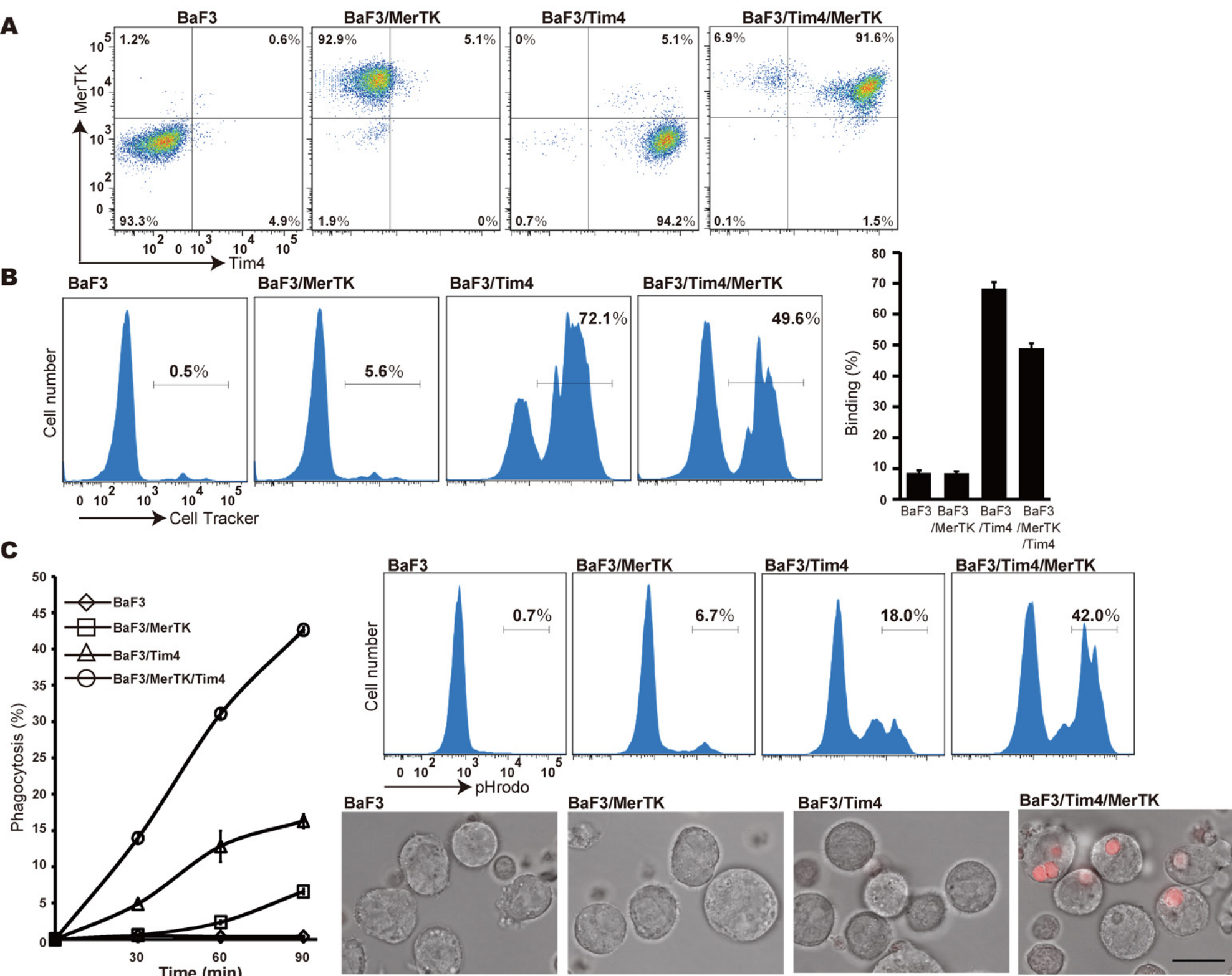
with CellTracker Orange-labeled apoptotic thymocytes (1×10^6 cells) at 37°C for 30 min, and subjected to flow cytometry. Numbers indicate the percentage of the CellTracker-positive cells. Experiments were performed in triplicate, and the average values are plotted with S.D. (bars) in the right panel. (C) Engulfment of apoptotic cells by Ba/F3 transformants. The Ba/F3 transformants expressing Tim4, MerTK, or both were incubated at 37°C for the indicated time with pHrodo-labeled apoptotic thymocytes, and analyzed by flow cytometry. The numbers indicate the percentage of pHrodo-positive cells. The experiments were performed in triplicate, and the average values are plotted with S.D. (bars). A representative FACS profile obtained with Ba/F3 cells that were incubated for 90 min with apoptotic cells is shown in the right panel. Ba/F3 cells were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 90 min, transferred to Lab-Tek chambered slides, and examined by fluorescence microscopy. Bright field and pHrodo (red) merged images are shown. Scale bar, 15 μ m.

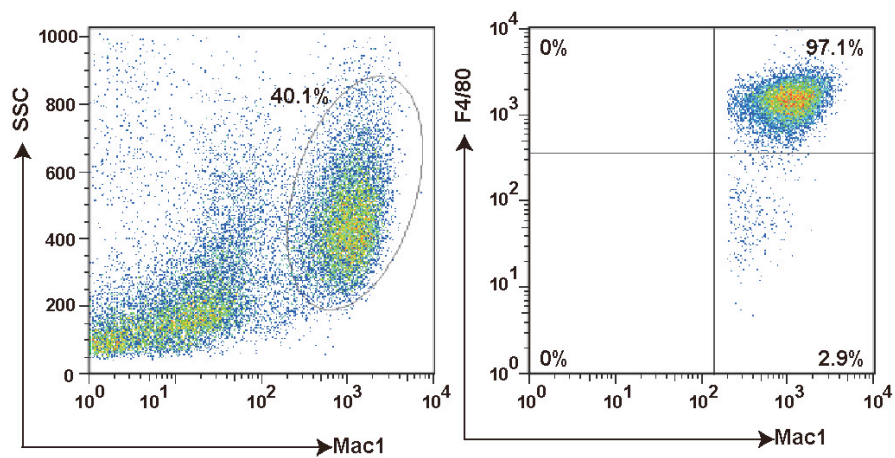
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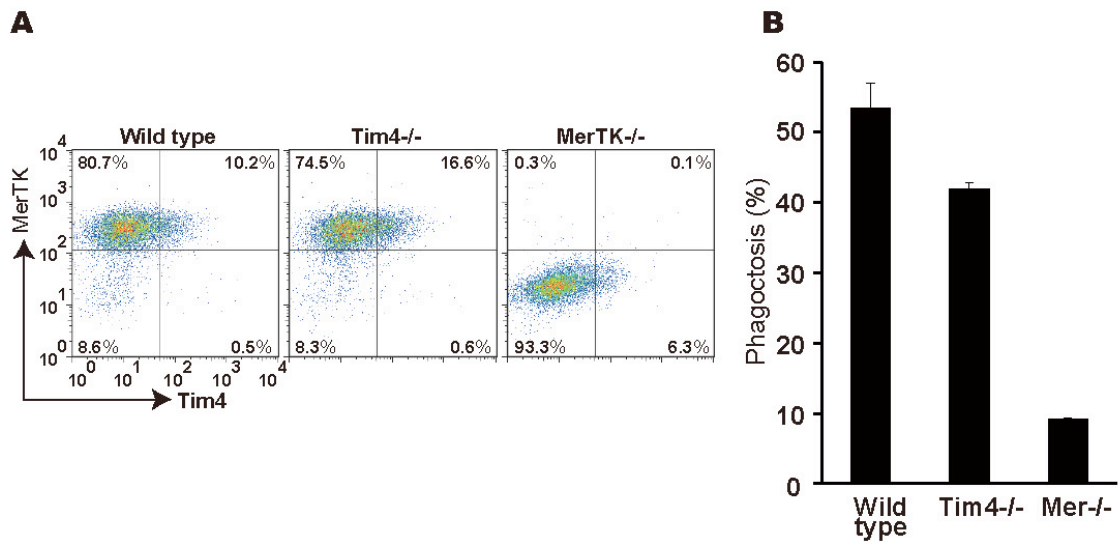




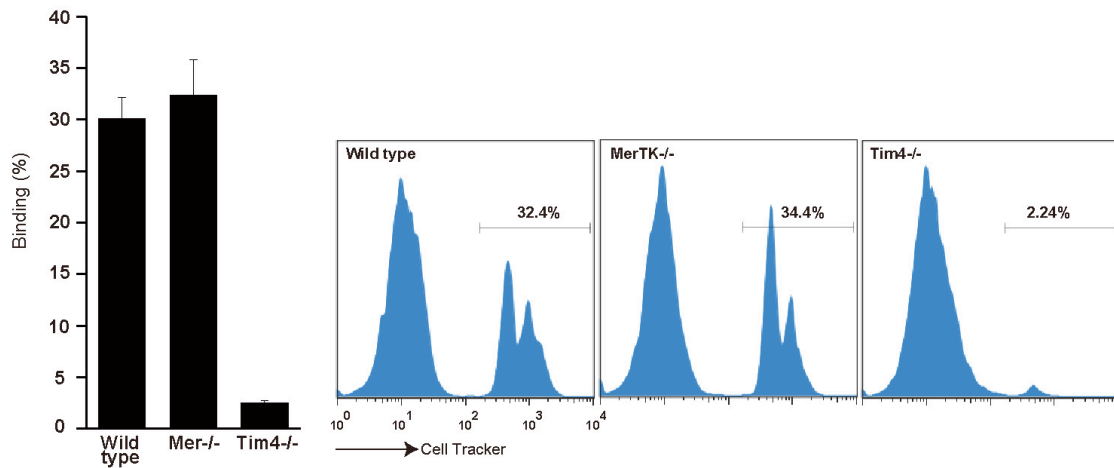




Supplementary Figure 1. Expression of Mac1 and F4/80 in rpMacs. Peritoneal cells from C57BL/6J mice were stained with APC-conjugated anti-mouse Mac1 mAb, and biotinylated anti-F4/80 mAb, followed by staining with Alexa488-conjugated streptavidin, and analyzed by flow cytometry. Left panel shows the staining profile for Mac1. In right panel, the Mac1-positive cells enclosed in left panel are analyzed for Mac1 and F4/80.

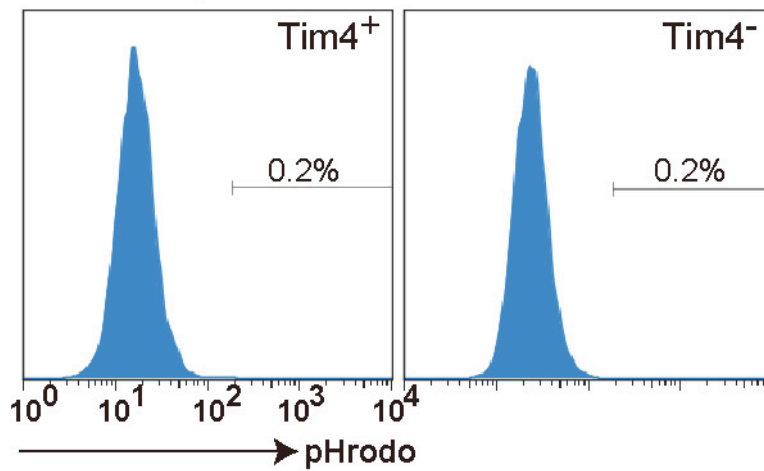


Supplementary Figure 2. Requirement of MerTK for the engulfment of apoptotic cells by mouse thioglycollate-elicited peritoneal macrophages. (A) The thioglycollate-elicited peritoneal macrophages (thio-pMacs) were prepared from the wild-type, *Tim4*^{-/-} and *MerTK*^{-/-} mice as described previously (Hanayama et al., 2002), and cultured in DMEM containing 10%FCS. Cells were stained with PerCP-Cy5.5-anti-mouse Mac1, biotinylated goat anti-MerTK, and hamster anti-mouse Tim4, followed by staining with Alexa488-streptavidin and APC-anti-hamster IgG. The stained cells were analyzed by flow cytometry. The expression profiles of MerTK and Tim4 in the Mac1-positive population are shown. (B) The thio-pMacs from wild-type, *Tim4*^{-/-}, and *MerTK*^{-/-} mice were incubated with pHrodo-labeled apoptotic thymocytes at 37°C for 120 min and the percentage of pHrodo-positive cells in Mac1-positive population was determined by flow cytometry. The experiments were performed three times, and the average values are plotted with the S.D. (bars).



Supplementary Figure 3. Thymocytes from 4 week old C57BL/6J mice were labeled with CellTracker Orange at 37°C for 30 min, and incubated with FasL in serum free DMEM at 37°C for 2 h to induce apoptosis. The cells were washed with PBS containing 0.5% BSA and 0.25% globulin, and suspended in PBS containing 0.5% BSA. Peritoneal cells from wild-type, *Tim4*^{-/-} and *Mer*^{-/-} mice were incubated with CellTracker-labeled cells at 37°C for 30 min, and analyzed by flow cytometry for the CellTracker-positive cells in Mac1-positive population. The number indicates the percentage of CellTracker-positive cells. The experiments were done in triplicate, and the average values with S.D. (bars) are plotted. At right, a representative CellTracker-staining profile of Mac1-positive cells is shown.

MerTK^{-/-} rpMacs



Supplementary Figure 4. No engulfment of apoptotic cells by MerTK^{-/-} rpMacs. The rpMacs (1×10^5 cells) from the MerTK^{-/-} mice were incubated at 37°C for 60 min with pHrodo-labeled apoptotic thymocytes (1×10^6 cells). The cells were stained with PerCP-Cy5-anti mouse Mac1, hamster anti-Tim4, followed by staining with APC-antihamster IgG, and analyzed by flow cytometry. The FACS profiles for pHrodo-positive cells in each Tim4⁺ and Tim4⁻ population of Mac1-positive cells are shown. The number indicates the percentage of pHrodo-positive cells in each population.