THE ROLE OF AXL/MERTK/TYRO3 RECEPTOR TYROSINE KINASES IN THE CLEARANCE OF APOPTOTIC CELLS

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in the Department of Microbiology and Immunology.

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ABSTRACT

HEATHER M. SEITZ: The Role of Axl/Mertk/Tyro3 Receptor Tyrosine Kinases in the Clearance of Apoptotic Cells

(Under the direction of Dr. Glenn K. Matsushima)

The phagocytosis of apoptotic cells is critical for maintaining normal development, tissue homeostasis, and lymphocyte maturation. While this phenomenon is well documented, a limited number of receptors have been identified and mechanisms have only been partly elaborated. We hypothesized that the Axl/Mertk/Tyro3 family of receptor tyrosine kinases are involved in the clearance of apoptotic cells by immune cells. Here we demonstrate that in addition to Mertk, Axl and Tyro3 are required for efficient phagocytosis of apoptotic cells in macrophages and dendritic cells in vitro. Macrophages rely primarily on Mertk and less on Axl and Tyro3; whereas in contrast, dendritic cells rely on Axl and Tyro3 rather than Mertk for engulfment of dying cells. The difference between macrophage and dendritic cell phagocytosis can be partly explained by ligand and receptor expression levels. In vivo, Mertk is critical for clearance of apoptotic cells in the thymus and retina, whereas in the spleen Axl and Tyro3 are most important for engulfment. Support for cooperation of the receptors in signaling can be found in data showing no detectable phosphorylation of Mertk in axl/tyro3−/− macrophages. This phosphorylation is thought to be important for downstream signaling of Vav1 and FAK to rearrange actin cytoskeleton or phagocytosis and migration. Indeed in the absence of tyro3 or axl/tyro3, there is lower association of Mertk with Vav1, and lower
phosphorylation of FAK in the absence of tyro3. Although our data suggests an interaction between the three receptors in this family, no interaction was detectable in primary cells or transiently transfected cell lines. The phagocytosis of apoptotic cells is thought to down regulate proinflammatory cytokines; however, in the absence of Mertk, Axl, and/or Tyro3, no defect in the down regulation of inflammatory cytokines in macrophages was observed when compared with wild type. Therefore, we conclude that usage of Axl, Mertk, and Tyro3 is dependent on cell type and is critical for efficient phagocytosis both in vitro and in vivo, however the downstream signaling initiated by these receptors is limited to engulfment, and does not include the down regulation of inflammatory cytokines.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABCA1</td>
<td>ATP-Binding Cassette Transporter 1</td>
</tr>
<tr>
<td>AC</td>
<td>Apoptotic Cell</td>
</tr>
<tr>
<td>ADAM10</td>
<td>A Disintegrin and Metalloproteinase Domain</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri-phosphate</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone Marrow derived Dendritic Cell</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CRQ</td>
<td>Croquemort</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular Domain</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyeraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAS6</td>
<td>Growth Arrest Specific gene 6</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide Exchange Factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine Triphosphate</td>
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<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>Immunoprecipitate</td>
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<tr>
<td>KLH</td>
<td>Keyhole Limpet Hemocyanin</td>
</tr>
<tr>
<td>LB</td>
<td>Latex Beads</td>
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<tr>
<td>LSM</td>
<td>Lymphocyte Separation Medium</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MBL</td>
<td>Mannose Binding Lectin</td>
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<tr>
<td>MFG-E8</td>
<td>Milk Fat Globule E8</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inhibitory Protein</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear Factor- kappa B</td>
</tr>
<tr>
<td>NZB/W</td>
<td>New Zealand Black/White mouse</td>
</tr>
<tr>
<td>OP</td>
<td>Opsonized cells</td>
</tr>
<tr>
<td>ONL</td>
<td>Outer Nuclear Layer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulphonylfluoride</td>
</tr>
<tr>
<td>PSR</td>
<td>Phosphatidylserine Receptor</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PTX3</td>
<td>Pentraxin 3</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal Pigment Epithelium</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
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<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythmatosus</td>
</tr>
<tr>
<td>SM</td>
<td>Smith Antigen</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of Cytokine Signalling</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal UTP Nick End Labelling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WB</td>
<td>Western Blot</td>
</tr>
<tr>
<td>WCL</td>
<td>Whole Cell Lysate</td>
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CHAPTER 1

INTRODUCTION
1.1 The Clearance of Apoptotic Cells

The phagocytosis of apoptotic cells is fundamental for normal development, lymphocyte maturation, and efficient homeostasis throughout the body. In development cells must specialize into different tissues and remodeling occurs at a high rate. In this process apoptotic cells are generated and must be removed efficiently to allow for further specialization of the surrounding tissue(1). In mice, macrophages populate developing tissues at the time circulation is established and this pattern is then maintained throughout embryogenesis and into adulthood(2). Additional evidence is seen in Drosophila, where CRQ, (the mammalian homologue is CD36) is expressed immediately after the onset of apoptosis and its expression is restricted exclusively to the migratory phagocytic hemocytes during embryogenesis(3). Evidence from knockout mice, indicate that phosphatidylserine receptor (PSR), a protein thought to recognize phosphatidylserine on apoptotic cells, is important for development of the lung and brain tissues. Failure to do so results in death of PSR$^{-/-}$ animals at birth due to lack of oxygenation from underdeveloped lung tissue(4-6).

It is interesting, that although efficient tissue remodeling in development relies on swift clearance of apoptotic cells, many of the proteins involved in this process, with the exception of PSR, have been genetically deleted in mice with no resulting defect in development. This makes an important point that although clearance of apoptotic cells is critical in development, there are likely many redundant mechanisms to preserve this function in phagocytes. Similarly, non-professional phagocytes such as mesenchymal and epithelial cells may compensate to ensure efficient clearance in these mutant animals. This is illustrated clearly in the PU.1$^{-/-}$ mouse that lacks macrophages, developmental phagocytosis
of apoptotic cells is accomplished by mesenchymal cells that compensate for lack of macrophages (7). Although the phagocytosis by these cells is much less efficient, development of the footplate proceeds normally. It is not surprising that during development and in adult animals that multiple signaling pathways are involved in the phagocytosis of apoptotic cells as it is a critical process important in normal development.

Comparative biology has given insight into the conservation and importance of the clearance of apoptotic cells. In *c. elegans* there are seven genes that can be linked to defects in clearance of apoptotic cells, *ced-1, ced-2, ced-5, ced-6, ced-7, ced-10*, and *ced-12*. Very similar to the redundancy and complexity seen in mammalian systems, in *c. elegans*, there appears to be two partially redundant groups of proteins that mediate this function, group 1: CED-1, CED-6, and CED-7; and group 2: CED-2, CED-5, CED-10, and CED-12. This highlights the importance and redundancy of pathways that control this process. Although both pathways are required for efficient clearance, deletion studies delineated that group 1 is involved in the engulfment process, whereas group 2 participates in both engulfment and in migration of gonadal distal tip cells (8). Further, many of the clearance proteins in *c. elegans* have homologues in the mammalian system with the same function, i.e. CED-2 is CrkIII, CED-5 is Dock180, CED-12 is ELMO, CED-7 is ABCA-1, and CED-10 is Rac (8). The high evolutionary conservation of the proteins involved in phagocytosis of apoptotic cells is additional evidence indicative of its importance.

Another area where efficient clearance of apoptotic cells is paramount is in lymphoid organs. In the spleen and thymus, B cells and T cells undergo a highly specialized process of selection that ensures specific, non-self lymphocytes proceed to maturation. In this process, it is estimated that 90% of thymocytes do not express a rearranged TCRαβ complex capable
of low-affinity interaction with MHC and self-peptide, and therefore fail positive selection and die by neglect(9). Similarly in the bone marrow, immature B cells that bind to self-antigen die by apoptosis. Further, once in the periphery, B cells in the spleen that are self-specific or highly activated undergo apoptosis(10). Without efficient removal of these apoptotic cells, newly maturing lymphocytes would be bathed in apoptotic and nuclear debris that could cause stimulation of autoreactive T and B cells resulting in autoimmune disease.

During bacterial inflammation neutrophils are the first responders to pathogen invasion. Neutrophils rapidly phagocytose bacteria and respond by secreting inflammatory cytokines to aid in alerting additional innate and subsequently the adaptive immune response(11). However, for inflammation to be resolved, there needs to be a mechanism of down regulating this cell type, as they are full of cytotoxic granules and infiltrate in high numbers into infected tissues. Neutrophil responses are partly downregulated by induction of apoptosis. This occurs when no cytokines or pro-inflammatory signals are present in the normal aged neutrophils population, or as they ingest bacteria and respond to infection in acute inflammation(12). Swift removal of the neutrophils that have undergone apoptosis is necessary in order to prevent their release of granules and to ensure that inflammation can be resolved quickly. This efficient removal of apoptotic neutrophils is accomplished by macrophages, which are the second responders at sights of inflammation(11, 12). Therefore the efficient phagocytosis of apoptotic neutrophils in addition to other cell types is paramount in resolving inflammation and restoring homeostasis to tissues.

Different tissues require different strategies to cope with the life cycle of cells and the rate of apoptosis and normal cell turnover. The vast majority of cells that clear these apoptotic bodies are tissue specific, i.e.: the epithelial cells, tissue specific macrophages and
dendritic cells, and neutrophils. In organs such as the spleen and thymus, it is thought that because of the high number of dying cells, specialized professional phagocytes play an important role in keeping the organ clear of dying cells. This is important to consider, because non-professional and professional phagocytes may have very different mechanisms of recognition and clearance of apoptotic cells(13). Non-professional phagocytes, such as epithelial cells, endothelial cells, stromal cells of the bone marrow, and nurse cells of the thymus, are thought to be much less efficient and may only recognize apoptotic cells at later stages of apoptosis than professional phagocytes. Professional phagocytes such as macrophages, dendritic cells, and neutrophils are thought to have highly efficient recognition and engulfment machinery that can clear an apoptotic cell before many of the hallmark signs of apoptosis are evident. The large number of phagocytic receptors on macrophages compared with non-professional phagocytes supports this hypothesis(14).

**Mechanisms**

There are many reported receptors on phagocytes and opsonins on apoptotic cells that have been implicated in this process (Table 1). During apoptosis, one of the earliest events is the translocation of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the cell membrane. Although PS exposure is evolutionarily conserved and thought to be a major signal for recognition, other changes to the surface of the apoptotic cell are also thought to be important in recognition and engulfment. These include the loss of sialic acid residues, externalization of oxidized low density lipoproteins, and a putative thrombospondin binding moiety(15). Apoptotic cells can then be opsonized or decorated by binding of GAS6, Protein S, and MFG-E8 to PS; or by binding of C1q, mannose binding lectin (MBL),
pentraxins, and/or thrombospondin-1 directly (16). These molecular flags act as indicators to phagocytes that a cell is undergoing apoptosis and requires immediate engulfment.

The receptors that are thought to recognize these opsonins or molecular flags include integrin $\alpha_5\beta_5$, calreticulin/CD91, CD14, Class A scavenger receptors, vitronectin receptor and Mertk of the Axl/Mertk/Tyro3 receptor tyrosine kinase family (14). It is yet unclear if and how these various receptors and ligands interact and cooperate, or how they might be specific to cell types and stage of apoptosis recognition. Two groups of opsonins have been implicated in early and late recognition of apoptosis; PS is thought to be an early marker of apoptosis whereas SAP, C1q and MBL binding are thought to occur in later stages of apoptosis. This difference in kinetics may be important for the differential role receptors play in various cell types and tissues. Other receptors are reported to bind PS directly, such as the Phosphatidylserine receptor (PSR), and Scavenger receptor B1. A thorough understanding of which receptors are used by the different and the engagement of multiple ligands is lacking. Studies to address the issue of how cell types utilize each receptor would therefore be very informative.

While recognition and engulfment receptors are seemingly very heterogeneous, i.e. their structures and modes of recognition are very different; the signaling that must occur downstream is the same for all receptors. The phagocytosis of most particles including apoptotic cells requires activation of the cytoskeletal rearrangement complex involving actin. This process is mediated through PI3-kinase activation and recruitment to the cell membrane, whereby PI3-kinase can activate guanine-nucleotide exchange factors (GEFs), such as Vav, or they may become active through other mechanisms. Following GEF activation, CDC42, Rac, and Rho become GTP bound and activated to recruit CrkII and Dock180 that results in
Evidence for actin rearrangement involved in all engulfment receptors can be seen by inhibition of phagocytosis using cytocholasins that act by preventing actin filament assembly and extension. Evolutionary conservation is also further evidence of the importance of this signaling cascade. For example, as mentioned above in *c. elegans*, CED-2, 5, and 10 are mammalian homologues of CrkII, Dock180, and Rac respectively and similarly facilitate phagocytosis.

Another interesting downstream consequence of phagocytosis of apoptotic cells specifically is the down modulation of inflammation. Under normal circumstances, phagocytes that encounter bacteria, particles, or opsonized cells will engage and phagocytose that particle. The binding of receptors on phagocytes such as the toll-like receptors then elicits an appropriate inflammatory response. In the case of bacteria or endotoxin, this may be a release of TNF-α, IL-1β, or MIP-1α. However, when the same phagocyte encounters an apoptotic cell, anti-inflammatory cytokines are released such as TGF-β, Prostaglandin-E2, Platelet Activating Factor, and IL-10(18, 19). The effect of phagocytosing an apoptotic cells is very strong as it can shut down inflammatory cytokines that have been elicited at the same time or after stimulation with apoptotic cells. These include chemotactic cytokines such as IL-8 and MCP-1, as well as pro-inflammatory cytokines TNF-α, IL-1β, and IL-12(20, 21). This process is thought to dampen any stimulation of the adaptive immune response and stop further recruitment of antigen presenting cells in hopes of preventing any autoimmune response. Investigators are now looking at ways to harness this anti-inflammatory power therapeutically and have begun infusing apoptotic cells into patients with inflammatory disorders such as Graft vs. Host Disease(22).
Clearance of apoptotic cells in animal models of autoimmunity

The study of the many receptors and ligands involved in the phagocytosis of apoptotic cells have been facilitated by genetically altered animal models. The consequence of the genetic deletion of these receptors has provided insights into function (Table 2). In many of the knockout animals for receptors or ligands of phagocytosis, there is little *in vivo* effect with regards to autoimmunity or gross tissue abnormalities. For instance, while CD14 is important in tethering apoptotic cells to the phagocyte, genetic deletion of this construct results in phenotypically normal animals with no autoimmunity(23). In C1q deficient animals, there is widespread autoimmunity including auto-antibodies and glomerulonephritis, however in murine systems this requires a mixed background of 129/Ola(24). When *c1q−/−* animals were crossed onto a single C57BL/6 background the autoimmunity phenotype disappeared, suggesting that multiple genetic contributions found on the 129 background contributed to this phenotype(25). Phosphatidylserine receptor deficient mice have abnormal development characterized by many apoptotic cells in the brain and lungs, resulting in non-viable animals(4-6). This suggests that PSR’s role in development is critical compared with its function in lymphocyte maturation or homeostasis. An exception in which deleting genes results in autoimmunity is seen in animals lacking the ligand, MFG-E8, or receptors such as Mertk, Axl, and Tyro3, where there are auto antibodies and systemic autoimmunity suggesting that these are key receptors in the phagocytosis of apoptotic cells(26-28).

Further supporting the importance of phagocytosis of apoptotic cells in autoimmune diseases, many animal models of systemic lupus erythmatosus have been shown to have defects in clearance. Of the various mouse models, the Fas/lpr mouse and NZB/W mice have macrophages defective in clearing apoptotic cells(29, 30). In addition macrophages from
human SLE patients have also been demonstrated to have defects in phagocytosis of apoptotic cells(31, 32). While it is yet unclear the specific role phagocytosis of apoptotic cells has in development of SLE, it is certainly correlative and one important element that may contribute to the manifestation of the disease.

A model can be developed where apoptotic cells that are not efficiently cleared may induce a microenvironment where inappropriate signals to dendritic cells, macrophages, and B cells results in autoimmune disease. Exposure of the sub-cellular components such as DNA, histones, and Sm antigen may lead to the processing and presentation of these self-antigens to auto-reactive B and T cells. These lymphocytes could then mediate an auto-antibody response that causes the inappropriate immune responses to vascular tissue and organs leading to the pathogenesis of SLE(10). Therefore, complete understanding of macrophage and dendritic cell function in clearance of apoptotic cells and subsequent effects on B and T cells may be an important first step in developing new approaches to treat SLE.

A pronounced autoimmunity has been demonstrated in mice deficient in the Axl/Mertk/Tyro3 family of receptor tyrosine kinases(27). Yet the role of this family of receptors on macrophages and dendritic cells in the induction of autoantibodies and B cell tolerance is not understood. Previous work from our group has shown that Mertk is critical for phagocytosis of apoptotic cells by macrophages and mice deficient in mertk develop autoimmunity(28). Studies using the triple knockout of Mertk, Axl, and Tyro3 reported the presence of apoptotic bodies in several tissues, including brain, liver, spleen, and kidney(27). Thus the clearance of apoptotic cells in adult animals may rely heavily on Axl/Mertk/Tyro3 receptor tyrosine kinases and it will be important to define where these receptors operate and
to delineate mechanisms that lead to pathology. The following sections highlight the important characteristics to date for Axl, Mertk, and Tyro3.

**Table 1** Receptors, Opsonins, and Surface molecules on apoptotic cells that are involved in recognition and clearance. Adapted from (16)

<table>
<thead>
<tr>
<th>Receptor on Phagocyte</th>
<th>Opsonin</th>
<th>Binding on Apoptotic Cell</th>
<th>References</th>
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<tr>
<td>PSR</td>
<td>PS (6)</td>
<td>PS</td>
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<td>Vitronectin Receptor</td>
<td>MFG-E8</td>
<td>PS</td>
<td>(26, 33)</td>
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<tr>
<td>Mertk, Axl, and Tyro3</td>
<td>GAS6</td>
<td>PS</td>
<td>(28, 34)</td>
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<td>Tyro3</td>
<td>Protein S</td>
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<td>(35)</td>
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<tr>
<td>ABCA1</td>
<td></td>
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<td>Class A Scavenger Receptor</td>
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<td>C1q</td>
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</table>

PSR-Phosphatidylserine Receptor, PS-phosphatidylserine, MFG-E8-milk fat globule E8, GAS6-growth arrest specific gene 6, ABCA1-ATP binding cassette transporter, TSP-thrombospondin, PTX3- pentraxin 3.
**Table 2** Mice genetically modified for receptors or ligands important for phagocytosis of apoptotic cells (Modified from Gregory and Devitt, Immunology 2004, 113: 1-14)

<table>
<thead>
<tr>
<th>Locus Deleted</th>
<th>Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>c1q or c4</em></td>
<td>Systemic autoimmunity, increased number of apoptotic cells in glomeruli. *** Only on mixed background</td>
<td>(24, 45)</td>
</tr>
<tr>
<td><em>merk, axl, tyro3</em></td>
<td>Systemic autoimmunity in single knockouts and enhanced in triple mutant animals. Autoantibodies, splenomegaly</td>
<td>(27, 28, 46)</td>
</tr>
<tr>
<td><em>PSR</em></td>
<td>Abnormal development, apoptotic cells in brain and lungs.</td>
<td>(5, 6)</td>
</tr>
<tr>
<td><em>ABCA1</em></td>
<td>glomerulonephritis</td>
<td>(47)</td>
</tr>
<tr>
<td><em>MFG-E8</em></td>
<td>Systemic autoimmunity, autoantibodies.</td>
<td>(26)</td>
</tr>
<tr>
<td><em>CD14</em></td>
<td>Normal</td>
<td>(23)</td>
</tr>
</tbody>
</table>

PSR-phosphatidylserine receptor, ABCA1-ATP-binding cassette transporter, MFG-E8-milk fat globule E8.

1.2 *Axl* Receptor Tyrosine Kinase

The Axl receptor tyrosine kinase was discovered in three separate laboratories in 1991. Claudio Basilico’s group at New York University isolated “ark” for adhesion related kinase, from a library screen of transformed NIH3T3 cells using a cDNA probe of murine *bek*, the receptor for the fibroblast growth factor family(48). Edison Liu’s group at University of North Carolina utilized DNA from patients with chronic myelogenous leukemia to transform NIH3T3 cells(49) and isolated a similar gene giving it the name *axl* after the Greek word anexelekto, or uncontrolled. The third group Bartram’s group in Germany, identified a similar gene, UFO for it’s unknown function using DNA from patients with a chronic myeloproliferative disorder to transform NIH3T3 cells(50). They isolated and characterized the gene and called it UFO for its unknown function. All authors were attempting to find
new kinases that may be involved in transformation of cell lines. What they found instead was a unique kinase, the first in the family to be discovered.

Axl is a 2826 bp cDNA with the polypeptide encoded by 923 amino acids giving Axl the molecular weight of 102 kDa(48). The extra-cellular domain structure of Axl consists of two Ig-like domains followed by two fibronectin type III-like domains. There are also five potential N-linked glycosylation sites in the extracellular domain of Axl. The transmembrane region and an intracellular kinase domain have conserved ATP binding sites (537-542), as well as the DLAARN (666-671) and DFGL (684-687) sites that have been found in many receptor tyrosine kinases. Unique to Axl and this family of receptors is the amino acid sequence KWAIAES, which although conserved among family members has an unknown function to date(48). The original predictions were that Axl, because of the IgG like domains and fibronectin III-like domains, was involved with cell adhesion. While that held true, many other functions for this protein have subsequently discovered.

The tissue distribution for Axl is widespread. By Northern blot analysis, axl was found to be expressed in murine hematopoetic tissues, breast, tail, uterus, ovary, heart, lung, bone kidney, and brain(48). Further, Axl is highly expressed in tumor cell lines from breast, lung, prostatic carcinoma, thyroid carcinoma, and leukemia patients(51-55). Axl is also expressed on macrophages and dendritic cells (Chapter 2 and (56)).

The function of Axl has been associated with aggregation, cell growth, transformation, and proliferation based on a large variety of systems and cell types. Early work demonstrated that Axl mediated cellular aggregation in a kinase independent manner (57, 58). This data has been refuted in a report that did not find any aggregation affect of Axl with or without ligand stimulation in three different human hematopoetic cell lines(59).
While the role of Axl in aggregation and adherence appears to be cell line specific, Axl has also been implicated in cell growth and proliferation in a number of different systems and cell types. Original observations of Axl were based on it’s ability to transform NIH3T3 cells(49, 50). Further, Axl is highly expressed in tumor cell lines from breast, lung, prostatic carcinoma, thyroid carcinoma, and leukemia patients(51-55). In addition, although ligand stimulation with GAS6 was only found to be mitogenic in NIH3T3 cells, Axl overexpression has a weak mitogenic effect in other cell lines(60). Furthermore, Axl was found to be essential for glioma cell invasion and metastasis, as a dominant negative form of Axl was completely inhibitory to tumor formation and invasion(52).

The widespread expression of Axl did not lend any clues to the putative ligand, however studies demonstrated that the ligand for Axl is Growth arrest specific gene 6 (GAS6). GAS6 is a serum protein involved in the coagulation cascade and studies have shown that GAS6 induces phosphorylation of the Axl receptor in a dose dependent manner(61). This has led to studies of Axl function in vasculature, where endothelial cells, pericytes, and smooth muscle cells express Axl. Axl is reportedly involved in leukocyte migration, neointima formation, VEGF signaling in angiogenesis, survival of endothelial cells after laminar shear stress, vascular calcification, and flow-induced vascular remodeling (62-66). Many groups have proposed using Axl dominant negative constructs for mediating these cellular processes. Importantly, Axl dominant negative constructs have been used in two different tumor models to inhibit signaling and angiogenesis(52). Further, in an angiogenesis model, VEGFR-2 signaling was inhibited by GAS6 stimulation of Axl in endothelial cells. This was done through activation of SHP-2, demonstrating that Axl is important in downregulating signals including VEGFR-2(63). These advances shed light on
the importance of GAS6 and Axl in regulating angiogenesis and tumor processes beyond its role in cell growth and proliferation.

Axl is post-translationally cleaved by the protease ADAM10, to generate an active soluble ECD that is capable of binding ligand. This may be an important step in Axl regulation, or it may serve as another way Axl can activate signaling cascades. One report indicates that Axl is in fact constitutively bound to GAS6 in serum and this may serve to regulate the function of both the ligand and receptor(60, 67). This could be biologically significant if Axl bound GAS6 is still able to activate other receptors, or if Tyro3 and Mertk require Axl to be bound to GAS6 for efficient signaling and activation.

Signaling pathways downstream for Axl have been examined in a number of different cell types with varying results. Reports indicate using yeast two hybrid or standard immunoprecipitation methods found that Axl interacts with SHP2, Nck2, SOCS1, C1-TEN, RanBPM, and IL-15. Although these proteins have differing functions, many are involved with downregulation of cellular responses i.e.; SOCS1, C1-TEN, and SHP2(56, 63, 68). The biological relevance for these interactions is still unclear as these reports have all been in various cell lines and functional assays remain lacking. Many of the reported interactions were not ligand dependent and varied among cell lines. The only exception was the IL-15 interaction that was verified in murine dendritic cells in addition to cell lines. This interaction activated IL-15R and induced protection from TNF-α mediated cell death(56).

Another indicator of Axl being important for downregulation of cellular responses is seen in type 1 IFNs suppression of TNF-α. GAS6, as well as Protein S and apoptotic cells were shown to specifically activate Axl and subsequently activate TWIST to downregulate NF-
kB(69). Thus, the function of Axl is complicated and dependent on the presence or absence of ligand and cell type expression.

While much is known about Axl function in vitro, studies in vivo are still lacking to determine the impact Axl has on these various cell processes. There may be a large dichotomy between in vitro observations and in vivo function, as axl−/− mice are viable, have no vasculature disorders, and are equally susceptible to tumor formation compared with wild-type mice. While these mice display enhanced autoantibody production, many of the reported functions for Axl have not been verified in these mice. Axl function on immune cells has led to very interesting insights into function, however a thorough investigation of Axl function in primary cells is lacking. The data demonstrating that Axl is critical for downregulating cytokines and cellular responses is in line with our evidence that this receptor is critical for maintaining immune homeostasis and preventing autoimmunity (unpublished data).

1.3 Mertk Receptor Tyrosine Kinase

Mertk was the most recent member of this family to be discovered. The human isoform was originally isolated by Snodgrass’s group at University of North Carolina-Chapel Hill from a B lymphoblastoid cDNA library using an anti-phosphotyrosine expression cloning(70). The murine homologue was discovered by the same group, who screened a murine spleen cDNA library for homologues of human mer and the chicken viral oncogene v-eyk(71). Wood’s group isolated the same protein by screening a human glioma expression library with anti-phosphotyrosine antibodies and found a protein they named Nyk(72).
Similarly to Axl, the search for an oncogene led them to Mertk, which plays a role not only in oncogenesis but in many other cellular processes.

The cDNA for Mertk is 3564 bp that translates into 994 amino acids with a molecular weight of 110 kDa. The domain structure of Mertk is consistent with that for Axl and Tyro3, including two Ig-like domains and two fibronectin III-like domains extracellularly. Also similar to Axl and Tyro3 the extracellular domain contains 15 N-linked glycosylation sites. Intracellularly Mertk, like Axl and Tyro3 contains the consensus ATP binding site (589-594), as well as DLAARN (718-723), DGFL (736-739), and the family’s signature sequence KWAIAES (760-766) which is unique to this family(71).

Expression of this receptor tyrosine kinase is widespread with highest expression seen by Northern blot heart, brain, lung, liver, muscle, kidney, and testis. Mertk expression has been demonstrated in monocytes, epithelium including the retinal pigment epithelium (RPE), and distinct cell types in reproductive tissues(71). Further, while Mertk is not expressed in lymphocytes including B and T cells, T and B cell lymphomas express Mertk at various levels. Mertk is also highly expressed in a variety of other cancers including mantle cell lymphoma, B cell leukemia, alveolar rhabdomyosarcoma, gastric, and pituitary adenomas(73-78).

The only known ligand for Mertk is GAS6, similar to Axl. Also similar to Axl, Mertk has been shown to be activated with and without ligand stimulation, therefore the role of GAS6 in Mertk activation may be complex. Protein S, which is a serum protein, expressed by a number of cell types appears to bind Tyro3 but not Mertk(79). Although GAS6 is currently the one ligand identified to bind Mertk, the binding dissociation constant of 29nM is very weak and it is plausible that other yet unidentified ligands may bind Mertk with
greater affinity(80). GAS6 binds to the other family members, Axl (0.4 nM) and Tyro3 (2.9 nM) with greater affinity but the functional consequence when all three receptors are expressed is not clear.

Mertk was originally thought to be an adhesion receptor, and while that has been demonstrated in cell lines, many other functions for this receptor have been elucidated. Initial reports on function for Mertk, demonstrated it’s ability to transform cell lines. Mertk was found to have strong mitogenic and transforming potential in vitro due to high expression in cancers and it’s transforming ability. Further, a truncated form of Mertk with the first 210 amino acids removed resulted in a constitutively active form of the protein, this autophosphorylation site was carefully mapped and found to be $Y^{749}SGDY^{753}Y^{754}R$, which is a similar site to that of other growth factors including, insulin-like growth factor-1, hepatocyte growth factor receptor, platelet-derived growth factor, and colony stimulating factor-1 receptor(81). Another group discovered that the Grb2 site was critical for Mertk’s ability to transform cell lines(73). Mutating $Y^{867}$ within the Grb2 binding site prevented IL-3 independent growth in an IL-3 dependent cell line. As the ligand for Mertk was not yet discovered, a chimeric protein was generated with a CD8 extracellular domain fused with Mertk intracellular domains (CDMer). They found that activation of CDMer increased activity of MAPK and Erk but this was independent of the Grb2 binding site. They also saw a PI3 Kinase dependent activation of NF-kB upon CDMer stimulation(73). Thus, the multiple phosphorylation sites on the intracellular domain of Mertk suggest many signaling pathways are regulated by Mertk.

The first in vivo function of Mertk was found after generating the Mertk$^{kd}$ mouse(82). This mouse was genetically engineered to have a kinase deficient phenotype, but subsequent
studies on the function of Mertk in these animals found that no protein was generated either by Western blot or flow cytometry (unpublished data). The \textit{mertk}^{kd} mouse has enhanced susceptibility to endotoxic shock, in fact the LPS LD\textsubscript{100} was \(\frac{1}{2}\) that found in wild-type mice\(\textsuperscript{(82)}\). This was found to be mediated through NF-kB activation and TNF-\(\alpha\) production, as \textit{mertk}^{kd} animals produce 3 fold higher TNF-\(\alpha\) than wild-type mice in serum when challenged with LPS \(\textsuperscript{(82)}\).

The next \textit{in vivo} function of Mertk was found in the Royal College of surgeon rat (RCS) in which the inherited blindness was found to be due to the mutation in Mertk. The RPE is an epithelial layer in the retina that is responsible for the removal of apoptotic like outer segments of growing rods and cones. Mutations in Mertk prevent the ingestion of these outer segments that are shed by the rods and cones and their accumulation destroys the homeostasis of the retina. Subsequently, the \textit{mertk}^{kd} mouse and humans that possess certain mutations in Mertk also are blind and suffer from retinitis pigmentosa. Thus, Mertk is critical for normal maintenance of the retina and preventing retinal degeneration.

Recently, Mertk was found to influence the clearance of apoptotic cells. This was deduced when GAS6, the ligand for Mertk, Axl, and Tyro3 was shown to bind to phosphatidylserine on the surface of an apoptotic cell. Therefore, it was hypothesized that perhaps another \textit{in vivo} function of Mertk was in recognition of apoptotic cells. Our group demonstrated in 2001 that macrophages from \textit{mertk}^{kd} mice were unable to clear apoptotic cells. Further, \textit{in vivo} experiments showed that \textit{mertk}^{kd} mice were also unable to clear an apoptotic cell burden in the thymus and to a lesser extent in the spleen. Subsequently, we reported that these mice were blind and that there was increased autoantibody production in these mice\(\textsuperscript{(28, 83-87)}\).
While Mertk’s function on immune cells has been best characterized on macrophages, the role of other family members is only partly known. There is little data to understand which subpopulations of monocytes express Mertk and how this receptor serves to regulate these cells. By examining both macrophages and dendritic cells we hope to provide clues as to Mertk’s function in the immune system.

1.4 Tyro3 Receptor Tyrosine Kinase

The third member of this receptor tyrosine kinase family is Tyro3. Tyro 3 was found by Lemke’s group at the Salk Institute using PCR of a rat peripheral nerve cDNA in searches for novel protein tyrosine kinases that regulate neural development. The cDNA encoding Tyro3 is 2259 bp that encodes an 875 amino acid protein with a molecular weight of 125 kDa. Tyro3, also known as Rse, Sky, brt, and tif, is similar to Axl and Mertk in domain structure and size. Extracellularly, Tyro3 contains two Ig-like domains and two fibronectin III-like domains. Intracellularly, the expected ATP binding site (510-515), DLAARN(640-645), and DFGL(658-662) sites as well as the KWAIAES (682-688) unique among these family members is found(88).

Tyro3 is highly expressed in large neurons of adult neocortex, hippocampus, and granule cell neurons in adult cerebellum(88). While expression of Tyro3 is 20-fold higher in neural tissues than non-neural tissues, Tyro3 is also expressed in monocytes, epithelium, reproductive tissues, and kidney(89, 90). While Axl, Mertk, and Tyro3 all bind GAS6, Tyro3 is unique in its ability to bind Protein S. While initial reports of this binding were across species and therefore disputed. We and others have shown that in the murine system Tyro3 does indeed bind murine Protein S(79, 91, 92). There is evidence that although
previous studies using human Tyro3 and human Protein S did not show binding, this may be due to the need for Protein S to be in high local concentrations(93). This could be important for receptor activation and regulation as Protein S is found ubiquitously in high concentrations in mouse serum, although Tyro3 is not constitutively phosphorylated. Requiring a high local concentration of Protein S would allow for Tyro3 signaling to be more tightly regulated. The usage of GAS6 and Protein S is interesting as they have contrary roles in the coagulation process. Protein S is well known to function as an anticoagulation factor and functions in the complement cascade, while GAS6 promotes coagulation and clot formation.

The function of Tyro3 has been studied in far fewer systems than that of Axl or Mertk; however, a role for Tyro3 in growth and differentiation of cells has been reported. The original report on Tyro3 found that it was able to transform Rat-2 fibroblasts when over expressed, similarly to the functions reported for Axl and Mertk(88). In the cultures of the retina, Tyro3 is believed to be important for the phosphorylation of Mertk in RPE which presumably triggers proper ingestion of shed outer segments(91). Data from this dissertation suggest that Tyro3 is not critical as deletion of this gene did not result in degeneration of the retina (our unpublished data). Another area of Tyro3 function is in bone resorption, where Tyro3 is expressed by osteoclasts, and GAS6 and Protein S stimulation caused phosphorylation of Tyro3 and increased pit area in a dose dependent manner(61) this was also found to be an important mechanism in a model for post menopausal osteoporosis caused by estrogen withdrawal in humans(94).

A role for Tyro3 in immune cells has not been demonstrated singly. Studies using the triple knockout have been helpful in elucidating the function of this receptor, however studies
with Tyro3 alone are lacking. Therefore, it is important to further understand not only the expression of Tyro3 on specific immune cells, but its function on these cells.

1.5 The Axl/Mertk/Tyro3 Family

Evidence has emerged recently that indicates that Axl, Mertk, and Tyro3 appear to cooperate together however; it is still unclear how this cooperation takes place. Unlike mertk$^{kd}$ mice, axl$^{-/-}$ and tyro3$^{-/-}$ mice showed no overt phenotypic abnormalities, but the loss of all three receptors has far more devastating consequences. The triple mutant mouse (axl$^{-/-}$, mertk$^{kd}$, tyro3$^{-/-}$) was originally generated from three different groups who each donated their single knockout. It is important to keep in mind then, that the studies on these triple knockouts came from mice that had three different genetic backgrounds. The first report from these mice demonstrated the function of Axl/Mertk/Tyro3 in spermatogenesis(95). The triple knockout mice are infertile, due mostly to a lack of functional sperm, in addition female triple mutant mice have difficulty maintaining pregnancy due to the autoimmune environment in these animals(95).

A second report highlighted the differences in the immune system in the triple knockout(27). They found enhanced spleen size, apoptotic bodies in virtually every organ examined, and high titres of autoantibodies. They also demonstrated that, while the lymphocytes do not express Mertk, Axl, or Tyro3, the triple mutant environment within the organs caused the lymphocytes to be hyperactivated. In addition to this report, the role of Axl, Mertk, and Tyro3 in natural killer cell differentiation was demonstrated using the triple knockout mouse. In this report, natural killer cells were found to express all three receptors and in the knockout mouse, normal natural killer cell differentiation was impaired(96).
Although the tissue expression of these receptors is different, it does overlap in many of the systems most affected in the triple knockout. Macrophages and dendritic cells express all three receptors. However, there are cell types where only one or two receptors are expressed, such as Tyro3 and Axl being highly expressed on Sertoli cells, Mertk expressed on Leydig cells, and in the retina Mertk and Tyro3 are expressed by retinal pigment epithelium whereas Axl is not. Receptor expression may be an important feature to consider when looking at this family of receptors, as the receptors singly, or in combination may have different function.

Another intriguing detail about the three receptors is ligand affinity and usage. While GAS6 has been demonstrated as a ligand for all three receptors, it has markedly different affinity for each receptor. Axl has the highest affinity, followed by Tyro3 and Mertk has a much lower affinity for this ligand. In addition, Tyro3 has been the only receptor shown to bind Protein S. The usage of ligands therefore may be a novel way to understand the function of these receptors.

The ligands, GAS6 and Protein S are both found in serum and involved in coagulation. This led to studies demonstrating that Mertk, Axl, and Tyro3 are critical for thrombus stabilization(97, 98). Using single knockout mice they found that in the absence of one receptor, expression of the other two receptors on the surface of platelets was reduced. This lends support to the idea that these receptors associate and function cooperatively(98). Additional support comes from the lack of compensation seen in the single knockouts. In platelet aggregation all three receptors are required for efficient thrombus formation.

Another model demonstrating a potential association between the three receptors is seen in the retina. The retinal pigment epithelium expresses both Mertk and Tyro3 and
Mertk has been shown previously to be essential for efficient clearance of outer segment debris by this cell layer (91, 99, 100). A recent report indicates that signaling through Tyro3 by Protein S may be critical for efficient clearance by retinal pigment epithelium. This report further supports the notion that without one receptor, expression of the other receptors is diminished; as they found lower levels of Tyro3 expressed on Mertk-kd retinal pigment epithelium (91). This data, along with data in the platelet model demonstrates that the three receptors may have some degree of association. Therefore studies examining the relationship among the three receptors on immune cells may illustrate important features of these receptors that are important for maintenance of normal immune homeostasis.
CHAPTER 2

MACROPHAGES AND DENDRITIC CELLS UTILIZE DIFFERENT
AXL/MERTK/TYRO3 RECEPTORS IN CLEARANCE OF APOPTOTIC CELLS

Submitted as: Seitz, H. M., Camenisch, T. D., Lemke, G., Earp, H. S., Matsushima, G. K.

Macrophages and Dendritic Cells Utilize Different Axl/Mertk/Tyro3 Receptors in the
Clearance of Apoptotic Cells. *Journal of Immunology.*
2.1 Abstract

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 auto-antibody 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 receptors also play a role. In contrast, apoptotic cell clearance in splenic and bone marrow-derived dendritic cells is prolonged compared with macrophages and relied primarily on Axl and Tyro3. The slower ingestion may be due to lower dendritic cell 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 be any role for Axl or Tyro3 in retinal homeostasis. Likewise, clearance of apoptotic thymocytes in vivo was dramatically reduced in mertk<sup>kd</sup> mice, but was normal in axl/tyro3<sup>−/−</sup> mice. However, axl/tyro3<sup>−/−</sup> mice were unable to clear an in vivo apoptotic cell burden in the spleen. Thus, cell and organ type specificity is clearly delineated, with dendritic cells and spleen relying primarily on Axl and Tyro3, retina and thymus requiring Mertk, and macrophages requiring all three family members for efficient phagocytosis of apoptotic cells. Surprisingly, in macrophages, tyrosine phosphorylation of Mertk in response to apoptotic cells is markedly diminished from axl/tyro3<sup>−/−</sup> mice, suggesting that the interactions of these receptors by heterodimerization may be important in some cells.
2.2 Introduction

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 cause lymphocyte activation, and autoantibody production(101). The phagocytosis of apoptotic cells has become an area of intense study, and many surface receptors have been implicated in the process of apoptotic cell recognition and engulfment(14). Knockout mice have been generated, deleting many of the implicated receptors and intermediate bridging molecules including: CD14, C4, Scavenger receptor A, Phosphatidylserine receptor, ABCA1, CD93, Milk fat globule E8 (MFG-E8), C1q, Vitronectin receptor, and Axl/Mertk/Tyro3 receptor tyrosine kinase family(6, 23, 24, 26, 28, 45, 102-106). However, only knockout mice lacking C1q, MFG-E8, and Axl/Mertk/Tyro3 receptor tyrosine kinases generate spontaneous autoimmunity(26, 28, 106).

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, Brt, Tif, Dtk)(71). Each member of the Axl/Mertk/Tyro3 receptor family shares a similar extra cellular 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(46). Mice lacking Mertk, or mertk<sup>kd</sup> (previously known as mer<sup>kd</sup>) mice show spontaneous autoantibody production(28, 46), splenomegaly, and enhanced TNF-α production in response to lipopolysaccharide(82). Further, 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(27). 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)(80). 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(107). The 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(108), but its role as a ligand for this family is still unclear, as Tyro3 is the only family member that binds to Protein S(92). 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(79). However, Protein S binds phosphatidylserine on apoptotic cells and can enhance phagocytosis, similar to GAS6(35).

The enhanced autoimmunity observed in the triple knockout mouse and the higher affinity Axl and Tyro3 have for the proposed ligands led us to further investigate the role of Axl and Tyro3 in clearance of apoptotic cells in different phagocytes(27, 80). In this report, we demonstrate that Axl and Tyro3 do function in apoptotic cell phagocytosis in both macrophages and dendritic cells, but their importance in terms of rate and extent of apoptotic cell phagocytosis varies depending upon the cell type. 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, Mertk 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. Yet the spleen relies on Axl and Tyro3 and not Mertk.

2.3 Materials and Methods

Animals. All mice were housed in specific pathogen-free Division of Laboratory Animal Medicine facilities in accordance with IACUC regulations. Male mice 8-10 weeks old were used in studies and wild-type mice are C57BL/6 (Jackson Laboratories and bred in house). merkkd, axl-/- and tyro3-/- mice were backcrossed onto the C57BL/6 background for six generations. Previously designated merkd mice are now designated merkkd in this report, as Mertk is the proper nomenclature. These merkkd mice show no detectable protein by Western blot or flow cytometry (unpublished results), therefore they are a functionally null mutation.

Macrophage in vitro phagocytosis. Peritoneal macrophages were isolated and phagocytosis assay were performed as previously described(28) with the following modifications. Peritoneal exudate cells were elicited by intraperitoneal injection with 3 ml of 3% thioglycollate (Fisher) for 72 hours followed by lavaging the peritoneum with 3 ml phosphate buffered saline. Resident macrophages were obtained by lavaging the peritoneum of naïve mice with phosphate buffered saline. Peritoneal exudate cells were plated at 1.5x10^5 cells/well of a 24 well plate. After 2 hours, non-adherent cells were washed off with PBS and fresh macrophage media (RPMI 1640 (Gibco) supplemented with 5% heat-inactivated FBS (Gemina Bio-products), 50 units penicillin G and 50 µg ml^-1 streptomycin sulphate (Gibco), sodium pyruvate (Sigma), and β-mercaptoethanol (Gibco)) was added. The resulting adherent peritoneal macrophages were then rested overnight at 37° C with 5% CO2.
To generate apoptotic thymocytes, we collected thymus from naïve wild-type animals and dissociated the tissue using forceps. The single cell suspension was then washed one time in PBS and resuspended in macrophage media with 2 µM dexamethasone (Sigma). After 5 hours of incubation at 37° C, 2 µM Cell Tracker Green or Cell Tracker Orange (Molecular Probes) was added to thymocytes and incubated for 30 minutes, cells were washed with PBS and then rested an additional 30 minutes in macrophage media. Annexin V-FITC (Trevigen) and VAD-FMK-FITC staining and analysis by flow cytometry revealed that thymocytes were 70-80% apoptotic. To generate opsonized thymocytes, freshly isolated thymocytes were incubated with murine anti-murine CD3 antibody (Serotec) for 30 minutes at 4° C with gentle rotation. After staining, cells were washed 3 times with PBS and then resuspended in media. Addition of fluorochrome-conjugated anti-mouse secondary antibody revealed greater than 80% of the thymocytes were labeled with CD3 antibody by flow cytometry. For phagocytosis assays, thymocytes were added at a 10:1 thymocyte:macrophage ratio for indicated timepoints. Macrophages were then washed extensively with phosphate buffered saline and fixed with 1% paraformaldehyde (Sigma). Phagocytosis was determined by first counting macrophages in brightfield and then overlaying image of fluorescent apoptotic cells to determine the number of macrophages with ingested apoptotic cells. Only apoptotic cells that were completely within boundaries of macrophage were scored as phagocytosed. 200 macrophages per timepoint in triplicate were counted for each genotype.

**Dendritic cell in vitro phagocytosis.** Bone marrow derived dendritic cells (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 LSM (MP) and centrifuged 500 x g for 15 minutes. The monocyte layer was collected, washed in PBS and resuspended in RPMI
1640 (Gibco) supplemented with 10% FBS, 50 units penicillin-G and 50 µg ml⁻¹ streptomycin sulphate (Gibco), 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 media was doubled and cytokines were readjusted to 10 ng/ml. On day 7, immature dendritic cells were washed once in PBS and plated in low cluster 24 well plates at 10⁶ cells/well. Splenic dendritic cells were isolated using anti-CD11c antibody bound to microbeads (Miltenyi Biotec). Splenic CD11c-positive cells were used immediately for phagocytosis. Apoptotic thymocytes were generated as described above and added to dendritic cells at a 10:1 ratio. At indicated time points, the cell suspension was plated on a glass coverslip and dendritic cells were adhered for 1 hour. Non-ingested thymocytes were washed off and dendritic cells were stained with anti-CD11c-FITC antibody (Pharmingen). Cells that were positive for both CD11c and ingested apoptotic cells were scored. To analyse pinocytosis, 1 mg/ml Albumin-FITC and 0.5 mg/ml dextran-FITC (Sigma) were added to dendritic cells for 30 minutes at 37°C or 4°C as a negative control. Cells were then washed and analysed by flow cytometry to determine uptake of albumin or dextran.

**Ligand binding assay and Western Blotting.** Axl, Mertk, or Tyro3 extracellular domain-Human IgG₁ Fc chimeras were purchased from R and D Systems. 10 ng of chimera was added to 100 µl normal mouse serum (Vector Laboratories) or PBS at room temperature for 1 hour with gentle rotation. Protein A sepharose (Amersham) was added to chimera/serum mixture and incubated an additional 30 minutes at room temperature with gentle rotation.

For phosphorylation experiments, thioglycollate elicited peritoneal cells were plated at 1 x 10⁷ cells per 100 mm petri dish. After 2 hours non-adherent cells were washed off and macrophages were rested for 7 days. Apoptotic cells were added at 10:1,
thymocytes:macrophage, ratio for 15 minutes and then macrophages were washed three times with PBS. Macrophages were lysed using IP lysis buffer (Tris Buffered Saline, 1% NP-40 (Pierce), 0.1 mg aprotinin, 0.1 mg α-1-antitrypsin, 0.1 mg leupeptin (Sigma), 1 mM PMSF (Sigma), 2 mM sodium orthovanadate (Sigma), and phosphatase inhibitor cocktails I and II (Sigma)) and lysates cleared by centrifugation. Non-specific proteins bound to sepharose were removed by incubating with Protein G sepharose (Amersham) alone for 30 minutes at 4° C followed by centrifugation. 0.5 μg anti-Mertk antibody (R and D systems) was added to cleared lysate and incubated 30 minutes at 4° C with gentle rotation. Protein G sepharose was added and lysates were incubated an additional 30 minutes at 4° C with gentle rotation. Proteins bound to sepharose were isolated by washing three times in lysis buffer. After final wash, SDS sample loading buffer was added and the sample was boiled, and resolved on an SDS-PAGE gel. Proteins were transferred onto PVDF membrane (Millipore) and blotted in 5% milk- Tris Buffered Saline with 0.1% Tween-20 (TBS-T) for 30 minutes. Primary antibody: anti-Protein S (Santa Cruz), anti-phosphotyrosine (Cell Signalling) or anti-Mertk (R and D systems) was added at 1:1000 overnight at 4° C. Blots were washed in TBS-T and incubated in anti-goat-HRP (Vector Laboratories) or anti-mouse-HRP (Vector Laboratories) for phosphotyrosine blots at 1:10,000 for 2 hours followed by further washing in TBS-T. Blots were incubated with ECL-Plus (Amersham) and visualized using Maximum Resolution film (Kodak).

**RT-PCR of GAS6 and Protein S.** Reverse transcriptase was performed mostly as described(109). Briefly, RNA was isolated from cells using the Trizol (Gibco BRL) reagent and 15 μg of crude RNA was treated with DNAse (Promega). After isolation, cDNA was synthesized for 60 minutes at 42° C using 5 μg RNA with 150 ng random hexamers and
MMLV reverse transcriptase (Invitrogen). PCR was then performed using 50 ng cDNA and primers specific for GAS6 (forward 5’ ACAGGCTCAACTACACCGAACAT 3’ and reverse 5’ TGACGGGTGCAGAAATCACCGATA 3’), Protein S (forward 5’ CGCGGTCTTGGACAAAGCAATGAA 3’ and reverse 5’ TGCCAGCTGGTGATAGGAATGTGA 3’), and GAPDH (forward 5’ CTACACTGAGGACCAGGTGTTGTCT 3’ and reverse 5’ GCGAACTTTATTGATGTTCAAA 3’). The PCR products were resolved on a 1.5% agarose gel using 100 bp molecular weight standards (Invitrogen).

**In vivo clearance of apoptotic cells and immunohistochemistry.** To look at the thymus in vivo, adult mice were injected intraperitoneally with 0.2 mg/25 g of dexamethasone (Sigma) as previously described(28). 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. 5 µm sections were cut and stained using the TUNEL method. Briefly, sections were deparaffinized with Histoclear (National Diagnostics) and graded ethanol to Tris Buffered Saline (TBS), permeabilized in TBS+0.1% Triton X-100 for 5 minutes 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) for 30 minutes at 37°C. The other half of thymus was dissociated and stained with VAD-FMK-FITC (Promega) to detect active Caspase 3 positive apoptotic cells. These cells were analysed on a Becton-Dickinson 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. They were then embedded in paraffin and 5 µm sections were cut and stained with hematoxylin and eosin as described previously(110).
To induce apoptosis in the spleen, KLH was injected IP as previously described(26). Briefly, 150 µg/25g of KLH in complete Freund’s adjuvant was injected twice, two weeks apart and four days after second injection, spleens were collected and snap frozen in OCT. 5 µm frozen spleen sections were cut and stained for TUNEL as described above. Tissues were analyzed using Image Pro Plus software, counting three fields per mouse, three mice per treatment group.

Statistical analysis. Statistics were calculated using JMPIN 4.0 software (SAS Institute Inc.). Tukey-Kramer HSD One-way analysis of variance (ANOVA) was performed at an alpha level of 0.01 or 0.05 to compare all variants in a data set.

2.4 Results

Mertk is critical for phagocytosis of apoptotic cells by macrophages(28), 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 mertk<sup>kd</sup> mice had a dramatic deficit in clearance of apoptotic cells in vitro. Although not as complete, macrophages from axl<sup>-/-</sup>, tyro3<sup>-/-</sup>, and axl/tyro3<sup>-/-</sup> mice were significantly less able (by ~50%) to phagocytose apoptotic cells when compared to macrophages from wild-type mice (Figure 2.1D). Representative confocal images demonstrate the apoptotic cells within the F4/80 positive macrophages (Figure 2.1A-C). No phagocytosis was observed in macrophages incubated at 4° C or with 2 µM Cytocholasin D indicating that measurements were of phagocytosis and not binding (Figure 2.1A-D, H).
In order to determine differences in the rate of ingestion, phagocytosis of apoptotic cells by macrophages was quantified over a time course. At 60 and 90 minutes macrophages from mice lacking \textit{axl/tyro3}^{+/−} had significantly lower levels of phagocytosis than wild-type macrophages (Figure 2.1E). Macrophages lacking Mertk remained incompetent even at 90 minutes. 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 minutes and then declined thereafter (Figure 2.1F). Similar to thioglycollate-elicited macrophages, resident peritoneal macrophages from \textit{axl/tyro3}^{−/−} mice had significant defects in phagocytosis at 60 and 90 minutes; although, they exhibited an intermediate response between macrophages from wild-type and \textit{mertk}^{kd} mice (Figure 2.1G).

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 (LB) or anti-CD3 antibody opsonized thymocytes (OP). There was no difference in phagocytosis by macrophages from wild-type mice versus macrophages from \textit{mertk}^{kd}, \textit{axl}^{−/−}, \textit{tyro3}^{−/−}, or \textit{axl/tyro3}^{+/−} mice (Figure 2.1H). This indicated that the defect in phagocytosis observed in \textit{mertk}^{kd} and \textit{axl/tyro3}^{+/−} macrophages was specific for apoptotic cells.

The mechanism for the phagocytosis of apoptotic cells by dendritic cells is not known; however it does not require Mertk(111). We postulated that Axl or Tyro3 might function on dendritic cells 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 dendritic cells were produced. There was no difference in co-stimulatory molecules CD80, CD86, MHC Class II or CD11c expression on dendritic cells from wild-
type versus those from $mertk^{kd}$, $axl/tyro3^{-/-}$ mice (Figure 2.2a). However, when immature bone marrow derived dendritic cells (BMDCs) were co-cultured with fresh or apoptotic thymocytes, dendritic cells from wild-type, and $mertk^{kd}$ mice cleared apoptotic cell with equal efficiency, while a significant decrease in phagocytosis was observed in dendritic cells from $axl^{-/-}$, $tyro3^{-/-}$, and $axl/tyro3^{-/-}$ mice (Figure 2.2B-D, E). Further, while BMDCs from $axl/tyro3^{-/-}$ mice never reached levels comparable with BMDCs from wild-type or $mertk^{kd}$ mice, BMDCs from $mertk^{kd}$ mice continued to ingest apoptotic cells up to 12 hours after being fed (Figure 2.2F). Confocal images were used to confirm that apoptotic cells were within CD11c positive dendritic cells and that the apoptotic cells had not been digested by the phagosome (Figure 2.2B-D).

To ensure that the BMDCs were indicative of those found in vivo, splenic dendritic cells were isolated and co-cultured with apoptotic cells. Similar to BMDCs, splenic dendritic cells from $axl/tyro3^{-/-}$ mice had a significant decrease in phagocytosis at 6 hours. In contrast, there was no significant difference between dendritic cells from wild-type and $mertk^{kd}$ mice (Figure 2.2G). A major characteristic ascribed to dendritic cells is pinocytosis where microparticles are continuously taken up from the environment for antigen presentation. To determine if this process was altered in $mertk^{kd}$ or $axl/tyro3^{-/-}$ mice, we incubated dendritic cells with FITC-conjugated albumin or FITC-conjugated dextran at 37º C or as a negative control at 4º C. There was no significant difference in the uptake of dextran or albumin in wild-type BMDCs versus BMDCS from $mertk^{kd}$ and $axl/tyro3^{-/-}$ mice. This indicated that pinocytosis is normal in BMDCs from $mertk^{kd}$ and $axl/tyro3^{-/-}$ mice (Figure 2.2H). However, these data suggest that macrophages and dendritic cells have a fundamental difference in
their ability to phagocytose apoptotic cells and this mechanism is mediated by different combinations of the Axl/Mertk/Tyro3 receptor family.

Preferential utilization of the three receptor tyrosine kinases by macrophages and dendritic cells 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(79), 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 (Figure 2.3A).

The selective usage of Axl/Mertk/Tyro3 receptors by phagocytes prompted an examination of whether dendritic cells or macrophages express different ligands. Using RT-PCR, we found that macrophages express both GAS6 and Protein S, while immature BMDCs only express Protein S (Figure 2.3B). 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 dendritic cells allows preferential interaction of only Tyro3.

The efficiency with which dendritic cells ingest apoptotic cells is markedly slower than macrophages. Dendritic cells require 6-9 hours to reach maximum numbers of ingested apoptotic cells (Figure 2.2F) whereas macrophages reach optimum numbers by 60 minutes (Figure 2.1E). 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; mean fluorescene intensities of 22.43, 15.38, and 11.44 respectively.
In contrast, dendritic cells have low levels of surface Mertk, Axl, and Tyro3; mean fluorescence intensity of 2.74, 7.51, and 4.74 compared with macrophages (Figure 2.3F-H). The low levels of Axl, Mertk, and Tyro3 expression on the surface of dendritic cells suggest these phagocytes may be less efficient at recognizing and binding apoptotic cells; hence, this could correlate with their slower rate of ingestion. The high level of Axl, Mertk, and Tyro3 on macrophages, on the other hand, is consistent with their greater efficiency in recognition, binding, and rate of ingestion of apoptotic cells.

To determine if 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 merktld mice are unable to clear an in vivo dexamethasone-induced apoptotic cell burden in the thymus(28). To determine whether Axl and Tyro3 function as co-phagocytic receptors in the thymus, we injected wild-type, merktld, axl−/−, tyro3−/−, and axl/tyro3−/− mice with dexamethasone and monitored apoptotic cell accumulation in the thymus. Eight hours is when the maximum level of apoptosis is reached in the thymus, and at this timepoint all animals had similar numbers of apoptotic cells in the thymus as observed by flow cytometry staining and TUNEL staining in tissues (Figure 2.4B and data not shown). This indicated that the induction of apoptotic cells by dexamethasone was similar in all genotypes.

At 24 hours, as previously shown, merktld mice were defective in clearance and therefore had an abundance of uncleared apoptotic cells in the thymus as evidenced by TUNEL+ staining (Figure 2.4A). In contrast, in axl−/−, tyro3−/−, and axl/tyro3−/− animals
clearance of apoptotic cells was 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 (Figure 2.4C). The fact that the axl<sup>−/−</sup>, tyro3<sup>−/−</sup>, and axl/tyro3<sup>−/−</sup> 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. Mertk was both necessary and sufficient in this context.

Recently, GAS6 has been implicated in efficient removal of outer segment debris in the retina, suggesting that the cognate receptors Axl, Mertk, and Tyro3 may be participating in the clearance of outer segments by retinal pigment epithelial cells(112). In addition, mutations in mertk have been linked with retinitis pigmentosa indicating a role for Mertk in maintaining normal retinal apoptotic material clearance and suggesting that Mertk functions in epithelial cells as well as in macrophages(28, 100). Retinal pigment epithelial cells lacking Mertk cannot clear apoptotic-like outer segment debris. This results in retinal degeneration, and destruction of the outer nuclear layer (ONL) by 6-7 weeks of age(100). To determine whether Axl and Tyro3, which have a higher affinity for GAS6 than Mertk, also contribute to retina homeostasis (as they do in macrophages – see Figure 2.2), we examined the retina of young and old adult axl<sup>−/−</sup>, tyro3<sup>−/−</sup> and axl/tyro3<sup>−/−</sup> knockout mice. At two months of age, there was no retinal degeneration in normal mice or in axl<sup>−/−</sup>, tyro3<sup>−/−</sup>, or axl/tyro3<sup>−/−</sup> mice (Figure 2.5). The retina of axl<sup>−/−</sup>, tyro3<sup>−/−</sup>, and axl/tyro3<sup>−/−</sup> mice had an ONL of between 12-14 nuclei similar to wild-type mice. Furthermore, older axl<sup>−/−</sup>, tyro3<sup>−/−</sup>, and axl/tyro3<sup>−/−</sup> mice were without evidence of retinal degeneration at 6 months of age (data not shown). In contrast, and as previously reported, mertk<sup>−/−</sup> mice had complete degeneration of the ONL at two months of age (Figure 2.5). 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 Mertk.

Another organ where high numbers of apoptotic cells are generated due to B cell activation and maturation is the spleen. We examined the role of Axl, Mertk, and Tyro3 in the clearance of an apoptotic cell burden in the spleen using the KLH model. We found that four days after KLH immunization apoptotic cells were efficiently cleared from wild type mice, whereas mertk<sup>kd</sup> mice had a slight increase in TUNEL positive cells (Figure 2.6). Interestingly, axl<sup>−/−</sup>, tyro3<sup>−/−</sup> mice had significantly higher numbers of TUNEL positive cells in the spleen compared with wild type and this effect was additive in the axl/tyro3<sup>−/−</sup> double knockout. Thus, in the spleen Axl and Tyro3 are most critical for efficient phagocytosis of apoptotic cells, whereas Mertk is not.

To further examine the cell type specificity, we studied Mertk phosphorylation in macrophages stimulated with apoptotic cells. Macrophages from wild-type mice possessing Axl and Tyro3 were able to phosphorylate Mertk at 15 minutes after apoptotic cell stimulation. In contrast, macrophages lacking Axl and Tyro3 had markedly diminished Mertk phosphorylation (Figure 2.7). These results suggest that in macrophages (and perhaps other cells), Axl and Tyro3, which have higher affinity for ligand, may be required for efficient Mertk tyrosine phosphorylation. Axl and/or Tyro3 may bind ligand and then activate Mertk by heterodimerization similar to ligand-dependent heterodimers found in other receptor families (e.g. EGFR family). Whether this is true in other cells (e.g. retinal epithelial cells) in which Mertk seems to function well in the absence of Axl and Tyro3 remains to be seen.
2.5 Discussion

The efficient clearance of apoptotic cells is paramount for maintaining tissue homeostasis. Patients and animal models of systemic lupus erythmatosus including, NZB/W, MRL, and lpr mice have phagocytes that are unable to clear apoptotic debris (29, 30, 113). Many genes have been implicated in apoptotic cell clearance, and a subset of these genes (such as \textit{mfg-e8} and \textit{mertk}), when deleted, lead to autoimmune phenotypes (26, 28). 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. Mertk 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 Mertk alone, e.g. autoantibody production, splenomegaly, and lymphocyte activation. This prompted further evaluation of the role that Axl and Tyro3 play in the phagocytosis of apoptotic cells.

In this study, we utilized single and double knockout mice for \textit{axl} and \textit{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 antigen-presentation and regulate adaptive immune responses, macrophages and dendritic cells. Macrophages from mice lacking \textit{axl}, \textit{tyro3}, or both \textit{axl/tyro3}, were less efficient in clearing apoptotic cells compared with wild-type macrophages (Figure 2.1). Macrophages from mice lacking \textit{mertk} exhibited a more dramatic deficit in clearance compared with macrophages from mice lacking \textit{axl} or \textit{tyro3} however, a 50\% reduction was significant and comparable to other receptor knockout models (6, 23, 26).
In contrast, while dendritic cells from mice lacking mertk had no defect in clearance of apoptotic cells (Figure 2.2E-G and (111)), dendritic cells from mice lacking axl or tyro3 had a dramatic deficit. Thus, Axl and Tyro3 are the two receptor tyrosine kinase family members that initiate ingestion of apoptotic cells by dendritic cells. Furthermore, later time points revealed dendritic cells from mice lacking mertk, had prolonged or extended phagocytic activity (Figure 2.2F), perhaps suggesting that Mertk may be playing a role to down regulate ingestion by dendritic cells. This is an important finding as numerous reports link the phagocytosis of apoptotic cells by dendritic cells to antigen presentation of apoptotic cell material(114).

In our studies, apoptotic cell phagocytosis by macrophages took only 60 minutes; dendritic cells took 6 hours. One plausible explanation for this slower ingestion was the lower expression of Axl, Mertk, and Tyro3 in dendritic cells, compared with macrophages (Figure 2.3). 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 (macrophages) versus professional antigen-presenting cells (dendritic cells). We also examined ligand expression for this family of receptors as a potential control point and found that while macrophages express both Protein S and GAS6, dendritic cells only express Protein S (Figure 2.3B and ref (67)). This could suggest that dendritic cells are more restricted and reliant on Tyro3 for phagocytosis, as Tyro3 was the only receptor shown to bind mouse Protein S (Figure 2.3A). The physiologic roles of Protein S and GAS6, which are present in the circulation, is to serve in the natural coagulation system and bind to phosphatidylserine on apoptotic cells, platelets, and endothelial cells(115). In vivo, it is certainly possible that the microenvironment of phagocytes contacting apoptotic cells and the
selective 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 (Figure 2.5A, C). It appears that mertk<sup>kd</sup> mice can clear apoptotic cells resulting from the physiologic negative and positive selection of thymocytes, as the untreated mertk<sup>kd</sup> mouse does not exhibit a large excess of apoptotic cells. It is possible that Axl and Tyro3 may be compensating for lack of Mer in mertk<sup>kd</sup> mice in normal homeostasis; however, our data suggest that Mertk is the primary molecule on thymic phagocytes critical for clearance of a large synchronous burden of apoptotic cells. It is of interest to note that, when all three receptors are missing apoptotic cell removal is hindered severely and multiple organs display uncleared apoptotic cells these include brain, testes, liver, and lymphoid tissue(27, 95).

In the retina, we also show that Mertk is critical for maintenance of the retinal tissues, whereas deletion of axl, tyro3, or both axl/tyro3 did not result in retinal degeneration (Figure 2.6). Furthermore, this observation is consistent with the literature that suggests a mutation in mertk is one primary cause of retinal degeneration(99, 100). These data suggest that degeneration of the retina reported in the triple mutant axl/mertk/tyro3<sup>x/c</sup> mice is primarily due to the absence of Mertk(95). A recent report demonstrates Tyro3 expression but not Axl expression in retinal pigment epithelial cells; however, our data suggest that Tyro3 is not critical for normal maintenance of the retina.
We have previously shown that GAS6 triggers Mertk tyrosine phosphorylation and downstream activation of Vav1, Rac1, and Cdc42(116). 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 which 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 (Figure 2.7). We are currently investigating whether receptor aggregation is different in macrophages versus dendritic cells. Alternative experiments to identify interaction among the Axl/Mertk/Tyro3 family of receptors have been difficult due to cross-reactivity of current antibodies; however, clues that these receptors act in combination have been demonstrated in triple axl/mertk/tyro3 knockout mice(27) and in recent studies using platelets, which also express all three receptors and appear to require heterodimerization for receptor tyrosine phosphorylation(98). Nonetheless, our report is the first to demonstrate multiple members of this receptor family are needed for macrophage ingestion of apoptotic cells.

Figures 2.4 and 2.5 demonstrate that Mertk alone functions \textit{in vivo} in the retina and thymus under normal homeostatic conditions (the retina) or stress-induced burden of apoptotic cells (the thymus). Thus, either Mertk does not require kinase activation for some functions or it can function at levels of tyrosine phosphorylation below the limits of detection of our \textit{in vivo} assays. To test whether there are kinase-independent functions, a “kinase-dead” Mertk will need to be knocked-in to the Mertk locus and the consequence for retinal, thymic, and macrophage apoptotic material/cell clearance assessed. More likely, heterodimerization between Axl, Mertk, and Tyro3 family members is important, but not an
absolute requirement. While the *in vitro* system (apoptotic cells and isolated macrophages) appears to show the need for heterodimers; *in vivo* Mertk can function well in the absence of Axl and Tyro3, perhaps by using other accessory molecules.

The spleen provided another immune organ to examine the phagocytosis of apoptotic cells. Interestingly we found that Axl and Tyro3, and not Mertk are critical for clearance of apoptotic B cells in the spleen (Figure 2.6). It is interesting to speculate the dendritic cells are therefore playing an important role in this organ in clearance. Previous reports have shown that tingible body macrophages are primarily responsible for clearance of the apoptotic cells in the spleen and we cannot rule out this cell type in our system. Further studies isolating specific populations of cells from the spleen will elucidate the role of cell type and receptor usage in the spleen. In the spleen *axl* and *tyro3* deficiency appear to have an additive effect compared with the single knockouts alone. This was not observed in macrophages or dendritic cells and this may lend insight into the function of these two receptors in this organ.

Many questions regarding Axl/Mertk/Tyro3 receptors and how they function differently on different phagocytes remain. Low levels of these receptors on dendritic cells have made detection of protein and phosphorylation difficult; therefore we cannot assess the requirement of Axl and Tyro3 for Mertk activation in dendritic cells. MFG-E8 is an intermediate protein that facilitates clearance of apoptotic cells in the spleen and is secreted by macrophages and dendritic cells and interacts with the integrin $\alpha_v\beta_3$(117). Other reports have shown that Mertk may also require the integrin $\alpha_v\beta_3$ for efficient signaling(118). 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. 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 dendritic cells. Axl is known to be cleaved by ADAM10 and is thought to exist primarily as a cleaved soluble form in murine dendritic cells(67). This soluble form of Axl (sAxl) has been found in complex with GAS6 in the serum of mice(67) 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 understanding 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 dendritic cells may provide clues for their biologic relevance. For macrophages, Axl and Tyro3 appear to be important for phosphorylation of Mertk, an event thought to be necessary for rapid activation of phagocytosis whereas dendritic cells, which do not require Mertk, are less efficient. The different engagement of this receptor family by macrophages versus dendritic cells may be partly dictated by the GAS6 or Protein S expression profile and the level of expression of these receptors on phagocytes. Dendritic cells express lower levels of Axl/Mertk/Tyro3 and this is also correlated to slower ingestion rates. 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. However, in the spleen Axl and Tyro3 are required for clearing an apoptotic cell burden whereas Mertk is not. Lastly, the cooperation among this receptor family may be important for efficient signaling.
as the different receptors have different ligand preference and affinity.
Figure 2.1 Macrophages lacking Axl and/or Tyro3 have a deficiency in clearance of apoptotic cells. A) Representative confocal images of WT B) mertk<sup>kd</sup>, or C) axl/tyro3<sup>−/−</sup> macrophages stained with F480-FITC and fed Cell Tracker Orange apoptotic cells. D) <i>In vitro</i> phagocytosis assay with thioglycollate-elicited PECs collected at 60 minutes after non-apoptotic (UT, open bars) or apoptotic (AT, closed bars) thymocytes were added. E) Thioglycollate-elicited macrophages from wild-type (●) mertk<sup>kd</sup> (■), or axl/tyro3<sup>−/−</sup> (▲) mice or F) resident macrophages were fed apoptotic thymocytes for indicated time points in minutes. G) Thioglycollate-elicited macrophages were fed 2 µM latex beads (LB, closed bars) or opsonized thymocytes (OP, open bars) for 60 minutes. WT- wild type Error bars are standard error, n > 3. * p < 0.05, ** p < 0.01.
Figure 2.2 Dendritic cells lacking Axl and/or Tyro3 have a deficiency in clearance of apoptotic cells. A) BMDCs were stained for CD11c-PE and MHC Class II-FITC, CD80-FITC, or CD86-FITC and analysed by flow cytometry. Data is gated on CD11c+ cells. This data is representative of 3 independent experiments. B) Representative image of phagocytosis assay, wild-type dendritic cells stained with CD11c-FITC, fed Cell Tracker Orange apoptotic cell. Images were taken on a Zeiss Confocal microscope. BMDCs (C, D) or splenic CD11c+ cells (E) were fed non-apoptotic thymocytes (UT, open bars) or apoptotic thymocytes (AT, closed bars) for 6 hours (C, E) or indicated time points in hours (D) and then plated on coverslips. BMDCs from wild-type (♦) mertk$^{kd}$ (■), or axl/tyro3$^{-/-}$ (▲) mice. F) BMDCs were treated with dextran or albumin at 4°C as a control or 37°C. Error bars are standard error, n > 3. * p < 0.05, ** p < 0.01.
Figure 2.3 Binding of Protein S to Tyro-3 and expression of GAS6, Protein S, Axl, Mertk, and Tyro3 by phagocytes. A) Mertk, Axl, and Tyro3 extracellular domains fused with Human IgG1 Fc domains (R and D systems) were incubated with or without normal mouse serum (NMS) and blotted with antibodies specific for Protein S (WB). B) Detection of GAS6 and Protein S by RT-PCR. cDNA was isolated from lung, immature BMDCs, macrophages (MΦ) or a monocyte cell line 32D. Absence of Taq polymerase (-Taq) is negative control for PCR and lack of reverse transcriptase (–RT) is negative control for cDNA preparation. Primers specific for GAS6, Protein S and GAPDH were used to examine expression by these cell types. MW is a 100 bp ladder. C-E) Thioglycollate-elicited peritoneal cells from wild-type mice were isolated and stained with F4/80-FITC (Pharmingen) and Mertk (R and D Systems), Axl (Santa Cruz), and Rse (Tyro-3, Santa Cruz) antibodies followed by anti-goat-PE (Caltag). Histograms are gated on F4/80 positive cells. F-H) Bone marrow-derived dendritic cells from wild-type mice on day 7 were harvested and stained for CD11c (Pharmingen) and receptors as indicated above. Histograms are gated on CD11c positive cells. Shaded sample is isotype control. Histograms are representative of at least three independent cell preparations.
Figure 2.4 Mertk is responsible for clearance of apoptotic cells in the thymus. Mice were injected with dexamethasone (closed bars) or PBS (open bars) for 8 hours (B), or 24 hours (A, C) and thymi were collected. Half the thymus was stained for TUNEL (A) and the other half was dissociated and stained with VAD-FMK and analyzed by flow cytometry (B and C) WT- wild type, M- mertk\textsuperscript{kd}, A- axl\textsuperscript{-/-}, T- tyro3\textsuperscript{-/-}, and AT- axl/tyro3\textsuperscript{-/-}. Data acquired from cytometry was gated on the whole/live cells in the suspension. Error bars represent standard error, n > 4. * p < 0.05
Figure 2.5  

Mertk is responsible for retina homeostasis. Eye’s were removed from 8 week old mice, fixed in 4% paraformaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin to examine morphology. ONL-outer nuclear layer is missing from retina in mertkkd mice, arrowhead indicates retinal pigment epithelium. Images are representative of 5 mice of each genotype. Scale bar is 50 microns.
Figure 2.6 Axl and Tyro3 are required for in vivo clearance in the spleen. Untreated mice, KLH-mice immunized with KLH, WT- wild type, M- mertk<sup>kd</sup>, A- axl<sup>+/+</sup>, T- tyro3<sup>+/+</sup>, and AT- axl<sup>+/+</sup>/tyro3<sup>+/+</sup>. Spleens were collected and stained for apoptotic cells using TUNEL method. This is representative of three experiments. Error bars represent standard error. * p < 0.05, ** p < 0.01
**Figure 2.7**

Macrophages were incubated with apoptotic cells for 15 minutes and then immunoprecipitated for Mer. Proteins were resolved and blotted with antibody specific for phosphotyrosine, stripped and reprobed for total Mer. This data is representative of two independent experiments. WT- wild type, M- mertk

**Figure 2.7 Axl and Tyro3 are required for efficient Mertk phosphorylation.**

Macrophages were incubated with apoptotic cells for 15 minutes and then immunoprecipitated for Mer. Proteins were resolved and blotted with antibody specific for phosphotyrosine, stripped and reprobed for total Mer. This data is representative of two independent experiments. WT- wild type, M- mertk, and AT-axl/tyro3/-. 
CHAPTER 3
ENGAGEMENT OF AXL/MERTK/TYRO3 RECEPTOR FAMILY MEMBERS AND THEIR REGULATION OF SIGNAL TRANSDUCTION IN PHAGOCYTOSIS AND CYTOKINE PRODUCTION
3.1 Abstract

Many receptor tyrosine kinases have been implicated as phagocytic receptors for apoptotic cells; however, little is known about how these receptors interact and signal for engulfment and down regulation of inflammatory cytokines. As demonstrated in Chapter 2, each of the Axl/Mertk/Tyro3 family of receptor tyrosine kinases is critical for efficient phagocytosis in macrophages. The genetic deletion of these receptor tyrosine kinases results in autoantibody production, suggesting the importance of these receptors in signaling after recognition of apoptotic cells. Using knockout animals and \textit{in vitro} cell lines, we demonstrate here that Axl, Mertk, and Tyro3 bind to the apoptotic cell. Both Vav1 and $\alpha_5\beta_5$ have been demonstrated previously to bind Mertk and become activated upon Mertk stimulation. We found that Tyro3 is required for efficient binding of Vav1 to Mertk, but no binding of $\alpha_5\beta_5$ to Mertk was observed. Downstream of receptor engagement is signaling for engulfment through intermediates such as FAK and Akt. We found that in macrophages, Mertk and Tyro3 are required for efficient FAK phosphorylation upon apoptotic cell stimulation, however phospho-Akt levels remained unchanged in macrophages deficient in Axl, Mertk, or Tyro3. Lastly, although Axl, Mertk, and Tyro3 are important in activating engulfment signals, the down regulation of inflammatory cytokines is not a function of this family of receptors. This is the first report of Axl, Mertk, and Tyro3 function in signaling for engulfment in primary cells. Thus, these data are an important step in understanding how Axl, Mertk, and Tyro3 deletion leads to dysfunctions in phagocytosis of apoptotic cells and autoimmune disease.
3.2 Introduction

Receptor tyrosine kinases are important for initiating signaling from a large variety of extracellular ligands. Many receptor tyrosine kinase families share ligands and signaling cascades, however there are very few receptor tyrosine kinase families that are known to interact. One such family is the EGF receptor family, which has 4 members that are known to heterodimerize to enhance ligand affinity and activation(119). The Axl/Mertk/Tyro3 family of receptor tyrosine kinases has been implicated in several biological processes including the phagocytosis of apoptotic cells(106).

Previous reports have shown that the ligands for these family members, Growth arrest specific gene 6 (GAS6) or Protein S (PS) bind specifically to phosphatidylserine exposed only on the surface of apoptotic cells. GAS6 and Protein S are thought to then engage Axl, Mertk, and/or Tyro3 and enhance phagocytosis of apoptotic cells. However, little is known of how the two ligands, GAS6 and PS, differentially bind and initiate signals through these receptors.

Reports from our group have demonstrated that multiple family members are required for efficient phagocytosis of apoptotic cells (Chapter 2). For example in murine macrophages, Mertk is not efficiently phosphorylated in macrophages lacking Axl and Tyro3 and macrophages from mice lacking axl, tyro3, or axl/tyro3 have a significant defect in phagocytosis. Further, in the retina, Mertk can be activated by Tyro3, presumably through Protein S signaling(91); however, Tyro3 appears not to be critical as we showed in the previous chapter that there is no anatomical deficit in vivo and the eyes of tyro3⁻/⁻ mice appear histologically normal. This lends support to the hypothesis that these receptors
interact with ligand to efficiently signal for the phagocytosis of apoptotic cells; however, the usage of each family member is complex and perhaps cell type specific.

Previous groups have shown that Mertk can associate with integrin $\alpha_5\beta_3$ and Vav1 in separate *in vitro* systems upon GAS6 stimulation(116, 118). Integrin $\alpha_5\beta_3$ and Vav1 have also been demonstrated to be involved in the phagocytosis of apoptotic cells(117, 120). Upon phagocytosis of apoptotic cells, the phagocyte must activate the cytoskeletal rearrangement complex involving the activation of PI3Kinase, Rac, Rho, and/or FAK, CrkII, and Dock180. In addition, evidence suggesting Mertk and Axl activate PI3K signaling cascades further support the notion that these receptors initiate downstream signals important for engulfment of apoptotic cells. It is currently unclear if Axl, Mertk, and Tyro3 are directly responsible for initiating this engulfment of apoptotic cells.

An important concept that stems from the phagocytosis of apoptotic neutrophils or macrophages is the down regulation of pro-inflammatory cytokines(20). Upon ingestion of apoptotic cells, but not foreign material or bacteria, the phagocyte is thought to reprogram its activation and initiate signals that shut down a pro-inflammatory cascade of cytokines(20). This process has been shown to involve activation of Akt, NF-kB, and subsequently TGF-$\beta$ expression(19, 121). Mertk is responsible for the down regulation of TNF-$\alpha$ signaling in response to LPS stimulation(82). Because these receptors are involved in phagocytosis of apoptotic cells, we hypothesized that Mertk, along with Axl and Tyro3 may have a dual function by playing a role in signaling for engulfment and in the down regulating proinflammatory cytokines as a consequence to phagocytosis of apoptotic cells.
3.3 Materials and Methods

**Chimera binding assays** Axl-Fc Chimera, Mertk-Fc Chimera, and Tyro3-Fc Chimera, in addition to irrelevant control BAFF-Fc chimeras were purchased from R and D systems. Apoptotic cells were generated by isolating thymocytes from naïve mice and treating a 5x10^6 cells/ml suspension with 2 µM dexamethasone (Sigma) for 6 hours. Apoptotic thymocytes or freshly isolated thymocytes were then washed twice in phosphate-buffered saline (PBS) and resuspended in complete macrophage media (RPMI 1640 (Gibco) supplemented with 5% heat-inactivated FBS (Gemina Bio-products), 50 units penicillin G and 50 µg/ml streptomycin sulphate (Gibco), sodium pyruvate (Sigma), and β-mercaptoethanol (Gibco)). Chimeric receptors were added at a concentration of 1 µg of receptor per 10^6 cells for 30 minutes at 37 C. After binding, thymocytes were washed three times in PBS and incubated in 1 µg of anti-human IgG1-FITC antibody per 10^6 cells. After secondary antibody staining the cells were again washed three times in phosphate buffered saline and fixed with 1% paraformaldehyde (Sigma) for 15 minutes. Cells were then analyzed for Fc chimera staining on a FACScan cytometer (Becton Dickinson) using Summit 4.3 software.

For blocking phagocytosis assay experiments, apoptotic or non-apoptotic thymocytes were first incubated with 2 µM Cell Tracker Green (Molecular Probes) for 30 minutes, washed with PBS and then rested in media for an additional 30 minutes. Chimeras or antibodies were then added at a final concentration of 0.25 µg/500 µl media and incubated with wild-type thioglycollate-elicited macrophages at a ratio of 10:1 thymocytes: macrophages. To block Fc receptors, anti-CD16/32 (Pharmingen) was added to macrophages at a dilution of 1:200 for 15 minutes before addition of thymocytes. Sixty minutes after addition of thymocytes coverslips with attached macrophages were washed five times with...
PBS and fixed in 1% paraformaldehyde. Three fields from each coverslip done in triplicate were counted for total macrophages and number of macrophages that had ingested an apoptotic cell. Phagocytosis is represented as the percent of macrophages ingesting an apoptotic cell at 60 minutes.

**Immunocytochemistry.** Anti-Mertk antibody was purchased from R and D Systems and directly conjugated using the monoclonal antibody labeling kit-AlexaFluor 555 (Molecular Probes). Anti-Axl antibody is from Sigma, and anti-goat-FITC is from Vector. Thioglycollate-elicited macrophages were plated on coverslips and allowed to adhere for 2 hours. Nonadherent macrophages were washed off and macrophages were allowed to rest overnight. Macrophages were then rinsed briefly in PBS and incubated in staining buffer (1:200 Fc Block, 2% normal donkey serum in phosphate buffered saline) for 30 minutes at 4°C. Coverslips were then incubated in primary antibody for 60 minutes at 4°C. For Axl staining, cells were washed three times with PBS and anti-goat-AlexaFluor 488 (Molecular Probes) was added for 30 minutes at 4°C. For double staining, Axl-stained coverslips were washed after addition of secondary antibody and stained for 30 minutes with directly conjugated anti-Mer-AlexaFluor 555 (R and D Systems). Coverslips were then fixed in 1% PFA and mounted using Vectashield with DAPI (Vector Laboratories). Pictures were taken on an Olympus BX40 microscope using a Olympus DP70 camera and analyzed using Image Pro Plus 5.0 software.

**Immunoprecipitations and Immunoblotting.** For immunoprecipitation (IP) experiments, cells were stimulated with apoptotic cells at a 10:1 apoptotic cell: macrophage ratio for indicated timepoints, and then lysed in 1 ml/10⁷ cells with IP lysis buffer (Tris Buffered Saline, 1% NP-40 (Pierce), 0.1 mg aprotinin, 0.1 mg α-1-antitrypsin, 0.1 mg leupeptin
(Sigma), 1 mM PMSF (Sigma), 2 mM sodium orthovanadate (Sigma), and phosphatase inhibitor cocktails I and II (Sigma)). Insoluble material was pelleted and cleared lysates were incubated with IP antibody for 1 hour, followed by addition of Protein G (Amersham) or Protein A/G Sepharose (Pierce) to lysates and incubation for an additional hour. Sepharose was pelleted and non-specific proteins washed in lysis buffer. After the last wash, the entire supernatant was removed and SDS loading dye was added, samples were boiled for 5 minutes and then entire supernatant was loaded onto SDS-PAGE gels. For whole cell lysates, protein was quantified using Biorad’s Dc protein assay and 50 µg of lysate was added.

After proteins were resolved on SDS-PAGE gels, they were transferred onto PVDF membranes and blocked using 5% Milk in TBS-T. Primary antibodies were added overnight at 4 C and secondary for two hours at room temperature. Blots were developed using ECL-Plus (Amersham) and HyBlot CL film (Danville).

**Cell lines and transfections**  CHO-K1 cells were purchased from ATCC, GAS6 expressing cell lines were made by stably transfecting CHO-K1 cells with pSEC-TAG2-GAS6 (a kind gift from Dr. Shelton Earp), these CHO-K1-GAS6 cells secrete more GAS6 into the supernatant than wild-type CHO-K1 as determined by Western blot (Data not shown). For immunoprecipitations experiments CHO-K1 or CHO-K1-GAS6 cells were transiently transfected using 6:1 Fugene: DNA ratio (Roche). Axl was isolated from murine kidney cDNA and cloned into PCMV-Tag2 (Stratagene). pIRES-EGFP-Mertk was a kind gift of Raymond Birge. Tyro3 cDNA was obtained from Dr. Stephen Goff and subcloned into pCDNA (Invitrogen). Cells were immunoprecipitated as described above 48 hours after transfection.
**ELISA and RNAse Protection Assay** For ELISA assays, high binding 96 well plates (Costar) were coated with anti-cytokine antibody at 100 ng/well in coating buffer (0.1M NaHCO3, pH 8.2) overnight at 4 C. Wells were washed in PBS-T (PBS with 0.05% Tween-20 (Fisher)) two times and excess PBS-T was completely removed from wells. Wells were then blocked with PBS containing 3% Bovine Serum Albumin (Sigma) for two hours at room temperature. Blocking solution was removed and 200 µl of cytokine containing supernatants were added to each well and incubated overnight at 4C. Wells were washed four times with PBS-T and biotinylated cytokine-specific antibody was added at 50 ng/well in blocking buffer. Wells were incubated in antibody for 45 minutes at room temperature, followed by six washes in PBS-T. Avidin-Peroxidase (Pharmingen) was added at 0.5 ug/ml for 30 minutes at room temperature, followed by washing 8 times in PBS-T. ABTS substrate (2.2’-azino-bis (3’ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citric acid, pH 4.35.) was activated by adding 10 µl 3% H2O2 to 10 ml of substrate and then immediately added to wells for 30 minutes. Plates were analyzed on a Elx808 Ultra microplate reader (Biotek) at an OD of 405nm.

RNAse protection assays were done with the RiboQuant kit probe set mCK-3b from Pharmingen. Briefly, RNA samples were collected using the Trizol reagent, followed by DNAse digestion and phenol chloroform purification, and precipitated using isopropanol and dried, hybridization buffer was then used to resuspend the RNA, and pre-labeled radioactive probe is added to RNA samples, heat shocked at 90 C and then allowed to slowly cool to 56 C and incubated overnight. Samples were then cooled to 37 C for 15 minutes and RNAse was added to digest non-hybridized RNA. Proteinase K was then added to inactivate the RNAse and the hybridized RNA samples were then purified using phenol chloroform.
Ethanol was used to precipitate the hybridized RNA and the complexes were then dried. Hybridized RNA was resuspended in loading buffer and loaded onto a 5% acrylamide sequencing gel. Samples were run to resolution using an unhybridized probe set as a ladder. After running, the gel was dried and exposed to a phosphorimager screen; data was analyzed using a Typhoon 9400 (GE Healthcare) and Molecular Dynamics software. Samples were normalized to GAPDH signal in each lane.

### 3.4 Results

The ligands, GAS6 and Protein S, have been shown to bind apoptotic cells and have been shown to bind to Axl, Mertk, and Tyro3, whereas Protein S bound only Tyro3. No one has demonstrated that Axl, Mertk, or Tyro3 bind to apoptotic cells through these intermediates. We therefore wanted to examine the ability of these receptors to bind apoptotic cells. Fc chimeric proteins that contain the extracellular domains of murine Axl, Mertk, or Tyro3 fused with the human IgG1 Fc region were used to determine binding. These Fc chimeric receptors were added to preparations of non-apoptotic or apoptotic thymocytes and then stained with an anti-human IgG1 FITC-conjugated secondary antibody. Mertk-Fc, Axl-Fc, and Tyro3-Fc bound the apoptotic cells better than irrelevant controls; however, Tyro3-Fc bound to a slightly greater number of apoptotic cells (Figure 3.1A). This is consistent with the ligand binding data suggesting that Tyro3 can bind Protein S in addition to GAS6 in order to bind the apoptotic cell(35, 79). Although specific binding to the apoptotic cells was above that observed with control Fc chimera protein, a much higher percentage of binding was expected, as our apoptotic cell preparations are ~80% Annexin V-positive, which indicates phosphatidylserine exposure (data not shown). The ligands GAS6
and Protein S have been shown to bind to phosphatidylserine and potentially bridge with Axl, Mertk, and/or Tyro3; however, binding was only ~60% of apoptotic cells. We can attribute this low value to either the number of Fc chimeric molecules added being sub-optimal or the different affinity of the Fc chimeras to the GAS6 or Protein S ligand compared to that of native Axl, Mertk, or Tyro3 receptor protein.

We next utilized these same Fc chimeric molecules to conduct ligand-blocking experiments of phagocytosis of apoptotic cells. We hypothesized that adding these receptor tyrosine kinase extracellular domain-Fc chimeric molecules would bind ligand-bound phosphatidylserine exposed on the surface of the apoptotic cell. The binding of Fc chimeric receptors to apoptotic cells would prevent the macrophages from being able to recognize and phagocytose the apoptotic cell efficiently. Therefore, the Fc chimeric receptors were added to the preparation of apoptotic thymocytes for 30 minutes before addition to the macrophages. The percentage of macrophages that had ingested apoptotic cells was contrary to expectations. Although addition of Axl and Mertk Fc chimeras did not significantly reduce phagocytosis, the addition of Tyro3 chimera led to a significant increase in the number of macrophages that had ingested apoptotic cells (Figure 3.1B). This was surprising, as the human Fc region in the Fc chimeric receptors were not expected to bind to murine Fc Receptors on macrophages. However, to rule out the possibility that Fc receptors were playing a role, anti-CD16/32 (FcγR) antibody or Fc Block was added to the phagocytosis assay (Figure 3C). This antibody specifically binds to the Fc γ receptor and subsequently prevents the capture of other antibodies. The addition of Fc Block to the phagocytosis assay reduced the levels of phagocytosis of apoptotic cells that were bound with Tyro3 Fc chimeric protein to wild-type control levels (Figure 3.1C). If the Fc regions of each chimera bound
equally to the Fc receptors found on these macrophages, then this suggests that the Tyro3-Fc chimera bound to more apoptotic cells than Axl and Mer-Fc chimeras.

An alternative method to block receptor binding is through the use of antibodies to either the ligand or receptor. In order to demonstrate which receptor and ligand was most critical for phagocytosis of apoptotic cells antibodies to Mertk, Axl, or Tyro3 were added to cultures containing macrophages from wild-type mice and apoptotic thymocytes (Figure 3.1D). These antibodies showed no significant reduction in the ability of macrophages to ingest the apoptotic cells. Similarly, anti-GAS6 or Protein S-specific antibodies were added to the phagocytosis assays in an attempt to block these interactions by occupying the ligand (Figure 3.1E). Our results were not conclusive; however, as no significant blocking of phagocytosis was observed with any antibody used (Figure 3.1E). As these blocking antibodies are polyclonal antibodies, it is plausible that the amount of antibody to the critical epitopes responsible for binding would be very small and may be inappropriate for blocking these. Therefore, these studies are inconclusive and do not demonstrate which receptor or ligand was most important for phagocytosis.

Tyro3 has the highest affinity for the apoptotic cell based on the Fc chimera studies done above. This is interesting, as the mertk<sup>kd</sup> mouse has macrophages that are completely unable to phagocytose apoptotic cells, whereas axl<sup>-/-</sup> and tyro3<sup>-/-</sup> mice have macrophages that are only partially deficient in phagocytosis (Chapter 2). This data, along with data from other groups showing that in the retina, Tyro3 is required for efficient Mertk function, suggests that perhaps these receptors interact to efficiently phagocytose apoptotic cells. In order to study this, we first examined whether the receptors were found on the same cell. We have previously demonstrated by flow cytometry that Axl, Mertk, and Tyro3 are all
expressed on macrophages and dendritic cells (Chapter 2); however, all commercially available antibodies that have been shown to be specific are produced in goats. This has prevented double-staining by flow cytometry to determine if the three receptors are co-localized on the same cell. An alternative approach was to directly conjugate the receptor-specific antibodies to avoid using a secondary antibody that was directed against goat Fc. The first attempt to directly conjugate the Mertk antibody to a FITC fluorochrome caused the antibody to no longer be specific and was determined to be unusable. Therefore, we directly conjugated this antibody to Alexa Fluor-555 (AF-555). This AF-conjugated antibody was shown to retain specificity and was able to bind and detect Mertk on the surface of macrophages on coverslips as well as in fresh frozen, acetone-fixed sections (Figure 3.2A first panel and data not shown). Macrophages were also stained with anti-Axl antibody in the second panel and co-staining of macrophages for Axl and Mertk suggested that these two receptors are on the same cell (Figure 3.2A overlay in the third panel). Unfortunately, the three commercially available anti-Tyro3 antibodies were not specific when compared with \( tyro3^- \) cells and these antibodies could not be used to determine if Tyro3 co-stained with Mertk and/or Axl. Furthermore, Axl and Mertk can be found singly on \(~70\%\) of macrophages, whereas coexpression of both receptors was seen on \(~70\%\) of macrophages.

In order to determine whether or not Axl, Mertk, and Tyro3 interacted in primary cells, coimmunoprecipitations were conducted on murine thioglycollate-elicited macrophages. Immunoprecipitation of one of the receptors and Western blotting for an associating receptor proved difficult due to the antibody reagents. Unfortunately, in optimizing this assay, it was determined that the antibodies against Axl and Tyro3 were nonspecific. In Figure 3.2B, titration of the amount of immunoprecipitating antibody to
reduce the non-specific bands detected by Western for Mertk antibody was successful; however Axl and Tyro3 antibodies could not be titrated effectively. Further, by titrating the immunoprecipititating antibody, we were concerned about losing detection of potential interacting receptors, as immunoprecipitating antibody should always be in excess of the target. These preliminary immunoprecipitation experiments demonstrated that primary cells could not be used to determine if interactions between these receptors exists.

A second approach to determine receptor interaction was to produce transfected cell lines with Axl, Mertk, or Tyro3 receptors, tagged with either FLAG epitopes or Myc epitopes, singly or in combination and used anti-FLAG or anti-Myc antibodies to detect interactions among the receptors. Therefore CHO-K1 cells expressing wild-type levels of GAS6 or CHO-K1 cells stably expressing higher levels of GAS6 were transfected with various combinations of the receptors i.e. Axl-FLAG and Mertk, Axl-FLAG and Tyro3 or Tyro3-FLAG and Mertk. We saw ample expression of all three constructs in the cell lines used (bottom panels of Figure 3.2C); however, we detected no interaction between the receptors by immunoprecipitation (top panel of Figure 3.2C). Although Axl and Tyro3 interactions were examined in Figure 3.2C, all other combinations of receptors were tried, and no interaction was seen in any combination of receptor expression (data not shown).

Previous reports on Mertk have demonstrated an in vitro interaction between Mertk and α,β integrin(118). Further a report by Mahajan and Earp suggests that Vav1 is constitutively bound to Mertk and upon stimulation Vav1 is phosphorylated and released from Mertk, presumably to signal Rac, Rho, and Cdc42(116). These previous experiments were done in NIH3T3 or 32D cells with chimeric Mertk constructs. In light of these reports, we wanted to investigate a role for Mertk in integrin and Vav1 signaling in primary
macrophages as part of the cascade leading to cytoskeletal rearrangements and phagocytosis. Primary murine macrophages were stimulated with apoptotic cells for 15 minutes and then immunoprecipitated for Mertk. Western blots were conducted to determine whether Mertk associated with Vav1 in primary macrophages (Figure 3.3A). In unstimulated wild-type macrophages, there was no detectable Vav1 bound to Mertk (Figure 3.3A lane 1); however upon stimulation, Vav1 associates with Mertk at 15’ (Figure 3.3A lane 5). In apoptotic cell stimulated macrophages, the absence of Tyro3, or Axl and Tyro3 resulted in very low but persistent levels of Vav1 bound to Mertk (Figure 3.3A lane 3, 4, 7, and 8). Therefore, although, Vav1 associates with Mertk in wild-type macrophages, we saw significantly less Vav1 association with Mertk in macrophages deficient in tyro3 or axl/tyro3.

We then examined whether α₁β₅ associated with Mertk. This experiment was based on reports of Mertk binding α₁β₅ in the NIH3T3 cell line. Therefore we used immunoprecipitations with anti-Mertk antibodies to look for interaction by Western blot for β₅. Macrophages were stimulated with both recombinant GAS6 (Figure 3.3B middle) or apoptotic cells for 15 minutes (Figure 3.3B right). Independent of stimulus used, we found no detectable α₁β₅ interaction with Mertk in primary murine macrophages.

Upon binding and phagocytosis of apoptotic cells, the macrophage initiates signaling cascades leading to actin cytoskeletal rearrangement. It is well documented that this process involves Rac, Rho, PI3K(21). There are also reports of FAK and Ikkα/β activation being required for efficient engulfment of apoptotic cells(117, 121). Because Axl, Mertk, and Tyro3 are critical for phagocytosis of apoptotic cells, we wanted to understand whether these receptors were responsible for activating the cytoskeletal rearrangement pathway. To determine the role of FAK, thioglycollate-elicited macrophages from wild-type or knockout
mice were then stimulated with apoptotic cells to measure phosphorylated FAK. In wild type macrophages, there was a large increase in phosphorylated FAK upon stimulation with apoptotic cells compared to unstimulated macrophages (Figure 3.4A lane 1 and 2). Further, macrophages deficient in Mertk or Tyro3 had less phosphorylated FAK than wild-type macrophages (Figure 3.4A lane 3 and 4, and lane 7 and 8). Macrophages deficient in Axl showed no decrease in phosphorylated FAK upon apoptotic cell stimulation, suggesting that Axl may not be responsible for FAK phosphorylation. This data demonstrates that Mertk and Tyro3 are important for FAK phosphorylation upon apoptotic cell stimulation.

Another molecular intermediate in this pathway that is involved in cytoskeletal rearrangement is PI3K activation. A downstream indicator of PI3K activation is an increase of phosphorylated Akt. In addition, phospho-Akt that leads to NF-kB activation and activation of NF-kB has been shown to be critical for the regulation of proinflammatory cytokines. We therefore examined macrophages for differences in pAkt levels after stimulation with apoptotic cells (Figure 3.4B). No differences were found between macrophages from wild-type and \( axl^{-/-}, mertk^{kd}, tyro3^{-/-}, \) or \( axl/tyro3^{-/-} \) mice in their ability to phosphorylate Akt. This contradicts data in dendritic cells that demonstrates a role for Mertk in activation of Akt upon GAS6 or apoptotic cell stimulation(121).

Lastly, one of the most important downstream effects of phagocytosis of apoptotic cells is the down regulation of inflammation. It is well documented that macrophages that have ingested apoptotic cells secrete anti-inflammatory cytokines such as TGF-\( \beta \) and IL-10, whereas the production of pro-inflammatory cytokines, even in the face of strong pro-inflammatory signals such as microbial pathogen associated molecular patterns (PAMPs) is shutdown(19). Examples of PAMPs used here are lipopolysaccharide (LPS) from Gram-
negative bacteria, peptidoglycan (PGN) from Gram-positive bacteria and zymosan from yeast. As the regulation of cytokines is an important process, we wanted to examine the role of Axl, Mertk, and Tyro3 in the down regulation of inflammation. First a dose titration was needed to determine the optimal concentration of pro-inflammatory stimulus without oversaturation and excessive activation of the macrophages that would make down regulation difficult to observe. Therefore, we titrated doses of LPS (Figure 3.5A), PGN (Figure 3.5B), and zymosan (Figure 3.5C) on macrophages from wild-type, mertk<sup>kd</sup>, axt<sup>-/-</sup>, tyro3<sup>-/-</sup>, and axl/tyro3<sup>-/-</sup> mice and examined TNF-α production. There were no significant differences observed in the levels of TNF-α produced in response to these microbial PAMP’s in the wild type or knockout mice (Figure 3.5A-C). Titration of the number of apoptotic cells needed to inhibit pro-inflammatory cytokine production required determination; therefore, we treated macrophages with a fixed dose of LPS, and then increased the number of apoptotic cells. The amount of TNF-α could be titrated down consistently with increasing numbers of apoptotic cells, and we found that a 40:1 ratio of apoptotic cells to macrophages gave complete inhibition of TNF-α production (Figure 3.5D). Further, there were no significant differences in the amount of TNF-α produced in the macrophages from knockout mice compared with wild-type mice. This was confirmed by examination of the amount of TNF-α RNA (Figure 3.5E). In RNAse Protection Assays (RPA), TNF-α RNA in macrophages increased upon stimulation with LPS. In contrast, the addition of apoptotic cells to the cultures dramatically inhibited expression of TNF-α RNA. This inhibition of TNF-α by addition of apoptotic cells was consistent with what we found in secreted protein in ELISAs (Figure 3.5D).
The phagocytosis of apoptotic cells results in the secretion of TGF-β. In Figure 3.5F, the addition of apoptotic cells to LPS-stimulated macrophages resulted in the secretion of the anti-inflammatory cytokine TGF-β. Although in our experiments, macrophages deficient in Mertk or Axl/Tyro3 showed a reduction in TGF-β RNA after ingestion similar to wild-type.

This suggests that although Axl, Mertk, and Tyro3 play a role in downstream signaling pathways involved in the engulfment of apoptotic cells, they are not required for cytokine regulation. The possibility exists however, that the receptors are able to compensate for the lack of one or two of the family members. Therefore, it is plausible that all three receptors must be eliminated in order to conclude whether cytokines are not regulated by these receptors and the triple knockout on a backcrossed C57BL/6 background mouse has yet to be generated.

3.5 Discussion

The proposed model of GAS6 or Protein S binding to the apoptotic cell, coupled with engagement of Axl, Mertk, and Tyro3 has not been directly demonstrated. Thus, no binding of Axl, Mertk, or Tyro3 to apoptotic cells has been shown. We attempted to look at the receptors binding affinity for apoptotic cells by utilizing Fc chimera constructs. We found that Tyro3 bound to a greater number of apoptotic cells, followed by Axl and Mertk (Figure 3.1A). Unfortunately the percent binding we observed was lower than expected values based on the percent of apoptotic cells in the population. The apoptotic cell preparations are routinely 80% Annexin V positive; however, we saw only 60% of apoptotic cells bound to the receptor Fc chimeras. Therefore more experimentation is needed to confirm the finding that Tyro3 binds a larger number of apoptotic cells than Axl and Mertk. This data makes
sense in light of the higher affinity of GAS6 for Axl and Tyro3 and it is plausible that Tyro3 may be binding to it’s alternative ligand, Protein S. Thus, Tyro3 could be using two ligands and thus increasing binding opportunities to apoptotic cells whereas Mertk or Axl presumably use only GAS6 that may present fewer interactions. In order to study this further, constructs should be made that do not have an Fc portion, to rule out any nonspecific binding due to differences in this part of the protein. Also, titrations with varying numbers of receptor and cells should be done to ensure that we are at saturating concentrations. Lastly, competitive binding with excess GAS6 and Protein S would be useful in delineating the role of these two intermediates in binding to the apoptotic cell.

The second method to study binding of Axl, Mertk, and Tyro3 with apoptotic cells was by blocking phagocytosis using the Fc chimeras and antibodies. We found that although no appreciable blocking was seen using any ligand antibody or Fc chimera construct, we found higher percent of phagocytosis in samples that contained Tyro3-Fc (Figure 3.1B-D). This data suggests that Tyro3 may be binding the apoptotic cell better than Axl or Mertk and then acting through Fc receptor-mediated phagocytosis to actually enhance phagocytosis. Since the Tyro-Fc chimera bound the surface of apoptotic cells similar to Axl-Fc or Mertk-Fc chimera (Figure 3.1A), we presume the higher phagocytosis with the Tyro-Fc chimera could be due to greater binding opportunities that results in increased tethering of the apoptotic cell to the macrophages to facilitate engulfment. We found that Fc receptor-mediated phagocytosis resulted in the increase of Tyro3-Fc ingested apoptotic cells and the inclusion of an antibody to block Fc receptor returned phagocytosis to wild-type levels (Figure 3.1C). These experiments should also be confirmed using constructs that do not
contain Fc regions. Nonetheless, these data suggest that Axl/Mertk/Tyro3 receptors can bind apoptotic cells along with other recognition receptors.

Data in Chapter 2, in addition to published reports in platelets and retina, suggest that these family members may interact for efficient signaling(91, 98). Therefore, we examined this interaction by first looking at Axl, Mertk, and Tyro3 expression on macrophages. We found that wild-type macrophages express both Axl and Mertk (Figure 3.2A). Unfortunately, cross-reactivity among the antibodies caused problems that prevented co-localization of Tyro3 with the Axl and Mertk receptors on macrophages. For further confirmation that receptors are co-localized on the same cell and could perhaps interact with one another, immunoprecipitations for the receptors was conducted. Unfortunately, crossreactive antibodies prevented examining interactions in primary cells (Figure 3.2B). We therefore attempted to examine receptor interactions in cell lines transfected with tagged constructs. Again we saw no interaction using tagged constructs (Figure 3.2C). This led us to the conclusion that these receptors do not interact, or do so transiently. Further experiments using crosslinking reagents will delineate if the interaction is transient.

Previously published work suggests that Mertk binds to Vav1 and the integrin $\alpha_\text{x} \beta_5$ in a ligand inducible manner(116, 118). These studies were done in cell lines and used chimeric constructs with artificial ligands so it is questionable whether this occurs in primary cells. We therefore wanted to confirm these findings in primary cells. We found that Mertk does indeed transiently associate with Vav1 upon apoptotic cell stimulation, and this interaction requires Tyro3 (Figure 3.3A). We did not see interaction with $\alpha_\text{x} \beta_5$ upon stimulation with GAS6 or apoptotic cells (Figure 3.3B). This data suggests that Mertk’s
primary role upon apoptotic cell stimulation in macrophages may be to activate Vav1 and not interact with $\alpha_\beta_5$.

FAK is downstream of integrin signaling and has been demonstrated to be important in actin cytoskeletal rearrangement. Although we found no association of Mertk and $\alpha_\beta_5$, we did see a difference in FAK activation upon apoptotic cell stimulation in wild-type compared with $\text{mer}tk^{kd}$ macrophages. Further, $\text{tyro}3^{-/-}$ macrophages also had less phosphorylated FAK than wild-type, whereas $\text{axl}^{-/-}$ macrophages had similar levels to wild-type (Figure 3.4A). While this is a discrepancy, there are other signaling intermediates besides integrins that can activate FAK in macrophages and thus this warrants further investigations.

Downstream of phagocytosis is PI3Kinase activation. PI3K signaling is important in actin cytoskeletal rearrangements as well as cytokine regulation. Because apoptotic cells have been shown to down regulate pro-inflammatory cytokines, we wanted to examine the PI3Kinase activity to determine if Axl, Mertk, or Tyro3 played a role. All three receptors presumably have a PI3K domain in their cytoplasmic tail. We found that there was no difference in phosphorylated Akt, a key signaling protein in the PI3kinase pathway. Further, Akt was not activated in macrophages upon stimulation with apoptotic cells alone in our hands (Figure 3.4B). This contradicts data seen in dendritic cells suggesting Mertk does signal through Akt in response to apoptotic cells. Explanations for the discrepancy are cell type, we used macrophages whereas Sen et al used dendritic cells, in addition to a different genetic strain of mouse our mice are backcrossed onto C57BL/6 background whereas Sen et al used mice on a Non-Obese Diabetic mouse background (NOD)(121).
As mentioned, down regulation of proinflammatory cytokines is an important downstream consequence of phagocytosis of apoptotic cells. Due to the role Axl, Mertk, and Tyro3 play in this process, we wanted to examine their role in cytokine regulation. We found that this family of receptors is not required for downregulation of proinflammatory cytokines and is somewhat contrary to the role suggest by earlier studies with Mertk(82). This was demonstrated by RNA and protein to ensure that regulation was happening at both steps (Figure 3.5).

Thus, we conclude that the receptor family members participate in recognizing apoptotic cells and that Tyro3 may have the greatest affinity for binding and tethering the apoptotic cell to the phagocytes. We have demonstrated that the engagement of apoptotic cells results in activation of Vav1 and may trigger FAK activity but appears not to activate Akt. However, the signaling pathway for phagocytosis appears to be independent from the regulation of cytokines. In fact, our data suggest that Axl/Mertk/Tyro3 receptors are not required for down regulation of proinflammatory cytokines.
Figure 3.1 Axl, Mertk, and Tyro3 bind to apoptotic cell. A) Flow cytometry data showing Fc chimera receptor constructs binding to apoptotic thymocytes. B) Blocking of phagocytosis with Fc chimera’s M- Mer Fc chimera added; A-Axl Fc Chimera; and T-Tyro3 Fc Chimera; non is non-apoptotic cells added to macrophages in absence of chimera, and apop is apoptotic cells added to macrophages C) Phagocytosis assay using Fc Block to prevent phagocytosis of Tyro3-Fc chimeric protein bound to apoptotic cells (AC). D) Anti-receptor antibody blocking phagocytosis assays E) Anti-ligand antibody blocking phagocytosis assays. PS-Protein S IC-isotype control for blocking antibody, AC or UNT-apoptotic cells alone NAC-non-apoptotic cells. Flow data is representative of two experiments, phagocytosis assay data is representative of three experiments.
Figure 3.2 Axl/Mertk/Tyro3 receptors do not interact on macrophages. A) Immuno-cytochemistry of peritoneal macrophages. Left panel- wild-type macrophages stained with anti-Mertk-AlexaFluor 555, middle panel- macrophages stained with anti-Axl and secondary antibody anti-goat AlexaFluor 488, right panel is overlay indicating macrophages expressing both Mertk and Axl (yellow). 20x field. B) Immunoprecipitation on primary macrophages. Wt-wild-type; kd-mertk\(^{kd}\); and ko-knockout (second panel is Tyro3 knockout, and third panel is Axl knockout). IP-immunoprecipitate, WB-Western blot. C) CHO-K1 or CHO-K1-GAS6 cells transfected with indicated constructs and immunoprecipitated for tagged protein. Data is representative of Axl/Mer co-transfections, and Mer/Tyro co-transfections. WCL-whole cell lysates of transfected cells.
Figure 3.3  Mertk association with Vav1 requires Tyro3.  A) Macrophages were stimulated with apoptotic cells for 15 minutes, and then immunoprecipitated for Mertk. Proteins were blotted for Vav1, stripped and reprobed for total Mertk.  B) Macrophages were stimulated with apoptotic cells or recombinant murine GAS6 for 15 minutes and then immunoprecipitated for Mertk. W-wild-type  A-axl/tyro3--/-. Proteins were blotted for beta5 integrin, stripped and reprobed for total Mertk.
Figure 3.4 Mertk and Tyro3 are required for efficient FAK phosphorylation upon apoptotic cells stimulation. A) Macrophages were treated with or without apoptotic cells (AC) for 15 minutes, after washing off unbound AC, whole cell lysates were blotted for phosphorylated FAK at tyrosine 861, p861Y-FAK. Blots were stripped and reprobed for total FAK. B) Macrophages were stimulated with apoptotic cells for 30 minutes and whole cell lysates were blotted for phospho-Akt. Blots were stripped and reprobed for total Akt.
Figure 3.5

A

LPS (ng/ml)

B

PGN (ng/ml)

C

Zymosan (µg/ml)

D

TNF-a (pg/ml)

wildtype mertkkd axl/-/ tyro3-/-

wildtype mertkkd axl/tyro3-/-

LPS (ng/ml)

PGN (ng/ml)

Zymosan (µg/ml)

TNF-a (pg/ml)

wildtype mertkkd axl/-/ tyro3-/-

wildtype mertkkd axl/tyro3-/-

LPS LPS+2X LPS+20X LPS+40X LPS+80X LPS+120X
Figure 3.5 Axl, Mertk, or Tyro3 are not required for downregulation of pro-inflammatory cytokines in response to apoptotic cells. A) LPS B) PGN, or C) Zymosan titration in micrograms on wild-type and knockout macrophages. Macrophages were stimulated for 3 or 18 hours and then supernatants were analyzed for TNF-alpha secretion. D) 1.0 ug/ml of LPS was used to stimulate macrophages in the presence of increasing concentrations of apoptotic cells. Fold increase (#X) represents number of apoptotic cells (apop) per macrophage. Supernatants were collected after 18 hours. E) TNF-alpha or F) TGF-beta relative RNA levels measured using RPA. Values were normalized to GAPDH in each sample. Error bars represent standard error. This data is representative of three experiments.
CHAPTER 4
DISCUSSION AND FUTURE DIRECTIONS
The phagocytosis of apoptotic cells is paramount for the maintenance of a number of physiologic processes including immune homeostasis. Although a large number of receptors, opsonins, and binding sites on apoptotic cells have been proposed to be involved in this process it is unclear how these receptors operate on various cell types and what their in vivo function is. Studies examining receptors on multiple cell types may provide insights to how different phagocytes recognize and engulf apoptotic cells and whether rates of phagocytosis has alteration in biological processes. The inability to phagocytose apoptotic cells has clear consequences, as a deficit in clearance has been linked to developmental abnormalities and survival, to organ dysfunction, and to autoantibody production and autoimmune disease. This process is conserved among flies, worms, mice, and humans which further illustrates the importance of examining the cellular and molecular mechanisms.

Our data demonstrates the importance of the Axl/Mertk/Tyro3 family of receptor tyrosine kinases in the clearance of apoptotic cells by both macrophages and dendritic cells. Previous reports indicated that Mertk was critical for the engulfment of apoptotic cells by macrophages but this receptor played no role in dendritic cell phagocytosis\(^{(28, 46)}\). The difference in phagocytosis by macrophages versus dendritic cells has now been explained by experiments elaborated in Chapter 2. In studies here with the single and double knockout mice, the function of apoptotic cell engulfment can now include Axl and Tyro3 family members. Interestingly, the combinatorial usage of the three receptor tyrosine kinases is different in macrophages and dendritic cells. Specifically, macrophages from \(axl^{-/-}\), \(tyro3^{-/-}\), and \(axl/tyro3^{-/-}\) mice have an \(~50\%\) reduction in phagocytosis of apoptotic cells (Figure 2.1). While this is a significant decrease in phagocytosis compared with wild type, it is not as
dramatic as the deficit in phagocytosis of apoptotic cells seen in the macrophages from $mertk^{kd}$ mice. Nonetheless, macrophages appear to utilize all three Axl/Mertk/Tyro3 receptor family members for engulfment.

While macrophages may be the most efficient cell type at phagocytosing apoptotic cells, dendritic cells also play an important role in the phagocytosis of apoptotic cells. Similar to previous findings (28, 46, 121), we found that dendritic cells from $mertk^{kd}$ mice had no defect in phagocytosis compared with wild type; however, dendritic cells from $axl^{-/-}$, $tyro3^{-/-}$, and $axl/tyro3^{-/-}$ mice had a complete deficit in phagocytosis of apoptotic cells (Figure 2.2). Although dendritic cells express Mertk, it is puzzling why there is a partition of function and suggests a second role for Mertk that may be more critical for dendritic cells such as cytokine regulation. This is the first demonstration that Axl and Tyro3 functions in engulfment of apoptotic cells and that murine dendritic cells rely solely on these two receptors.

In order to better understand the differences in receptor usage between macrophages and dendritic cells, we examined whether the two ligands for this receptor family, GAS6 and Protein S, were expressed differently among cell types. The ligands for Axl, Mertk, and Tyro3 are expressed differently on macrophages versus dendritic cells. While both ligands, GAS6 and Protein S, are ubiquitously found in the serum of normal mice, the concentration of these ligands in the local microenvironment may be greater and could provide the necessary threshold to promote receptor engagement and activation. We found through RT-PCR, that macrophages express both GAS6 and Protein S, while dendritic cells express only Protein S (Figure 2.3b). This may help to explain why macrophages are more efficient phagocytes, as they have GAS6 which can bind to each receptor and Protein S which binds
strongly to Tyro3 available. The combination of engaging multiple receptors may trigger signal transduction particularly through Mertk which is the major trigger for engulfment.

Another interesting observation on the differences between macrophages and dendritic cells is their surface receptor expression. We found that while macrophages and dendritic cells both express all three receptors, the level of receptor expression on macrophages was higher than that seen in dendritic cells (Figure 2.3c-h). While there were no differences in expression between Axl, Mertk, or Tyro3, the fact that dendritic cells have less surface expression of these receptors could explain why they are slower at ingesting apoptotic cells compared with macrophages. The lower level of receptor available for engagement would be consistent with poorer signaling for phagocytosis. Furthermore, each receptor may have inherent differences in their ability to trigger phagocytosis i.e. Mertk may be the most prominent receptor; however, studies were not conducted to compare Axl or Tyro3.

Previous reports in collaboration with Lemke’s laboratory described the presence of apoptotic bodies throughout the triple Axl/Mertk/Tyro3 knock out mice without knowing a reason for the widespread observations(27). Our laboratory reported the mechanism was due to Mertk functioning as a recognition and phagocytosis receptor specifically for apoptotic cells and not other particles(28). To understand the role Axl, Mertk, and Tyro3 play in vivo, we looked at thymus, retina, and spleen. We found that in the thymus, while mertk<sup>bd</sup> mice were significantly unable to clear apoptotic cells compared with wild type, there were no differences in axl<sup>−/−</sup>, tyro3<sup>−/−</sup>, or axl/tyro3<sup>−/−</sup> mice (Figure 2.4). This suggests that in the thymus, Mertk plays an important role in clearance, whereas Axl and Tyro3 do not. The cell type responsible for clearance in thymus is unknown, although macrophages and dendritic
cells are abundant in this tissue. We therefore expected to see a partial deficit in mice lacking Axl, Tyro3, or both. A possible explanation lies in the fact that under normal unstimulated conditions, the thymus in all genotypes, including mertk$^{kd}$ has no appreciable defect in clearance. Therefore, the compensation mechanisms in the thymus are highly redundant and efficient, which could account for the ability of axl$^{+/}$, tyro3$^{+/}$, and axl/tyro3$^{+/}$ mice to clear apoptotic cells normally due to the presence of Mertk. This assumption is reasonable in an organ in which 99 percent of the thymocytes undergo apoptosis and the swift removal of apoptotic debris is important to maintain proper tissue homeostasis.

Furthermore, while the axl$^{+/}$, tyro3$^{+/}$, and axl/tyro3$^{+/}$ mice are able to compensate, the macrophages in mertk$^{kd}$ mice do not have adequate compensation by other receptors or other cell types; hence the dramatic deficits in its absence.

Similar to the thymus, we examined the retina of the Axl and Tyro3 knockout animals as it has been previously established that mertk$^{kd}$ mice are blind due to the retinal pigment epithelia’s inability to phagocytose shed outer segments. We therefore thought it was important to examine the role of Axl and Tyro3 in the retina. In contrast to mertk$^{kd}$ we found that there was no evidence of retinal degeneration in mice lacking axl, tyro3, and axl/tyro3 which was compared with wild type (Figure 2.5). This demonstrates another organ where Mertk is critical, yet Axl and Tyro3 are not. Recent reports demonstrate Tyro3 but not Axl expression in addition to Mertk on retinal pigment epithelium(91). This report also shows that in mice lacking mertk there is lower expression of Tyro3 on the retinal pigment epithelium, and that Mertk can be phosphorylated by stimulation with Protein S, which is only thought to stimulate Tyro3. A previous report on rat RPE also suggested that Mertk was the critical receptor and found similar results that GAS6 and Protein S could promote
phagocytosis(112). This is interesting data to consider; however our finding of normal retina tissue in mice lacking Tyro3 refutes the importance of Tyro3 on retinal pigment epithelium or it suggests that an alternative mechanism can lead to the phosphorylation of Mertk. It is plausible the integrins on RPE might interact with Mertk and further studies to look closely at Mertk function in the absence of Tyro3 are warranted.

A third organ we examined in looking at in vivo function of these receptors was the spleen. This organ was chosen because of the large number of lymphocytes that undergo expansion and contraction and blood flow that must be maintained. We found that opposite of what is observed in the thymus mice lacking axl/tyro3 had a significant defect in the clearance of an apoptotic cell burden in the spleen compared with wild type. Mice deficient in MFG-E8, a ligand for integrins, was also shown to prevent proper phagocytosis of KLH-stimulated lymphocytes in the spleen. Interestingly, while mertkkd mice had higher levels of apoptotic cells they were not significant. In addition, the spleen is the first organ where lack of axl or lack of tyro3 is additive in the axl/tyro3 double knockout (Figure 2.7). One conclusion from this data is that in the spleen, dendritic cells along with macrophages are highly abundant and it is the dendritic cells that come in contact initially with the apoptotic B cells in this model. Perhaps the complete inability of dendritic cells and partial defect seen in macrophages in mice lacking axl/tyro3 overwhelms the system leading to uncleared persistent apoptotic cells. We do not know however, which cell type is ingesting the apoptotic cells, and therefore it is difficult to speculate on mechanism in the spleen. Conditional knockout of each Axl/Mertk/Tyro3 receptor in either macrophages or dendritic cells may provide insights as which cell type is contributing to the clearance in the spleen.
Evidence from studies in platelets and retina suggest that association of the three receptors is required for optimal function. Because we see a dramatic defect in macrophages from merk<sup>kd</sup> mice, we wanted to examine first the role of Mertk in both wild type and axl/tyro3<sup>−/−</sup> mice. We found that Mertk was phosphorylated upon stimulation of apoptotic cells only in wild type macrophages (Figure 2.6). We detected no Mertk phosphorylation with a phospho-specific antibody (data not shown) or a global phosphotyrosine antibody after Mertk immunoprecipitations in macrophages from mice lacking Axl and Tyro3 (Figure 2.6). This suggests that association of Axl or Tyro3 receptors with Mertk is required for optimal activation of Mertk. And while we cannot rule out that there was a low undetectable level of Mertk phosphorylation in macrophages from mice lacking axl and tyro3, it supports the defect in phagocytosis observed in these mice.

In addition to published reports, our data suggests that Axl, Mertk, and Tyro3 cooperate together to mediate efficient phagocytosis of apoptotic cells. While extensive studies have been done examining the affinity of GAS6 for each receptor, there has been little study on how Protein S binds. What is currently known is that Protein S binds only to Tyro3. We have demonstrated that murine Protein S binds to murine Tyro3, which had not been demonstrated previously (Figure 2.5). Both Protein S and GAS6 have been shown to bind apoptotic cells as well, therefore our hypothesis is that these ligands serve to opsonize the apoptotic cell and promote recognition and binding with the Axl, Mertk, and Tyro3 receptors on phagocytes. Although there is strong evidence to suggest this link, there is no direct evidence of Axl, Mertk, or Tyro3 binding to the surface of an apoptotic cell, through a ligand or not. Therefore we sought to examine in Chapter 3, the ability of extracellular domains of these receptors to bind to the apoptotic cell, in the presence of normal mouse
serum. We found that Tyro3 constructs had the highest percent of bound apoptotic cells, this was followed by Axl and Mertk (Figure 3.1). This data is consistent with the ligand-binding data suggesting that Mertk and Axl can only utilize GAS6. Tyro3, which has been shown to have the greater affinity for GAS6 among the receptor family members, was the best binder. In addition to binding through GAS6, Tyro3 has the ability to use Protein S that can bind to phosphatidylserine on apoptotic cells and can weakly trigger phagocytosis alone or possibly help activate Mertk for a stronger response. While these experiments did demonstrate binding to the apoptotic cell, the percentage of cells with bound receptor was low. We expected to see numbers similar to the number of Annexin V positive cells in the population (~80%). Annexin V binds to phosphatidylserine, the proposed binding site of both Protein S and GAS6. This could be due to the \textit{in vitro} nature of the assay, or having the Fc chimeric receptor constructs, secondary antibody or ligand not in optimum levels. Further titrations of these reagents will aid in resolving this issue.

Another method to address the role each ligand and receptor plays in the phagocytosis of apoptotic cells is to block the interaction using specific antibodies. This technique has been used in many other systems including CD14, C1q, and Scavenger Receptor binding\cite{38, 39, 102}. We therefore utilized commercially available antibodies to GAS6, Protein S, Axl, Mertk, and Tyro3 to block interactions of phagocytes with apoptotic cells. Unfortunately, we saw no appreciable blocking with any antibody used (Figure 3.1). This makes interpretation difficult, as the antibodies used were polyclonal and therefore the specificity of the epitopes of these antibodies is unknown, as is the abundance of antibodies specific for any one epitope. Therefore we cannot make any definitive conclusions based on these antibody-blocking studies.
An alternative way to block receptor binding is to use a decoy receptor. We utilized the Axl-Fc, Mertk-Fc, or Tyro3-Fc chimera constructs to hopefully absorb all available ligands in cultures or occupy the binding sights of ligand to prevent phagocytosis of apoptotic cells. While the intention of the assay was to block interaction, only a modest blocking was observed using Axl-Fc and Mertk-Fc constructs, however the Tyro3-Fc chimera actually enhanced phagocytosis (Figure 3.1). One theory to explain this data is that Tyro3 is binding preferentially to the apoptotic cell and acting to opsonized the cell with Fc regions. This would cause enhanced phagocytosis, as Fc receptor-mediated phagocytosis is highly efficient in macrophages and independent of Mertk. To test this theory, we blocked Fc receptor-mediated phagocytosis with an antibody to the Fcγ receptor (Figure 3.1). This effectively blocked the enhancement of phagocytosis that we saw with Tyro3-Fc chimera indicating that this construct was acting non-specifically through Fc receptor-mediated phagocytosis. One conclusion that can be drawn from this data is that Tyro3 binds to the apoptotic cell better than Axl or Mertk, as was demonstrated when the binding was analyzed by flow cytometry. This conclusion assumes that all constructs were comparably made and proteins were all folded properly to allow for similar ligand binding. In addition, this conclusion assumes that the Fc portion of each chimera construct is equally able to bind and activate the Fc Receptor complex. Because of these assumptions, binding and blocking experiments using non-Fc chimeric molecules is necessary. While cell lines producing these molecules have been generated, the proteins still need to be purified, quantified, and tested for ligand binding.

An additional piece of evidence to demonstrate interaction among the family members is that they are expressed on the same cell. Previous surface staining has only been
done using single receptors. This is because each of the commercially available antibodies are only available unconjugated to fluorochromes and all are prepared in goats, limiting the usable repertoire of secondary antibodies to identify each receptor singly. Attempts to label the antibodies to fluorochromes ourselves have been largely unsuccessful. The only antibody we have shown to be labeled and maintain specificity is the anti-Mertk antibody conjugated to Alexa Fluor-555. Using this antibody we show that Axl and Mertk are expressed on the same macrophage (Figure 3.2). Unfortunately no private or commercially available antibody to Tyro3 exists that demonstrates specificity in staining.

To examine directly at the ability of these receptors to associate we used immunoprecipitation experiments. We again ran into challenges with specificity of commercial antibodies, and therefore utilized cell lines transiently transfected with tagged constructs. We found no detectable association between the receptors (Figure 3.2). While this data suggests that no interaction exists, we still cannot rule out a transient association, or perhaps another intermediate that is associating with multiple receptors. Studies using cross-linking agents will help us better understand if there is a transient association and how close the receptors might be on the cell surface; however, initial attempts have not revealed binding partners.

The literature suggests that Mertk associates with Vav1 and/or α,β integrin during activation, and that this interaction is critical for Mertk phagocytosis function(116, 118). This previous work was done in transfected cell lines, with overexpressed constructs. Therefore, it was important to understand if these mechanisms were an artifact of experimental conditions or whether such associations exist in primary cells. In primary macrophages, Mertk associates with Vav1 but not α,β (Figure 3.3). Our data differs from
published results in that Mertk does not constitutively associate with Vav1 in resting primary macrophages. Thus, we saw no association in unstimulated macrophages, and a transient binding and release of Vav1 at 15 and 30 minutes respectively. This is consistent with the literature demonstrating a role for Vav1 in actin remodeling for engulfment(116).

Further studies need to be done to prove or eliminate an association with integrins. They have been shown to be involved in phagocytosis in multiple cell types including RPE and transfected cell lines. It has been suggested that cytoskeletal rearrangements are dependent on cooperation of integrin and Mertk to trigger phosphorylation and to induce phagocytosis(118). These experiments would include looking at other integrins such as αvβ3, using crosslinking agents to detect any transient associations and look at the effect of stimulation of integrins with MFG-E8, which is a ligand that has been demonstrated to trigger phagocytosis of apoptotic cells.

The binding of the apoptotic cell and the downstream signaling must occur to trigger phagocytosis. A known signaling protein in this pathway, FAK, has been demonstrated to be downstream of integrin signaling and activated by Mertk. We saw lower FAK phosphorylation levels in tyro3−/− and axl/tyro3−/− macrophages compared with wild type suggesting a deficit in FAK activation (Figure 3.4). This may be due to the lower level of Mertk phosphorylation in these cells, as previous work showed Mertk phosphorylation directly affecting FAK phosphorylation in cell lines. This need for Mertk phosphorylation by Tyro3 or phosphorylation of integrins has been implied in our study and others(27, 91). Thus, the interactions and phosphorylation events that trigger receptor family members requires further investigation and the development of additional reagents will facilitate future studies.
While multiple points along the signaling pathway suggest a role for Axl, Mertk, and Tyro3 in signaling for engulfment of apoptotic cells another critical feature of this process is the downregulation of cytokines. Mertk has been previously demonstrated to be required to down regulate NF-kB upon LPS stimulation in an endotoxic shock model(82). Akt is upstream of NF-kB activation and is phosphorylated upon apoptotic cell stimulation(121). We saw no difference in phospho-Akt levels or downregulation of cytokines in macrophages from mertk\textsuperscript{kd} or axl/tyro3\textsuperscript{-/-} mice compared with wild type (Figure 3.4b and 3.5). This was examined using multiple PAMPs and multiple titrations and ratios of apoptotic cells. This data suggesting a lack of suppression of cytokines was also confirmed using both RNA and protein assessments. Our results demonstrates that in macrophages, Mertk, Axl, and Tyro3 serve in engulfment of cells only. In contrast, Mertk has been shown to be important in the downregulation of NF-kB and the reduction in IL-12(121). Studies using triple knockout axl/mertk/tyro3\textsuperscript{-/-} animals would be beneficial to understanding if compensation is occurring in single or double knockout mice used in our studies, and would definitively determine if each member of this family of receptors is required for downregulating proinflammatory cytokines.

We remain puzzled by the differences in Mertk usage between macrophages and dendritic cells. Unfortunately the commercially available antibodies to Mertk are not strong enough to allow detection of appreciable Mertk protein in dendritic cells; therefore understanding the phosphorylation status in these cells has been difficult. We also do not know the expression pattern or usage of Axl, Mertk, and/or Tyro3 on cell types other than macrophages and dendritic cells. Other phagocytic cell types, such as microglia, thymic epithelial cells, neutrophils, and osteoclasts have not been examined for expression or defects
in phagocytosis. Understanding the role of Axl, Mertk, and Tyro3 in each specific cell and tissue type may develop a comprehensive picture of how these receptors are utilized and that their function may change depending on the cell type.

Another important area of phagocytosis of apoptotic cells that is still understudied is the apoptotic cells themselves. In all of our in vitro studies we have used dexamethasone-induced apoptotic thymocytes from wild type animals. While this choice makes sense biologically because of the role macrophages play in clearing thymocytes in vivo, many other apoptotic cell types occur in the body and are phagocytosed. It would therefore be important to understand how each type of apoptotic cell and necrotic cell attract phagocytes and trigger their recognition and phagocytosis. Various cell types may produce different “eat me” signals that we do not fully understand. A comprehensive examination of different apoptotic cell types would aid in understanding which stage of apoptosis and which markers of apoptosis are recognized by Axl, Mertk, and Tyro3 receptors. The regulation, signal transduction, and additional function of this family of receptor tyrosine kinases warrant further examination.
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