Mouse macrophages show different requirements for phosphatidylinerse receptor Tim4 in efferocytosis

Yuichi Yanagihashi*a, Katsumori Segawaa, Ryota Maeda*b, Yo-ichi Nabeshima*b, and Shigekazu Nagataa

Laboratory of Biochemistry and Immunology, World Premier International Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan, and aDepartment of Molecular Life Science, Foundation for Biomedical Research and Innovation, Kobe, Hyogo 650-0047, Japan

Contributed by Shigekazu Nagata, July 9, 2017 (sent for review April 4, 2017; reviewed by Martin Herrmann and Masato Tanaka)

Protein S (ProS) and growth arrest-specific 6 (Gas6) bind to phosphatidylinerse (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 (Fc) region, transiently transfected HEK293T cells, and performed efferocytosis by mouse resident peritoneal macrophages and cultured microglia in a Tim4-dependent manner. To support efferocytosis, ProS and Gas6 bind to phagocytes via their Gla domain in a Ca\(^{2+}\)-dependent manner. Axl strongly binds mProS and mGas6. Tim4 is a membrane protein that strongly binds PtdSer. Here, we showed that Tim4 mediates efferocytosis and 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-positive skin macrophages required Tim4 for TAM-stimulated efferocytosis, whereas efferocytosis by thiglycollate-elicited peritoneal macrophages or primary cultured microglia was TAM-dependent, but not Tim4-dependent. These results indicate that Tim4 and TAM collaborate for efficient efferocytosis in certain macrophage populations.

Significance

Every day, billions of cells undergo apoptosis, expose phosphatidylinerse (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, 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 TIM4 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-positive skin macrophages was strongly dependent on Tim4. In contrast, thiglycollate-elicited peritoneal macrophages and cultured microglia did not require Tim4 for the TAM-mediated efferocytosis, suggesting that these phagocytes have an alternative 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 support 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 IgG–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. S1A), 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 Protein S (ProS) and Gas6, and expressed 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). These receptor tyrosine kinases include Tim4, belonging to this category (12). We recently 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 NIH 3T3-derived cell line that did not express TIM4 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-positive skin macrophages was strongly dependent on Tim4. In contrast, thiglycollate-elicited peritoneal macrophages and cultured microglia did not require Tim4 for the TAM-mediated efferocytosis, suggesting that these phagocytes have an alternative system for enhancing the process.

Author contributions: Y.Y., K.S., Y.-I.N., and S.N. designed research; Y.Y. and R.M. performed research; Y.Y., R.M., and Y.-I.N. analyzed data; and Y.Y., K.S., and S.N. wrote the paper. Reviewers: M.H., University of Erlangen-Nuremberg; and M.T., Tokyo University of Pharmacy and Life Sciences. Conflict of interest statement: S.N. and Masato Tanaka are coauthors on a 2017 review article.

Significance

Every day, billions of cells undergo apoptosis, expose phosphatidylinerse (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-positive skin macrophages required Tim4 for the efficient efferocytosis, whereas thiglycollate-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.

www.pnas.org/cgi/doi/10.1073/pnas.1705365114

PNAS Early Edition | 1 of 6
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. S1A). 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. S1B), suggesting that they mainly existed as a dimer. ProS, and probably Gas6 as well, is known to be present in heterogeneous 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<sup>2+</sup>-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 $K_{d}$s 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. S3A), 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. S3B). We therefore knocked out Axl, Tyro3, and Gas6 genes in NIH 3T3 using the CRISPR/Cas9 system (19) (Fig. S3C). The resulting Axl<sup>−/−</sup>Tyro3<sup>−/−</sup>Gas6<sup>−/−</sup> NIH 3T3 [triple knockout (TKO)] lost the ability to engulf apoptotic cells in response to 10 nM Gas6 (Fig. S3A), 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. 2A), and their efferocytosis ability was assayed (Fig. 2B). The mAxl-expressing cells responded well to mGas6 but not at all to mProS, consistent with our finding that mGas6 but not mProS was 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 mAxl 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. S4A). 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. S4B). As shown in Fig. 3, TKO cells expressing mTim4 did not respond to mProS or mGas6, 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 mAxl 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.

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

<table>
<thead>
<tr>
<th>TAM receptor</th>
<th>Analysis methods</th>
<th>$k_{on} \times 10^{4}$ (M&lt;sup&gt;−1&lt;/sup&gt;s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>$k_{off} \times 10^{-4}$ (s&lt;sup&gt;−1&lt;/sup&gt;)</th>
<th>$K_{d}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axl</td>
<td>SPR</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Axl</td>
<td>BLI</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Mer</td>
<td>SPR</td>
<td>1.04 ± 0.01</td>
<td>6.00 ± 0.02</td>
<td>57.8 ± 0.58</td>
</tr>
<tr>
<td>Mer</td>
<td>BLI</td>
<td>2.37 ± 0.02</td>
<td>9.39 ± 0.09</td>
<td>39.6 ± 0.81</td>
</tr>
<tr>
<td>Tyro3</td>
<td>SPR</td>
<td>6.00 ± 0.05</td>
<td>9.70 ± 0.03</td>
<td>16.2 ± 0.23</td>
</tr>
<tr>
<td>Tyro3</td>
<td>BLI</td>
<td>8.82 ± 0.12</td>
<td>22.0 ± 0.19</td>
<td>24.9 ± 0.61</td>
</tr>
<tr>
<td>ProS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gas6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.
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 Mer−/− 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. 4A). Western blotting detected a high level of Mer, but little Axl and no Tyro3 protein (Fig. 4B). 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. 4C). In agreement with a previous report (13), the resident peritoneal macrophages expressed Tim4 mRNA and protein (Fig. 4A and B), and the efferocytosis by these macrophages was completely Tim4 dependent (Fig. 4C).

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. 5A). 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...
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. S5A), 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. S5B). This region of mAxl has no similarity with mMer or mTyro3, which agrees with the reduced affinity of mGas6 to mMer or mTyro3.
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, BA11, CD300a, Stabilin-1, and Stabilin-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-αβ3-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 effector cells to be studied. Pathogens, in particular the enveloped virus, expose PtdSer (42) and are known to efficiently bind to Tim4-expressing cells (43). This 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 transfomants highly expressing the Flag-tagged mProS or mGas6 were established and cultured in the presence of 10 μg/mL vitamin K$_1$.

19. Phylogenetic reconstruction of TIM-4-expressing tissue resident macrophages. SI Materials and Methods.

ACKNOWLEDGMENTS. We thank M. Fuji for secretarial assistance. This work was supported in part by grants-in-aid from the Japan Society for the Promotion of Science (to Y.Y., K.S., and S.N.) and by Core Research for Evolutional Science and Technology (Grant JPMCR14M4 to S.N.).