Mouse macrophages show different requirements for phosphatidylserine receptor Tim4 in efferocytosis

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Protein S (ProS) and growth arrest-specific 6 (Gas6) bind to phosphatidylserine (PtdSer) and induce efferocytosis upon binding TAM-family receptors (Tyro3, Axl, and Mer). Here, we produced mouse ProS, Gas6, and TAM-receptor extracellular region fused to IgG fragment crystallizable region in HEK293T cells. ProS and Gas6 bound Ca^{2+} dependently to PtdSer (K_d 20–40 nM), Mer, and Tyro3 (K_d 15–50 nM). Gas6 bound Axl strongly (K_d < 1.0 nM), but ProS did not bind Axl. Using NIH 3T3-based cell lines expressing a single TAM receptor, we showed that TAM-mediated efferocytosis was determined by the receptor-binding ability of ProS and Gas6. Tim4 is a membrane protein that strongly binds PtdSer. Tim4 alone did not support efferocytosis, but enhanced TAM-dependent efferocytosis. Resident peritoneal macrophages, Kupffer cells, and $CD169^+$ skin macrophages required Tim4 for TAM-stimulated efferocytosis, whereas efferocytosis by thioglycollate-elicited peritoneal macrophages or primary cultured microglia was TAM dependent, but not Tim4 dependent. These results indicate that TAM and Tim4 collaborate for efficient efferocytosis in certain macrophage populations.

efferocytosis | macrophages | Tim4 | apoptosis | engulfment

Apoptotic cells are quickly engulfed by macrophages, a process called efferocytosis (1), to prevent the release of noxious materials from dying cells that may cause systemic autoimmune diseases (2, 3). To be recognized by macrophages, apoptotic cells expose phosphatidylserine (PtdSer) on their surface as an "eat me" signal.

Various proteins have been proposed to specifically bind PtdSer for efferocytosis (4). Among them, Protein S (ProS) and growth arrest-specific 6 (Gas6) are secreted glycoproteins with about 40% amino acid sequence identity (5). They have a similar structure, consisting of a "Gla" domain containing 11 γ-carboxyglutamic acids, a loop region, four epidermal growth factor-like repeats, and a sex hormone-binding globulin-like structure composed of two globular laminin G-like (LG) domains. ProS and Gas6 bind to PtdSer via their Gla domain in a Ca^{2+}-dependent manner (6). ProS is a serum protein (350 nM in human plasma) (7) and negatively regulates blood coagulation (8). Gas6 is present in the serum at a much lower level (less than 0.2 nM in human plasma) than ProS (9).

To support efferocytosis, ProS and Gas6 bind on phagocytes to a subfamily of receptor tyrosine kinases called "TAM," from the first letter of the three members (Tyro3, Axl, and Mer) (10). Mouse (m)TAM receptors, which are all type I membrane proteins, have an overall identity of about 40% in their amino acid sequence. Their extracellular regions consist of two Ig-like domains and two fibronectin type III-like domains. ProS and Gas6 bind to TAM receptors via an interaction of their LG domain with Ig-like domains of TAM receptors (11).

In addition to TAM, phagocytes express proteins that directly bind PtdSer (2, 4). T-cell immunoglobulin and mucin domains containing protein 4 (Tim4) belongs to this category (12). We previously showed that Tim4 collaborates with the ProS–Mer system to elicit efferocytosis in mouse resident peritoneal macrophages (13). Here, we prepared recombinant mProS and mGas6 and found that the affinity of mProS and mGas6 to PtdSer was three to eight times weaker than Tim4's affinity to PtdSer. Both mProS and mGas6 bound to mMer and mTyro3 with similar affinities (K_d of 20–50 nM), but their affinity to mAxl was extremely different. Whereas mGas6 bound mAxl tightly, the binding of mProS to mAxl was undetectable. We then prepared an NIH 3T3-derived cell line that did not express TAM and performed efferocytosis with NIH 3T3 expressing a single type of mTAM receptor. We found that efferocytosis proceeded in accordance with the affinity of ProS and Gas6 to the TAM receptors and was strongly enhanced by Tim4 expression. Finally, we found that the TAM-mediated efferocytosis by resident peritoneal macrophages, Kupffer cells, and $CD169^+$ skin macrophages was strongly dependent on Tim4. In contrast, thioglycollate-elicited peritoneal macrophages and cultured microglia did not require Tim4 for the TAM-mediated efferocytosis, suggesting that these phagocytes have another system for enhancing the process.

Results

Preparation of TAM Receptors and Ligands. Receptors and ligands are often species specific. The reported interactions between TAM receptors and their ligands have been somewhat confusing, due to the use of proteins from different species in the analyses (5). To study the interaction between TAM receptors and their ligands, we prepared all of the reagents as recombinant mouse proteins. For mouse TAM receptors, the extracellular region was fused to human IgG1– fragment crystallizable (Fc) region, transiently expressed in HEK293T cells in serum-free medium, and purified by protein A-Sepharose in the presence of 1% Triton X-100. The purified proteins showed a single homogeneous band of 85, 120, or 90 kDa in SDS/PAGE under reducing conditions (Fig. S1*A*), but behaved as a dimer of 260, 300, or 250 kDa under nonreducing conditions. To produce mouse TAM ligands, HEK293T cells were stably transformed with an expression vector for Flag-tagged

Significance

Every day, billons of cells undergo apoptosis, expose phosphatidylserine (PtdSer), and are engulfed by macrophages in a PtdSer-dependent manner. Here, we present that Tim4, a PtdSer receptor, strongly enhances Protein S- or growth arrest-specific 6-induced efferocytosis by TAM receptor-expressing phagocytes. Resident peritoneal macrophages, Kupffer cells, and $CD169^+$ skin macrophages required Tim4 for the efficient efferocytosis, whereas thioglycollate-elicited peritoneal macrophages and cultured microglia did not. These results indicate that the efferocytosis by different macrophages may have different physiological outcomes and, therefore, would contribute to the understanding of macrophage heterogeneity.

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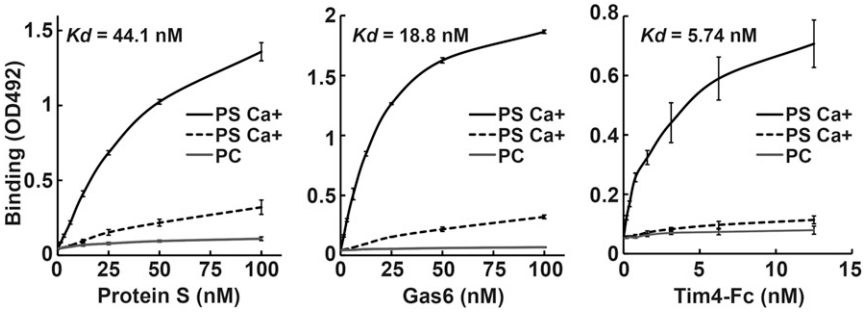

Fig. 1. Binding of mProS and mGas6 to phosphatidylserine. Microtiter plates (96 wells) were coated with PtdCho or PtdSer and incubated with the indicated concentrations of Flag–mProS, Flag–mGas6, or mTim4–Fc. Bound proteins were quantified by ELISAs. The assay was performed in triplicate, and the average values are plotted with SD (bar). K_ds were calculated from the binding curves through sigmoid fitting using Gnu R open access software (R Development Core Team).

mProS or mGas6. Clones that secreted high levels of the recombinant protein were identified by Western blotting with anti-Flag mAb and grown in serum-free medium supplemented with vitamin K to support γ-carboxylation of the Gla domain (14). The proteins were purified by anti-Flag affinity chromatography in the presence of 1% Triton X-100, followed by HiTrapQ chromatography to remove proteins that were not γ-carboxylated. The purified mProS and mGas6 had an apparent molecular mass of 70 and 80 kDa, respectively, and were homogeneous (Fig. S1*A*). On gel filtration using Superdex 200, most of the protein eluted as a single peak with an apparent molecular mass of about 190 kDa (Fig. S1*B*), suggesting that they mainly existed as a dimer. ProS, and probably Gas6 as well, is known to be present in heterogenous multimeric forms in the serum (15) and to oligomerize upon binding PtdSer (16). The homogeneous dimeric structure of our preparations might have been due to the 1% Triton X-100 treatment to remove membranous materials.

As reported for rat Gas6 (6) and bovine ProS (17), lipid overlay and solid-phase binding assays indicated that mProS and mGas6 specifically bound PtdSer in a Ca^{2+}-dependent manner (Fig. 1 and Fig. S2), whereas they had no affinity for other phospholipids, including PtdCho, PtdEtn, and sphingomyelin. Tim4–Fc, a fusion protein of the extracellular region of mTim4 and human IgG1–Fc (12), showed a similar specificity for PtdSer, with a three to eight times stronger affinity than that of mProS or mGas6 for PtdSer (Fig. 1).

Interaction Between Mouse TAM Ligands and Their Receptors. We next analyzed the interaction between TAM ligands (mProS and mGas6) and the Fc fusion proteins of TAM receptors (mTyro3–Fc, mAxl–Fc, and mMer–Fc) using surface plasmon resonance (SPR) (18) and biolayer interferometry (BLI) (Table 1). Although BLI gave 1.5- to 2-fold higher association (k_{on}) and dissociation (k_{off}) rate constant values for all of the combinations, the K_ds obtained by both technologies were similar, or at most 1.5-times different, and indicated that mGas6 bound to mAxl very tightly, with a K_d less than 1 nM, whereas ProS had no ability to bind mAxl. On the other hand, mProS and mGas6 bound to mTyro3 and mMer with similar affinities or with at most a 3-fold difference (K_d of 15–50 nM).

Establishment of NIH 3T3 Expressing a Single TAM Receptor. To examine the effect of TAM system on efferocytosis, we performed efferocytosis assays in a serum-free medium (Fig. S3*A*), because serum carries a high level of ProS. A total of 10 nM mProS did not support efferocytosis by NIH 3T3, but the same concentration of mGas6 strongly supported the process. Lew et al. (16) recently reported that various immortalized cell lines express at least one TAM receptor. Real-time RT-PCR showed that the NIH 3T3 expressed a high level of Axl, low levels of Tyro3, ProS, and Gas6, and very little Mer (Fig. S3*B*). We therefore knocked out *Axl*, *Tyro3*, and *Gas6* genes in NIH 3T3 using the CRISPR/Cas9 system (19) (Fig. S3*C*). The resulting $Axl^{-/-}Tyro3^{-/-}Gas6^{-/-}$ NIH 3T3 [triple knockout (TKO)] lost the ability to engulf apoptotic cells in response to 10 nM Gas6 (Fig. S3*A*), indicating that the endogenous Axl was responsible for the Gas6-induced efferocytosis in NIH 3T3 cells.

To examine the ability of each TAM receptor to support efferocytosis, the TKO cells were then singly transformed with mAxl, mMer, or mTyro3 (Fig. 2*A*), and their efferocytosis ability was assayed (Fig. 2*B*). The mAxl-expressing cells responded well to mGas6 but not at all to mProS, consistent with our finding that mGas6 but not mProS bound to mAxl (Table 1). The half-maximal concentration of mGas6 for increasing efferocytosis was about 0.1 nM, which was five times lower than the K_d for the interaction between mGas6 and mAxl–Fc (Table1), supporting the idea that TAM signaling is activated more strongly by a PtdSer-engaged TAM ligand than a free ligand (16, 20). In agreement with the weak ability of mProS and mGas6 to bind mMer and mTyro3, they moderately supported efferocytosis by mMer- or mTyro3-expressing cells.

Effect of Tim4 on TAM-Mediated Efferocytosis. We previously reported that Tim4 strongly enhances Mer-mediated efferocytosis in a reconstituted system using Ba/F3 cells that grow in suspension (13). NIH 3T3 did not express Tim4 (Fig. S4*A*). Therefore, to examine the effect of Tim4 on the NIH 3T3-based efferocytosis, TKO cells and their transformants expressing mAxl, mMer, or mTyro3 were further transformed with mTim4 (Fig. S4*B*). As shown in Fig. 3, TKO cells expressing mTim4 did not respond to mProS or

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Table 1. Kinetic parameters for the interaction of ProS and Gas6 with TAM family proteins

		ProS			Gas6		
TAM receptor	Analysis methods	$k_{on} \times 10^4$ ($M^{-1}\cdot s^{-1}$)	$k_{off} \times 10^{-4}$ (s^{-1})	K_d (nM)	$k_{on} \times 10^4$ ($M^{-1}\cdot s^{-1}$)	$k_{off} \times 10^{-4}$ (s^{-1})	K_d (nM)
Axl	SPR	N.D.	N.D.	N.D.	37.6 ± 0.19	3.00 ± 0.01	0.80 ± 0.01
Axl	BLI	N.D.	N.D.	N.D.	77.7 ± 1.52	4.84 ± 0.08	0.62 ± 0.02
Mer	SPR	1.04 ± 0.01	6.00 ± 0.02	57.8 ± 0.58	5.47 ± 0.04	11.9 ± 0.03	21.7 ± 0.27
Mer	BLI	2.37 ± 0.02	9.39 ± 0.09	39.6 ± 0.81	11.3 ± 0.06	15.1 ± 0.09	13.4 ± 0.19
Tyro3	SPR	6.00 ± 0.05	9.70 ± 0.03	16.2 ± 0.23	10.9 ± 0.17	25.3 ± 0.12	23.3 ± 0.54
Tyro3	BLI	8.82 ± 0.12	22.0 ± 0.19	24.9 ± 0.61	21.3 ± 0.27	64.8 ± 0.32	30.5 ± 0.61

The association (k_{on}) and dissociation (k_{off}) rate constants for each ligand–receptor pair were determined by BLI (biolayer interferometry) and SPR (surface plasmon resonance). N.D., not detected.

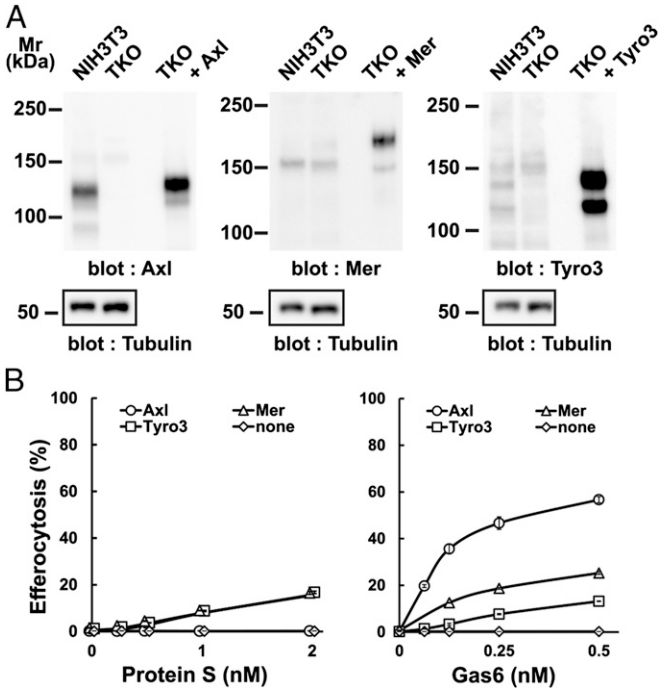

Fig. 2. TAM receptor-mediated engulfment of apoptotic cells. (*A*) *Axl*$^{-/-}$*Tyro3*$^{-/-}$*Gas6*$^{-/-}$ NIH 3T3 (TKO) cells were infected by lentiviruses carrying the cDNA for mAxl, mMer, or mTyro3, and stable transformants were established. NIH 3T3, TKO, and TKO transformants expressing the indicated mTAM receptor were analyzed by Western blotting with an anti-mAxl, anti-mMer, anti-mTyro3, or anti–α-tubulin Ab. (*B*) TKO cells and their derivatives were incubated with pHrodo-apoptotic thymocytes in DMEM containing 5 mg/mL BSA and the indicated concentration of mProS or mGas6, and analyzed by flow cytometry for pHrodo. The experiments were performed in triplicate, and the average percentage of pHrodo^{+} cells was plotted with SD (bar) as efferocytosis.

mGas6 for efferocytosis. In mAxl-expressing cells, mTim4 did not evoke mProS-supported efferocytosis, but strongly enhanced mGas6-supported efferocytosis. Specifically, the Tim4 expression reduced the concentration of mGas6 required for mAxl-mediated efferocytosis by at least 10 times. A much stronger enhancing effect of Tim4 on efferocytosis was observed with mMer-expressing cells, in which Tim4 strongly enhanced not only mGas6-, but also mProS-supported efferocytosis. In mTyro3-expressing cells transformed with mTim4, efferocytosis took place constitutively, or without TAM ligand, and mProS or mGas6 did not further stimulate it. This finding is difficult to interpret, because we could not detect the direct association between Tim4 and Tyro3 by the immunoprecipitation followed by Western blotting using antibodies against Tim4 and Tyro3. In any events, these results indicated that Tim4 alone could not support the engulfment of apoptotic cells, but strongly enhanced TAM system-mediated efferocytosis.

Different Requirements of Tim4 for Efferocytosis by Mouse Tissue Macrophages. We previously reported that *Mertk*$^{-/-}$ or *Tim4*$^{-/-}$ mouse resident peritoneal macrophages cannot engulf apoptotic cells (13). Here we found by real-time RT-PCR that resident peritoneal macrophages expressed not only Mer, but also Axl mRNA (Fig. 4*A*). Western blotting detected a high level of Mer, but little Axl and no Tyro3 protein (Fig. 4*B*). The Axl's, but not Mer's extracellular region is known to be cleaved off (21), suggesting that resident peritoneal macrophages express both Axl and Mer. Efferocytosis using resident peritoneal macrophages in the presence of increasing concentrations of TAM ligands showed that mGas6 and mProS stimulated the efferocytosis with a dose–response similar to that obtained with TKO cells expressing Mer and Tim4 (Fig. 4*C*). In agreement with a previous report (13), the resident peritoneal macrophages expressed Tim4 mRNA and protein (Fig. 4 *A* and *B*), and the efferocytosis by these macrophages was completely Tim4 dependent (Fig. 4*C*).

To examine whether other phagocytes require the Tim4 and TAM system for efferocytosis, CD169^{+} skin macrophages, Kupffer cells, primary cultured microglia, and thioglycollate-elicited peritoneal macrophages were prepared. These phagocytes expressed Mer mRNA and protein, although at significantly different levels among them (Fig. 5). That is, Mer mRNA (and protein) was 10–20 times more abundant in Kupffer cells than in thioglycollate-elicited peritoneal macrophages. Kupffer cells expressed high levels of Axl mRNA and protein. The other phagocytes expressed a low level of Axl mRNA, and Tyro3 mRNA could not be detected in any of the phagocytes examined (Fig. 5*A*). The Tim4 mRNA and protein were detected in CD169^{+} skin macrophages and Kupffer cells, but not in the cultured microglia and thioglycollate-elicited peritoneal macrophages (Fig. 5). Accordingly, the ProS- or Gas6-promoted efferocytosis by skin macrophages and Kupffer cells was reduced if Tim4 was absent (Fig. 6). In particular, Kupffer cells fully required Tim4 for efferocytosis and responded well to a low concentration of mProS or mGas6 for

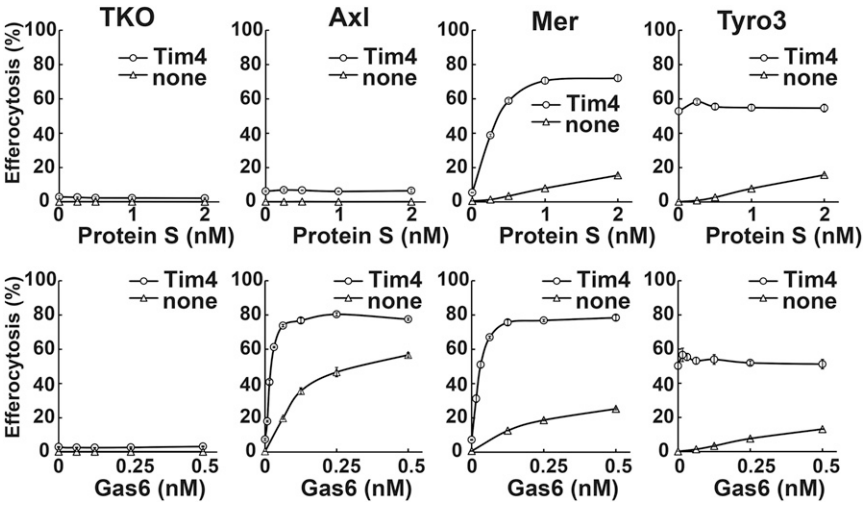

Fig. 3. Enhancement of TAM-mediated efferocytosis by Tim4. TKO, TKO-mAxl, TKO-mMer, and TKO-mTyro3, and their transformants expressing mTim4 were incubated with pHrodo-apoptotic thymocytes in DMEM containing 5 mg/mL BSA and the indicated concentrations of mProS or mGas6, and analyzed by flow cytometry. Experiments were performed in triplicate, and the average percentage of pHrodo^{+} cells was plotted with SD (bar) as efferocytosis.

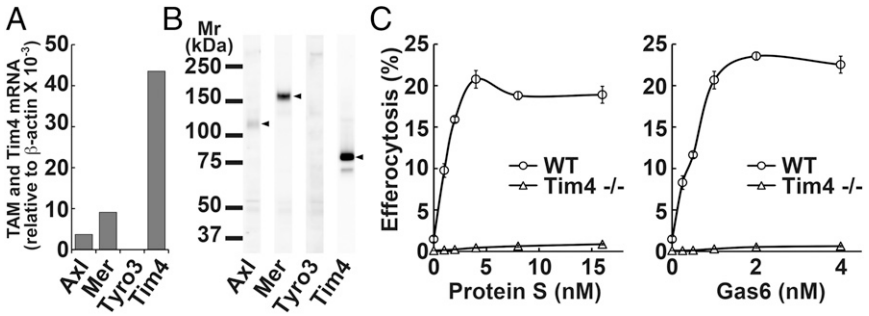

Fig. 4. Tim4 and TAM ligand-dependent efferocytosis by resident peritoneal macrophages. (*A*) RNA from resident peritoneal macrophages was analyzed by real-time RT-PCR for mAxl, mMer, mTyro3, and mTim4. The mRNA level is expressed relative to β-actin mRNA level. (*B*) Cell lysates were analyzed by Western blotting with an anti-mAxl, anti-mMer, anti-mTyro3, or anti-mTim4. (*C*) Tim4 dependency of TAM-mediated efferocytosis. Resident peritoneal macrophages from wild-type or *Tim4*$^{-/-}$ mice were coincubated with pHrodo-apoptotic thymocytes in DMEM containing 5 mg/mL BSA and the indicated concentrations of mProS or mGas6. After incubation, cells were detached, stained with APC-anti-CD11b, and analyzed by flow cytometry for pHrodo. The experiments were performed in triplicate, and the average percentage of CD11b^{+} cells that were pHrodo^{+} was plotted with SD (bar) as efferocytosis.

efferocytosis. The requirement of Kupffer cells' efferocytosis for Tim4 seems to be higher than that observed with TKO expressing Axl or Mer, which may indicate that Axl or Mer at the endogenous level almost absolutely require Tim4. On the other hand, although mProS and mGas6 efficiently stimulated microglia and thioglycollate-elicited peritoneal macrophages to engulf apoptotic cells, the lack of Tim4 had no effect on the mProS- or mGas6-stimulated efferocytosis.

Discussion

In this report, we showed that mGas6 has a high affinity for mAxl, whereas mProS has almost no affinity for mAxl, and that they both bind to mMer and mTyro3 with a similar affinity. The X-ray structure analysis of the complex of LG domain of Gas6 and Ig domain of Axl (11) and a bioinformatics study (22) indicated that nine functional residues in Gas6 (Met-297, Arg-305, Arg-310, Leu-311, Val-389, Lys-399, Arg-411, Asp-452, and Asn-462 in mGas6 numbering) are at the interface with Axl. An alignment of the amino acid sequence of mGas6 with that of mProS shows that none of these nine amino acids is conserved in mProS (Fig. S5*A*), which may explain why mProS is unable to bind mAxl. The major interaction site of Axl with Gas6 is the region of amino acid positions 70–100 (11) (Fig. S5*B*). This region of mAxl has no similarity with mMer or mTyro3, which agrees with the reduced affinity of mGas6 to mMer or mTyro3.

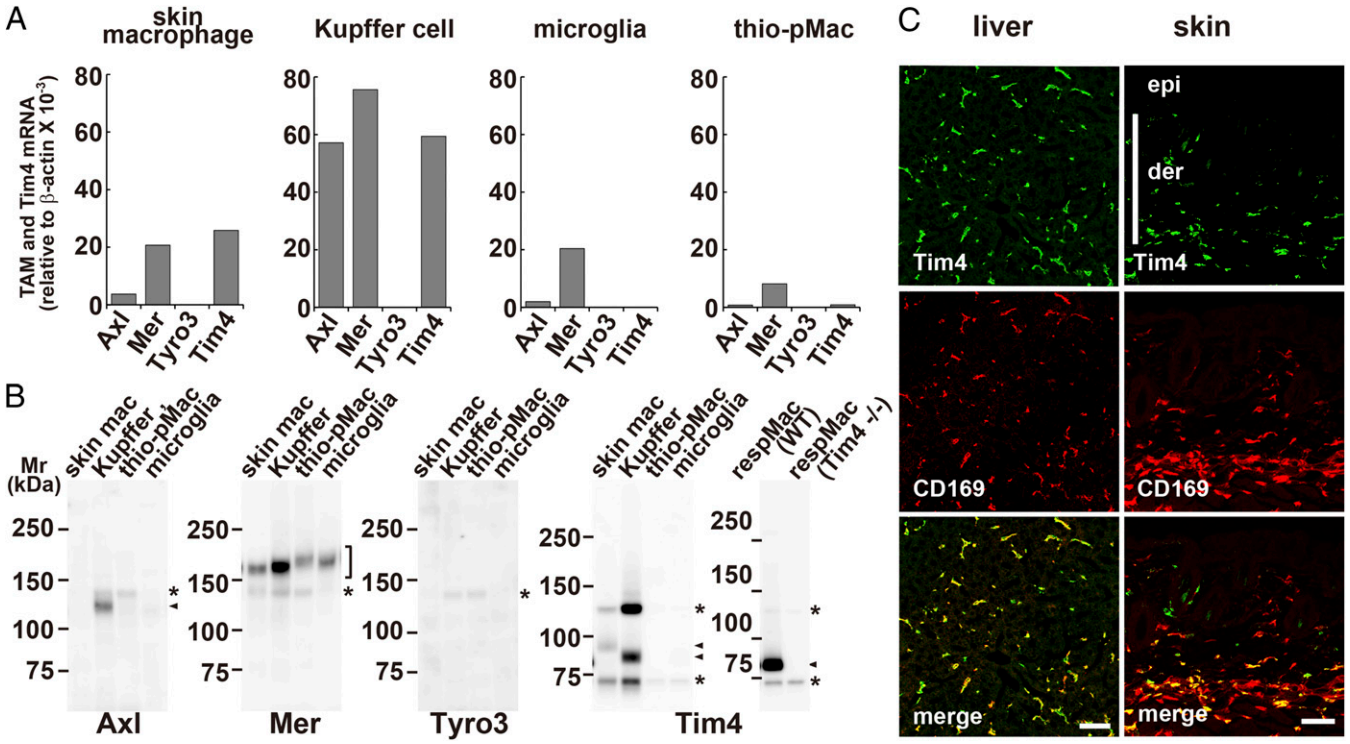

Fig. 5. Different expression of TAM receptors and Tim4 by tissue macrophages. (*A*) RNA from mouse skin macrophages (skin mac), Kupffer cells (Kupffer), microglia from glial culture, and thioglycollate-elicited peritoneal macrophages (thio-pMac), was subjected to real-time RT-PCR for Axl, Mer, Tyro3, and Tim4. Each mRNA level is expressed relative to β-actin mRNA level. (*B*) Cell lysates from the indicated macrophages were analyzed by Western blotting with antibodies against mAxl, mMer, mTyro3, or mTim4. Arrowheads and asterisks indicate specific and nonspecific bands, respectively. (*C*) Cryosections of liver and skin were stained with antibodies against Tim4 (green) and CD169 (red). (*Lower*) Merged images. der, dermis; epi, epidermis. (Scale bar, 50 μm.)

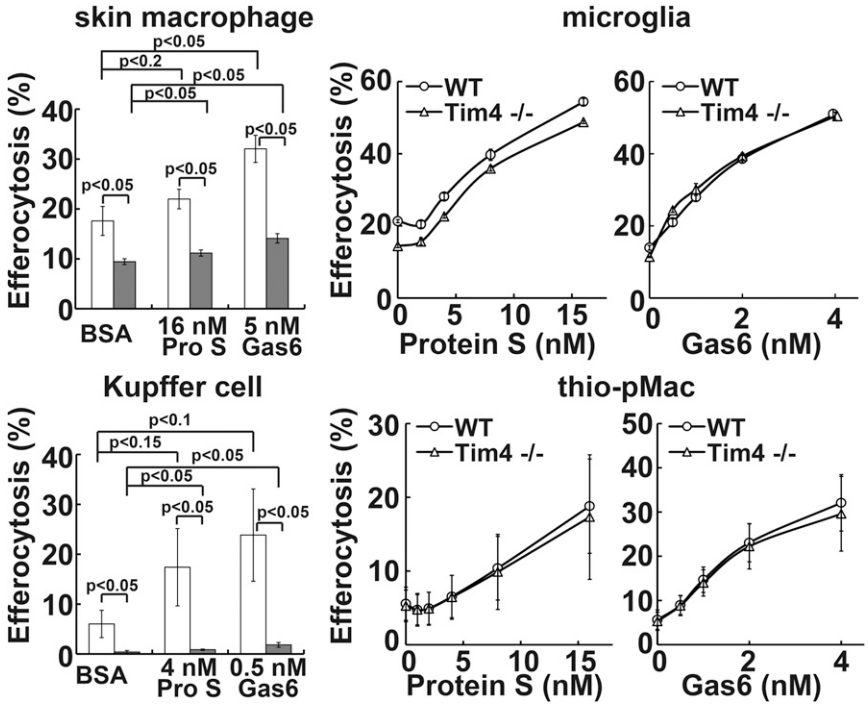

Fig. 6. Different requirement of Tim4 for efferocytosis by tissue macrophages. The indicated tissue macrophages from wild-type (open column) or *Tim4*$^{-/-}$ (closed column) mice were seeded into 24-well plates, and the adherent cells were coincubated with pHrodo-apoptotic thymocytes at 37 °C for 1 h (thioglycollate elicited peritoneal macrophages, thio-pMac) or 2 h (others) in DMEM containing 5 mg/mL BSA and the indicated concentrations of mProS or mGas6. Cells were detached and stained with PE/Cy7-anti-CD11b and AlexaFluor647-anti-CD169 (skin macrophages), APC-anti-F4/80 (Kupffer cells), or APC-anti-CD11b (thio-pMac). The percentage of CD11b^{+}CD169^{+} cells (skin macrophages), F4/80^{high} and autofluorescence-positive cells (Kupffer cells), or CD11b^{+} cells (thioglycollate elicited peritoneal macrophages) that was pHrodo^{+} was determined by flow cytometry. For microglia, the percentage of pHrodo^{+} cells was determined for the total cell population. Experiments were performed in triplicate for skin macrophages and primary microglia or independently three times for Kupffer cells and thioglycollate-elicited peritoneal macrophages, and the average value was plotted with SD (bar) as efferocytosis. *P* values are shown.

Many groups including ours have used established cell lines to reconstitute efferocytosis (12, 23–25). This practice led to the identification of many different molecules (MFG-E8, Tim-4, Tim-1, BAI1, CD300a, Stabillin-1, and Stabillin-2) as being able to bind PtdSer exposed on apoptotic cells and enhance efferocytosis (4). On the other hand, we also noticed that the reconstitution of efferocytosis strongly depends on the host cells. For example, the expression of Tim4 alone fully supports efferocytosis in NIH 3T3 (12), but it does not confer efferocytosis ability on Ba/F3 or resident peritoneal macrophages (13). Here, we found that when Tim4 was expressed alone in TKO cells, mProS or mGas6 failed to stimulate efferocytosis. However, this ability was restored by expressing one of the TAM receptors, indicating that Tim4 requires a TAM receptor to stimulate efferocytosis. It will be important to determine whether the other molecules proposed as PtdSer receptors (4) act by themselves or require TAM receptors for efferocytosis.

We previously showed that the expression of Tim4 or Mer alone in Ba/F3 or resident peritoneal macrophages does not support efferocytosis, but that efferocytosis can occur when both Tim4 and Mer are expressed (13). However, Ba/F3 expressing Tim4 alone but not Mer alone efficiently recruited apoptotic cells, leading us to conclude that Tim4 acts at the tethering step, whereas Mer functions at the tickling or internalization step. A similar result was obtained here with mProS-stimulated efferocytosis. That is, mMer- or mTyro3-expressing TKO cells weakly responded to mProS for efferocytosis, and this response was strongly enhanced by coexpressing Tim4, supporting the idea that the Tim4 and Mer systems collaborate for efficient efferocytosis. The mGas6-stimulated efferocytosis, in particular with Axl-expressing TKO cells, was observed without Tim4, suggesting that the higher affinity of Gas6 to PtdSer and to Axl may obviate the need for the tethering step. However, even under these conditions, the expression of Tim4 strongly enhanced the efferocytosis. The number of apoptotic cells attached to Tim4-expressing cells far exceeded that observed with Tim4-nonexpressing cells in the presence or absence of Gas6 (Fig. S6), supporting the idea that Tim4 enhances efferocytosis by functioning at the tethering step.

We found here that Kupffer cells, resident peritoneal macrophages, and CD169^{+} skin macrophages express and require Tim4 for efferocytosis. Kupffer cells are a major cell population in the liver (about 15%) (26), where they clear aged red blood cells and engulf infected or damaged hepatocytes to accelerate liver resolution (27, 28). Resident peritoneal macrophages invade the liver to repair damaged tissues (29), indicating that resident peritoneal macrophages perform a similar function as Kupffer cells. One of the functions of skin CD169^{+} macrophages is to resolve inflammation by clearing recruited immune cells (30, 31). Aged erythrocytes, damaged hepatocytes, and recruited dying immune cells all expose PtdSer to be recognized by macrophages (32), indicating that the collaborative function of Tim4 and TAM receptors in these macrophages plays an important role in clearing the damaged cells.

The thioglycollate-elicited peritoneal macrophages required a TAM receptor to elicit efferocytosis, in agreement with a previous report by Scott et al. (33). However, unlike resident peritoneal macrophages, they did not require Tim4 for efferocytosis. We and others showed that peritonitis-induced inflammatory macrophages require MFG-E8, which acts as a bridge between PtdSer-exposing apoptotic cells and integrin-$\alpha_v\beta_3$-expressing macrophages (25, 34, 35). Unlike the resident peritoneal macrophages, which have a tolerogenic role (36), the apoptotic cells engulfed by inflammatory macrophages provide antigens to stimulate the immune system (37). It is possible that the different requirement of Tim4 or MFG-E8 for efferocytosis explains the different properties of resident versus inflammatory macrophages. The microglia in brain appear to express Tim4, although very weakly or in only a limited population (38, 39). However, the cultured microglia did not express Tim4, but efficiently engulfed apoptotic cells using the TAM system. Because bone-marrow-derived macrophages express Mer, and can engulf apoptotic cells without Tim4, Dransfield et al. (40) proposed that Mer functions at both the tethering and tickling steps of efferocytosis. Although this possibility cannot be ruled out, we prefer a model in which other molecules collaborate with TAM receptors for efficient efferocytosis in the cultured microglia. In this study, the Tim4 expression appeared to be restricted to tissue-resident macrophages. However, tissue-resident macrophages are

heterogeneous (41). Whether resident macrophages in other tissues, such as lung, spleen, and intestines, express and require Tim4 for efficient efferocytosis remains to be studied. Pathogens, in particular the enveloped virus, expose PtdSer (42) and are known to efficiently bind to Tim4-expressing cells (43). It will be interesting to study whether Tim4-expressing resident macrophages, such as peritoneal macrophages and Kupffer cells, are targets of these PtdSer-exposing pathogens.

Materials and Methods

Recombinant Proteins. For the TAM–Fc fusion proteins, DNA fragments coding for chimeric molecules between the extracellular region of TAM and the human IgG1 Fc constant region were prepared by recombinant PCR, inserted into pEF-BOS-EX, and introduced into HEK293T cells. Triton X-100 was added to the conditioned medium to a final concentration of 1%, and the Fc fusion proteins were purified by Protein A–Sepharose. To produce mProS and mGas6, stable 293T cell transformants highly expressing the Flag-tagged mProS or mGas6 were established and cultured in the presence of 10 μg/mL vitamin K_1. The conditioned media were treated with 1% Triton X-100, and the recombinant proteins were purified by anti-Flag mAb-agarose.

SPR and BLI. SPR analyses were performed using a ProteOn XPR36 Protein Interaction Array System (Bio-Rad), whereas BLI analyses were performed using an Octet RED96 System (ForteBio).

Isolation of Tissue Macrophages. Resident and thioglycollate-elicited peritoneal macrophages were isolated as described (13, 34). Primary microglia, Kupffer cells, and $CD169^+$ skin macrophages were prepared essentially as described (44–47) with some modifications. All mouse studies were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University. Full details for *Materials and Methods* are given in *SI Materials and Methods*.

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