MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation

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Running title: Signal transduction for efferocytosis and cell proliferation

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Abstract

Apoptotic cells expose phosphatidylserine (PtdSer) on their surface, leading to efferocytosis, i.e. their engulfment by resident macrophages that express the PtdSer receptor T-cell immunoglobulin mucin receptor 4 (TIM4) and TAM family receptor tyrosine kinase receptors (MERTK, AXL, and TYRO3). TAM family receptors stimulate cell proliferation, and the many aspects of the growth signaling pathway downstream of TAM family receptor have been previously elucidated. But the signaling cascade for the TAM receptor-mediated efferocytosis has been elusive. Here, we observed that efferocytosis by mouse resident peritoneal macrophages was blocked by inhibitors against the MERTK, MAPK/ERK kinase (MEK), AKT Ser/Thr kinase (AKT), focal adhesion kinase (FAK), or signal transducer and activator of transcription 6 (STAT6) pathway. Accordingly, apoptotic cells stimulated the phosphorylation of MERTK, ERK, AKT, FAK, and STAT6, but not of IκB or STAT5. A reconstituted efferocytosis system using MERTK- and TIM4-expressing NIH3T3-derived cells revealed that the juxtamembrane and C-terminal regions of MERTK have redundant roles in efferocytosis. The transformation of murine interleukin-3 (IL-3)-dependent Ba/F3 cells (a pro B cell line) with MERTK and TIM4 enabled them to proliferate in response to apoptotic cells in a PtdSer-dependent manner. This apoptotic cell–induced MERTK-mediated proliferation required both MERTK’s...
juxtamembrane and C-terminal regions, and was blocked by inhibitors of not only ERK, AKT, FAK, and STAT6, but also of NF-κB and STAT signaling. These results suggest that apoptotic cells stimulate distinct sets of signal transduction pathways via MERTK to induce either efferocytosis or proliferation.

Vast numbers of surplus or toxic cells are generated during animal development (1, 2). These cells undergo apoptosis, expose phosphatidylserine (PtdSer) on their surface as an “eat me” signal, and are cleared by phagocytes (3, 4). This process also occurs at the resolution phase of inflammation in adult tissues (5). In addition, large numbers of senescent cells such as aged neutrophils and red blood cells are cleared by macrophages in a PtdSer-dependent manner (3). The engulfment of apoptotic or senescent cells, called “efferocytosis (6)”, is essential for preventing these cells from undergoing secondary necrosis, which can cause cells to release their contents, thereby activating the immune system (3, 5, 7, 8).

The PtdSer exposed on apoptotic and senescent cells is recognized by soluble PtdSer-binding proteins such as Protein S (PROS), GAS6, and MFG-E8 (9-11), and by PtdSer receptors (for example, TIM1 and TIM4) expressed on phagocytes (12). PROS and GAS6 bind to PtdSer on apoptotic cells, and to TAM family receptor kinases (TYRO3, AXL, and MERTK) on macrophages, and act as a bridge between the apoptotic cells and phagocytes (13, 14). Macrophages are the most prominent phagocytes that perform efferocytosis, and express at least one of the TAM receptor kinases. We recently showed that a set of resident macrophages such as resident peritoneal macrophages (rpMac), Kupffer cells, and skin macrophages, express TIM4 and TAM kinase for efficient efferocytosis (15, 16). The extracellular region of TIM4 binds PtdSer with high affinity (12), and its cytoplasmic region is dispensable for efferocytosis (17). On the other hand, the affinity of PROS or GAS6 for PtdSer is weaker than that of TIM4 (16), but the TAM receptors’ cytoplasmic region is indispensable for efferocytosis (18). These results support a two-step model for efferocytosis (19), in which TIM4 mediates the tethering of the apoptotic cell to the macrophage, followed by TAM receptor-mediated internalization of the apoptotic cell.

TAM receptor kinases were originally identified as oncogenes expressed in various cancer cells, in particular, myeloid leukemia cells (14, 20). PROS or GAS6 induces the dimerization of TAM family receptors, which activates their kinase activity, followed by the phosphorylation of tyrosine residues in their cytoplasmic region (21). Many signaling molecules, such as ERK, p38 MAPK, FAK, AKT, NF-κB, and STAT6, have been identified as downstream components of MERTK in the MERTK-mediated growth promotion or chemoresistance of cancer cells (22-24). In contrast, the signaling cascade for TAM-mediated efferocytosis has been elusive.

In this report, we expressed MERTK and TIM4 in interleukin (IL)-3-dependent Ba/F3 cells, and found that these cells survived in the absence
of IL-3 in a PtdSer-dependent manner, and their growth was strongly enhanced by the presence of apoptotic cells. We then found that the efferocytosis with resident peritoneal macrophages was inhibited by inhibitors against MEK, AKT, FAK, or STAT6, but not against NF-κB or STAT5 pathway. On the other hand, the apoptotic cells-induced cell growth was efficiently blocked not only by inhibitors of MEK, AKT, FAK, or STAT6, but also by inhibitors against NF-κB or STAT5 pathways. Using NIH3T3-derived cell lines expressing TIM4 and MERTK mutants, we showed that MERTK’s membrane-proximal and C-terminal tail regions were not required for efferocytosis. While, the apoptotic cell-stimulated growth signaling required the membrane-proximal and C-terminal tail regions of MERTK in addition to its kinase domain. These results indicated that the apoptotic cells can stimulate the cell growth via MERTK, and overlapping distinct signaling molecules are involved in MERTK-mediated efferocytosis versus MERTK-mediated growth promotion.

Results

Apoptotic cell-activated cell proliferation

We previously showed that apoptotic cells are engulfed by MERTK- and TIM4-expressing macrophages or NIH3T3 cells (16). Since MERTK is known to mediate growth signaling (20), we examined the effect of TIM4 on MERTK-mediated cell growth using mouse IL-3-dependent Ba/F3 cells. The Ba/F3 cells were transformed with MERTK or TIM4 alone, or with both MERTK and TIM4, and cultured in RPMI containing 10% FCS and IL-3. The culture medium was then deprived of IL-3 overnight, and the transformants were kept in the medium lacking IL-3. As shown in Fig 1A, not only the parental and TIM4-expressing Ba/F3, but also the transformants expressing only MERTK died within 24 h, suggesting that the PROS in the 10% serum (about 30 nM) (9) was not sufficient to support the MERTK-mediated cell growth. On the other hand, about 60% of the cells expressing both TIM4 and MERTK (TIM4/MERTK) survived for 24 h, and this percentage did not change after 72 h, suggesting that the same number of cells died and proliferated.

The percentage of Trypan blue-positive dead cells in the culture of TIM4/MERTK transformants was about 70%, and the presence of D89E mutant of MFG-E8 that could mask PtdSer on apoptotic cells (11) dose-dependently inhibited the cell survival (Fig. 1A). These results suggested that PtdSer on the apoptotic cells generated by IL-3 deprivation supported the growth of the neighboring cells. To examine this possibility, the Ba/F3 cell transformants were co-cultured with FASL-treated apoptotic thymocytes. As shown in Fig. 1B, apoptotic thymocytes stimulated the growth of Ba/F3 cells expressing both TIM4 and MERTK, but not of the cells expressing one or the other. This effect of apoptotic cells on the Ba/F3 transformants was dose-dependent, and was reduced by masking PtdSer with D89E.

To further confirm that Ba/F3 cells expressing MERTK and TIM4 proliferated in response to
apoptotic cells, they were cultured for 72 h in the absence of IL-3, and pulsed for 4 h with Bromodeoxyuridine (BrdU). As shown in Fig. 1C, more than 50% Ba/F3 cells were labelled with BrdU in the presence of IL-3, while no BrdU-positive cells were observed in the absence of IL-3. On the other hand, about 0.8% of Ba/F3 cells expressing TIM4 and MERTK were labelled with BrdU in the absence of IL-3, and this percentage increased to 10.4% when the culture were supplemented with apoptotic thymocytes.

**Effect of signal transducer inhibitors on efferocytosis and cell proliferation**

Mouse rpMacs express both TIM4 and MERTK, and efficiently engulf apoptotic cells in a TIM4- and MERTK-dependent manner (15, 16). To analyze the signaling molecules required for efferocytosis, rpMacs were treated with various inhibitors against signal transducers, and then incubated with apoptotic thymocytes in the presence of the inhibitor. As shown in Fig. 2A, a low concentration of CH5451098, an inhibitor against MERTK and AXL (25), suppressed efferocytosis in a dose-dependent manner, confirming that MERTK is essential for the efferocytosis by rpMacs (15, 16). Among the signaling components that are reported to be activated by TAM kinase (20, 22), inhibitors against MEK (PD98059) (26), PI3K (LY294002) (27) for AKT pathway, FAK (PF-00562271) (28), or STAT6 pathway (AS1517499) (29) efficiently blocked efferocytosis. The concentrations of these inhibitors required to block efferocytosis were comparable to those reported to inhibit the signal transduction in cells. In contrast, CAPE and SH4-54, inhibitors against NF-κB (30) and STAT3/STAT5 (31), respectively, did not inhibit the efferocytosis by rpMacs at concentrations that should inhibit cellular signal transduction.

To examine which signal transducers were involved in the apoptotic cell-stimulated MERTK-mediated cell growth, 2.5 x 10⁶ Ba/F3 cell transformants expressing TIM4 and MERTK were cultured in the absence of IL-3 for 24 h with or without 2.5 x 10⁶ apoptotic thymocytes in the presence of specific inhibitors for signal transducers. As shown in Fig. 2B, the number of TIM4/MERTK-Ba/F3 cells decreased to 60-70% in the absence of apoptotic thymocytes, while this number remained almost unchanged in their presence. In accordance with the requirement for MERTK’s kinase activity, an inhibitor of MERTK’s kinase activity blocked the apoptotic cell-stimulated cell growth in a dose-dependent manner. The inhibitors against MEK, AKT, FAK, and STAT6 pathways that blocked efferocytosis (Fig. 2A) also inhibited the cell growth, although a higher concentration of the PI3K inhibitor was needed to inhibit the cell growth than efferocytosis. Notably, inhibitors against NF-κB or STAT5 also efficiently blocked the apoptotic cell-stimulated IL-3-independent growth of Ba/F3 cell transformants expressing TIM4 and MERTK.

**Phosphorylation of the signaling molecules activated by apoptotic cells**

MERTK is autophosphorylated at tyrosine...
residues when it is activated (32). To examine whether apoptotic cells induced the activation of MERTK, rpMacs from wild-type and MerTK−/− mice were incubated with or without a 10-times excess of apoptotic thymocytes for 10 min. The cell lysates were then immunoprecipitated with an anti-mouse MERTK Ab, and the precipitates were analyzed by western blotting. As shown in Fig. 3A, a band of about 200 kDa was detected with anti-MERTK in the wild-type (WT) but not the MerTK−/− (KO) rpMacs. Mouse MERTK is heavily glycosylated (15 N-glycosylation sites), which may explain why its apparent Mr was much greater than that calculated from its amino acid sequence (Mr. 110,155). Western blotting of the immunoprecipitates with an anti-phosphotyrosine mAb (clone 4G10) revealed the 200-kDa band in the rpMacs that had been treated with apoptotic cells. These results indicated that apoptotic cells activated the tyrosine kinase activity of MERTK in rpMacs.

The cell lysates from the apoptotic cell-treated rpMacs were then analyzed by western blotting using antibodies against the phosphorylated signal transducers. The anti-phospho-ERK1/2 recognizes the phosphorylated Thr202/Tyr204 of MAPK (ERK1/2) generated by MAPKK (33). Activated AKT is detected by an antibody that recognizes phosphorylated Ser473, which is phosphorylated by the mTOR-Rictor complex (34). FAK is a cytoplasmic tyrosine kinase, and is activated by integrin clustering, leading to its auto-phosphorylation at Tyr397 (35). STAT5 and STAT6 are transcription factors that are activated by various cytokines via JAK kinases, which phosphorylate Tyr694 of STAT5 and Tyr641 of STAT6 (36-38). Finally, IKK phosphorylates Ser32 of IκB, leading to activation of the transcription factor, NFκB (39). As shown in Fig. 3B, ERK1/2 phosphorylated at T202/Y204, AKT phosphorylated at S473, FAK phosphorylated at Y397, and STAT6 phosphorylated at Y641 were detected in the rpMacs incubated with apoptotic cells, but not in the untreated cells. None of the phosphorylated signaling molecules were detected in MerTK−/− rpMacs, indicating that their apoptotic cell-induced phosphorylation was MERTK-dependent. In contrast, neither IκB phosphorylated at S32 nor STAT5 phosphorylated at Y694 was detected in the apoptotic cell-treated rpMacs. These results indicated that apoptotic cells induced the activation of ERK1/2, AKT, FAK, and STAT6 in rpMacs, which agreed with our observation that inhibitors against these signaling pathways suppressed efferocytosis (Fig. 2).

Different MERTK cytoplasmic domains for efferocytosis and cell growth

The cytoplasmic region of mouse MERTK consists of 386 amino acids, of which 271 form a tyrosine kinase domain (UniProt: http://www.uniprot.org/uniprot/Q60805) (Fig. 4A). The lysine residue at position 614 of MERTK is involved in forming the cleft for ATP-binding, and is essential for the MERTK-kinase activity (40). To confirm that MERTK’s tyrosine-kinase activity was essential for MERTK-mediated efferocytosis, the lysine-614 was replaced by methionine.
(K614M) (Fig. 4A). The juxtamembrane region of tyrosine kinase receptors including MERTK and AXL is well conserved, and is involved in clustering anionic lipids, such as for signal transduction (41). Mouse MERTK carries two tyrosine residues (Y867 and Y924) at its C-terminal tail region (Fig. 4A), and the phosphorylated Y867 is reported to be involved in the activation of NF-κB and PI3 kinase (40). To examine the requirement of these regions for efferocytosis and cell growth, three mutants of MERTK lacking either the juxta-membrane (ΔN; amino acids 531-559) or the C-terminal tail region (ΔC; amino acids 853-994), or both the regions (ΔNΔC) were constructed.

We previously reported that Axl−/−Tyro3−/−Gas6−/− NIH3T3 (TKO) cells expressing TIM4 and MERTK behave like rpMacs, and efficiently engulf apoptotic cells (16). To examine the ability of MERTK mutants to engulf apoptotic cells, TKO cells expressing TIM4 were transformed with wild-type or mutant MERTK (Supplementary Fig. S1), and then subjected to efferocytosis assays. As reported previously (16), the TKO transformants expressing TIM4 and wild-type MERTK efficiently engulfed the apoptotic cells (Fig. 4B). In contrast, the K614M mutant completely abolished the cells’ ability to perform MERTK-mediated efferocytosis. On the other hand, although the ΔN and ΔC mutants supported efferocytosis as efficiently as the wild-type MERTK did, the efferocytosis activity of the ΔNΔC mutant carrying only the kinase domain in the cytoplasmic region (aa 582-852) (Fig. 4A) was significantly reduced (Fig. 4B), suggesting that either the N-terminal juxta-membrane region or the C-terminal tail region of MERTK was necessary for the full efferocytosis activity.

To examine which of the regions of MERTK was responsible for the apoptotic cell-stimulated cell proliferation, Ba/F3 cells expressing TIM4 were transformed with wild-type or mutant MERTK (Supplementary Fig. S2). As shown in Fig. 4C, in addition to the kinase-dead mutants (K614M mutants), the deletion mutant of the N-terminal juxta-membrane region (ΔN) completely lost the ability to support IL-3-independent Ba/F3 cell growth. The ability of the C-terminal deletion mutant (ΔC) of MERTK to support cell growth was reduced to about 20% of the ability of wild-type MERTK. These results indicated that, unlike the signal transduction for efferocytosis, not only the tyrosine kinase activity but also signals from the juxta-membrane region and C-terminal tail region of MERTK were required for the MERTK-supported cell growth.

**Activation of signaling molecules via the kinase domain of MERTK**

We next examined the effect of the MERTK mutations on its tyrosine kinase activity and on the activation of signaling molecules. As shown in Fig. 5A, the K614M mutant of MERTK was not auto-phosphorylated at tyrosine residues in TKO cells upon the addition of apoptotic cells, confirming that the mutant lost the kinase activity. On the other hand, the ΔN or ΔC mutant was tyrosine-phosphorylated at a similar efficiency as
wild-type MERTK by apoptotic cells, confirming that the tyrosine-kinase activity was not disrupted by the ΔN or ΔC mutation. Accordingly, the addition of apoptotic cells stimulated the phosphorylation of ERK1/2, AKT, and STAT6 in TKO cells expressing the wild-type but not the kinase-dead mutants MERTK (K614M) (Fig. 5B). The activated forms of the signaling molecules of ERK1/2 and AKT were observed in the apoptotic cell-treated cells expressing the ΔN, ΔC, or ΔNΔC mutant. In contrast, STAT6 phosphorylated at Tyr641 was observed in the cells expressing the ΔN or ΔC mutant of MERTK, but not in the cells expressing the ΔNΔC mutant, suggesting that in addition to the kinase activity of MERTK, a signal from the N-terminal juxtamembrane region or the C-terminal tail region of MERTK is required to activate STAT6. In contrast to ERK, AKT, and STAT6, FAK phosphorylated at Y397 was observed in the parental TKO cells without apoptotic-cell stimulation (data not shown), suggesting that FAK might have been activated through a pathway other than MERTK, such as the integrin system that was constitutively activated in TKO cells.

Discussion

Like other processes of programmed cell death, the removal of cell corpses was genetically studied in C. elegans, in which dying cells are engulfed by neighboring cells (42). That study identified three partially redundant signaling pathways, CED-1/CED-6/CED7/CED-10, CED-2/CED-5/CED-12/CED-10, and ABL-1/API-1/CED-10 (43). Like mammalian cells, PtdSer is exposed on the surface of dying cells in C. elegans by the caspase (CED-3)-dependent phospholipid scramblase (CED-8), an ortholog of mammalian XKR8 (44, 45). CED-1 appears to indirectly bind PtdSer via a soluble bridging protein called TTR-52 (46). However, how CED-1 activates downstream signaling molecules has not been elucidated. A pathway consisting of CED12/ELMO and CED10/RAC was reported to elicit efferocytosis in experiments using Chinese hamster ovary (CHO) cell lines (47). In addition, BAI1 (Brain-specific angiogenesis inhibitor 1), an adhesion GPCR family receptor, was proposed to activate the ELMO-RAC pathway for efferocytosis in this CHO system. However, whether any macrophages use this system for efferocytosis is not clear.

We recently reported that a set of resident mouse macrophages uses MERTK and TIM4 to elicit efficient efferocytosis (16). Roles of MERTK-activated Phospholipase C γ2 and FAK in efferocytosis were previously reported using the J774 mouse macrophage cell line or HEK293T cells that were transiently transfected with MERTK cDNA (48-50). MERTK is known to activate many other signaling molecules, and we found here that at least MEK/ERK, AKT, FAK, and STAT6 pathways were necessary for efficient efferocytosis. Efferocytosis takes place at lamellipodia of the engulfing cells, where activated RAC1 is recruited to form a phagocytic cup with polymerized actin patches to encapsulate apoptotic cells (51). When the apoptotic cells are
internalized, the phagocytic cup closes, accompanied by the de-polymerization of actin bundles. This elaborate process of efferocytosis has features in common with cell motility (52). Cell motility consists of multiple processes, including cell protrusion, cell retraction, adhesion, and vesicle exocytosis, and is regulated by multiple signaling molecules (53). For example, downstream of FAK, ERK localizes to lamellipodia and phosphorylates MLCK (myosin light chain kinase) to regulate the turnover of focal adhesions (54). Meanwhile, ERK phosphorylates Paxillin, and phosphorylated Paxillin serves as a scaffold for FAK to activate PI3K for RAC activation (55). STATs are transcription factors, and are activated by various cytokines via JAK kinases (56). However, recent studies indicate that some STAT members also act outside the nucleus, such as at mitochondria and focal adhesions (56-58). Thus, it is likely that interplay occurs among these kinases (ERK, FAK, AKT, STAT6, and probably more) to regulate RAC1 to polymerize or de-polymerize actin for efferocytosis. Using specific inhibitors for these kinases, it should be possible to dissect this process, to study each step in more detail.

Here, we found that the apoptotic cells stimulate the proliferation of the cells expressing MERTK and TIM4. This growth-promoting activity was fully dependent on PtdSer. The MERTK-mediated efferocytosis is known to produces TGFβ to stimulate the cell proliferation (59). Although this possibility cannot be ruled out, considering the oncogenic property of MERTK (14, 20), we prefer that the apoptotic cells directly activates MERTK for the cell proliferation. As shown for the PtdSer-dependent efferocytosis (15, 16), it is likely that the PtdSer-exposing apoptotic cells are recruited to MERTK-expressing cells by TIM4 for the MERTK-mediated cell growth. The apoptotic cells are then cross-linked with MERTK on the responder cells via an interaction between PtdSer and PROS, and activate the MERTK to promote cell growth. Notably, TIM4 is expressed together with MERTK or AXL in various tumors such as histiocyte sarcoma, histiocytic and dendritic cell neoplasms, lung cancer, and glioma (60-62), and it has been proposed to contribute to tumorigenicity. It will be interesting to examine whether these primary tumor cells respond to apoptotic cells for their growth.

Many groups have studied the signal transduction pathway for MERTK-mediated cell growth, and reported that not only the kinase domain, but also the juxtamembrane and C-terminal tail regions are necessary to mediate the signal (20). The tyrosine residues in these regions are auto-phosphorylated, and phosphorylated tyrosines serve as binding sites for signaling molecules. In fact, we found that deleting either the juxtamembrane or the C-terminal tail region of MERTK severely reduced its growth-promoting activity. This was in sharp contrast to the effect of these mutations on efferocytosis. In addition to the signaling molecules required for efferocytosis, signals from the NF-κB and STAT6 pathways were required for MERTK-mediated cell growth, suggesting that the juxtamembrane region and C-
terminal tail regions are responsible for these signaling pathways. It is also possible that signaling through the MEK, AKT, FAK, and STAT6 pathways has different roles between growth promotion and efferocytosis. Since MERTK is strongly expressed in various tumor cells, inhibitors against MERTK are considered to be anti-tumor agents (63-65). However, these inhibitors would also block MERTK-mediated efferocytosis, which could lead to autoimmune disease (3, 66). Our results indicating that MERTK-mediated cell growth requires additional signaling pathways may be useful for developing safer and more useful reagents for cancer therapy.

**Experimental procedures**

**Mice, cell lines, recombinant proteins, antibodies, and reagents**

C57BL/6J mice were purchased from Japan SLC and CLEA Japan. MerTK−/− mice were from the Jackson Laboratory. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan.

Mouse interleukin-3 (IL-3)-dependent Ba/F3 cells were maintained in RPMI1640 containing 10% fetal bovine serum (FBS) and 45 units/mL IL-3 as described previously (67). Axl−/−Tyro3−/− Gas6−/− (TKO) NIH3T3 cells were described previously (16). TKO NIH3T3 cells and Plat-E cells(68) were maintained in DMEM containing 10% FBS.

Recombinant Leucine-zippered human Fas ligand (FASL) was produced in COS7 cells and purified as described (69). In brief, COS7 cells were transfected with a FASL expression plasmid by electroporation, and cultured in DMEM containing 1% FBS for 48 h. The supernatant was subjected to (NH₄)₂SO₄ precipitation at 60% saturation, and dialyzed against PBS. The hamster anti-TIM4 Ab (clone Kat5-18) was described previously (12). Other antibodies and reagents were as follows: biotinylated goat anti-mouse MERTK, R&D Systems; rabbit anti-ERK1/2 and anti-phospho-ERK1/2 (Thr202/Tyr204), anti-p38 and anti-phospho-p38 (Thr180/Tyr182), anti-AKT and anti-phospho-AKT (Ser473), anti-FAK and anti-phospho-FAK (Tyr397), anti-IkB and phospho-IkB (Ser32), anti-STAT5 and anti-phospho-STAT5 (Tyr694), anti-STAT6 and anti-phospho-STAT6 (Tyr641), Cell Signaling; HRP-conjugated mouse anti-phosphotyrosine (4G10), Merck Millipore; HRP-mouse anti-Flag, Sigma-Aldrich. pHrodo Red succinimidyl ester (pHrodo); Life Technologies. The following chemicals were used to inhibit signaling pathways: PD98059 (MEK), PF-00562271 (FAK), Caffeic acid phenethyl ester (CAPE) (NF-κB), and SH4-54 (STAT5) from Selleck Chemicals; LY294002 (PI3K) from Cell Signaling; AS1517499 (STAT6) from Sigma-Aldrich; and CH5451098 (MERTK and AXL) from Chugai Pharmaceutical Co. Ltd.

**Transformation**

Lentiviral expression vectors (CSII-EF, pCAG-HIVgp, pENV-IRES-puro, and pRSV-Rev) were from H. Miyoshi, Riken Resource Center. Mouse MERTK cDNA (NM_008587.1) was described
previously (15). Deletion mutants of MERTK, ΔN (Δ531-559), AC (Δ853-894), and ΔNAC (Δ531-559 and Δ853-894), were constructed using In-Fusion HD Cloning Kits (Takara Clontech). The kinase dead K614M mutant (40) was prepared by recombinant PCR (70). MERTK and its mutants were Flag-tagged at the C terminus and inserted into the CSII-EF vector. pMxs-puro-TIM4 and pNEF-BOS-EX-TIM4 were described previously (12, 15).

The Ba/F3 transformants expressing MERTK and TIM4 (15), and TKO transformants expressing MERTK and TIM4 (16) were described previously. MERTK mutants were expressed in Ba/F3-TIM4, or TKO-TIM4 cells using a lentiviral vector system. Briefly, HEK293T cells were co-transfected using Fugene 6 (Promega) with the CSII-EF vector carrying cDNA for MERTK or its mutants, pCAG-HIVgp, pENV-IRES-puro, and pRSV-Rev. After culturing for 48 h, viruses in the supernatant were used to infect TKO cells. For the infection of Ba/F3 cells, viruses in the supernatant were concentrated by centrifugation at 6000 x g for 16 h at 4°C and used for spin-infection. Transformants were stained with anti-MERTK, and if necessary, sorted using a FACSAria II (BD Biosciences).

**Efferocytosis assay**

Efferocytosis was assayed as described previously (15, 16) with rpMacs or TKO cells as the phagocytes, and with apoptotic thymocytes as the prey. In brief, thymocytes from 4-week-old mice were treated at 37°C for 1.5 h with 100 units/mL FASL, and washed with PBS. The cells were stained with 0.1 μg/mL pHrodo for 30 min at room temperature and washed with DMEM containing 10% FBS. For efferocytosis with rpMacs, peritoneal cells were isolated from 8-10-week old mice, and seeded at 5 x 10^5 cells on a 12-well plate. After incubation for 2 h at 37°C in DMEM containing 10% FBS, the cells were washed with PBS to remove non-adherent cells, and subjected to the efferocytosis assay with 2.5 x 10^6 pHrodo-labeled apoptotic thymocytes. To examine the effect of inhibitors, rpMacs were pre-incubated with the inhibitor for 30 min, and then the efferocytosis assay was performed at 37°C for 60 min in the presence of the inhibitor. For the efferocytosis assay with TKO cells, 6 x 10^4 TKO cells were seeded in a 24-well plate, and cultured for 24 h at 37°C. The pHrodo-labeled apoptotic thymocytes (6 x 10^5) were added to TKO cells, and the mixture was incubated at 37°C for 60 min in DMEM containing 10% FBS. After incubation, the TKO cells were washed with PBS, detached with trypsin, stained with 0.5 μM Sytox blue (Life Technologies) in 20 mM CHES buffer (pH 9.0) containing 150 mM NaCl, and analyzed by flow cytometry with a FACSCanto II (BD Biosciences).

**Immunoprecipitation and western blotting**

TKO transformants (1 x 10^6 cells) in a 6-cm plate were incubated at 37°C for 6 h in DMEM containing 10% FBS, and starved overnight in serum-free DMEM. The cells were then incubated with 1 x 10^7 apoptotic thymocytes in 2 mL of DMEM containing 1 μg/mL PROS for 10 min at
37°C, washed with cold PBS, and lysed in 1 mL of lysis buffer [25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 5% glycerol, a cocktail of protease inhibitors (complete mini EDTA-free, Roche), and a cocktail of phosphatase inhibitors (PhosSTOP, Roche)]. After centrifugation at 15,000 rpm for 10 min at 4°C, the supernatants were mixed with Protein G Dynabeads (Life Technologies) (10 µL/sample) to which the goat anti-MERTK Ab had been conjugated by incubating for 3 h at 4°C in TBS-T [25 mM Tris-HCl buffer (pH 7.5), 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20]. The mixture was rotated for 3 h at 4°C, washed with lysis buffer, suspended in 30 µL of sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, 2% β-mercaptoethanol), and incubated at 95°C for 5 min. After removing the beads, the eluates were subjected to western blotting.

For western blotting, the samples were separated by 7.5% or 10% SDS-PAGE, and transferred to a PVDF membrane (Merck Millipore). After incubation for 1 h at room temperature in blocking buffer consisting of TBS-T and 5% skim milk or 5% BSA (Probumin, Merck Millipore), the membranes were incubated overnight at 4°C with the primary antibody in the blocking buffer. The membranes were then incubated for 1 h at room temperature with the secondary antibody, and signals were detected with Immobilon Western Chemiluminescent HRP substrate (Merck Millipore).

**Proliferation assay**

Ba/F3 cells (5 x 10⁶ cells) were incubated at 37°C overnight in 10 mL of RPMI1640 containing 10% FBS and 45 units/ml mouse IL-3, washed with RPMI1640 containing 10% FBS, and cultured overnight in RPMI1640 containing 10% FBS. The cells were then cultured at 2.5 x 10⁵ cells/mL in 24-well plates with 2.5 x 10⁶ apoptotic thymocytes in RPMI1640 containing 10% FBS, and counted with a hemocytometer after staining with trypan blue. The DNA synthesis was assayed by incorporation of BrdU. In brief, Ba/F3 cells were cultured for 72 h in the absence of IL-3, pulsed for 4 h with 10 µM BrdU (Sigma-Aldrich), and fixed at room temperature for 20 min with 1% PFA. After permeabilization with 0.3% saponin, the cells were treated at 37°C for 1 h with 300 µg/ml DNase I, and stained with anti-BrdU (Abcom) followed by incubation with Alexa fluor 488-anti-rat IgG (Molecular probe).

**Statistical analysis**

All data were expressed as the mean with SD. Differences between groups were examined for statistical significance using Student’s *t*-test.

Author contributions-------C.N. and S.N. conceived the project and designed experiments; C.N. carried out experiments; C.N., Y.Y., K.S., and S.N. analyzed the data; C.N. and S.N. wrote the manuscript.
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Conflict of interests------ The authors declare that they have no conflict of interests.

Abbreviations used are: PtdSer, phosphatidylserine; MFG-E8, Milk Fat Globule-EGF Factor 8; IL, interleukin; rpMacs, resident peritoneal macrophages; PROS, Protein S.

References
1. Fuchs, Y., and Steller, H. (2011) Programmed cell death in animal development and disease. Cell 147, 742-758
2. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell death in development. Cell 96, 245-254
3. Nagata, S. (2018) Apoptosis and the clearance of apoptotic cells. Annu. Rev. Immunol. 36, 489-517
4. Birge, R. B., Boeltz, S., Kumar, S., Carlson, J., Wanderley, J., Calianese, D., Barcinski, M., Brekken, R. A., Huang, X., Hutchins, J. T., Freimark, B., Empig, C., Mercer, J., Schroit, A. J., Schett, G., and Herrmann, M. (2016) Phosphatidylserine is a global immunosuppressive signal in efferocytosis, infectious disease, and cancer. Cell Death Differ. 23, 962-978
5. Arandjelovic, S., and Ravichandran, K. S. (2015) Phagocytosis of apoptotic cells in homeostasis. Nat. Immunol. 16, 907-917
6. deCathelineau, A. M., and Henson, P. M. (2003) The final step in programmed cell death: phagocytes carry apoptotic cells to the grave. Essays Biochem. 39, 105-117
7. Muñoz, L. E., Lauber, K., Schiller, M., Manfredi, A. A., and Herrmann, M. (2010) The role of defective clearance of apoptotic cells in systemic autoimmunity. Nat. Rev. Rheumatol. 6, 280-289
8. Kawano, M., and Nagata, S. (2018) Lupus-like autoimmune disease caused by a lack of Xkr8, a caspase-dependent phospholipid scramblase. Proc. Natl. Acad. Sci. USA 280, 2132-2137
9. Hafizi, S., and Dahlbäck, B. (2006) Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J. 273, 5231-5244
10. Nakano, T., Ishimoto, Y., Kishino, J., Umeda, M., Inoue, K., Nagata, K., Ohashi, K., Mizuno, K., and Arita, H. (1997) Cell adhesion to phosphatidylserine mediated by a product of growth arrest-specific
11. Hanayama, R., Tanaka, M., Miwa, K., Shinohara, A., Iwamatsu, A., and Nagata, S. (2002) Identification of a factor that links apoptotic cells to phagocytes. Nature 417, 182-187
12. Miyanishi, M., Tada, K., Koike, M., Uchiyama, Y., Kitamura, T., and Nagata, S. (2007) Identification of TIM4 as a phosphatidylserine receptor. Nature 450, 435-439
13. Lemke, G. (2017) Phosphatidylserine Is the Signal for TAM Receptors and Their Ligands. Trends Biochem. Sci. 42, 738-748
14. Rothlin, C. V., Carrera-Silva, E. A., Bosurgi, L., and Ghosh, S. (2014) TAM Receptor Signaling in Immune Homeostasis. Annu. Rev. Immunol. 33, 355-391
15. Nishi, C., Toda, S., Segawa, K., and Nagata, S. (2014) TIM4- and MerTK-mediated engulfment of apoptotic cells by mouse resident peritoneal macrophages. Mol. Cell. Biol. 34, 1512-1520
16. Yanagihashi, Y., Segawa, K., Maeda, R., Nabeshima, Y.-i., and Nagata, S. (2017) Mouse macrophages show different requirements for phosphatidylserine receptor TIM4 in efferocytosis. Proc. Nat. Acad. Sci. USA 114, 8800-8805
17. Park, D., Hochreiter-Hufford, A., and Ravichandran, K. (2009) The phosphatidylserine receptor TIM-4 does not mediate direct signaling. Curr. Biol. 19, 346-351
18. Scott, R. S., McMahon, E. J., Pop, S. M., Reap, E. A., Caricchio, R., Cohen, P. L., Earp, H. S., and Matsushima, G. K. (2001) Phagocytosis and clearance of apoptotic cells is mediated by MER. Nature 411, 207-211
19. Hoffmann, P. R., deCathelineau, A. M., Ogden, C. A., Leverrier, Y., Bratton, D. L., Daleke, D. L., Ridley, A. J., Fadok, V. A., and Henson, P. M. (2001) Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells. J. Cell Biol. 155, 649-659
20. Graham, D. K., DeRyckere, D., Davies, K. D., and Earp, H. S. (2014) The TAM family: phosphatidylserine- sensing receptor tyrosine kinases gone awry in cancer. Nat. Rev. Cancer 14, 769-785
21. Lemke, G. (2013) Biology of the TAM receptors. CSH Perspect. Biol. 5, a009076
22. Cummings, C. T., DeRyckere, D., Earp, H. S., and Graham, D. K. (2013) Molecular pathways: MERTK signaling in cancer. Clin. Cancer Res. 19, 5275-5280
23. Kasikara, C., Kumar, S., Kimani, S., Tsou, W.-I., Geng, K., Davra, V., Sriram, G., Devoe, C., Nguyen, K.-Q. N., Antes, A., Krantz, A., Rymarczyk, G., Wilczynski, A., Empig, C., Freimark, B., Gray, M., Schlunegger, K., Hutchins, J., Kotenko, S. V., and Birge, R. B. (2017) Phosphatidylserine sensing by TAM receptors regulates AKT-dependent chemoresistance and PD-L1 expression. Mol. Cancer Res. 15, 753-764
24. Schoumacher, M., and Burbridge, M. (2017) Key Roles of AXL and MER Receptor Tyrosine Kinases
in Resistance to Multiple Anticancer Therapies. *Curr. Oncol. Rep.* 19, 1-14
25. Pettazzoni, P., Viale, A., Shah, P., Carugo, A., Ying, H., Wang, H., Genovese, G., Seth, S., Minelli, R., Green, T., Huang-Hobbs, E., Corti, D., Sanchez, N., Nezi, L., Marchesini, M., Kapoor, A., Yao, W., Francesco, M. E. D., Petrocchi, A., Deem, A. K., Scott, K., Colla, S., Mills, G. B., Fleming, J. B., Hefferman, T. P., Jones, P., Toniatti, C., DePinho, R. A., and Draetta, G. F. (2015) Genetic events that limit the efficacy of MEK and RTK inhibitor therapies in a mouse model of KRAS-driven pancreatic cancer. *Cancer Res.* 75, 1091-1101
26. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J. Biol. Chem.* 270, 27489-27494
27. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* 269, 5241-5248
28. Roberts, W. G., Ung, E., Whalen, P., Cooper, B., Hulford, C., Autry, C., Richter, D., Emerson, E., Lin, J., Kath, J., Coleman, K., Yao, L., Martinez-Alsina, L., Lorenzen, M., Berliner, M., Luzzio, M., Patel, N., Schmitt, E., LaGreca, S., Jani, J., Wessel, M., Marr, E., Griffr, M., and Vajdos, F. (2008) Antitumor activity and pharmacology of a selective focal adhesion kinase inhibitor, PF-562,271. *Cancer Res.* 68, 1935-1944
29. Nagashima, S., Yokota, M., Nakai, E.-i., Kuromitsu, S., Ohga, K., Takeuchi, M., Tsukamoto, S.-i., and Ohta, M. (2007) Synthesis and evaluation of 2-\{(2-(4-hydroxyphenyl)-ethyl)amino\}pyrimidine-5-carboxamide derivatives as novel STAT6 inhibitors. *Bioorg. Med. Chem.* 15, 1044-1055
30. Natarajan, K., Singh, S., Burke, T. R., Grunberger, D., and Aggarwal, B. B. (1996) Caffeic acid phenethyl ester is a potent and specific inhibitor of activation of nuclear transcription factor NF-kappa B. *Proc. Natl. Acad. Sci. USA* 93, 9090-9095
31. Haftchenary, S., Luchman, H. A., Jouk, A. O., Veloso, A. J., Page, B. D. G., Cheng, X. R., Dawson, S. S., Grinshtein, N., Shahani, V. M., Kerman, K., Kaplan, D. R., Griffin, C., Aman, A. M., Al-awar, R., Weiss, S., and Gunning, P. T. (2013) Potent Targeting of the STAT3 Protein in Brain Cancer Stem Cells: A Promising Route for Treating Glioblastoma. *ACS Med. Chem. Lett.* 4, 1102-1107
32. Ling, L., Templeton, D., and Kung, H. J. (1996) Identification of the major autophosphorylation sites of Nyk/Mer, an NCAM-related receptor tyrosine kinase. *J. Biol. Chem.* 271, 18355-18362
33. Nishimoto, S., and Nishida, E. (2006) MAPK signalling: ERK5 versus ERK1/2. *EMBO Rep.* 7, 782-786
34. Hresko, R. C., and Mueckler, M. (2005) mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* 280, 40406-40416
35. Sulzmaier, F. J., Jean, C., and Schlaepfer, D. D. (2014) FAK in cancer: mechanistic findings and clinical applications. Nat. Rev. Cancer. 14, 598-610
36. Gouilleux, F., Wakao, H., Mundt, M., and Groner, B. (1994) Prolactin induces phosphorylation of Tyr694 of Stat5 (MGF), a prerequisite for DNA binding and induction of transcription. EMBO J. 13, 4361-4369
37. Reich, N. C. (2014) STATs get their move on. JAK-STAT 2, e27080-27010
38. Mikita, T., Campbell, D., Wu, P., Williamson, K., and Schindler, U. (1996) Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. Mol. Cell. Biol. 16, 5811-5820
39. Hinz, M., and Scheidereit, C. (2013) The IkB kinase complex in NF-κB regulation and beyond. EMBO Rep. 15, 46-61
40. Georgescu, M. M., Kirsch, K. H., Shishido, T., Zong, C., and Hanafusa, H. (1999) Biological effects of c-Mer receptor tyrosine kinase in hematopoietic cells depend on the Grb2 binding site in the receptor and activation of NF-kappaB. Mol. Cell. Biol. 19, 1171-1181
41. Hedger, G., Sansom, M. S. P., and Koldso, H. (2015) The juxtamembrane regions of human receptor tyrosine kinases exhibit conserved interaction sites with anionic lipids. Sci. Rep. 5, 9198
42. Reddien, P. W., and Horvitz, H. R. (2004) The engulfment process of programmed cell death in caenorhabditis elegans. Annu. Rev. Cell Dev. Biol. 20, 193-221
43. Pinto, S. M., and Hengartner, M. O. (2012) Cleaning up the mess: cell corpse clearance in Caenorhabditis elegans. Curr. Opin. Cell Biol. 24, 881-888
44. Suzuki, J., Denning, D. P., Imanishi, E., Horvitz, H. R., and Nagata, S. (2013) Xk-related protein 8 and CED-8 promote phosphatidylserine exposure in apoptotic cells. Science 341, 403-406
45. Chen, Y.-Z., Mapes, J., Lee, E.-S., Skeen-Gaar, R. R., and Xue, D. (2013) Caspase-mediated activation of Caenorhabditis elegans CED-8 promotes apoptosis and phosphatidylserine externalization. Nat. Commun. 4, 1-9
46. Kang, Y., Zhao, D., Liang, H., Liu, B., Zhang, Y., Liu, Q., Wang, X., and Liu, Y. (2012) Structural study of TTR-52 reveals the mechanism by which a bridging molecule mediates apoptotic cell engulfment. Genes & Develop. 26, 1339-1350
47. Gumienny, T. L., Brugnera, E., Tosello-Trampont, A. C., Kinchen, J. M., Haney, L. B., Nishiwaki, K., Walk, S. F., Nemergut, M. E., Macara, I. G., Francis, R., Schedl, T., Qin, Y., Van Aelst, L., Hengartner, M. O., and Ravichandran, K. S. (2001) CED-12/ELMO, a novel member of the CrkII/Dock180/Rac pathway, is required for phagocytosis and cell migration. Cell 107, 27-41
48. Todt, J. C. (2004) The receptor tyrosine kinase MerTK activates phospholipase C during recognition of apoptotic thymocytes by murine macrophages. J. Leuk. Biol. 75, 705-713
49. Tibrewal, N., Wu, Y., D’apoz;ello, V., Akakura, R., George, T. C., Varnum, B., and Birge, R. B. (2008) Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and down-modulation of lipopolysaccharide-inducible NF-kappaB transcriptional activation. *J. Biol. Chem.* 283, 3618-3627

50. Wu, Y., Singh, S., Georgescu, M.-M., and Birge, R. B. (2005) A role for Mer tyrosine kinase in alphavbeta5 integrin-mediated phagocytosis of apoptotic cells. *J. Cell Sci.* 118, 539-553

51. Nakaya, M., Kitano, M., Matsuda, M., and Nagata, S. (2008) Spatiotemporal activation of Rac1 for engulfment of apoptotic cells. *Proc. Natl. Acad. Sci. USA* 105, 9198-9203

52. Elliott, M. R., and Ravichandran, K. S. (2016) The dynamics of apoptotic cell clearance. *Dev. Cell* 38, 147-160

53. Tanimura, S., and Takeda, K. (2017) ERK signalling as a regulator of cell motility. *J. Biochem.* 162, 145-154

54. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F. (2004) FAK–Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat. Cell Biol.* 6, 154-161

55. Ishibe, S., Joly, D., Liu, Z.-X., and Cantley, L. G. (2004) Paxillin Serves as an ERK-Regulated Scaffold for Coordinating FAK and Rac Activation in Epithelial Morphogenesis. *Mol. Cell* 16, 257-267

56. Stark, G. R., and Darnell Jr, J. E. (2012) The JAK-STAT Pathway at Twenty. *Immunity* 36, 503-514

57. Sehgal, P. B. (2014) Non-genomic STAT5-dependent effects at the endoplasmic reticulum and Golgi apparatus and STAT6-GFP in mitochondria. *JAK-STAT* 2, e24860-24810

58. Silver, D. L., Naora, H., Liu, J., Cheng, W., and Montell, D. J. (2004) Activated signal transducer and activator of transcription (STAT) 3: localization in focal adhesions and function in ovarian cancer cell motility. *Cancer Res.* 64, 3550-3558

59. Stanford, J. C., Young, C., Hicks, D., Owens, P., Williams, A., Vaught, D. B., Morrison, M. M., Lim, J., Williams, M., Brantley-Sieders, D. M., Balko, J. M., Tonetti, D., Earp III, H. S., and Cook, R. S. (2014) Efferocytosis produces a prometastatic landscape during postpartum mammary gland involution. *J Clin Invest* 124, 4737-4752

60. Dorfman, D. M., Hornick, J. L., Shahsafaei, A., and Freeman, G. J. (2010) The phosphatidylserine receptors, T cell immunoglobulin mucin proteins 3 and 4, are markers of histiocytic sarcoma and other histiocytic and dendritic cell neoplasms. *Hum. Pathol.* 41, 1486-1494

61. Zhang, Q., Wang, H., Wu, X., Liu, B., Liu, W., Wang, R., Liang, X., Ma, C., and Gao, L. (2015) TIM-4 promotes the growth of non-small-cell lung cancer in a RGD motif-dependent manner. *Br. J. Cancer* 113, 1484-1492
62. Xu, L., Xiao, H., Xu, M., Zhou, C., Yi, L., and Liang, H. (2011) Glioma-derived T Cell Immunoglobulin- and Mucin Domain-containing Molecule-4 (TIM4) Contributes to Tumor Tolerance. *J. Biol. Chem.* 286, 36694-36699

63. Schlegel, J., Sambade, M. J., Sather, S., Moschos, S. J., Tan, A.-C., Winges, A., DeRyckere, D., Carson, C. C., Trembath, D. G., Tentler, J. J., Eckhardt, S. G., Kuan, P.-F., Hamilton, R. L., Duncan, L. M., Miller, C. R., Nikolaishvili-Feinberg, N., Midkiff, B. R., Liu, J., Zhang, W., Yang, C., Wang, X., Frye, S. V., Earp, H. S., Shields, J. M., and Graham, D. K. (2013) MERTK receptor tyrosine kinase is a therapeutic target in melanoma. *J. Clin. Invest.* 123, 2257-2267

64. McIver, A. L., Zhang, W., Liu, Q., Jiang, X., Stashko, M. A., Nichols, J., Miley, M. J., Norris-Drouin, J., Machius, M., DeRyckere, D., Wood, E., Graham, D. K., Earp, H. S., Kireev, D., Frye, S. V., and Wang, X. (2017) Discovery of Macrocyclic Pyrimidines as MerTK-Specific Inhibitors. *ChemMedChem* 12, 207-213

65. Cummings, C. T., Zhang, W., Davies, K. D., Kirkpatrick, G. D., Zhang, D., DeRyckere, D., Wang, X., Frye, S. V., Earp, H. S., and Graham, D. K. (2015) Small Molecule Inhibition of MERTK Is Efficacious in Non-Small Cell Lung Cancer Models Independent of Driver Oncogene Status. *Mol. Cancer Ther.* 14, 2014-2022

66. Cohen, P. L., Caricchio, R., Abraham, V., Camenisch, T. D., Jennette, J. C., Roubey, R. A. S., Earp, H. S., Matsushima, G., and Reap, E. A. (2002) Delayed apoptotic cell clearance and lupus-like autoimmunity in mice lacking the c-mer membrane tyrosine kinase. *J. Exp. Med.* 196, 135-140

67. Toda, S., Hanayama, R., and Nagata, S. (2012) Two-step engulfment of apoptotic cells. *Mol. Cell. Biol.* 32, 118-125

68. Morita, S., Kojima, T., and Kitamura, T. (2000) Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther.* 7, 1063-1066

69. Shiraishi, T., Suzuyama, K., Okamoto, H., Mineta, T., Tabuchi, K., Nakayama, K., Shimizu, Y., Tohma, J., Ogihara, T., Naba, H., Mochizuki, H., and Nagata, S. (2004) Increased cytotoxicity of soluble Fas ligand by fusing isoleucine zipper motif. *Biochem. Biophys. Res. Commun.* 322, 197-202

70. Higuchi, R. (1990) Recombinant PCR. in *PCR protocols: A guide to methods and applications*, Academic Press, San Diego. pp 177-188
Figure 1. PtdSer-dependent cell proliferation. A, PtdSer-dependent growth stimulation of TIM4- and MERTK-expressing Ba/F3. Ba/F3 cells (2.5 x 10^5) expressing TIM4, MERTK, or both TIM4 and MERTK were cultured in 0.5 mL of RPMI1640 containing 10% FBS. After incubation for the indicated periods, the number of viable cells was counted after staining with trypan blue. At right, Ba/F3 cells expressing TIM4 and MERTK were cultured for 72 h in the medium containing the indicated concentration of D89E, and the number of viable cells was expressed as the percentage of that in the absence of D89E. The experiments were carried out 3 times, and average values were plotted with SD (bars). The values were statistically analyzed by Student’s t-test. **P < 0.01, ***P < 0.001. B, The apoptotic cells-stimulated PtdSer-dependent cell growth. Ba/F3 cells (2.5 x 10^5) expressing TIM4, MERTK, or both TIM4 and MERTK were cultured in 0.5 mL of the medium for the indicated periods in the presence of 2.5 x 10^6 apoptotic thymocytes (Left). In Center and Right, Ba/F3 cells (2.5 x 10^5) expressing both TIM4 and MERTK were cultured in 0.5 mL of the medium for 72 h in the presence of the indicated concentration of apoptotic thymocytes (Center) or in the presence of 6.25 x 10^5 apoptotic thymocytes and the indicated concentration of D89E. After incubation, the trypan blue-negative viable cells were counted. At Right, the number of viable cells was expressed as the percentage of that in the absence of D89E. The experiments were carried in triplicate, and average values were plotted with SD (bars). *P < 0.01, **P < 0.001; Student’s t-test. C, DNA synthesis of Ba/F3 cells expressing TIM4 and MERTK without IL-3. 2.5 x 10^5 Ba/F3 cells (a, d and e) and Ba/F3 expressing MERTK and TIM4 (b and c) were cultured at 37°C for 72 h without (a, b and c) or with (d and e) IL-3 in the absence (a, b, d and e) or presence (c) of 2.5 x 10^6 apoptotic thymocytes. The culture was supplemented with (a, b, c and d) or without (e) 10 μM BrdU, and further incubated for 4 h. The incorporated BrdU was then detected with FITC-labelled anti-BrdU.
Figure 2. Effect of various inhibitors on MERTK-mediated efferocytosis and cell growth. A, Effect of various inhibitors on efferocytosis by rpMacs. Resident peritoneal cells were pre-treated with the indicated concentration of each inhibitor, and subjected to an efferocytosis assay with pHrodo-labeled apoptotic thymocytes in the presence of the inhibitor. Efferocytosis (the percentage of pHrodo-positive cells) was determined by flow cytometry, and is expressed relative to that observed without inhibitors. The experiment was done in triplicate, and the average values are plotted with SD (bar). The values were statistically analyzed by Student’s t-test against the value without inhibitor. # $P < 0.01$, ## $P < 0.001$. B, Effect of various inhibitors on the apoptotic cell-promoted cell proliferation. Apoptotic thymocytes were added to Ba/F3 cells expressing wild-type MERTK and TIM4, and the mixture was incubated in the presence of the indicated concentrations of the inhibitor. After incubating for 24 h, the number of viable Ba/F3 cells was counted. The experiments were carried out three times, and the average values were plotted with SD (bars). ** $P < 0.01$, *** $P < 0.001$; Student’s t-test against the value without inhibitor. The cell number is expressed relative to that observed in the presence of apoptotic cells without inhibitors. The cell number in the absence of inhibitors was 3-5 x 10⁵ cells/mL.
Figure 3. Activation of signaling molecules induced by apoptotic cells. Adherent resident peritoneal cells (2.0 x 10^6 cells) from wild-type (WT) or MerTK^-/- (KO) mice were incubated at 37°C for 10 min with (+) or without (-) 1.0 x 10^7 apoptotic thymocytes, washed with PBS, and lysed in lysis buffer. A, MERTK was immunoprecipitated with anti-MERTK, dissolved in SDS-sample buffer, and one quarter of aliquots were analyzed by western blotting with an HRP-anti-phosphotyrosine mAb (upper panel), or a biotinylated anti-MERTK Ab followed by incubation with HRP-streptavidin (middle panel). In bottom, the membrane was stained with Coomassie Brilliant Blue (CBB). B, The cell lysates from 1.5 x 10^6 cells were analyzed by western blotting using antibodies against the indicated phosphorylated (upper panel) or non-phosphorylated molecules (lower panel) followed by HRP-conjugated anti-rabbit IgG. The phosphorylated amino acid residues recognized by the antibody are indicated in parenthesis. In bottom, membranes were stained with CBB. #, non-specific band. The western blots were performed several times, and the band intensity of the phosphorylated kinase was quantitated by densitometry. When the addition of apoptotic thymocytes in the wild-type macrophages caused the apparent change of the band intensity, the fold-change is shown with SD. *P<0.03; Student t-test.
Figure 4. Effect of MERTK deletion mutations on efferocytosis and cell growth. 

A. Schematic diagrams of MERTK mutants. At top, the structure of MERTK is shown schematically. Immunoglobulin-like domains (IG1 and IG2), fibronectin type III-like domains (FN1, and FN2), the transmembrane region (TM), and the tyrosine kinase domain are boxed with the amino acid positions indicated at the borders. The amino acid positions 530 and 560 indicate the exon-intron junction used to construct the ΔN mutant. Three tyrosine (Y) residues at positions 544, 867, and 924, and a lysine (K) residue at position 614 are indicated. In the K614M mutant, the lysine (K) residue at 614 was mutated to methionine (M). In the ΔN and ΔC mutants, the amino acid region from 531 to 559, proximal to TM, or the amino acid region from 853 to 994 in the C-terminus was deleted, respectively. In the ΔNΔC mutant, both the N-terminal juxtamembrane and the C-terminal regions were deleted.

B. Effect of MERTK mutations on efferocytosis. TKO cell transformants (6 x 10^4 cells) expressing wild-type or the indicated mutant MERTK together with TIM4 were incubated with 6 x 10^5 pHrodo-apoptotic thymocytes at 37°C for 60 min, and then analyzed by flow cytometry for pHrodo-positivity. The experiments were carried out in triplicate, and the average percentage of pHrodo-positive cells was plotted as efferocytosis (%) with SD (bars). **P < 0.01, ***P < 0.001; Student's t-test.

C. Effect of various mutations of MERTK on the apoptotic cell-promoted cell growth. Ba/F3/TIM4 cell transformants (6 x 10^4 cells) expressing wild-type or the indicated mutant MERTK were incubated for 24, 48, or 72 h in the absence (left) or presence (right) of 2.5 x 10^5 apoptotic thymocytes, and the viable Ba/F3 cells were counted. The experiments were performed in triplicate, and average values were plotted with SD (bars). The values were statistically analyzed by Student’s t-test against that obtained with wild-type MERTK. **P < 0.01, ***P < 0.001.
Figure 5. Effect of MERTK mutations on the activation of MERTK and signaling molecules. TKO transformants (1.0 x 10⁶ cells) expressing the wild-type or the indicated mutant MERTK were incubated with (+) or without (-) 1.0 x 10⁷ apoptotic thymocytes at 37°C for 10 min in DMEM containing 1 µg/mL Protein S. After removing unengulfed thymocytes, the cells were lysed with 1.0 mL of lysis buffer containing 1% Triton X-100. A, MERTK in the cell lysates (1 mL) was immunoprecipitated with an anti-MERTK Ab, dissolved in 60 µl of SDS sample buffer, and 15-µl aliquots were subjected to western blotting with an HRP-anti-phosphotyrosine mAb (upper panel) or a biotinylated anti-MERTK Ab followed by streptavidin-HRP (lower panel). In bottom, membranes were stained with CBB. B, The cell lysates (15 µl) were analyzed by western blotting using HRP-antibodies against the indicated phosphorylated (upper panel) or non-phosphorylated proteins (lower panel). The phosphorylated amino acid residues recognized by the antibodies are shown in parenthesis. In bottom, membranes were stained with CBB. Western Blots were performed several times. The band intensity was quantified by densitometry, and compared between those obtained with or without apoptotic cells. When the difference was apparent, the induction fold is indicated with SD. *P<0.05, Student t-test.
MERTK tyrosine kinase receptor together with TIM4 phosphatidylserine receptor mediates distinct signal transduction pathways for efferocytosis and cell proliferation

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