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TAM receptors and the clearance of apoptotic cells

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The Tyro3, Axl, and Mer (TAM) receptor tyrosine kinases and their ligands Gas6 and Protein S are required for the optimal phagocytosis of apoptotic cells in the mature immune, nervous, and reproductive systems. Genetic analyses in mice, rats, and humans reveal that this receptor-ligand system plays an especially important role in the phagocytosis that is triggered by the "eat-me" signal phosphatidylserine. Deficiencies in TAM signaling lead to human retinal dystrophies and may contribute to lupus and other human autoimmune diseases. The TAM system appears to interact and cooperate with several other phagocytic networks, including scavenger receptor and integrin-based systems, and may serve as a signaling hub that integrates these systems.

Keywords: Gas6; Protein S; TAM receptors; tyrosine kinase; apoptosis

TAM receptors and their ligands

Tissue homeostasis and renewal requires both the birth of new cells and the death of old ones. In almost all settings, "out with the old" complements "in with the new." Cells that are aberrant, aged, or infected must not only be killed but their corpses must also be efficiently cleared from tissues. Multicellular organisms have therefore developed a series of systems to recognize, dispose of, and recycle dead cells and components thereof.[1] Many of these systems are evolutionarily ancient and function in *C. elegans* and *Drosophila*. However, one system—the TAM receptor tyrosine kinases and their ligands[2]—is much more recently evolved, and is specialized for the regular (periodic) removal of apoptotic cells from fully differentiated tissues and organs in vertebrates and prevertebrate chordates. This signaling system plays especially important roles in the adult reproductive, nervous, and immune systems.

The three TAM receptors—Tyro3, Axl, and Mer—are receptor-configured protein tyrosine kinases (Fig. 1).[2–4] Of the 58 receptor PTKs in the human genome,[5] the three TAMs are among the few that are specific to vertebrates. These receptors are normally silent—that is, the steady-state activity of their intracellular tyrosine kinases is low. They are activated by the binding of two closely related ligands—Gas6 and Protein S (ProS)[6–8]—that also are specific to vertebrates and prevertebrate chordates. Both the ligands and the receptors hetero- and homo-dimerize (Fig. 1), and receptor dimerization, which is facilitated by ligand binding is required for activation.

Gas6 and ProS share a distinctive arrangement of structural motifs (Fig. 1).[2,6] Each protein has a ~60 amino acid "Gla domain" at its amino terminus, a region rich in glutamic acid residues that are γ-carboxylated in a vitamin K–dependent reaction.[9] These Gla domains bind the phospholipid phosphatidylserine (PtdSer), which is also an important feature of the *in vivo* function of Gas6 and ProS during the phagocytosis of apoptotic cells.[10–13] The Gla domain is followed by four epidermal growth factor (EGF)-like modules and then by two tandem laminin G domains, which are related to those of the sex hormone binding globulin (SHBG). This SHBG-like module is both necessary and sufficient for TAM receptor binding and activation.[14,15] Overall, Gas6 and protein S share ~42% amino acid identity.

Given their structure and their action during phagocytosis (see below), Gas6 and ProS are sometimes referred to as "bridging molecules" or

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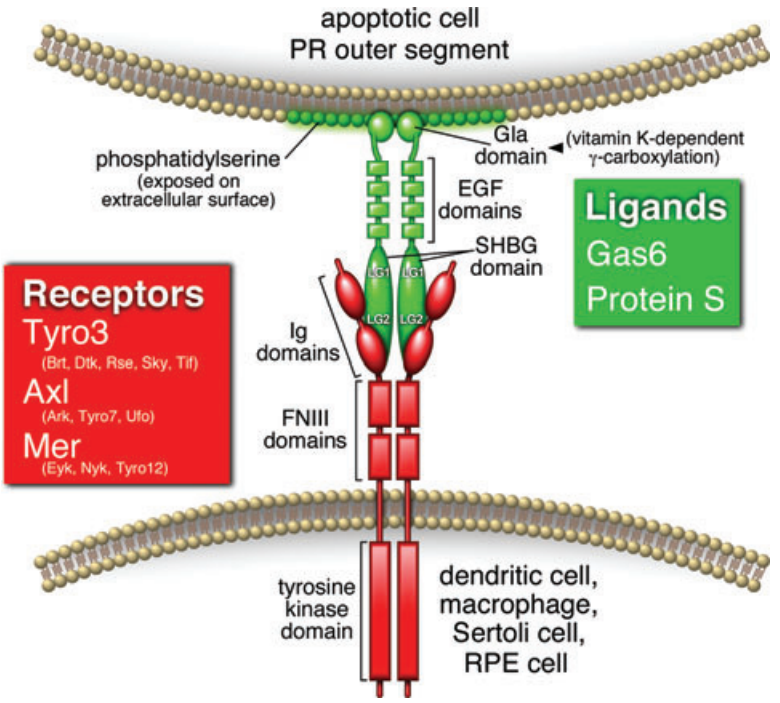

Figure 1. The three TAM receptors and their two ligands. Tyro3, Axl, and Mer (officially c-Mer) are receptor protein-tyrosine kinases (red) expressed by dendritic cells, macrophages, immature NK cells of the immune system, Sertoli cells of the testis, retinal pigment epithelial (RPE) cells of the eye, endothelial cells of the vasculature, and other cells.[2] Older alternative names for each receptor are also indicated. (The corresponding NCBI gene names are *Tyro3*, *Axl*, and *Mertk*.) TAM receptor dimers bind to their two ligands, Gas6 and Protein S (green), through interaction between the two N terminal Ig domains of the receptors and the two C terminal laminin G (LG) regions—which together make up the "SHBG domain"—of the ligands. Through their N terminal Gla domains, Gas6 and ProS bind to phosphatidylserine, which is displayed on the extracellular surface of apoptotic cells and on photoreceptor (PR) outer segments. These Gla domains require vitamin K–dependent γ-carboxylation of glutamic acid residues for full TAM ligand bioactivity. Adapted from Lemke and Rothlin.[2]

opsonins. However, this is in one respect a misrepresentation. The TAM ligands are really not classical opsonins or bridges, in the sense of serving either as a passive structural link between a phagocyte (e.g., a macrophage) and the cell that it is engulfing, or as an opsonin for coating antigen or neutralizing charge. Rather Gas6 and ProS must bind to the PtdSer on an apoptotic cell and at the same time bind to a TAM receptor expressed on the surface of the phagocyte (Fig. 1)—and in so doing *activate* its intracellular tyrosine kinase. In macrophages and dendritic cells, the most prominently expressed TAMs are Axl and Mer,[16] and Mer activation (evidenced by its autophosphorylation) triggers a signal transduction cascade that mobilizes and reconfigures components of the actin cytoskeleton required for apoptotic cell engulfment. In the absence of TAM receptor tyrosine kinase activation and downstream signal transduction, there is no effective TAM-mediated phagocytosis.

TAM receptors and apoptotic cell clearance

The role that TAM receptors and their ligands play in the phagocytosis of apoptotic cells was revealed by genetics—primarily the reverse genetics of mouse knock-outs.[17–25] Remarkably, and unlike any other receptor tyrosine kinase family, the TAM gene family in the mouse can be ablated in its entirety without any major effect on embryonic development.[17] When *Tyro3*$^{-/-}$*Axl*$^{-/-}$*Mer*$^{-/-}$ triple knock-out (TAM TKO) mice are born, and for the first 2–3 weeks thereafter, they are largely indistinguishable from their wild-type counterparts.[17] This is in marked contrast to many other receptor PTKs, which play essential roles in embryonic development, and whose single gene knock-outs are

therefore embryonic lethal mutations. Beginning at ~3 weeks, a series of degenerative phenotypes develop in the male reproductive system, in the retina, and throughout the hematopoetic and immune systems of the TAM TKOs. These degenerative phenotypes eventually become debilitating, although TAM triple mutants are typically viable for more than a year in a standard mouse colony.[17]

Adult TAM TKO males are infertile, with testes that are only one-third the size of wild-type males.[17] At 5 weeks after birth—approximately 1 week after the onset of active sperm production—the seminiferous tubules of the testes of these mice are seen to be filled with apoptotic cell corpses. The pile-up of these corpses results in the eventual death of nearly all germ cells, from spermatogonial stem cells to mature sperm.[17] This phenotype is not developmental in nature, in that at 2 weeks after birth, all of the normal cell types have differentiated and are present in the TAM TKO testes; it is only with the onset of sexual maturity that the defect is revealed and develops. The degenerative phenotype is cell nonautonomous with respect to the dying germ cells and is due to the loss of TAM receptor function from Sertoli cells.[17] Among the most important roles played by these somatic support cells of the testes is the phagocytosis of apoptotic germ cells that are generated during meiosis. It has been estimated that more than half of the spermatogenic population normally dies by apoptosis during each cycle of spermatogenesis, and so the clearance of these cell corpses (on the order of 10^8/day in a human male) by Sertoli cells is critical. Sertoli cells express all three TAM receptors and both TAM ligands, and in the absence of TAM signaling, phagocytosis of apoptotic germ cells in the testes is almost completely abrogated.[17,26–28]

A very similar phenotype is seen in the retina of TAM receptor mouse mutants, and in this instance, it is Mer that appears to make the major contribution. As adults, both TAM TKOs and *Mer*$^{-/-}$ single mutants are blind, owing to the nearly complete absence of photoreceptors (PRs).[17,29,30] This is again a degenerative, rather than a developmental, phenotype; at 2 weeks after birth, the wild-type and *Mer*$^{-/-}$ retinae are indistinguishable from each other, with a normal complement and organization of retinal cell types in both wild-type and mutant.[29,30] Beginning at ~3 weeks after birth, however, apoptotic cells are seen specifically in the PR layer in the mutants, and by 10 weeks, essentially all PRs in *Mer*$^{-/-}$ mice have died. This is again a cell nonautonomous effect with respect to PRs, in that these cells do not express the TAMs. Rather, both Mer and Tyro3 are expressed by cells of the retinal pigment epithelium (RPE).[29] Like Sertoli cells in the testes, RPE cells in the retina are highly phagocytic. Unlike Sertoli cells however, they do not normally engulf apoptotic cells. Rather, the apical microvilli of RPE cells engulf and metabolize only part of a living cell—the distal ends of PR outer segments that are the rhodopsin-containing organelles in which light is first detected. PRs synthesize and insert a new rhodopsin-containing membrane at the proximal base of their outer segments every day, and the distal tips (the oldest parts) of these organelles are correspondingly phagocytozed by RPE cells—in a circadian rhythm—in order to maintain a constant outer segment length. In *Mer*$^{-/-}$ mice, RPE cells are born and appear to differentiate normally, but they fail to perform this daily phagocytosis of outer segments.[29,30] PRs nonetheless continue to synthesize the membrane and extend their outer segments, which are constrained by the limited space of the retina and are thus increasingly distorted in their growth. The failure in RPE phagocytosis leads to the nonautonomous apoptotic death of all PRs in the retina and to blindness. Unlike the situation with developing germ cells in the testes, this apoptosis of PRs does not normally occur in the adult retina but is instead triggered by the failure of RPE cells to phagocytose PR outer segments in the retinae of the mutants. Mutations in the *Mer* gene have also been found to account for a rare form of inherited retinitis pigmentosa in humans[25] and for the PR death that occurs in the *RCS* rat, a long-standing animal model of hereditary retinal degeneration.[23,24]

In the immune system, TAM receptor signaling—again prominently through Mer—is required for the phagocytosis of apoptotic cells by macrophages and dendritic cells.[20,31] Most populations of these cells express Mer and Axl but little or no Tyro3. It is interesting that different sets of phagocytically active cells express different combinations of TAM receptors—all three in Sertoli cells, Tyro3 and Mer in RPE cells, and Axl and Mer in most DCs and macrophages[32]—but that Mer expression seems to be a constant feature of each of these phagocytic cells. Anomalously high levels of uncleared apoptotic cells (as visualized by TUNEL, annexin, or caspase staining) are not immediately obvious in the primary and

secondary lymphoid organs of *Mer*$^{-/-}$ mice. However, if apoptotic cells are induced in these mice—either through injection of a toxic level of dexamathasone (to induce apoptosis in cortical thymocytes) or through direct injection of apoptotic cells—then a markedly slower rate of apoptotic cell clearance by macrophages is observed in the mutants relative to wild-type.[20] This is dependent on Mer kinase activation and on the presence of PtdSer. Also, this can be triggered by the binding of either Gas6 or ProS to Mer. Correspondingly, the key active component in serum that stimulates the phagocytosis of apoptotic cells by macrophages has been found to be ProS,[33] and this ProS stimulation requires Mer binding and activation.[34] These effects of the loss of TAM signaling on phagocytosis do not reflect any deficiency in the ability of *Mer*$^{-/-}$ phagocytes to *bind* apoptotic cells, which they do just as well as wild-type.[20] Rather, they arise from a defect in apoptotic cell engulfment and internalization. This again speaks to the fact that the TAM receptor ligands Gas6 and ProS are more than simple bridging molecules. At the same time, TAM mutant macrophages do not exhibit any defects in phagocytic processes that are *not* PtdSer dependent; the phagocytosis of either latex beads or bacteria is not compromised by the loss of TAM signaling. Indeed, phagocytosis of labeled bacteria and latex beads is actually *enhanced* in TAM triple mutant macrophages since these cells are generally activated.[18]

TAM receptors are specialized for "homeostatic phagocytosis"

It is important to emphasize that the TAM-dependent phagocytosis of apoptotic cells is a specialized process that is largely restricted to adult tissues. Analysis of the *Tyro3*/*Axl*/*Mer* triple mutants indicates that the TAMs are not involved in the clearance of the enormous number of apoptotic cells that are generated during embryonic development. There is only modest expression of TAM in vertebrate embryos before birth; all three receptors are strongly upregulated in their expression levels only after birth (in the mouse between the first and second postnatal week), and expression is maintained at high levels into and throughout adult life.[3] As noted above, when TAM triple mutant mice are born, they are indistinguishable from wild-type neonates in most respects.[17] Thus, the apoptotic cells that are generated during the development of embryonic tissues and organs are cleared normally in these triple mutants. The TAM system instead appears to be particularly important for the phagocytosis of apoptotic cells in adult, fully-differentiated tissues and organs that are subject to constant challenge, remodeling, and renewal. As is the case for both spermatogenesis and PR growth, these remodeling processes are frequently carried out in a cyclic fashion, according to a regularized (often circadian) schedule.[35] We have termed this form of remodeling "homeostatic phagocytosis,"[2] to indicate that it occurs as part of a regularized process of tissue balance and renewal. There are many tissues in which such a regularized process occurs. One especially notable instance is the daily generation of new red blood cells in higher vertebrates. During the end-stage differentiation of mammalian erythrocytes, the nuclei of maturing cells are extruded, and these extruded nuclei must be engulfed and turned over by specialized macrophages in the blood islands of the bone marrow. Extruded erythrocyte nuclei represent an enormous clearance burden, since it has been estimated that in humans 2×10^{11} new red blood cells are generated each day.[1,36] The role of the TAMs in this process has not yet been investigated.

TAM receptors and other clearance mechanisms

How central are the TAMs and their ligands to the phagocytosis of apoptotic cells in the immune system and elsewhere? What is their importance relative to other recognition and engulfment pathways? And what is their potential interaction with these pathways? There is in fact considerable evidence for such interaction.

A series of recognition or "eat-me" signals related to phosphatidylserine have been described,[1] and healthy cells use an intricate enzymatic machinery to maintain an asymmetry in the membrane distribution of this phospholipid. A set of "flippases" and "floppases" ensure that PtdSer is almost exclusively expressed in the *inner* leaflet of the plasma membrane bilayer in normal cells.[37] These enzymes are disabled during apoptosis, and externally displayed PtdSer is therefore among the most distinctive and potent recognition features of apoptotic cells.[38] The masking of PtdSer inhibits apoptotic cell phagocytosis.[39]

As diagrammed in Figure 1, the TAM receptors can recognize PtdSer through their ligands Gas6 and

ProS; the C-terminal SHBG domains of these ligands bind to and activate the TAM receptors and their amino-terminal Gla domains bind to PtdSer. In this way, Gas6 and ProS do indeed "bridge" a TAM-expressing phagocyte to a PtdSer-expressing target cell, but as noted above, this "bridging" is more than purely structural, since phagocytosis requires TAM receptor activation. In this context, it is especially interesting to note that prevertebrate chordates, such as *Ciona intestinalis*, express—in addition to a conventional TAM-like receptor and ligand pair—a hybrid protein in which an intracellular TAM tyrosine kinase and transmembrane domain are directly linked to a TAM-ligand-like Gla domain.[2,40] This shuffled protein therefore combines both TAM receptor and ligand domains in a single molecule, and as such may function as a direct PtdSer receptor. *Ciona* does not have a blood coagulation cascade, and so ProS and closely related Gla domain–containing proteins that bind to PtdSer on the platelet surface and regulate blood coagulation in vertebrates may have been co-opted from phagocytosis signaling pathways rather than the other way around.

Other apoptotic cell recognition and engulfment ("find me" and "eat me") systems, some of which may not depend on PtdSer binding, have been analyzed. These include the Tim (T cell immunoglobulin- and mucin-domain-containing molecule) family of proteins (Tim-1, Tim-3, and Tim-4[41,42]) and the MFG-E8 bridging protein, which both binds to PtdSer and to integrin receptor systems expressed on the surface of phagocytes.[43,44] Other recognition systems important for apoptotic cell engulfment include the scavenger receptors.[45] It is therefore of interest to note that for several of these systems, an association with and dependence upon Mer activity have been demonstrated. The scavenger receptor SR-A I/II (CD204), for example, both physically associates with and signals through Mer in macrophages, and this Mer interaction is essential for the optimal phagocytosis of apoptotic cells.[46] Similarly, an association of Mer with integrin-based engulfment systems has also been demonstrated. In 293T cells, αvβ3/5 integrin-dependent activation requires Mer, and in CS-1 melanoma cells, Mer fails to stimulate phagocytosis of apoptotic cells in the absence of either β5 or β3.[47] In RPE cells, MFG-E8-stimulation of PR outer segment phagocytosis through αvβ5 integrins has been demonstrated to require a physical association and physiological integration with Mer.[48,49] It is noteworthy that RPE phagocytosis differs from other clearance systems in that the membrane particles being cleared are parts of living, rather than dying cells; yet the same "eat me" recognition and engulfment signals used in the clearance of apoptotic cells are used for RPE phagocytosis of PR outer segments.

Finally, the well-known ability of (immunosuppressive) nuclear hormone receptor agonists—including glucocorticoids, LXR agonists such as oxysterols, and PPARγ/δ agonists—to stimulate the phagocytosis of apoptotic cells by macrophages may be tied to their ability to upregulate macrophage expression of Mer. Glucocorticoids, for example, have been shown to induce ProS-dependent phagocytosis of apoptotic cells by macrophages; this glucocorticoid induction appears to be Mer dependent, since it is prevented by pretreatment with Mer blocking antibodies.[50] Similarly, activation of liver X receptors (LXRs) in macrophages stimulates the phagocytosis of apoptotic cells, and this stimulation also appears to be Mer dependent, since LXR stimulation of phagocytosis is largely abrogated in macrophages in which Mer expression is reduced by siRNA treatment.[51] Together, all of these results suggest that, in many settings, signal transduction that is triggered by activation of TAM family receptors, most notably Mer, is likely to play a central integrative role in the phagocytic clearance of apoptotic cells.

Conflict of interest

The authors declare no conflict of interest.

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