Diversification of TAM receptor tyrosine kinase function

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The clearance of apoptotic cells is critical for both tissue homeostasis and the resolution of inflammation. We found that the TAM receptor tyrosine kinases Axl and Mer had distinct roles as phagocytic receptors in these two settings, in which they exhibited divergent expression, regulation and activity. Mer acted as a tolerogenic receptor in resting macrophages and during immunosuppression. In contrast, Axl was an inflammatory response receptor whose expression was induced by proinflammatory stimuli. Axl and Mer differed in their ligand specificities, ligand-receptor complex formation in tissues, and receptor shedding upon activation. These differences notwithstanding, phagocytosis by either protein was strictly dependent on receptor activation triggered by bridging of TAM receptor–ligand complexes to the ‘eat-me’ signal phosphatidylserine on the surface of apoptotic cells.

Billions of apoptotic cells are generated each day in the body’s tissues, and the rapid clearance of these dead cells is essential for both tissue homeostasis and resolution of the inflammatory response to infection. Inefficient clearance can lead to tissue damage and the development of autoimmunity1. However, the mechanisms responsible for the clearance of apoptotic cells in inflammatory settings versus homeostatic settings are unknown. We sought to determine whether individual members of the TAM family of receptor tyrosine kinases (RTKs)—TYRO3, Axl and Mer—might be specialized to function in these very different environments.

The TAM RTKs are known to regulate the innate immune response3–5, mediate the homeostatic phagocytosis of apoptotic cells and membranes in adult tissues6–9, facilitate the infection of target cells by enveloped viruses10,11 and contribute to the progression and metastasis of human cancers12–14. However, the specific roles of individual TAM receptors, together with their ligands GAS-6 and protein S, are poorly understood.

Genetic studies have shown that TAM signaling has an especially important role in sentinel cells of the immune system1,4, where the principal TAM receptors are Axl and Mer. Activation of either Mer or Axl in these cells has been found to dampen activation of the immune system3, and the upregulation and activation of Axl in dendritic cells (DCs) is an intrinsic negative feedback inhibitor of the innate immune response6,15. Accordingly, deficiencies in TAM signaling result in profound autoimmunity3,4.

We found that Axl and Mer were dedicated to function in inflammatory and tolerogenic settings, respectively. Macrophage expression of Mer was upregulated by immunosuppressive agents such as dexamethasone (Dex), whereas Axl was inhibited by such agents. Conversely, proinflammatory agents upregulated expression of Axl and inhibited expression of Mer. We found that Mer and Axl specifically mediated the phagocytosis of apoptotic cells in homeostatic environments and inflammatory environments, respectively, and that catalytic activation of these receptors was required for these events. Notably, this activation had to be induced by a TAM ligand whose binding bridged a phagocyte TAM receptor to the ‘eat-me’ signal phosphatidylserine (PtdSer) on the target apoptotic cell.

We further demonstrated a difference between Mer and Axl in their ligand dependence: both GAS-6 and protein S drove Mer-dependent phagocytosis, but only GAS-6 was able to drive Axl-dependent phagocytosis. GAS-6 was constitutively prebound to Axl in tissues in vivo without substantial activation of the receptor, and the presence of GAS-6 in these tissues was dependent on the coexpression of Axl but was independent of Mer and TYRO3. Finally, activation-induced proteolytic cleavage of the extracellular domain of Axl liberated Axl–GAS-6 complexes, which resulted in the rapid removal of both receptor and ligand from tissues. These features of TAM biology must be taken into account in the design and application of any TAM-targeted therapy.

RESULTS

Differences in the expression of Axl and Mer

We analyzed TAM expression in both mouse bone marrow–derived macrophages (BMDMs) and dendritic cells (BMDCs) in vitro, and in subsets of cells of the immune system in vivo. In BMDCs, prepared with the growth factor GM-CSF16, Axl had far more abundant expression than that of Mer (Fig. 1a). These cultures also had low expression of TYRO3. In contrast, BMDMs had abundant expression of Mer, minimal expression of Axl and no expression of TYRO3 (Fig. 1a).

The Axl mRNA copy number (per nanogram of total RNA ± s.d.) was 29 ± 4 and 28 ± 9 in BMDC cultures and BMDM cultures, respectively, which suggested that most of the difference between these cells was post-transcriptional; in contrast, for Meritk mRNA, these numbers were 3 ± 1 and 37 ± 6, respectively. Tyrosine autophosphorylation of Mer was stimulated by both protein S and GAS-6, but Axl was activated only by GAS-6 (Fig. 1b).

In vivo, we observed several tissues in which the expression patterns of Axl and Mer paralleled those of cultured macrophages and...
DCs. For example, in the spleen, CD68⁺ tingible body macrophages were mainly Mer⁺ (ref. 17), whereas CD11c⁺ white pulp DCs were mostly Axl⁺ (ref. 18) (Fig. 1e). Cortisone and aldosterone also induced Mer expression in BMDMs (Supplementary Fig. 1). We also observed some populations, such as red pulp macrophages in the spleen and Kupffer cells in the liver, in which Axl and Mer were also observed at low expression levels (Supplementary Fig. 1). We also observed populations that were enriched only in macrophages, such as red pulp macrophages in the spleen and Kupffer cells in the liver, in which Axl and Mer were also observed at low expression levels (Supplementary Fig. 1). We also observed some populations, such as red pulp macrophages in the spleen and Kupffer cells in the liver, in which Axl and Mer were also observed at low expression levels (Supplementary Fig. 1). We also observed some populations, such as red pulp macrophages in the spleen and Kupffer cells in the liver, in which Axl and Mer were also observed at low expression levels (Supplementary Fig. 1).

**Induction of Mer expression by tolerogenic stimuli**

The differences in the expression of Axl and Mer in macrophages and DCs was matched by reciprocal responses to tolerogenic stimuli. The immunosuppressive glucocorticoid dexamethasone (Dex) has been shown to upregulate expression of Mer in human monocyte-derived macrophages19, and we found that CD11c⁺CD11b⁺MHCIIC⁺ cells in the lung had abundant expression of Axl but low expression of Mer (Supplementary Fig. 1). We also observed some populations, such as red pulp macrophages in the spleen and Kupffer cells in the liver, in which Axl and Mer were coexpressed (Supplementary Fig. 1). Finally, we detected divergent expression of Mer and Axl in BMDM cultures: individual BMDM cultures expressed either Mer or Axl, but not both (Supplementary Fig. 2a, top row).

We found that Dex-mediated inhibition of LPS-induced tumor necrosis factor (TNF) (Supplementary Fig. 4b), Dex-mediated changes in gene expression (Supplementary Fig. 4c) and Dex inhibition of signaling via mitogen-activated protein kinases and the kinase Akt (Supplementary Fig. 4d) were all independent of Axl and Mer.

**Induction of Axl expression by inflammatory stimuli**

The polarization of macrophages into a ‘classically activated’ M1 phenotype is stimulated by Toll-like receptor (TLR) ligands and interferon-γ (IFN-γ); their polarization into an ‘alternatively activated’ M2 phenotype is stimulated by interleukin 4 (IL-4) and IL-13, and their polarization into a ‘regulatory tolerogenic’ phenotype is stimulated by anti-inflammatory agents23. We found that the expression of Axl by BMDMs was potentiated by inflammatory mediators of classical M1 activation, which in general had modest inhibitory effects on Mer expression. LPS, for example, elevated Axl mRNA abundance over a time course that closely followed the induction of Nos2 mRNA, which encodes inducible nitric oxide synthetase (Fig. 2a). There was almost no basal expression of Nos2 mRNA before stimulation (Fig. 2a). Over the same time course, expression of Gas6 mRNA and Pros1 mRNA, which encode Gas6 and protein S, respectively, was modestly reduced (Fig. 2a), as published before24. We surveyed a panel of pattern-recognition receptor ligands for their ability to regulate Axl and Mer expression in BMDMs (Fig. 2b).

Axl expression was elevated by many of these inflammatory mediators, the most potent of which were ligands for TLR3, TLR4 and RIG-I, such as LPS and poly(I:C) (Fig. 2b,c). TNF and IFN-α also elevated Axl expression (Fig. 2c). TLR ligands induce Axl expression in DCs via type I interferons, as DCs deficient in interferon receptors fail to upregulate Axl in response to poly(I:C)25. Accordingly, we found that the upregulation of Axl expression by IFN-α in BMDMs was slightly faster than its upregulation by poly(I:C) (Supplementary Fig. 5). IFN-γ potently induced the expression of both Axl and Mer (Fig. 2c). However, individual BMDMs in IFN-γ-treated cultures were again either Mer⁺ or Axl⁺; only a small minority of these cells coexpressed both receptors (Supplementary Fig. 2a, bottom row).

Although the results reported above were consistent with Axl being a marker of M1 activation, we found that IL-4 also elevated Axl expression and inhibited Mer expression in BMDMs (Fig. 2d). As expected, these reciprocal changes in receptor expression were paralleled by reciprocal changes in the autophosphorylation of Mer and
Axl in response to recombinant GAS-6 (Fig. 2d). Through analysis with surface biotinylation, we verified that Dex-mediated stimulation of Mer and LPS-mediated stimulation of Axl were both associated with increased expression of these receptors on the cell surface (Fig. 2e). Together these observations indicated that Axl and Mer had divergent profiles of expression and regulation in inflammatory settings versus tolerogenic settings. In general, the induction of Mer expression was accompanied by the inhibition of Axl, and vice versa.

**TAM specialization during phagocytosis**

Genetic analyses have shown that Mer is required for the phagocytosis of apoptotic cells in various tissues, but a possible role for Axl in this process has been less well studied. We first examined the mobilization of Axl and Mer to the surface of BMDMs in contact with apoptotic thymocytes. We labeled the apoptotic cells with a cytoplasmic dye (CellTracker Orange) and incubated them with BMDMs for 30 min. In the absence of a TAM ligand, neither Axl nor Mer was localized to the site at which the BMDM associated with the apoptotic cell (Fig. 3a, left). However, upon the addition of either GAS-6 or protein S to Mer⁺ BMDMs or of GAS-6 (but not protein S) to Axl⁺ BMDMs, we observed substantial relocation of Mer and Axl to the membrane surrounding entrapped apoptotic cells (Fig. 3a–c). Poly(I:C)-treated Axl⁺ BMDMs formed a readily apparent phagocytic cup during the engulfment of apoptotic cells, as visualized by scanning electron microscopy (Fig. 3d).

To assess quantitatively the ability of Axl to mediate the phagocytosis of apoptotic cells, we analyzed wild-type, Axl⁻/⁻ and Mertk⁻/⁻ macrophages by a flow cytometry–based phagocytosis assay that exploits pHrodo³², a pH-sensitive fluorescent dye (Supplementary Fig. 2a, bottom). Phagocytic activity in these untreated BMDM cultures treated with Dex, poly(I:C) or IFN-γ for these experiments. We found that untreated (Mer-expressing) BMDMs had a modest phagocytic index that was increased by either GAS-6 or protein S, and that the phagocytosis of apoptotic cells was Mer dependent and Axl independent (Fig. 3e). Stimulation of phagocytosis was significantly enhanced in Dex-treated cells (Fig. 3f). In contrast, phagocytosis by poly(I:C)-treated, Axl-expressing BMDMs was stimulated only by GAS-6, and this stimulation was entirely Axl dependent (Fig. 3g). IFN-γ-treated BMDM cultures contained cells with high expression of either Axl or Mer (Supplementary Fig. 2a, bottom). Phagocytic activity in these
cultures also was potentiated by GAS-6, and this potentiation was completely absent only in Axl−/− Mer−/− cultures (Fig. 3i). Togethe these results demonstrated that both Axl and Mer functioned as phagocytic receptors in vitro, but that they acted in different settings and relied on different ligands.

Basal phagocytosis was decreased in Dex-treated cells and elevated in poly(I:C)-treated cells, and neither of these effects was TAM dependent (Fig. 3f,g). We therefore measured changes in mRNAs encoding other known factors that mediate the recognition and engulfment of apoptotic cells in response to both pro- and anti-inflammatory stimuli. We found that many of these were coregulated with either Axl or Mer. For example, mRNA encoding the C-type lectin LOX-1 (ref. 28) was coregulated with Axl (Fig. 3h), whereas mRNA encoding the PtdSer receptor BAI1 (ref. 29) was coregulated with Mer (Fig. 3h).

These results indicated that activated macrophage populations and tolerogenic macrophage populations used distinct cohorts of phagocytic mediators to recognize and engulf apoptotic cells.

To assess the ability of Axl to mediate the phagocytosis of apoptotic cells by macrophages in vivo, we intraperitoneally administered pHrodo-labeled apoptotic cells 16 h after injection of poly(I:C) and allowed 1 h for phagocytosis before isolating peritoneal macrophages. CD11b+ peritoneal macrophages had high expression of Axl in response to injection of poly(I:C) (Fig. 3j). We found that poly(I:C)-treated Axl+/− macrophages phagocytized apoptotic cells less efficiently than their wild-type counterparts did (Fig. 3k).

Figure 3 Axl is a phagocytic receptor in activated macrophages. (a–c) Immunostaining of Mer and Axl in cells from BMDM cultures treated for 24 h with 0.1 μM Dex (a) or 10 μg/ml of poly(I:C) (b,c) and then incubated for 30 min with apoptotic cells (AC) stained with CellTracker Orange in the presence or absence of TAM ligands (GAS-6 or protein S); c is an enlargement of the area outlined (dashed line) in b. Open arrowheads, non-engulfed apoptotic cells attached to TAM-negative membrane; filled arrowheads, engulfed apoptotic cells surrounded by TAM-positive membrane. Scale bars, 20 μm (a,b) or 5 μm (c). (d) Scanning electron microscopy of poly(I:C)-treated BMDM cells (as in b,c). Scale bar, 1 μm. (e–g,l) Phagocytosis in wild-type, Axl−/−, Mer−/− and Axl−/− Mer−/− BMDM cultures left untreated (Ctrl; e) or treated for 24 h with 0.1 μM Dex (f), 10 μg/ml of poly(I:C) (g) or 250 U/ml of IFN-γ (h), then incubated for 1 h with pHrodo-labeled apoptotic cells with or without TAM ligands and analyzed by flow cytometry. *P < 0.05, **P < 0.01 and ***P < 0.001 (unpaired two-tailed t-test). (h) Quantitative RT-PCR analysis of genes encoding various proteins (left margin) in BMDMs left untreated or treated for 24 h with 0.1 μM Dex or 100 ng/ml of LPS; results (log2) were normalized to that of mRNA encoding cyclophilin A and are presented relative to those of untreated cells. P < 0.05 (two-tailed t-test). (i) Axl expression on CD11b+ peritoneal macrophages collected from wild-type mice by peritoneal lavage 16 h after intraperitoneal injection of saline or 100 μg poly(I:C), measured by flow cytometry. (j) Phagocytosis by CD11b+ macrophages from wild-type mice (n = 7) or Axl−/− mice (n = 7) given intraperitoneal injection of 100 μg poly(I:C) for 16 h, followed by injection of pHrodo-labeled apoptotic cells for 1 h; macrophages were collected by peritoneal lavage and quantified by flow cytometry. Each symbol represents an individual mouse; small horizontal lines indicate the mean (±s.e.m.). P = 0.0004 (two-tailed t-test). Data are representative of two independent experiments with ten images per condition (a–d), two independent experiments with three mice per condition (f), two independent experiments with duplicate cultures for each genotype and each condition (e–g,i) or three independent experiments (h) or are pooled from two independent experiments (k).
We also assayed the potential role of TYRO3 in the phagocytosis of apoptotic cells by BMDCs. BMDCs had minimal expression of TYRO3, and this expression was further downregulated by proinflammatory stimuli. The regulation and activation of Axl and Mer in BMDCs paralleled that observed for BMDMs (Supplementary Fig. 7a,b). Through the use of BMDMs from Tyro3−/− mice, we found that TYRO3 was not required for Dex-induced Mer-dependent phagocytosis of apoptotic cells or for poly(I:C)-induced Axl-dependent phagocytosis of apoptotic cells (Supplementary Fig. 7c,d). The minimal TYRO3 expressed by BMDCs therefore did not have a substantial role in the phagocytosis of apoptotic cells.

**TAM activity during phagocytosis**

We assessed the ability of apoptotic cells, in concert with TAM ligands, to modulate TAM receptor kinase activity in BMDMs. GAS-6 and protein S were produced by BMDMs (Fig. 1e), and culture medium supplemented with 10% serum contained ~30 nM GAS-6 and protein S. The addition of apoptotic cells to BMDMs for 30 min potentiates autophosphorylation of Mer and Axl, whereas no Axl activation was observed in those BMDMs with or without 10 nM GAS-6 and 300 nM BMS-777607, quantified by flow cytometry. *P < 0.01 and **P < 0.001 (unpaired two-tailed t-test). Data are representative of two independent experiments (a–c) or from two independent experiments with duplicate cultures for each condition (d); mean and s.d.

**Axl-dependent GAS-6 expression in vivo**

We observed that the maintenance of GAS-6 in many tissues was entirely dependent on simultaneous expression of Axl. Although we readily detected GAS-6 by immunohistochemistry in sections of spleen, small intestine, liver and lung from wild-type mice, its expression was lost in those tissues from Axl−/− mice (Fig. 5a). This effect was specific to Axl, as the presence of GAS-6 was unaltered in those tissues in Mertk−/− mice (Fig. 5a) and Tyro3−/− mice (data not shown). Immunoblot analysis of GAS-6 in splenic lysates confirmed the results obtained by in vivo immunostaining (Fig. 5b). Consistent with the hypothesis that most or all splenic GAS-6 is normally bound to Axl, we found that the basal phosphorylation of Axl was slightly higher in wild-type spleens than in GAS-6−/− splenic lysates (Fig. 5c), but this basal Axl activation was still far below that observed upon the addition of GAS-6 in vitro (Fig. 1b) or upon the addition of activating antibody to Axl in vivo (reported below).

When we costained sections of wild-type splenic red pulp with antibody to Axl (anti-Axl) and anti-GAS-6, we observed perfect colocalization (Fig. 5d). Similarly, when we stained poly(I:C)-treated BMDM cultures, we found that BMDMs displaying surface Axl were always the same cells that displayed surface GAS-6, and vice versa (Fig. 5e). Specific steady-state binding of GAS-6 to Axl may have accounted for the higher basal activation of Axl than that of Mer (Fig. 4a,c). The absence of GAS-6 in Axl−/− spleens (Fig. 5a) was not due to an inability of Axl−/− splenic macrophages to express the Gas6 gene, as we saw no difference in the Gas6 mRNA in Axl−/− spleens and wild-type spleens (Fig. 5f). The translation of GAS-6 protein also occurred normally in Tyro3−/− and Mertk−/− BMDMs in culture (Fig. 1e). Finally, the ‘missing GAS-6’ of Axl−/− tissues did not accumulate in the circulation, as the low concentration of GAS-6 normally present in serum was unchanged in Axl−/− mice (Fig. 5g).

Together these data indicated that maintenance of a GAS-6 protein reservoir in many tissues was dependent on the surface expression of Axl and that in these tissues GAS-6 was normally prebound to Axl. We know of no equivalent dependence for other RTKs and their ligands.
Antibody-mediated TAM activation

RTKs are activated by ligand-driven dimerization and multimerization of receptor subunit monomers. Thus, antibodies generated against RTK ectodomains often act as ligand-independent RTK activators through their ability to drive receptor dimerization. Although cross-activation of Mer has been achieved with a combination of primary and secondary antibodies, directly activating antibodies to the TAM receptors have not yet been reported. We found that affinity-purified, polyclonal anti-Axl (AF854; R&D Systems), anti-Mer (AF591; R&D Systems) and anti-TYRO3 (AF759; R&D Systems) activated their respective receptors (Fig. 6a and data not shown). In contrast to GAS-6, which activated all three TAM receptors, anti-Mer and anti-Axl displayed absolute receptor specificity (Fig. 6b).

We assessed the utility of these antibodies as TAM activators in vivo. We first injected anti-Axl intravenously and monitored activation and expression of Axl and Mer in the spleen. We found that splenic Axl was activated within 15 min after injection and that its activity returned to baseline by 24 h (Fig. 6c). Mer was not activated by anti-Axl, and control immunoglobulin G (IgG) had no effect on the activation or expression of Axl (Fig. 6c). As noted before, we found that strong activation of Axl led to the rapid cleavage of the Axl ectodomain from the cell surface, a consequent loss of steady-state Axl, and the appearance of soluble Axl ectodomain (Fig. 6c).

We next assessed whether varying doses of the antibodies could activate their receptors in liver, lung and spleen. We detected dose-dependent activation of Axl in spleen and lung, again associated with splenic cleavage of Axl, which was especially notable at the highest antibody dose (Fig. 6d). In the liver, cleavage of Axl was so robust that we were unable to detect any remaining Axl protein 1 h after injection of either 50 µg or 10 µg of the antibody (Fig. 6d). We also observed dose-dependent activation of Mer in these tissues at 1 h after injection of anti-Mer (Fig. 6e). The activation of Mer was greatest in liver and lung and, in contrast to results obtained for Axl, it was not associated with cleavage or loss of Mer protein (Fig. 6e). These results indicated that activating antibodies functioned as specific tools for the activation of individual TAM receptors.

TAM activation alone is insufficient for phagocytosis

The TAM-activating antibodies allowed us to assess whether TAM activation in the absence of ligand could promote phagocytosis. We found that the addition of either activating antibody to Axl or activating antibody to Mer alone, in the absence of added GAS-6, had no stimulatory effect on the phagocytosis of apoptotic cells (Fig. 6f,g). Moreover, the addition of these receptor-activating antibodies in the presence of GAS-6 actually inhibited the GAS-6-stimulated phagocytosis of apoptotic cells (Fig. 6f,g). This inhibition could have resulted from competition between the antibody and GAS-6 for receptor binding or, for anti-Axl, antibody-induced cleavage of Axl. Thus, TAM activation was necessary for the phagocytosis of apoptotic cells by macrophages (Fig. 4d), but activation in the absence of a tripartite receptor–ligand–apoptotic cell (PtdSer) bridging interaction was not sufficient for phagocytosis.

Axl activation inhibits inflammatory responses in vivo

As the activation of Axl in DCs inhibits the production and signaling of type I interferons, we hypothesized that treatment with the activating antibody to Axl, even though it did not promote phagocytosis, might be anti-inflammatory in vivo. To test this possibility, we gave mice intraperitoneal injection of LPS (or saline as a control) together with either the activating antibody to Axl or control IgG, then measured mRNAs encoding type I interferons in the spleen 2 h after injection. We observed marked suppression of Ifnb1 mRNA and Ifna4 mRNA in mice given injection of anti-Axl but not in those given injection of control IgG (Fig. 6h,i). These results suggested that activating antibodies to Axl might be a viable approach to TAM-specific immunosuppressive therapeutics. Overall, our results
demonstrated that Axl and Mer were operationally distinct receptors. Our data revealed a pronounced diversification of the expression, activity, function, ligand use and proteolytic processing of TAM receptors (Supplementary Fig. 8).

**DISCUSSION**

We found that Axl and Mer segregated into distinct niches of expression and function: Mer acted mainly in settings of steady-state and induced tolerance, whereas Axl was specialized to act in the feedback inhibition of inflammation. Inflammatory stimuli that elevated Axl expression tended to decrease Mer expression, and immunosuppressive stimuli that elevated Mer tended to decrease Axl expression. Axl expression was induced in both M1-polarized macrophages and M2-polarized macrophages, which suggested that it acts as a response receptor for nearly any inflammatory insult or tissue injury. Both Axl and Mer functioned as immunosuppressive phagocytic receptors, but they operated in inflammatory environments and tolerogenic environments, respectively.

When activated by ligand binding, Axl promotes the cleavage of its extracellular domain from the cell surface through the activation of proteases. We found that such cleavage, which generated ‘soluble Axl’, also occurred when Axl was activated by crosslinking antibodies.

Elevated soluble Axl in blood has been reported to mark multiple human disease and trauma states, including aortic aneurysm, lupus flares, pneumonia infection, preeclampsia, coronary bypass and insulin resistance. We suggest that cleavage of Axl and the generation of a soluble Axl–GAS-6 complex are triggered by the inflammation-induced exposure of PtdSer in these settings and may be a broadly useful diagnostic biomarker for inflammation in human disease.

Although Mer also acts to suppress inflammation, it does so in two settings that are very different from the inflammatory environment in which Axl operates. The first is in normal tissues that are subject to continuous cellular renewal throughout life, and in which billions of apoptotic cells are generated and cleared on a regular, often circadian, schedule. The second is during enhanced immunotolerance induced by corticosteroids and LXR agonists. We showed that the ability of these agents to stimulate phagocytosis of apoptotic cells was entirely dependent on their ability to upregulate macrophage expression of Mer.

Our data indicated that the pattern of Mer polarization in homeostatic settings and Axl polarization in inflammatory settings extended to various other phagocytic mediators. These results suggested that distinct subgroups of phagocytic receptors are specialized to...
orchestrate the clearance of apoptotic cells in different environments. The cleavage of Axl upon its activation suggests that Axl may be required only for initial stages of the phagocytosis of apoptotic cells. This is in agreement with work suggesting that Axl is a tethering receptor for apoptotic cells and that it acts together with CD91 (LRP-1 (‘low-density lipoprotein receptor–related protein 1’)) for engulfment. Consistent with that suggestion, we observed that CD91 was coregulated with Axl by Dex.

The segregation of Axl and Mer extended to their ligands. Unlike the other TAM receptors, Axl and GAS-6 were codependent: Axl uniquely depended on GAS-6 for its activation, and GAS-6 required Axl for its stable maintenance in vivo. The constitutive presence of an Axl–GAS-6 complex in tissues would suggest that exposure of PtdSer may be the actual trigger for Axl activation. Indeed, the low basal activity of Axl in complex with GAS-6 was substantially enhanced by exposure to PtdSer-rich membranes.

Their divergence notwithstanding, both Axl and Mer mediated the PtdSer-dependent phagocytosis of apoptotic cells, and our results have elucidated several features of the bridging model for this process. In this model, GAS-6 or protein S binds concomitantly a TAM receptor on the phagocyte surface and PtdSer on the apoptotic cell.

First, we showed that activation of TAM kinase activity was necessary for phagocytosis, which indicated that TAM receptors serve as more than passive docking sites for apoptotic cells on the surface of phagocytes. Second, we showed that activation of TAM kinases was not sufficient for the phagocytosis of apoptotic cells. And third, we observed rapid mobilization of the receptors to sites of apoptotic cell contact upon the addition of purified ligands. Thus, the interposition of TAM ligand between the macrophage and its phagocytic target was obligatory.

TAM receptor divergence is relevant to human therapy. TAM inhibitors are in development for cancer therapies and the treatment of infection with enveloped viruses, whereas TAM activators have been proposed as treatments for autoimmune indications. The fact that Mer functions on a daily basis throughout decades of adult life suggests that its long-term inhibition in the course of a cancer therapy should be evaluated for side effects, including the development of impaired vision, diminished male fertility and autoimmune disease. Long-term inhibition of Axl may result in fewer adverse reactions. In several settings, antibody-based therapies may have the advantage of absolute receptor specificity. Notably, the finding that downregulation of Axl is brought about through ectodomain cleavage calls into question the utility of the activation of Axl as a vehicle for the delivery of cytotoxic drugs in Axl-overexpressing tumors. At the same time, the use of activating antibodies to Axl—for example, in the potential treatment of lupus or arthritis flares—should have the advantage of being self-limiting owing to this receptor cleavage. Our results indicate that such antibodies may also be effective in the control of inflammation subsequent to infection. These and related considerations suggest that modulation of TAM receptors is an especially promising approach to the treatment of human disease.

**METHODS**

Methods and any associated references are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

A.Z. designed and performed the experiments; P.G.T. aided in the design and execution of in vivo experiments; E.D.L. prepared purified recombinant GAS-6; I.D. aided in the design of the flow-cytometry-based phagocytosis assay; G.L. contributed to the design of the experiments and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Mice. The Tyro3+/−, Axl+/−, and Mertk−/− strains and the Gas6−/− strain were as published before. All lines, except for the TAM TKOs, have been backcrossed for >9 generations to a C57BL/6 background. All animal procedures were conducted according to guidelines established by the Salk Institutional Animal Care and Use Committee. Mice (8–12 weeks of age, both males and females) were randomly allocated to experimental groups (three to five mice per group) and investigators were blinded to group allocation during the experiment. Investigators were not blinded to sample identity. Group size was based on previous literature.

Reagents and antibodies. Dexamethasone, hydrocortisone, cortisone, aldobesterone, 17β-estradiol, estrone, estriol, progesterone, DMSO and β-1,3-glucan from Euglena gracilis were from Sigma-Aldrich. CellTracker Orange (CMRA) was from Life Technologies. Pam3CSK4, HKLm, poly(I: C), LPS from Escherichia coli, ST-FLA, FSL-1, gardiquimod, CgP, MDP, IE-DAP and pppdsRNA-Lyo vector were from Invivogen. LPS from Salmonella minnesota R595 was from List Biological Laboratories.

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BMDM and BMDC cultures. Bone marrow cell cultures were differentiated with ice-cold DPBS and were lysed on ice in a buffer containing 50 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.07 M sucrose, 0.1% β-mercaptoethanol, and protease and phosphatase inhibitors (Roche). Tissues were ‘snap frozen’ in liquid nitrogen before lysis. For immunofluorescence analysis, equal amounts of protein (10 µg) in LDS sample buffer (Invitrogen) were separated by electrophoresis through 4–12% Bis-Tris polyacrylamide gels (Novex, Life Technologies) and were transferred to PVDF membranes (Millipore). For immunofluorescence analysis, cell lysates were incubated overnight at 4 °C with antibodies (0.2 µg antibody (identified above) for 0.5 mg protein in cell lysate). Protein G–Sepharose (Invitrogen) was added for 2 h and immunoprecipitates were washed twice with 1 ml of lysis buffer containing 0.5 M NaCl and once with 1 ml of 50 mM Tris-HCl pH 7.5. Immunoprecipitates were eluted in LDS buffer, separated by electrophoresis through polyacrylamide gels and transferred to PVDF membranes. Nonspecific binding was blocked with TBST (50 mM Tris-HCl pH 7.5, 0.15 M NaCl and 0.25% Tween-20) containing 5% BSA, and membranes were incubated overnight at 4 °C with primary antibodies (identified above) diluted 1,000-fold in blocking buffer. Blots were then washed in TBST and incubated for 1 h at 22–24 °C with secondary horseradish peroxidase–conjugated antibodies (identified above) diluted 5,000-fold in 5% skim milk in TBST. After repeating the washes, signal was detected with enhanced chemiluminescence reagent.

Enzyme-linked immunosorbent assay. Enzyme-linked immunosorbent assay for measurement of TNF (eBiosiences) and GAS-6 (R&D Systems) was done according to manufacturers’ instructions.

Surface biotinylation. Cell surfaces were biotinylated as described. Cells were washed three times with ice-cold PBS, pH 8.0, and were incubated for 30 min at 4 °C with 1 mg/ml Sulfo-NHS-LC-Biotin (Thermo) in PBS, pH 8.0. Cells were then washed three times with ice-cold 100 mM glycine in PBS and lysed.

Immunocytochemistry and immunohistochemistry. For immunohistochemistry, tissues were fresh frozen and cut into sections 11 µm in thickness, air-dried and stored desiccated at −70 °C. Before being stained, sections were fixed for 3 min with ice-cold acetone and washed in PBS, then nonspecific binding was blocked by incubation for 1 h in blocking buffer (PBS containing 0.1% Tween-20, 5% donkey serum and 2% IgG-free BSA). Slides were then washed in 0.1% Tween-20 in PBS and incubated overnight at 4 °C with 1 µg/ml primary antibody (identified above) in blocking buffer. Slides were then washed five times 5 min in PBS 0.1% Tween-20 and were incubated for 2 h at 22–24 °C in the dark with Hoechst and fluorophore–coupled donkey (Jackson) secondary antibodies (identified above) diluted 1:400 in blocking buffer. Slides were washed, sealed with Fluoromount-G (SouthernBiotech) and stored at 4 °C. For immunocytochemistry cells were plated on coverslips and treated with the appropriate stimuli. For fixed labeling, cells were first fixed for 10 min in 4% PFA and washed with PBS. Nonspecific binding was then blocked by incubation of coverslips for 30 min in blocking buffer with 0.1% Triton X-100; coverslips were washed in 0.1% Tween-20 in PBS and were incubated for 1 h at 22–24 °C with 1 µg/ml primary antibody (identified above). Coverslips were washed five times in PBS 0.1% Tween-20 and were incubated for 1 h at 22–24 °C in the dark with Hoechst stain and fluorophore–coupled donkey secondary antibody (identified above) diluted 1:400 in blocking buffer. Coverslips were washed and mounted on slides with Fluoromount-G. For live labeling, cells were incubated for 30 min at 4 °C with primary antibody (identified above) diluted to 1 µg/ml in cold medium. Cells were washed three times with cold DPBS and fixed for 10 min with 4% PFA. Nonspecific binding was blocked and coverslips incubated with secondary antibodies, washed and mounted as above. Images were obtained with a Zeiss LSM 710 microscope with Plan-Apochromat 20×/0.8 M27 and 63×/1.40

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Oil DIC M27 objectives at the Salk Waitt Advanced Biophotonics Center Core Facility.

**Scanning electron microscopy.** Cells on coverslips were fixed overnight at 4 °C in 3% glutaraldehyde and 3% paraformaldehyde in PBS, pH 7.5. Samples were then dehydrated in a graded ethanol series on ice. After dehydration, the coverslips were loaded into Teflon sample holders and processed in an automated critical point drier (Leica EM CPD300; Leica), which was set to perform 25 exchange cycles of CO₂ at medium speed and 20% stirring. All additional fill, heating, and venting steps were performed at medium speed as well. After drying, the coverslips were carefully removed and were made to adhere to double-sided carbon tabs on aluminum stubs. The mounted samples were then ‘sputter coated’ (Leica SCD500; Leica) with approximately 7 nm of platinum while being rotated. Samples were then imaged on a scanning electron microscope (EVO HD, Zeiss Ltd.) at 3 kV for optimal contrast. The entire procedure was performed at the Waitt Advanced Biophotonics Center Core Facility, Salk Institute.

**RT-qPCR.** Total cellular RNA was isolated with an RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen). DNA was removed by on-column digestion with DNase (Qiagen). An RT Transcriptor First Strand cDNA Synthesis Kit (Roche) with anchored oligo(dT) primers (Roche) was used for reverse transcription. Quantitative PCR was run in a 384-well plate format on a ViiA 7 Real-Time PCR System (Applied Biosystems) with 2× SYBR Green PCR Master Mix (Applied Biosystems). Primers are in Supplementary Table 1. Expression was analyzed by the threshold cycle (ΔCt) method.

**Phagocytosis assay.** Differentiated BMDMs were plated on a 48-well plate at 70% confluency and were incubated for 24 h in DMEM containing indicated stimuli. For the generation of apoptotic cells, thymocytes were isolated from 3- to 6-week-old mice, red blood cells were lysed with ACK buffer and remaining cells were incubated for 6 h in RPMI medium containing 5% FCS and 2 μM Dex to induce apoptosis. This routinely resulted in 70% apoptotic and ≤5% necrotic cells. Apoptotic cells were then stained for 30 min with 100 ng/ml pHrodo-SE (Invitrogen) as published. Labeled cells were washed twice in PBS containing 1% BSA (to block remaining pHrodo-SE) and 1 mM EDTA (to remove any bound GAS-6 and protein S) and once with DMEM. Apoptotic cells were then incubated for 10 min with recombinant mouse GAS-6 or purified human protein S and were added to macrophages at a ratio of 10:1 (apoptotic cells/phagocytes) and were incubated for 1 h at 37 °C. BMDMs were then briefly washed in DPBS and were incubated for 10 min at 37 °C in trypsin (0.25%), then were placed on ice and detached by vigorous pipetting. Phagocytosis was assessed by flow cytometry with post-acquisition data analysis with FlowJo software (TreeStar). pHrodo fluorescence was measured with excitation at 561 nm and emission filters for phycoerythrin (574-590 nm) on an LSR II (BD Biosciences) at the Flow Cytometry Core of the Salk Institute. For *in vivo* phagocytosis assay, pHrodo-labeled apoptotic cells were injected intraperitoneally. After 1 h peritoneal cavity was washed with ice cold PBS and the percent of CD11b⁺pHrodo⁺ phagocytic macrophages was quantified by flow cytometry.

**Activating antibodies in vivo.** Male 8- to 12-week-old mice were given intravenous injection (retro-orbital injection, 0.2 ml final volume) or intraperitoneal injection (0.3 ml final volume) of the appropriate concentration of activating antibody to Axl (AF854; R&D Systems) or to Mer (AF591; R&D Systems) or control IgG (AB-108-C; R&D Systems). Mice were killed at specified time points after injection and tissues were ‘snap frozen’ in liquid nitrogen for further analysis of protein and mRNA.

**Data analysis.** All experiments were done in duplicate or triplicate and were repeated at least three times. Replicate numbers were chosen to be adequate for the statistical method used. Data had normal distribution and equal variance. A two-tailed Student’s *t*-test was used for statistical analysis. Differences with a *P* value of <0.05 were considered statistically significant.

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