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*J Immunol* 2007; 178:5635-5642; doi: 10.4049/jimmunol.178.9.5635
http://www.jimmunol.org/content/178/9/5635

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Macrophages and Dendritic Cells Use Different Axl/Mertk/Tyro3 Receptors in Clearance of Apoptotic Cells

Heather M. Seitz,* Todd D. Camenisch,‡ Greg Lemke,§ H. Shelton Earp,§ and Glenn K. Matsushima2*†‡¶


The clearance of apoptotic cells is important for regulating tissue homeostasis, inflammation, and autoimmune responses. The absence of receptor tyrosine kinases (Axl, Mertk, and Tyro3) results in widespread accumulation of apoptotic cells and autoantibody production in mice. In this report, we examine the function of the three family members in apoptotic cell clearance by different phagocytic cell types. Mertk elimination nearly abolished macrophage apoptotic cell phagocytosis; elimination of Axl, Tyro3, or both, reduced macrophage phagocytosis by approximately half, indicating that these also play a role. In contrast, apoptotic cell clearance in splenic and bone marrow-derived dendritic cells (DCs) is prolonged compared with macrophages and relied primarily on Axl and Tyro3. The slower ingestion may be due to lower DC expression of Axl and Tyro3 or absence of GAS6 expression, a known ligand for this receptor family. In vivo, phagocytosis of apoptotic material by retinal epithelial cells required Mertk. Unlike macrophages, there did not appear to any role for Axl or Tyro3 in retinal homeostasis. Likewise, clearance of apoptotic thymocytes in vivo was dramatically reduced in merkkd mice, but was normal in axl/tyro3−/− mice. Thus, cell and organ type specificity is clearly delineated, with DCs relying on Axl and Tyro3, retina and thymus requiring Mertk, and macrophages exhibiting an interaction that involves all three family members. Surprisingly, in macrophages, tyrosine phosphorylation of Mertk in response to apoptotic cells is markedly diminished from axl/tyro3−/− mice, suggesting that the interactions of these receptors by heterodimerization may be important in some cells.

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1 This work was supported in part by National Institute for Allergy and Infectious Disease Grant 5-S1702.

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www.jimmunol.org

Received for publication August 25, 2006. Accepted for publication February 13, 2007.

The phagocytosis of apoptotic cells is important during development, lymphocyte maturation, and normal cell turnover. Without efficient apoptotic cell clearance, dying cells accumulate, undergo secondary necrosis, and release intracellular and nuclear contents into the extracellular environment. Released self-molecules may become antigenic and cause lymphocyte activation and autoantibody production (1). Knockout mice have been generated, deleting many of the implicated receptors and intermediate-bridging molecules including the following: CD14, C4, scavenger receptor A, phosphatidylserine receptor, ABCA1, CD93, milk-fat globule (MFG3)-E8, C1q, vitronectin receptor, and Axl/Mertk/Tyro3 receptor tyrosine kinase family (3–13). However, only knockout mice lacking C1q, MFG-E8, and Axl/Mertk/Tyro3 receptor tyrosine kinases generate spontaneous autoimmunity (9, 12, 13).

Mertk (also known as Eyk, Nyk, and Tyro-12) belongs to a family of receptor tyrosine kinases that include Axl (also known as ARK, Ufo, Tyro-7) and Tyro3 (also known as Rse, Sky, Btr, Tif, Dtk) (14). Each member of the Axl/Mertk/Tyro3 receptor family shares a similar extracellular domain structure and a signature KWAIAES sequence in the cytoplasmic kinase domain. Mertk, Axl, and Tyro3 are widely expressed in adult tissues although their function in many of these tissues remains unclear (15). Mice lacking Mertk, or merkkd (previously known as merkd) mice show spontaneous autoantibody production (12, 15), splenomegaly, and enhanced TNF-α production in response to LPS (16). Furthermore, mice lacking all three receptors display a hyperimmune phenotype illustrated by enhanced autoantibody production, splenomegaly, and lymphocyte activation greater than that seen in mice lacking Mertk alone (17). One ligand for these receptors is growth arrest-specific gene 6 (GAS6), which has the highest affinity for Axl, followed by Tyro3 and then Mertk (Kd of 0.4, 2.9, and 29 nM, respectively) (18). GAS6 binds to phosphatidylserine on the outer leaflet of an apoptotic cell and may serve as a bridging molecule between Axl/Mertk/Tyro3 family receptors on phagocytes and the apoptotic cell (19). However, affinity of the GAS6 phosphatidylserine complex for Axl, Mertk, and Tyro3 has not been measured. Another potential ligand is protein S, a serum protein involved in the coagulation cascade (20), but its role as a ligand for this family is still unclear, because Tyro3 is the only family member that binds to protein S (21). This finding was made using Tyro3 and protein S from different species, and, until now, binding of murine Tyro3 to murine protein S has yet to be reported (22). However, protein S binds phosphatidylserine on apoptotic cells and can enhance phagocytosis, similar to GAS6 (23).
The lack of apoptotic cell clearance in the mertk<sup>nld</sup> mouse resulting in autoantibodies coupled with the enhanced autoimmunity observed in the triple knockout mouse led us to investigate whether Axl and Tyro3 are also involved in clearance of apoptotic cells in different phagocytes (17, 18). In this report, we demonstrate that Axl and Tyro3 do function in apoptotic cell phagocytosis but are more important in dendritic cells (DCs) and to a lesser extent in macrophages. In contrast, Merk is the primary phagocytic receptor for apoptotic cells in macrophages, but it does not have this function in DCs as published previously (12, 24). We also demonstrate that Axl and Tyro3 preferentially bind the ligands GAS6 and protein S, respectively, and, surprisingly, that in the absence of Axl and Tyro3, Merk phosphorylation is markedly reduced in response to apoptotic cells. However, we show that in vivo Mertk, even in the absence of Axl and Tyro3, is fully competent to clear apoptotic cell/material in the thymus and the retina.

Materials and Methods

**Animals**

All mice were housed in the specific pathogen-free Division of Laboratory Animal Medicine facilities in accordance with Institutional Animal Care and Use Committee regulations. Male mice 8–10 wk old were used in studies and wild-type mice are C57BL/6 (The Jackson Laboratory; bred in house). mertk<sup>nld</sup>, axl<sup>−/−</sup>, and tyro3<sup>−/−</sup> mice were backcrossed onto the C57BL/6 background for six generations. Previously designated mertk<sup>nld</sup> mice are now designated mertk<sup>nld</sup> in this report, because Merk is the proper nomenclature. These mice show no detectable protein by Western blot or flow cytometry (unpublished results), therefore they have a functionally null mutation.

**Macrophage in vitro phagocytosis**

Peritoneal macrophages were isolated and phagocytosis assay were performed as previously described (12) with the following modifications. Peritoneal exudate cells were elicited by i.p. injection with 3 ml of 3% thioglycollate (Sigma-Aldrich) and lavaged 72 h following a peritoneum lavage with 3 ml of PBS. Resident macrophages were obtained by lavaging the peritoneum of naive mice with PBS. Peritoneal exudate cells were plated at 1.5 × 10<sup>5</sup> cells/well of a 24-well plate. After 2 h, nonadherent cells were washed off with PBS and fresh macrophage medium (RPMI 1640 (Life Technologies) supplemented with 5% heat-inactivated FBS (Gemini Bioproducts), 50 U penicillin G, and 50 µg/ml 1 streptomycin sulfate (Invitrogen Life Technologies), sodium pyruvate (Sigma-Aldrich), and 2-ME (Invitrogen Life Technologies)) was added. The resulting adherent peritoneal macrophages were then rested overnight at 37°C with 5% CO<sub>2</sub>. To generate apoptotic thymocytes, we collected thymus from naive wild-type mice and dissociated the tissue using forceps. The single-cell suspension was then layered on a density gradient of lymphocyte separation medium (Molecular Probes) added to thymocytes and incubated for 30 min, resulting in autoantibodies coupled with the enhanced autoimmunity observed in the triple knockout mouse led us to investigate whether Axl and Tyro3 are also involved in clearance of apoptotic cells in different phagocytes (17, 18). In this report, we demonstrate that Axl and Tyro3 do function in apoptotic cell phagocytosis but are more important in dendritic cells (DCs) and to a lesser extent in macrophages. In contrast, Merk is the primary phagocytic receptor for apoptotic cells in macrophages, but it does not have this function in DCs as published previously (12, 24). We also demonstrate that Axl and Tyro3 preferentially bind the ligands GAS6 and protein S, respectively, and, surprisingly, that in the absence of Axl and Tyro3, Merk phosphorylation is markedly reduced in response to apoptotic cells. However, we show that in vivo Mertk, even in the absence of Axl and Tyro3, is fully competent to clear apoptotic cell/material in the thymus and the retina.

DC in vitro phagocytosis

Bone marrow-derived DCs (BMDCs) were isolated by harvesting femurs from mice and flushing out bone marrow with PBS. Bone marrow cells were then layered on a density gradient of lymphocyte separation medium (MP) and centrifuged 500 × g for 15 min. The monocyte layer was collected, washed in PBS, and resuspended in RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FBS, 50 U penicillin-G, and 50 µg/ml 1 streptomycin sulfate (Invitrogen Life Technologies), 10 ng/ml GM-CSF, and 10 ng/ml IL-4 (PeproTech). Monocytes were plated on low cluster 6-well plates (Costar). On day 3, the medium was doubled and cytokines were adjusted to 10 ng/ml. On day 7, immature DCs were washed once in PBS and plated in low cluster 24-well plates at 10<sup>5</sup> cells/well. Spleenic DCs were isolated using anti-CD11c Ab bound to microbeads following the manufacturer’s protocol (Miltenyi Biotec). Spleenic CD11c-positive cells were used immediately for phagocytosis. Apoptotic thymocytes were generated as described above and added to DCs at a 10:1 ratio. At indicated time points, the cell suspension was plated on a glass coverslip and DCs were adhered for 1 h. Noningested thymocytes were washed off and DCs were stained with anti-CD11c-FITC and anti-phosphatase inhibitor-1 cells that were positive for both CD11c and ingested apoptotic cells were scored. To analyze pinocytosis, 1 mg/ml albumin-FITC and 0.5 mg/ml dextran-FITC (Sigma-Aldrich) were added to DCs for 30 min at 37°C or 4°C as a negative control. Cells were then washed and analyzed by flow cytometry to determine uptake of albumin or dextran.

**Ligand binding assay and Western blotting**

Axl, Merk, or Tyro3 extracellular domain-human IgG1 Fc chimeras were purchased from R&D Systems. Ten nanograms of chimera was added to 100 µl of normal mouse serum (Vector Laboratories) or PBS at room temperature for 1 h with gentle rotation. Protein A Sepharose (Amersham Biosciences) was added to chimera/serum mixture and incubated for an additional 30 min at room temperature with gentle rotation.

For phosphorylation experiments, thioglycollate-elicited peritoneal cells were collected once in PBS and plated in low cluster 24-well plates at 10<sup>7</sup> cells per 100-mm petri dish. After 2 h, nonadherent cells were washed off and macrophages were rested for 7 days. Apoptotic cells were added at 10:1, thymocytes:macrophage, ratio for 15 min and then macrophages were washed three times with PBS. Macrophages were lysed using immunoprecipitation (IP) lysis buffer (TBS, 1% Nonidet P-40 (Pierce), 0.1 mg of aprotinin, 0.1 mg of a-antitrypsin, 0.1 mg of leupeptin (Sigma-Aldrich), 1 nM PMFS (Sigma-Aldrich), 2 mM sodium orthovanadate (Sigma-Aldrich) and lysates cleared by centrifugation. Non specific proteins bound to Sepharose were removed by incubating with protein G Sepharose (Amersham Biosciences) alone for 30 min at 4°C followed by centrifugation. Anti-Mer Ab (0.5 µg) (R&D Systems) was added to cleared lysate and incubated 30 min at 4°C with gentle rotation. Proteins bound to Sepharose were isolated by washing these three times in IP lysis buffer. After a final wash, SDS sample-loading buffer was added, and the sample was boiled and resolved on a SDS-PAGE gel. Proteins were transferred onto polyvinylidene difluoride membrane (Millipore) and blotted in 5% milk-TBS with 0.1% Tween 20 (TBS-T) for 30 min. Primary Ab, anti-protein S (Santa Cruz Biotechnology), anti-phosphoantigen (Cell Signaling), or anti-Mer (R&D Systems) was added at 1:1,000 overnight at 4°C, followed by addition of anti-g-HRP (Vector Laboratories) or anti-mouse-HRP (Vector Laboratories) for phosphateblots at 1:10,000 for 2 h followed by further washing in TBS-T. Blots were incubated with ECL-Plus (Amersham Biosciences) and visualized using Maximum Resolution film (Kodak).

**RT-PCR of GAS6 and protein S**

Reverse transcriptase was performed mostly as described previously (25). Briefly, RNA was isolated from cells using the TRizol (Invitrogen Life Technologies) reagent and 15 µg of crude RNA was treated with DNase (Promega). After isolation, cDNA was synthesized for 50 min at 42°C using 5 µg of RNA with 150 ng of random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). PCR was then performed using 50 ng of cDNA and primers specific for GAS6 (forward, 5′-TACAGGCCTCTAAGTACCC-3′ and reverse, 5′-TGACGGGTGCAGAAATCACCGATA-3′), protein S (forward, 5′-GGCCTTTGAGACAAAGCATTGAA-3′ and reverse, 5′-TGCCAGCCTGGTGATAGGAATGTGA-3′), and GAPDH (forward, 5′-CTACACTGAGACCGGATTCTAG-3′ and reverse, 5′-GCGAATTCTTGTAGGTATCATC-3′). The PCR mixture contained 0.2 mM dNTPs and 1.5 mM MgCl<sub>2</sub>. The PCR products were resolved on a 1.5% agarose gel using Maximum Resolution film (Kodak).

In vivo clearance of apoptotic cells and immunohistochemistry

Adult mice were injected i.p. with 0.2 mg/25 g of dexamethasone (Sigma-Aldrich) as described previously (12). At indicated time points, the thymus...
was removed and half was fixed in 10% buffered formalin overnight at 4°C, followed by processing and embedding in paraffin. Sections (5 μm) were cut and stained using the TUNEL method. Briefly, sections were deparaffinized with Histoclear (National Diagnostics) and graded ethanol TBS. Sections were then permeabilized in TBS + 0.1% Triton X-100 for 5 min and nuclear material was exposed using Proteinase K. After washing out detergents and enzyme, sections were incubated with dUTP-FITC (Roche) and TdT enzyme (Invitrogen Life Technologies) for 30 min at 37°C. The other half of the thymus was dissociated into a single-cell suspension and stained with VAD-FMK-FITC (Promega) to detect active caspase 3-positive apoptotic cells. These cells were analyzed on a BD Biosciences FACScan cytometer using Summit software. To examine the histopathology of the retina, eyes were removed from adult mice and fixed in 4% paraformaldehyde overnight at 4°C. The tissues were then embedded in paraffin and 5-μm sections were cut and stained with H&E as described previously (26).

**Statistical analysis**

Statistics were calculated using JMP IN 4.0 software (SAS Institute). Tukey-Kramer HSD one-way ANOVA was performed at an alpha level of 0.01–0.05 to compare all variants in a data set.

**Results**

Mertk is critical for phagocytosis of apoptotic cells by macrophages (12), but the role of Axl and Tyro3 has not been adequately studied. Primary peritoneal macrophages were isolated and exposed to apoptotic thymocytes. Greater than 40% of the macrophages from wild-type mice ingested apoptotic cells. In contrast, macrophages from mertkkd mice had a dramatic deficit in clearance of apoptotic cells in vitro. Although not as complete, macrophages from axl−/−, tyro3−/−, and axl/tyro3−/− mice were significantly less able (by ~50%) to phagocytose apoptotic cells when compared with macrophages from wild-type mice (Fig. 1A).

To determine differences in the rate of ingestion, phagocytosis of apoptotic cells by macrophages was quantified over a time course. At 60 and 90 min, macrophages from mice lacking axl/ tyro3−/− had significantly lower levels of phagocytosis than wild-type macrophages (Fig. 1B). Macrophages lacking Mertk remained incompetent even at 90 min. To ensure the phagocytosis was not affected by the thioglycollate used to elicit the macrophages, we isolated resident peritoneal macrophages and compared phagocytosis. Resident macrophages from wild-type mice initiated phagocytosis of apoptotic cells readily at 30 min and then declined thereafter (Fig. 1C). Similar to thioglycollate-elicited macrophages, resident peritoneal macrophages from axl/tyro3−/− mice had significant defects in phagocytosis at 60 and 90 min, although they exhibited an intermediate response between macrophages from wild-type and mertkkd mice (Fig. 1C).

Other mechanisms of phagocytosis were examined to determine whether Axl and Tyro3 were involved. Macrophages from wild-type or mutant mice were given latex beads or anti-CD3 Ab opsonized thymocytes. There was no difference in phagocytosis by macrophages from wild-type mice vs macrophages from mertkkd, axl−/−, tyro3−/−, or axl/tyro3−/− mice (Fig. 1D). This finding indicated that the defect in phagocytosis observed in mertkkd and axl/tyro3−/− macrophages was specific for apoptotic cells.

To ensure that the phagocytosis assay was measuring ingested cells as opposed to bound/noningested cells, macrophages from wild-type, mertkkd, and axl/tyro3−/− mice were treated with apoptotic cells at 4°C or with 2 μM cytochalasin D, which prevents phagocytosis and ingestion. After extensive washing, there were no ingested apoptotic cells within macrophages after incubation at 4°C or with cytochalasin D (Fig. 1E, III and IV). To ensure that binding was not different among the genotypes, macrophages that were not washed extensively after incubation with apoptotic cells were examined. There was no difference in the number of apoptotic cells bound to the macrophages from wild-type, mertkkd, and axl/tyro3−/− (Fig. 1E, cross-hatched bars).

The mechanism for the phagocytosis of apoptotic cells by DCs is not known; however, it does not require Merkt (24). We postulated that Axl or Tyro3 may function as receptors important in the recognition and phagocytosis of apoptotic cells. Bone marrow from femurs of wild-type and knockout mice was cultured and day 7 immature DCs were produced. Characterization of these CD11c+ BMDCs by flow cytometry showed no difference in co-stimulatory molecules CD80, CD86, or MHC class II expression.

**FIGURE 1.** Macrophages lacking Axl and/or Tyro3 have a deficiency in clearance of apoptotic cells. A, In vitro phagocytosis assay with thioglycollate-elicited peritoneal exudate cells collected at 60 min after nonapoptotic (UT, □) or apoptotic (AT, ■) thymocytes were added. B, Thioglycollate-elicited macrophages from wild-type (●), mertkkd (■), or axl/tyro3−/− (▲) mice or resident macrophages from wild-type (●), mertkkd (■), or axl/tyro3−/− (▲) mice (C) were fed apoptotic thymocytes for indicated time points in minutes. D, Thioglycollate-elicited macrophages were fed 2 μM latex beads (LB, ■) or CD3 opsonized nonapoptotic thymocytes (OP, □) for 60 min. E, Thioglycollate-elicited macrophages were fed nonapoptotic (NAC) or apoptotic cells (AC) for 60 minutes with 2 μM Cytochalasin D (AC+CytoD) or incubated at 4°C (AC 4°C). Bound AC were incubated with Cytochalasin D for 30 min. Error bars are SE; n > 3. *, p < 0.05 and **, p < 0.01 compared with wild-type. These data are representative of four independent experiments. 
between DCs from wild-type vs those from merk\textsuperscript{kd} and axl/tyro3\textsuperscript{−/−} mice (Fig. 2A). To determine whether Axl or Tyro3 plays a role in phagocytosis of apoptotic cells by DCs, immature BMDCs were cocultured with fresh or apoptotic thymocytes and the amount of phagocytosis was quantified. BMDCs from wild-type, and merk\textsuperscript{kd} mice cleared apoptotic cell with equal efficacy. In contrast, a significant inhibition in phagocytosis was observed in DCs from axl\textsuperscript{−/−}, tyro3\textsuperscript{−/−}, and axl/tyro3\textsuperscript{−/−} mice (Fig. 2B). Furthermore, whereas BMDCs from axl/tyro3\textsuperscript{−/−} mice never reached levels comparable with BMDCs from wild-type or merk\textsuperscript{kd} mice, BMDCs from merk\textsuperscript{kd} mice continued to ingest apoptotic cells up to 12 h after being fed (Fig. 2C).

To ensure that the BMDCs were indicative of those found in vivo, splenic DCs were isolated and cocultured with apoptotic cells. Although the CD11c\textsuperscript{+} CD8\textsuperscript{−} population is thought to be the primary phagocytic DC in the spleen, we examined the number of these cells in mice from wild-type, merk\textsuperscript{kd}, and axl/tyro3\textsuperscript{−/−} and found no significant differences (mean fluorescence intensity (MFI) of 11.72, 14.39, and 13.99, respectively). Similar to BMDCs in Fig. 2B, splenic DCs from axl/tyro3\textsuperscript{−/−} mice had a significant decrease in phagocytosis at 6 h. In contrast, there was no significant difference between splenic DCs from wild-type and merk\textsuperscript{kd} mice (Fig. 2D). A major characteristic ascribed to DCs is pinocytosis, where microparticles are continuously taken up from the environment for Ag presentation. To determine whether this process was altered in merk\textsuperscript{kd} or axl/tyro3\textsuperscript{−/−} mice, we incubated DCs with FITC-conjugated albumin or FITC-conjugated dextran at 37°C or as a negative control at 4°C. There was no significant difference in the uptake of dextran or albumin in wild-type BMDCs vs BMDCs from merk\textsuperscript{kd} and axl/tyro3\textsuperscript{−/−} mice. This result indicated that pinocytosis is normal in BMDCs from merk\textsuperscript{kd} and axl/tyro3\textsuperscript{−/−} mice (Fig. 2E).

To demonstrate our quantification of ingested apoptotic cells can discriminate apoptotic cells merely bound on the cell surface of phagocytes, BMDCs from wild-type, merk\textsuperscript{kd}, and axl/tyro3\textsuperscript{−/−} mice were incubated with apoptotic cells in the presence of 2 μM cytochalasin D to inhibit actin rearrangements. Only very low levels of phagocytosis in the presence of cytochalasin D was observed, suggesting that our phagocytosis assays are measuring ingested and not just externally bound apoptotic cells (Fig. 2F, □). In addition, there was no difference in binding between genotypes when BMDCs incubated with apoptotic cells were not extensively washed (Fig. 2F, cross-hatched bars). These data suggest that macrophages and DCs have a fundamental difference in their ability to phagocytize apoptotic cells, and this mechanism is mediated by different combinations of the Axl/Mertk/Tyro3 receptor family.

Preferential use of the three receptor tyrosine kinases by macrophages and DCs may be dictated by the presence of specific ligands. Due to conflicting reports in the literature about whether or not same species Tyro3 and protein S are binding partners (22), we examined whether murine Tyro3 extracellular domain interacts with murine serum-derived protein S. In these experiments, only Tyro3 extracellular domain interacted with murine protein S, whereas Mertk and Axl had undetectable amounts of protein bound (Fig. 3A). The selective usage of Axl/Mertk/Tyro3 receptors

FIGURE 2. DCs lacking Axl and/or Tyro3 have a deficiency in clearance of apoptotic cells. A, BMDCs were stained for CD11c-FITC and MHC class II-FITC, CD80-FITC, or CD86-FITC and analyzed by flow cytometry. These data are representative of three independent experiments. B and C, WT or knockout mice were fed nonapoptotic thymocytes (UT, □) or apoptotic thymocytes (AT, △) for 6 h (B and D) for indicated time points in hours (C) and then plated on coverslips. BMDCs are indicated as wild-type (●), merk\textsuperscript{kd} (■), or axl/tyro3\textsuperscript{−/−} (▲) mice. D, BMDCs were treated with FITC-conjugated dextran or FITC-conjugated albumin at 4°C as a control or 37°C. E, BMDCs were treated with FITC-conjugated dextran or FITC-conjugated albumin at 4°C as a control or 37°C. F, BMDC’s were fed non-apoptotic (NAC) or apoptotic cells (AC) for 6 hours in the presence of 2 μM Cytochalasin D (AC+CytoD). Bound AC were fed to BMDCs incubated in CytoD for 1 hour and BMDCs were not washed before counting. Error bars are SE; n > 3, *p < 0.05 and **p < 0.01 compared with wild-type. These data are representative of three independent experiments.
by phagocytes prompted an examination of whether DCs or macrophages express different ligands. Using RT-PCR, we found that macrophages express both GAS6 and protein S, whereas immature BMDCs only express protein S (Fig. 3B). The differential expression of GAS6 and protein S by these phagocytes may partly explain why selected receptors are playing a role in phagocytosis. Thus, the expression of both protein S and GAS6 by macrophages may afford engagement of all three receptors, whereas the expression of only protein S by DCs allows preferential interaction of only Tyro3.

The efficiency with which DCs ingest apoptotic cells is markedly slower than macrophages. The maximum percentage of phagocytic DCs require 6–9 h (Fig. 2C) and reach optimum numbers by 60 min (Fig. 1A). One plausible explanation is the relative cell surface expression of Axl, MerTk, and Tyro3 on these phagocytes. Macrophages have high levels of MerTk, Axl, and Tyro3 on their surface; MFI of 22.43, 15.38, and 11.44, respectively (Fig. 3, C–E). In contrast, DCs have low levels of surface MerTk, Axl, and Tyro3; MFI of 2.74, 7.51, and 4.74 compared with macrophages (Fig. 3, F–H). The low levels of MerTk, Axl, and Tyro3 expression on the surface of DCs suggest that these phagocytes may be less efficient at recognizing, binding, and ingesting apoptotic cells; hence, this could correlate with their slower rate of phagocytosis. The high level of Axl, MerTk, and Tyro3 on macrophages, in contrast, is consistent with their greater efficiency in recognition, binding, and rate of ingestion of apoptotic cells.

To determine whether Axl and Tyro3 are necessary for phagocytosis in other cell types and in vivo, we examined the thymus, an organ in which continuous cell death occurs as a consequence of maturation and negative selection of thymocytes. Administration of the glucocorticoid dexamethasone to mice induces thymocyte death and provides a model to experimentally monitor the clearance of apoptotic cells. Previously, our group has shown that merkt−/− mice are unable to clear an in vivo dexamethasone-induced apoptotic cell burden in the thymus (12). To determine whether Axl and Tyro3 function as cophagocytic receptors in the thymus, we injected wild-type, merkt−/−, axl−/−, tyro3−/−, and axl/tyro3−/− mice with dexamethasone and monitored apoptotic cell accumulation in the thymus as exemplified in Fig. 4A. The maximum level of apoptosis is typically observed at 8 h in the thymus. At this time point, all animals had similar numbers of apoptotic cells in the suspension. Error bars represent SE; n ≥ 3. *p < 0.05 compared with wild-type. These data are representative of four independent experiments.
apoptotic cells similar to wild-type. Apoptotic cell number in each genotype was confirmed by dissociating the thymus and staining for the active caspase-3 marker VAD-FMK-FITC by flow cytometry (Fig. 4C). The fact that the axl−/−, tyro3−/−, and axl/tyro3−/− and wild-type mice showed no difference in the number of apoptotic cells indicated that Axl and Tyro3 were not required for the clearance of a large burden of apoptotic cells in the thymus. Mer was both necessary and sufficient in this context.

Recently, GAS6 has been implicated in the efficient removal of outer segment debris from photoreceptors in the retina, suggesting that the cognate receptors Axl, Mer, and Tyro3 may be participating in the clearance of outer segments by retinal pigment epithelial cells (27). In addition, mutations in mertk have been linked with retinitis pigmentosa, indicating a role for Mer in maintaining normal retinal apoptotic material clearance and suggesting that Merk functions in epithelial cells as well as in macrophages (12, 28). Retinal pigment epithelial cells lacking Merk cannot clear apoptotic-like outer segment debris. This results in retinal degeneration in the photoreceptor layer (PRL) and destruction of the outer nuclear layer (ONL) by 6–7 wk of age (28). To determine whether Axl and Tyro3, which have a higher affinity for GAS6 than Merk, also contribute to retina homeostasis (as they do in macrophages; see Fig. 2), we examined the retina of young and old adult axl−/−, tyro3−/−, and axl/tyro3−/− knockout mice. At 2 mo of age, there was no retinal degeneration in normal mice or in axl−/−, tyro3−/−, or axl/tyro3−/− mice (Fig. 5). The retina of axl−/−, tyro3−/−, and axl/tyro3−/− mice had an intact PRL and an ONL of between 12 and 14 nuclei similar to wild-type mice. Furthermore, older axl−/−, tyro3−/−, and axl/tyro3−/− mice were without evidence of retinal degeneration at 6 mo of age (data not shown). In contrast, and as previously reported, mertk−/− mice had complete degeneration of the photoreceptors and ONL at this age (Fig. 5). Thus, the homeostasis of the retina and the clearance of outer segment debris by retinal pigment epithelial cells does not require Axl or Tyro3 and appears to rely solely on functional Merk.

To further examine the cell type specificity, we studied Merk phosphorylation in macrophages stimulated with apoptotic cells. Macrophages from wild-type mice possessing Axl and Tyro3 were able to phosphorylate Merk at 15 min after apoptotic cell stimulation. In contrast, macrophages lacking Axl and Tyro3 had markedly diminished Merk phosphorylation (Fig. 6). These results suggest that in macrophages (and perhaps other cells), Axl and Tyro3, which have higher affinity for ligand, may be required for efficient Merk tyrosine phosphorylation. Axl and/or Tyro3 may bind ligand, then activate Merk by heterodimerization similar to ligand-dependent heterodimers found in other receptor families (e.g., epidermal growth factor receptor family). Whether this is true in other cells (e.g., retinal epithelial cells) in which Merk seems to function well in the absence of Axl and Tyro3 remains to be seen.

**Discussion**

The efficient clearance of apoptotic cells is paramount for maintaining tissue homeostasis. Patients and animal models of systemic lupus erythematosus including, New Zealand Black/White, MRL, and lpr mice have phagocytes that are unable to clear apoptotic debris (29–31). Many genes have been implicated in apoptotic cell clearance, and a subset of these genes (such as mfg-e8 and mertk), when deleted, lead to autoimmune phenotypes (9, 12). Thus, the relationship between apoptotic cell phagocytosis and immune homeostasis is important, and the exact mechanism by which different phagocytes recognize and ingest apoptotic debris merits further study. Merk is emerging as a pivotal cell surface receptor that bridges innate immune responses and regulation of autoimmune disease, but mice lacking all three Axl/Mertk/Tyro3 receptors have an enhanced phenotype compared with mice lacking Merk alone, e.g., autoantibody production, splenomegaly, and lymphocyte activation. This finding prompted further evaluation of the role that Axl and Tyro3 play.

In this study, we used single and double knockout mice for axl and tyro3 to help elucidate their function in clearance of apoptotic cells. Because different organs require different cell types to engulf apoptotic debris and maintain tissue homeostasis, we first chose two phagocytes that also serve in Ag presentation and regulate adaptive immune responses, macrophages, and DCs. Our original report suggested that Merk was critical for the phagocytosis of apoptotic cells by macrophages (12), and we find in this study that Axl and Tyro3 function as phagocytic receptor. However, macrophages lacking axl, tyro3, or both axl/tyro3 were less efficient in clearing apoptotic cells compared with wild-type macrophages (Fig. 1, A–C). In contrast, whereas DCs from mice lacking mertk had no defect in clearance of apoptotic cells as previously noted (24), DCs from mice lacking axl or tyro3 had a dramatic deficit. Thus, Axl and Tyro3 are the two receptor tyrosine kinase family members that are critical for the ingestion of apoptotic cells by DCs.

It is unclear why receptor usage among these two cell types differs so dramatically, but perhaps these discrepancies are correlated with their different functions as efficient phagocytes vs professional APCs. In our studies, apoptotic cell phagocytosis by
macrophages took only 60 min; DCs took 6 h. Although the notion of macrophages as more efficient phagocytes than DCs has been observed for many particles, one plausible explanation for this slower ingestion of apoptotic cells was the lower expression of Axl, Mertk, and Tyro3 in DCs, compared with macrophages (Fig. 3, C–H). Thus, the lower levels of phagocytic receptors on DCs may prevent adequate receptor activation. Alternatively, Axl and Tyro3, which are critical on DCs, may be less efficient at triggering phagocytosis. Furthermore, later time points revealed that DCs from mice lacking *mertk* had prolonged or extended phagocytic activity (Fig. 2C), perhaps suggesting that Mertk may be playing a role to down-regulate ingestion and become a nonphagocytic DC.

We also examined ligand expression for this family of receptors as a potential control point that regulates phagocytosis. We found that whereas macrophages express both protein S and GAS6, DCs only express protein S (Fig. 3B and Ref. 32). This finding could suggest that DCs are more restricted and reliant on Tyro3 for phagocytosis, because Tyro3 was the only receptor shown to bind mouse protein S (Fig. 3A). The physiologic role of protein S, which is present in the circulation, is known to serve in the natural coagulation system and binds to phosphatidylserine on apoptotic cells, platelets, and endothelial cells (33). In vivo, it is certainly possible that the microenvironment of phagocytes contacting apoptotic cells and the selective local production of protein S and GAS6 ligand may dictate engagement of specific receptors and partly regulate rates of phagocytosis.

In tissues such as the thymus, where phagocytes and apoptotic cells are present, we examined whether Axl and Tyro3 affect clearance of apoptotic cells. Mertk was critical for clearance of an abundant apoptotic cell burden in the thymus, but Axl and Tyro3 were not required. Mice lacking *axl*, *tyro3*, or both *axl/tyro3* cleared the burden of apoptotic cells as efficiently as wild-type mice (Fig. 4, A and C). It appears that *mertk*−/− mice can clear apoptotic cells resulting from the physiologic negative and positive selection of thymocytes, because the untreated Mertk mouse does not exhibit a large excess of apoptotic cells. It is possible that Axl and Tyro3 may be compensating for the lack of Mer in *mertk*−/− mice in normal homeostasis; however, our data suggest that Mertk is the primary molecule on thymic phagocytes critical for clearance of synchronous, large burden of apoptotic cells (Fig. 4, A and C). Even in the genetic absence of Axl and Tyro3, Mertk is sufficient to allow ingestion of large amounts of apoptotic cells and maintain normal tissue homeostasis (Fig. 4C). When all three receptors are missing and apoptotic cell removal is therefore hindered severely, multiple organs are affected including brain, testes, liver, and lymphoid tissue (17, 34) above that seen in *mertk*−/− mice alone.

In the retina, we also show that Mertk is critical for the maintenance of the retinal tissues, whereas deletion of *axl*, *tyro3*, or both *axl/tyro3* did not result in retinal degeneration (Fig. 5). Furthermore, this observation is consistent with the literature that suggests a mutation in *mertk* is one primary cause of retinal degeneration (28, 35). These data suggest that degeneration of the retina reported in the triple mutant *axl/mertk/tyro3−/−* mice is primarily due to the absence of Mertk (34). Currently, it is not known whether retinal epithelial cells express Axl or Tyro3; however, our data suggest that these two receptor family members are not critical for normal maintenance of the retina.

We have previously shown that GAS6 triggers Mertk tyrosine phosphorylation and downstream activation of Vavα, Rac1, and Cdc42 (36). The complexity and, perhaps, the cell type specificity are emphasized by our findings regarding Mertk tyrosine phosphorylation in monocytes. We show in this report that, in macrophages that exhibit a role for Mertk, Axl, and Tyro3, there may be a need for at least two family members to trigger Mertk tyrosine phosphorylation. Binding of apoptotic cells to macrophages only results in robust phosphorylation of Mertk in the presence of Axl and/or Tyro3. We are currently investigating whether receptor aggregation is different in macrophages vs DCs. Alternative experiments to identify interaction among the Axl/Mertk/Tyro3 family of receptors have been difficult due to cross-reactivity of current Abs; however, clues that these receptors act in combination have been demonstrated in triple *axl/mertk/tyro3* knockout mice (17) and in recent studies using platelets, which also express all three receptors and appear to require heterodimerization for receptor tyrosine phosphorylation (37). Nonetheless, our report is the first to demonstrate that multiple members of this receptor family are needed for macrophage ingestion of apoptotic cells.

Many questions regarding Axl/Mertk/Tyro3 receptors and how they function differently on different phagocytes remain. Low levels of these receptors on DCs have made detection of protein and phosphorylation difficult; therefore, we cannot assess the requirement of Axl and Tyro3 for Mertk activation in DCs. MFG-E8 is an intermediate molecule that facilitates clearance of apoptotic cells in the spleen and is secreted by macrophages and DCs and interacts with the integrin αβ (38). Other reports have shown that Mertk may also require the integrin αβ for efficient signaling (39). Therefore, it remains controversial which combination of receptors, ligands, and signaling is required for proper induction of phagocytosis of apoptotic bodies. In our report, we cannot exclude the possibility that integrins may be cooperating with Axl, Mertk, and Tyro3 to facilitate this process as IPs to demonstrate that interactions have been difficult with these cell types and with potential cross-reactive Abs. Secondly, Axl and Tyro3 appear to have different ligand affinity and yet they both are required for efficient phagocytosis by macrophages and most importantly by DCs. Axl is known to be cleaved by ADAM10 and is thought to exist primarily as a cleaved soluble form in murine DCs (32). This soluble form of Axl (sAxl) has been found in complex with GAS6 in the serum of mice (32) to the extent that no free GAS6 was detected. These studies further indicated that sAxl might enhance GAS6 expression and stability. Therefore, a more complex relationship between sAxl/GAS6, Tyro3, and Mertk may exist, and an understanding of how this receptor family may interact is an area currently under investigation.

In summary, our report documents a novel role for Axl and Tyro3 receptors in the clearance of apoptotic cells, and the combinatorial usage by macrophages and DCs may provide clues for their biologic relevance. For macrophages, Mertk remains a key receptor; however, Axl and Tyro3 participate to a lesser extent and appear to be important for phosphorylation of Mertk, an event thought to be necessary for rapid activation of phagocytosis. In contrast, DCs that express Mertk but do not require Mertk for the phagocytosis apoptotic cells are mostly dependent on Axl and Tyro3. The different engagement of this receptor family by macrophages vs DCs may be partly dictated by the GAS6 or protein S expression profile and the level of expression of these receptors on phagocytes. DCs express lower levels of Axl/Mertk/Tyro3 and this may result in slower ingestion rates. Nonetheless, we suggest that the reliance of DCs on Axl and Tyro3 for the clearance of apoptotic cells may be correlated to the induction of autoimmunity in these knockout mice. In vivo, we have also demonstrated that Mertk compensates for the lack of Axl and Tyro3 in the thymus and retina, making Mertk most important for these organs in maintaining homeostasis. Lastly, the cooperation among this receptor family may be important for efficient signaling because the different receptors have a different ligand preference and affinity.
Acknowledgments
We thank Stephen Goff and Qing Kevin Zhang at Columbia University (New York, NY) for providing the axl−/− mice, and Jeff Frelinger for critical reading of the manuscript.

Disclosures
The authors have no financial conflict of interest.

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