

# **REGULATION OF INNATE IMMUNE RESPONSES BY MERTK**

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## ABSTRACT

JULIE CLARKE WILLIAMS: Regulation of Innate Immune responses by Mertk  
(Under the direction of Glenn K. Matsushima)

The Tyro3/Axl/Mertk(TAM) family of receptors is important for phagocytosis of apoptotic cells, cytokine regulation, and suppression of autoimmune disease. Mice lacking Mertk present with a Systemic Lupus Erythematosus-like syndrome; however, the etiology of the autoimmune phenotype in the *mertk*<sup>-/-</sup> mice is unknown. As such, our hypothesis is that Mertk is regulating innate immune responses that prevent the initiation of autoimmune disease. This dissertation examines the role of the TAM family of receptors in 1) the clearance and killing of bacteria, 2) the regulation of immune cells in the peritoneal cavity and 3) the regulation of cytoplasmic dsDNA-induced cell death through the regulation of p202. Chapter 2 shows the TAM receptors are dispensable for phagocytosis and killing of bacteria. Chapter 3 shows, Mertk regulates the immigration and the number of immune cells in the peritoneal cavity, particularly the T cells and B cells. In addition, this increase in peritoneal cells correlates to autoantibody formation found only in *mertk*<sup>-/-</sup> mice and not *axl*<sup>-/-</sup> or *tyro3*<sup>-/-</sup>. Finally, Chapter 4 shows that Mertk suppresses expression of the lupus susceptibility factor, p202, and that p202 may rescue macrophages from cytoplasmic dsDNA-induced cell death. These studies have clarified the literature with regards to TAM receptor family and bacteria clearance, have provided new insights to the autoimmune phenotype associated with the under studied peritoneal cavity, and have established a novel mechanism by which Mertk regulates death of APCs

which may be critical in preventing the development of autoimmune disease.

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## LIST OF ABBREVIATIONS

AIM2	Absent in Melanoma 2
APC	Antigen Presenting Cell
ASC	Apoptosis Spec associated protein containing a CARD domain
AT	poly dA:dT
BAFF	B cell activating factor
BMMΦ	Bone marrow derived macrophage
BrdU	5-bromo-2'-deoxyuridine
CRP	C reactive protien
CT	Calf Thymus DNA
CTL	Cytotoxic T lymphocyte
DC	Dendritic Cell
dsDNA	double stranded DNA
EBV	Epstein Barr Viruse
GAP	GTPase activating proteins
Gas6	Growth arrest specific factor 6
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
FAK	Focal adhesion kinase
IFN	Interferon
IFNR	Interferon receptor
IKK	Iκβ kinase

IL	Interleukin
IRAK	IL-1R associated kinase
IRF3	Interferon regulatory factor 3
LBP	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
MΦ	Macrophage
MFG-E8	Milk fat globule E8
MHC	Major histocompatibility complex
MNDA	myeloid nuclear differentiation antigen
MyD88	myeloid differentiation primary-response factor 88
Nba2	New Zealand Black Autoimmunity 2
NF-κB	Nuclear Factor kappa B
NOD	Nonobese diabetic
PAMP	Pathogen associated molecular pattern
PEC	Peritoneal Exudate Cells
PGE2	Prostaglandin E2
PLCγ	Phospholipase Cγ
RILP	rab-7 interacting lysosomal protein
SAP	Serum ameloid protein
SLE	Systemic Lupus Erythematosus
Sm	Smith antigen
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and transactivator

SR-A	Scavenger receptor A
TAB	TAK 1 binding protein
TAK	Transforming growth factor $\beta$ activated kinase
TAM	Tyro3/Axl/Mertk family
TBK	TRAF-family member associated NF- $\kappa$ B activator binding kinase
TGF- $\beta$	Transforming growth factor beta
Th	T helper
Thio-PEC	Thioglycollate elicited peritoneal exudate cells(macrophage)
TIR	Toll and IL-1 receptor
TLR	Toll like receptor
TNF $\alpha$	Tumor necrosis factor alpha
TRAF	tumor necrosis factor receptor associated factor 6
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TRIF	TIR-domain-containing adaptor protein inducing IFN- $\beta$
UV	Ultraviolet
WT	Wild-type

## **Chapter 1:**

### **Introduction**

## **1.1 The Tyro3/Axl/Mertk family**

The Tyro3/Axl/Mertk(TAM) family of receptor tyrosine kinases is one of the last families of receptors to be described and functionally delineated. These cell surface receptors are expressed in many different cell types, including neural, reproductive, vascular smooth muscle, cancer, osteoclast, and hematopoietic cells (1-6). The work presented in this dissertation focuses on the function of these receptors by hematopoietic-derived cells, macrophages (MΦ) and dendritic cells (DC). This family of receptors is characterized by two extracellular immunoglobulin(IgG)-like domains, two extracellular fibronectin three-like domains, and an intracellular kinase domain containing a KWIAIES motif (7, 8). Tyro3 is an 880 amino acid long protein that is derived from a 2643 base pair processed sequence found on chromosome 2 in mice. While Axl is similar in length to Tyro3 at 888 amino acids and 2667 base pair sequence, the gene is located on chromosome 7 in mice. Mertk is larger than Axl and Tyro3 and consists of 994 amino acids from 2985 base pairs, but is found on chromosome 2 along with Tyro3 (NCBI database). The amino acid sequence homology between Mertk and Axl is 45.1%, comparing Mertk with Tyro3 is about 42.9%, and between Axl and Tyro 3 is 44.7%. In addition, in the cytoplasmic region, most of the 15 tyrosine residues are conserved with the exception of two fewer in Axl and three less in Tyro3. This difference in homology suggests that there may be functional differences regarding interactions with potential ligands, interactions with other receptors and in the signaling capacity of the three family members.

### **1.1a Ligands for TAM family**

One of the ligands for the TAM receptor family is growth arrest specific factor 6 (Gas6) (9). Gas6 is a serum protein produced by several cell types and is primarily involved in amplifying platelet aggregation (10). More recently, Gas6 is thought to act as a bridging molecule between a receptor and phosphatidylserine present on apoptotic cells (11-14). This ligand causes phosphorylation of all three TAM family members (9, 15-17). Gas6 is expressed by MΦ and is also found pre-bound to apoptotic thymocytes (18, 19). Gas6 has a relative binding affinity of Axl > Tyro3 >> Mertk (20). The binding of Gas6 to the TAM receptor family members and subsequent phosphorylation is thought to activate phagocytosis of the apoptotic cell (11, 12).

Protein S is another ligand for the TAM family members. Protein S and Gas6 share 40% sequence homology and are thought to function similarly with regard to binding of apoptotic cells and bridging to the TAM receptors to trigger phagocytosis (10). In addition Protein S and Gas6 are both vitamin K dependent proteins found in plasma but Gas6 lacks a critical loop and does not have the anti-coagulation properties of Protein S (10). However, while MΦ express Gas6, both MΦ and DC can express Protein S (18). Protein S appears to be the more important ligand for phagocytosis of rod outer segments in the eye by retinal pigment epithelial cells, which express Mertk (21). Early studies on binding of Protein S to TAM family members remains unclear but favors Protein S binding preferentially to Tyro3 but not Axl or Mertk. A recent report suggests that Protein S can bind Mertk only after oxidation and this may be critical for bridging phosphatidylserine on apoptotic cells to Mertk (22).

### **1.1b Mertk expression**



Mertk is a receptor tyrosine kinase that is in the TAM family. Mertk is an acronym for monocyte, epithelial cells and reproductive tissue which express high levels of mRNA and it has tyrosine kinase activity. Northern analysis of mouse tissue revealed expression of Mertk in the heart, brain, spleen, lung, liver, muscle, kidney and testis (7). Mertk is expressed on MΦ, DC, NK, and NKT cells, but not neutrophils (23). Mertk is also not expressed on normal T and B cells, though it is found on some lymphocytic cancer cell lines (7). In concordance with this, overexpression of Mertk in NIH 3T3 cells causes transformation (24). Mertk is also expressed in the murine monocytic cell lines, RAW 264.7 and J774.1 (7, 25).

### **1.1c Mertk: downstream signaling**

In earlier reports when a ligand for Mertk had not yet been identified, many of the original Mertk signaling studies performed used chimeric proteins that dimerized without stimulation. Nonetheless, these studies identify the autophosphorylation sites in the Mertk intracellular domain. These sites are found to be tyrosine 749, 753, and 754 in the human protein which correspond to 744, 748, and 749 of the murine protein (26). The use of chimeric proteins identify the ATP-binding site at lysine 614 which is critical for kinase activity (27). In addition, overexpression of chimeric Mertk proteins identifies a number of downstream signaling pathways that are activated by Mertk including signaling through PI3K, Shc, Grb2, Raf-1, MAPK, Phospholipase Cγ(PLCγ), and NF-κB (24, 27). While some of these signaling intermediates are confirmed using appropriate ligands, others may be the result of over expression or the particular cell type.

Previously published data from our lab also implicates Mertk as a receptor involved in the clearance of apoptotic cells (28). MΦ lacking Mertk have the ability to bind, but not ingest apoptotic cells (28). In contrast, DC lacking Mertk phagocytize AC similarly to wild-type, whereas DC lacking Axl and Tyro3 have a decreased ability to clear apoptotic cells (18, 23, 29). Signaling by Mertk after activation with Gas6 activates the Guanine Nucleotide Exchange Factor (GEF) Vav1 (12). Upon stimulation of cells with Gas6, Mertk is phosphorylated and subsequently phosphorylates Vav, leading to its activation (12). Vav is a GEF for the Rho family GTPases, and in human monocytes, Gas6 activates Rac, Rho, and Cdc42 to become GTP bound, resting monocytes show no active Rac, Rho, or Cdc42 (12). This is somewhat curious, because Vav is not a GEF for Cdc42 (30). Therefore, there must be another signal stimulated via Gas6 that activates this Rho family GTPase.

A second mechanism by which Mertk activates phagocytosis of apoptotic cells is through cooperation with scavenger receptor A (SR-A) or integrin  $\alpha_v\beta_5$  (31, 32). SR-A and Mertk are phosphorylated and associate in a time-dependent manner upon stimulation with apoptotic cells (25, 31). Anti-SR-A antibodies inhibit apoptotic cell-induced phosphorylation of Mertk and the activation of PLC $\gamma$ 2 (31). Inhibition of PLC $\gamma$ 2 blocks phagocytosis of apoptotic cells (25). Thus, the SR-A dependent phosphorylation of Mertk and subsequent activation of PLC $\gamma$ 2 is a signaling pathway critical for phagocytosis of apoptotic cells.

Integrin  $\alpha_v\beta_5$  associates with Mertk and facilitates the phagocytosis of apoptotic cells.  $\alpha_v\beta_5$  activity is triggered by the binding of its ligand, Milk Fat Globule EGF factor 8 (MFG-E8) (33). When both  $\alpha_v\beta_5$  and Mertk are present, there is a synergistic activation

for the phagocytosis of apoptotic cells (32). The engagement of Mertk results in the autophosphorylation of tyrosine 867 which subsequently phosphorylates focal adhesion kinase(FAK) in a Src kinase-dependent manner. Phosphorylation of FAK allows it to interact with  $\alpha_v\beta_5$  (32). This integrin  $\alpha_v\beta_5$  and FAK complex then recruits p130/CrkII/Dock180 complex which in turn activates Rac, one of the Rho family GTPases discussed previously. Finally, tyrosine 867 found on the intracellular domain of Mertk is implicated in this pathway and virtually all of the signaling pathways discussed in this section (34).

One last function of Mertk is that it acts in the negative regulation of lipopolysaccharide (LPS) signaling and cytokine expression. An in depth discussion of LPS/Toll-like receptor can be found in section 1.2a. Early work suggests Mertk is important for inhibiting the production of Tumor Necrosis Factor  $\alpha$ (TNF $\alpha$ ) by LPS-stimulated M $\Phi$ s (35). Evidence from work completed in DC shows that Mertk is necessary for the inhibition of LPS-mediated signaling by apoptotic cells (36). The interaction of Mertk and PI3K as well as Src kinase upon ligand-binding has been established, and both are important in down-regulating nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling and subsequent Interleukin-12 (IL-12) production. However only PI3K is implicated in modulating NF- $\kappa$ B signaling and subsequent TNF $\alpha$  production (36). Similarly, another TNF family cytokine, B cell activating factor (BAFF), appears to be regulated by Mertk. Mice deficient in Mertk have excessive levels of BAFF in their spleens and DC. High levels of this cytokine is implicated in autoimmune disease such as systemic lupus erythematosus(SLE) (discussed in more in 1.3c); however, the BAFF

produced by DCs from *mertk*<sup>-/-</sup> mice does not provide any survival advantage to B cells suggesting other mechanisms are likely contributing to B cell autoimmunity (37).

#### **1.1d *mertk*<sup>-/-</sup> mouse**

Our lab has previously generated a mouse in which the kinase domain of Mertk is replaced with a neomycin cassette previously termed *mer*<sup>kd</sup> or *mertk*<sup>kd</sup> now known as *mertk*<sup>-/-</sup> (35). Although this mouse was predicted to express a signaling dead Mertk protein, no protein is detectable by Western blot of MΦ whole cell lysates (unpublished data Matsushima Lab, (38)). However, experiments on the *mertk*<sup>-/-</sup> mouse provide one of the first physiologic functions for Mertk, the negative regulation of the LPS response and the control of pro-inflammatory cytokines. MΦ from *mertk*<sup>-/-</sup> mice show extended nuclear NF-κB binding to the cis-acting site of the TNFα promoter during stimulation with LPS (35). In addition, upon LPS challenge, *mertk*<sup>-/-</sup> mice are susceptible to endotoxic shock and death. Serum TNFα levels are markedly increased and *mertk*<sup>-/-</sup> mice show increased intestinal inflammation as compared to wild type (35). The pretreatment of *mertk*<sup>-/-</sup> mice with anti-TNFα antibody significantly reduces lethal endotoxic shock. These data suggest that Mertk is a negative regulator of LPS signaling.

Mice lacking Mertk have enlarged spleens which contain apoptotic cell fragments (35, 37). Spleens of TAM family triple knock-out mice are larger than their *mertk*<sup>-/-</sup> counterparts on a mixed genetic background (39). This notion is challenged in recent studies included in Chapter 3 in which backcrossed mice from our colony now show similar splenic weights in *mertk*<sup>-/-</sup> and TAM<sup>-/-</sup> mice. However, *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> mice have splenic weights which are similar to wild-type mice. Although *mertk*<sup>-/-</sup> mice have

greater splenic weight, this increase can not be attributed to an increase in hematopoietic cells as similar numbers of nucleated cells are found in both *mertk*<sup>-/-</sup> and wild-type mice (37). The only cell type elevated are DCs, which comprise on average less than 3% of the total splenic cellular population (37). Data from the Lemke lab also indicates increased splenic DC populations in their TAM<sup>-/-</sup> mice (40). It is plausible that the increase in splenic weight is primarily attributable to the red blood cell population.

In addition, a mutation in *Mertk* is implicated in vision loss of the Royal College of Surgeons (RCS) rat, a model of retinitis pigmentosa. In these rats, loss of vision is due to the failure of retinal pigment epithelial cells to clear outer segments shed by the photoreceptor cells (41). Similar to the RCS rats, *mertk*<sup>-/-</sup> mice are also blind, however mice lacking the family members *Axl* and *Tyro3* are not (18). This has important implications for *Mertk* as a critical regulator of photoreceptor homeostasis and *mertk*<sup>-/-</sup> mice are a model to study this mechanism of blindness which has been reported in humans (41).

Correlating to a defect in apoptotic cell clearance, *mertk*<sup>-/-</sup> mice develop autoantibodies with age and present with a SLE-like syndrome. This syndrome is marked in humans as well as mice by development of autoantibodies to chromatin, single and double-stranded DNA(dsDNA) among other antigens (29, 42). Although an earlier report describes individual TAM family members as contributing to autoantibody production, our data in Chapter 3 suggests only *Mertk* is critical for autoantibody formation and not *Axl* or *Tyro3* (43). A more detailed description of the SLE like syndrome is found in section 1.3f.

### **1.1e *Axl* expression and function**

The TAM family member Axl is expressed on a variety of cell types. Northern blot analysis shows expression in breast, tail, uterus, ovary, heart, lung, bone, kidney, and brain(44). Important to this work, Axl is also expressed on MΦ and DC (18). In addition to expression in normal tissue, Axl also has transforming qualities when over-expressed in cell lines (45). Concurrently, Axl is highly expressed in many different human cancers including but not limited to leukemia, Gastric, colon, prostate, lung, breast, liver, melanoma, and glioblastoma (46). Axl also has a role in erythropoiesis (47).

Interestingly, Axl expression is inducible on human DC by type I Interferon (IFN). This expression can be suppressed by TLR stimulation and the suppression is dependent on proteolytic cleavage of Axl by the metalloproteinase ADAM10 (48, 49). This proteolytic cleavage results in soluble Axl which can be found bound to Gas6 in mouse serum (48). In addition, human DC are rescued from growth factor deprivation-induced cell death by Gas6 stimulation (49). Axl associates with IL-15 receptor to protect cells from TNFα-stimulated cell death (50).

While Axl associates with two membrane-bound receptors, IL-15R and Interferon receptor 1 (IFNR1), there are a number of intracellular signaling molecules that can also associate with Axl (40, 50). These intracellular signaling components include, PI3K, Grb2, SHP2, PLCγ, (Suppressor of cytokine signaling 1) SOCS1, HCK2, RanBPM, and C1-TEN (51). With so many possible binding partners, the intracellular signaling options are almost limitless. It is possible that ligand binding to receptors, receptor-receptor interactions, or cell type determine the functional consequences of signaling downstream of Axl.

While the *axl*<sup>-/-</sup> mice are viable, there are few studies involving the *axl*<sup>-/-</sup> singly. Our lab shows that unlike mice lacking Mertk, *axl*<sup>-/-</sup> mice do not have retinal degeneration, however, MΦ lacking Axl are defective in phagocytosis of apoptotic cells, though not to the extent of cells lacking Mertk. DC however appear to be more reliant on Axl than MΦ for the phagocytosis of apoptotic cells (18). Another study finds slightly elevated autoantibody production in the *axl*<sup>-/-</sup> mice however unlike the *merlk*<sup>-/-</sup> or TAM<sup>-/-</sup> mice, these mice do not have increase splenic weights (43). Taken together these data suggest that Axl plays a less prominent role in autoimmunity than Mertk.

### **1.1f Tyro3 expression and Function**

The TAM family member Tyro3 is most highly expressed in the brain (52). However it is also expressed on epithelium, kidney, reproductive tissues and most important to this work, MΦ (53, 54). Low levels of Tyro3 are also detected on bone marrow-derived DC (18). Tyro3 is expressed in cancer, including myeloid leukemia and multiple myeloma (46).

Studies involving the signaling via Tyro3 are much more limited than that of the other TAM family members. Tyro3 is important for the binding of the ligand Protein S and subsequent activation of Mertk in retinal pigmented epithelial cells (21). Tyro3 associates with PI3K, Src, and RanBPM; however, no downstream signaling events have been specifically linked to Tyro3 (51).

Mice lacking Tyro3 (*tyro3*<sup>-/-</sup>) are also viable, though similar to Axl, studies investigating this single knock out are few. Our lab shows that similar to *axl*<sup>-/-</sup> and unlike *merlk*<sup>-/-</sup> mice, *tyro3*<sup>-/-</sup> are not blind and do not have retinal degeneration (18). Again,

similar to *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup> MΦ have a decreased ability to phagocytize apoptotic cells though again not as dramatically as *merck*<sup>-/-</sup>. DC lacking Tyro3 also behave like *axl*<sup>-/-</sup> DC and show less phagocytosis of apoptotic cells (18). One other group shows slightly elevated autoantibody production and normal splenic weights in the *tyro3*<sup>-/-</sup> mice (43). Overall, it appears that mice lacking Tyro3 have similar experimental outcomes as those lacking Axl.

## **1.2 Responses to Infection**

During a typical course of infection, both bacteria and apoptotic cells are present (55). First, bacteria interact with resident macrophages that detect the pathogen through cell surface receptors including Toll-like receptors (TLRs), complement receptors and scavenger receptors. Engagement of these receptors initiates signaling cascades that leads to phagocytosis and activation of several transcription factors including NF-κB. Transcription resulting from NF-κB activation includes the pro-inflammatory cytokines TNFα and IL-1 among others (56). These cytokines in combination with chemokines act to call in “help” to combat the infection. In most cases, depending on location of the infection, the “help” is in the form of neutrophils and macrophages. The first wave of responders is the neutrophils partly due to their large percentage of the white blood cells in blood (>45%), which migrate into the area of infection in the first 24 hours. After these neutrophils engulf bacteria, they begin to die by apoptosis. About 48 hours after the initial exposure to bacteria, macrophages become more prominent in the infected area, where they interact with bacteria as well as dead and dying neutrophils. It is believed that the macrophage is a key modulator in resolving inflammation.



### **1.2a Toll-like receptor signaling in response to infections**

One important family of pattern recognition receptors are the TLRs. To date, there are 10 human TLRs and 13 murine TLRs. Each TLR whose function is known recognizes a different subset of pathogen associated molecular patterns (PAMPs) (57). In general, TLR2 will recognize peptidoglycan of Gram-positive bacteria, TLR4 will bind Lipopolysaccharide (LPS) of Gram-negative bacteria, TLR5 recognizes flagellin, TLR7 can bind double-stranded RNA, and TLR9 will sense CpG DNA. TLR2, TLR4 and TLR5 will sense PAMPs in extracellular compartments while TLR7 and TLR9 are found in endosomes and sense nucleic acids from pathogens captured in phagosomes (57).

TLR4 binds LPS in a complex with MD-2, CD14, and lipopolysaccharide-binding protein (LBP), all which enhance TLR4 signal transduction (58). Upon binding of LPS to TLR4, there are two pathways that become activated, these are termed myeloid differentiation primary-response factor 88 (MyD88)- dependent and MyD88-independent pathways (57). For the MyD88-dependent pathway, MyD88 interacts with the TLR through their Toll/IL-1R (TIR) domains. MyD88 subsequently recruits IL-1R associated kinase 4 (IRAK4) which interacts with IRAK1 (57). Upon MyD88/IRAK4/IRAK1 complex formation, IRAK1 becomes hyperphosphorylated and this phosphorylation engages the binding of tumor necrosis factor receptor associated factor 6 (TRAF6). Binding of TRAF6 to IRAK1 allows these two molecules to dissociate from the receptor complex and bind to transforming growth factor  $\beta$  activated kinase 1 (TAK1), TAK1 binding protein 1 (TAB1), and TAB2 (57). At this point IRAK1 is degraded and the TRAF6/TAK1/TAB1/TAB2 complex moves into the cytosol. Translocation to the

cytosol allows the complex to associate with ubiquitin ligases that ubiquitinate TRAF6 (57). Ubiquitination of TRAF6 activates TAK1 and TAK1 can now phosphorylate the I $\kappa$ B kinase (IKK) complex. The IKK complex then phosphorylates the inhibition of nuclear factor  $\kappa$ B (I $\kappa$ B). Phosphorylation of I $\kappa$ B causes its dissociation from NF- $\kappa$ B, allowing NF- $\kappa$ B to translocate to the nucleus and upregulate gene expression (57). Genes regulated by NF- $\kappa$ B in this context are typically pro-inflammatory cytokines such as TNF $\alpha$  and IL-1 $\beta$ .

For the MyD88-independent signal transduction, ligand binding recruits the adaptor molecule TIR-domain-containing adaptor protein inducing IFN- $\beta$  (TRIF) via TRIF-related adaptor molecule (TRAM) to the TLR (57). Once recruited to the receptor, TRIF activates IKK $\epsilon$  and TRAF- family member associated NF- $\kappa$ B activator-binding kinase (TBK1) which subsequently phosphorylate the interferon regulatory factor 3(IRF3). IRF3 then moves to the nucleus and binds to the interferon regulatory elements of certain genes. These genes include the IFN- $\beta$  and other IFN regulatory genes (57).

Not mentioned previously, TLR3 recognizes dsRNA typically found in some viruses. TLR3 is found in endosomal compartments and upon binding of dsRNA, an adaptor molecule, TRIF, will activate TBK-1 and IKK $\epsilon$  (57). TLR3 is the only TLR which exclusively uses the My-D88-independent signaling pathway. In addition, dsRNA can be recognized in the cytosol by RIG-I, which through its CARD domain, can activate TBK-1 and IKK $\epsilon$ . In both pathways, TBK-1 and IKK $\epsilon$  activate IRF-3 which facilitates transcription of type I interferons (59).

## **1.2b Phagocytosis of Bacteria**

During the course of an infection, the macrophage first senses pathogen-associated molecular pattern of bacteria through many different receptors including Toll-like receptors (TLR), mannose receptors, scavenger receptors, CD36 as well as Fc and complement mediated interactions (60, 61). Upon recognition, these receptors signal for phagocytosis by the myosin contraction and rearrangement of the actin cytoskeleton; however, each receptor-mediated pathway may track through different subcellular mechanisms (62). This actin rearrangement is mediated by the Rho family of GTPases, Rac, Rho, and Cdc42 (63). The activation status of this family of molecules cycles from “on”, GTP bound, to “off”, GDP bound. The GTPases are regulated by GTPase activating proteins (GAPs) and GEFs (63). The GAPs stimulate intrinsic GTPase activity thus pushing the Rho GTPases to the off state. In contrast, the GEFs assist the exchange of GDP to GTP and thereby activate the Rho GTPases. One important GEF for the Rho family GTPases are the Vav proteins (64, 65). Vav proteins are themselves regulated by their phosphorylation status. This phosphorylation status of Vav is controlled by binding to phosphorylated cell surface receptors (65). Thus, upon activation, Vav is phosphorylated and acts as a GEF for Rho and Rac, but not the structurally similar Cdc42 (30). Rho and Rac are now in their active form and cause the cytoskeleton rearrangement necessary for phagocytosis. Also, TLR activation of macrophages can lead to Vav phosphorylation (66, 67), providing a further link between TLRs and phagocytic machinery.

Once the phagosome is formed around the bacteria, it begins to mature by fission and fusion events with lysosomes and late endosomes (61). Although protein markers of phagolysosome maturation such as LAMP 1 and 2 are identified, the regulation of this

vesicle fusion event is poorly understood (61). Currently the small GTPases rab-5 and rab-7 as well as rab-7 interacting lysosomal protein(RILP) are implicated as factors regulating phagosome-lysosome fusion (68, 69). The TLRs are also implicated as modulators of phagosome maturation, and this maturation is dependent on p38 mitogen-activated protein kinase (70). With the addition of this recent evidence, Colin Watts proposes that the p38 regulated protein, guanine nucleotide dissociation inhibitor (GDI), a rab affecter, could be the link between TLR signals and phagosome maturation (71). The penultimate consequence of phagocytosis and phagosome maturation is for the break down of antigens. The presentation of antigens by major histocompatibility complex (MHC) is ultimately critical for the induction of adaptive immunity which further facilitates removal of pathogens by ushering antibody-mediated phagocytosis and the provision of cytokines such as IFN $\gamma$  to increase phagocyte killing.

### **1.2c Phagocytosis of Bacteria and Apoptotic cells**

One important role for the TAM receptors is phagocytosis(18, 28). This process is critical to resolving an infection, both by phagocytosis of bacteria and of infected dead cells. Phagocytosis is mediated by a combination of interactions with cell surface receptors and ligands expressed on the surface of the phagocytic particle. Interestingly, many of the receptors involved in phagocytosis recognize foreign or non-self molecules of bacteria and apoptotic cells. These include SR-A, CD14, integrins, complement receptors and Fc receptors (72-75). In addition to cell surface proteins, intracellular machinery used for phagocytosis of bacteria and apoptotic cells also overlaps.

### **1.2d Phagocytosis of apoptotic cells**

As stated previously, many receptors used for phagocytosis of bacteria are also engaged for phagocytosis of apoptotic cells (75). Once an apoptotic cell is targeted for phagocytosis, the intracellular phagocytic machinery used for bacteria is employed. Similarly to bacteria, apoptotic cells are engulfed via activation of the Rho GTPases. Phagosome maturation proceeds similarly to that of a phagosome containing bacteria with one important distinction (76, 77). This distinction is with regard to the MHC molecules that fuse with the maturing phagosome. Maturation of the phago-lyso-endosome is critical for antigen presentation of particles from phagosomes that contain bacteria. However, importantly the lack of MHC fusion to phago-lyso-endosomes containing apoptotic cells prevents antigen presentation of self antigens found in the apoptotic cell (76). Improper presentation of self-antigens could potentially lead to autoimmune disease.

### **1.2e Regulation of Cytokine Signaling by Apoptotic cells**

Phagocytosis of apoptotic cells regulates the production of a number of cytokines and chemokines. Some of these cytokines are upregulated directly by apoptotic cells, and some are downregulated after or with an inflammatory stimulation such as LPS. For example, macrophages given apoptotic cells alone will upregulate the anti-inflammatory factors transforming growth factor- $\beta$  (TGF- $\beta$ ) and Prostaglandin E<sub>2</sub>(PGE<sub>2</sub>)(78). In addition, the same group shows that macrophages given apoptotic cells and LPS together have decreased levels of IL-1 $\beta$ , TNF $\alpha$ , and IL-10 when compared to cells exposed to LPS alone (78). Therefore, the downregulation of pro-inflammatory cytokines is attributed to

a feedback loop mediated by apoptotic cell-induced production of TGF- $\beta$  and PGE2 (78). In addition, another group found that apoptotic cells inhibit TNF $\alpha$  and IL-6 production by macrophages (79). Moreover, apoptotic cells also suppress LPS or IFN stimulation of the chemokines CXCL9 and CXCL10 (79). In addition, a negative feedback pathway is activated by the transcription of the SOCS1 and SOCS3 genes which are increased upon apoptotic cell stimulation (79). These SOCS proteins are known to inhibit both the TLR-signaling pathway as well as the Jak/Stat signaling intermediates (80, 81).

The SOCS proteins are directly implicated in TAM receptor-mediated inhibition of TLR-signaling components (40, 82, 83). Recently it has been shown that a ligand of the TAM receptors, Gas6, stimulates SOCS1 and SOCS3 upregulation (40). It is believed that this upregulation of SOCS interferes with TLR or type I interferon receptor signaling (IFNR) (40). SOCS1 can inhibit TLR signaling by binding MAL to mediate polyubiquitination; IRAK which inhibits phosphorylation of IKK $\alpha$ ; the p65 subunit of the NF- $\kappa$ B complex in the MyD88-dependent signaling. Alternatively, SOCS1 can bind Tyk2 within the IFNR complex of the MyD88-independent pathway to prevent phosphorylation of Stat1(82). SOCS proteins can also compete for Signal transducer and activator of transcription 1 (STAT1) binding to the IFNR and thus preventing phosphorylation and activation of STATs. It is believed that inhibition is partly accomplished by SOCS3 binding STAT3 binding sites on cytokine receptors, thereby uncoupling STAT3 and preventing its phosphorylation (84, 85). In addition, SOCS3 may bind TRAF6 in the MyD88-dependent pathway and TRAF3 in the MyD88-independent pathway thereby inhibiting NF- $\kappa$ B and IRF3 respectively (83). Correspondingly, TAM<sup>-/-</sup> DC produce greater amounts of TNF $\alpha$  and IL-6 in response to TLR stimulation.

Consistent with these findings, Gas6 stimulation also reduces DC responses to TLR ligands and IFN stimulation (40).

A brief discussion of cytokines regulated by Mertk is found in section 1.1c. Relevant to this discussion, it has been found by our lab and others that Mertk is important for the ability of the apoptotic cell to regulate production of the cytokines TNF $\alpha$ , IL-1 $\beta$ , and IL-12 (19, 35, 36)(unpublished data, Matsushima Lab). LPS and apoptotic cell-stimulated DCs lacking Mertk on the NOD background have similar production of TNF $\alpha$  and IL-12 when compared to stimulated DCs that did not receive apoptotic cells (19, 36). Whereas LPS and apoptotic cell-stimulated wild-type NOD DC have lower TNF $\alpha$  and IL-12 when compared to cells stimulated with LPS alone. This exposure to apoptotic cells and subsequent DC inhibition induces Mertk PI3K/Akt signaling and more recently recruitment of Src to Mertk leading to Stat3 activation (36, 86). Thus, Mertk is critical for apoptotic cell induced down regulation of TNF $\alpha$  and IL-12 by DC.

### **1.3 Systemic Lupus Erythematosus**

SLE is an autoimmune disease of unknown etiology with both genetic and environmental contributions (42). Because this disease has a wide range of symptoms, the American college of rheumatology identifies 11 criteria for diagnosis of SLE (42). These criteria include Malar rash, Discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, hematologic disorder, immunologic disorder (anti-dsDNA, anti-Sm) and anti-nuclear antibody production. At least 4 of these criteria must be present in

order for the patient to be diagnosed with SLE. However, antibodies to dsDNA and Sm antigen are present in greater than 95% of patients (42).

A role for genetic contributions to SLE is evident in skewing of disease variance by sex as well as familial associations. SLE is more common in women than men, 9:1, and also more common and severe in African Americans (42). Certain HLA alleles are linked to a prevalence of SLE and genetic alterations in the complement components and Fc receptors are also linked to SLE. More monozygotic twins both have SLE than dizygotic twins. Family members of SLE patients are more likely to be diagnosed with SLE, although 20% of family members of SLE patients have increased autoantibody titers but do not progress to full blown disease (42). This is one clear indication that genetics is not the only factor contributing to the onset of SLE.

Many environmental factors are correlated with SLE. Ultraviolet(UV) light causes disease associated flares in 70% of patients (87). UV light has the potential to induce apoptosis, thus providing increased availability of autoantigens. Oral hormone ingestion is also associated with SLE as women taking oral contraceptives or hormone replacement are twice as likely to be diagnosed (87). In addition, a role for infection in SLE etiology has been proposed. SLE patients are more likely to be infected with Epstein-Barr Virus(EBV) than age-matched controls, and interestingly they also have higher viral titers (87, 88). Another piece of evidence that points to EBV as a potential factor contributing to SLE is that EBV Nuclear antigen-1 is a known molecular mimic to self antigens and is capable of inducing cross-reactive antibodies to Sm and Ro (89). Given that SLE patients also tend to have elevated circulating type I IFN, a cytokine



known to induce anti-viral host mechanisms, EBV seems a likely candidate for involvement in SLE pathology and etiology.

### **1.3a Antigen presenting cells in SLE**

Antigen-presenting cells (APC) including MΦ and DC have been shown to play a variety of roles in the pathogenesis of SLE including phagocytosis of apoptotic and necrotic cells, antigen presentation to T and B cells, maintenance of tolerance, and modulation of cytokine and chemokine production. The role of the APC varies as interactions between APC and lymphocytes occur under non-autoimmune scenarios as well. Impairment in clearance of apoptotic and necrotic cell material is seen in both murine models of SLE and human SLE. In fact, one study found increased apoptotic cells in lymphnodes of SLE patients (90). Defects in the complement-mediated phagocytosis components are also strongly correlated with SLE, as 93% of patients with complete C1q deficiency develop SLE early in life (91). However, most defects associated with C1q and SLE are due to autoantibody production and hypercatabolism (92, 93). The dysfunction of DNase I is also associated with SLE in humans. DNase I is necessary for the digestion of chromatin in apoptotic and necrotic cells as well as removal of DNA from interstitial compartments. DNase I activity is lower in SLE patients and this correlates with increased disease severity(94). Another human genetic polymorphism associated with SLE and clearance of self materials is in C-reactive protein (CRP). Two polymorphisms found in the CRP and Serum ameloïd protein(SAP) loci are associated with low levels of these proteins and SLE (95). Clearly the disposal of self material plays a role in SLE pathogenesis.

Deficiencies in clearance of dying cells are found in mouse models of SLE as well. Confirming the previously described human genetic predispositions, mice lacking C1q, DNase I, and SAP, all have SLE like phenotypes including autoantibody production and glomerular nephritis (96). In addition, mice lacking secreted IgM show delayed debris clearance and manifest disease in the form of antinuclear antibodies and deposition of complement and renal IgG (97). Mice lacking phosphatidylserine receptor are neonatal lethal; characterized by accumulation of apoptotic cells in the brain and lung (98). MFG-E8 knock out mice show impaired uptake of apoptotic B cells resulting in antinuclear antibodies, anti-dsDNA antibodies and glomerular nephritis (99). Lastly and most important to this work, mice lacking Mertk also show defects in apoptotic cell clearance and produce anti-nuclear and anti-dsDNA antibodies (28, 29, 37).

### **1.3b TLR signaling and SLE**

Shown recently, signaling by TLR3, 7, 9 contributes to the progression of SLE. These TLRs reside in the endosomal compartments of the cell and recognize single-stranded and double-stranded nucleic acids. As discussed earlier, TLR3 recognizes double-stranded RNA and uses the MyD88-independent TRIF adaptor molecule to upregulate type I IFN (57). TLR7 recognizes single-stranded RNA and uses the MyD88 adaptor for down-stream signaling. TLR9 recognizes CpG-rich double-stranded DNA and also uses the MyD88 adaptor for down-stream signaling. These receptors are normally thought of as viral pathogen-sensing receptors. Due to the restricted endosomal localization of these nucleic acid-sensing TLRs, they do not normally engage self nucleic acid.

One hallmark of SLE is the deposition antibody-nucleic acid complexes. In B cells, upon engagement of the BCR, the antibody-nucleic acid complexes found are internalized and TLR7, and 9 are present to engage the nucleic acid and transmit downstream signaling (100, 101). As such, these complexes may increase the production of autoantibody.

In DC, these antibody-nucleic acid complexes can be recognized by both Fc receptors and TLRs (102). DC can be activated by anti-nucleosome and chromatin complex as well as anti-DNA complexes to produce IFN $\alpha$  (103, 104). However, similar to the BCR-TLR dual engagement, the Fc receptor-TLR dual engagement is necessary for activating plasmacytoid dendritic cells (pDC) as Fc crosslinking with out TLR engagement will not activate pDC (105). Discussed in more detail below, the activation of pDC is a critical step for the production of IFN $\alpha$ . Dual activation of TLR and Fc receptor or BCR can lead to aberrant cytokine production and autoantibody producing cell activation.

### **1.3c Cytokines involved in SLE**

A variety of cytokines play a role or are elevated in both murine and human SLE. An overview of these cytokines can be found in Table 1. To introduce the potential roles for these cytokines, it is necessary to first introduce the concept of T helper (Th) subsets, Th1 and Th2. Th0 cells are driven by APCs to become Th1 cells that produce IFN- $\gamma$  as well as TNF $\alpha$ . Th1 cells then proliferate in the presence of IL-2 and perpetuate their own phenotype by producing IFN- $\gamma$ . In contrast Th1, Th0 cells can be driven by APCs to become Th2 which primarily produce IL-4. Th2 cells perpetuate their phenotype by

producing IL-4, IL-5, and IL-10. Interestingly, IFN $\gamma$  produced by Th1 cells actually inhibits Th2 cell differentiation, and IL-4 produced by Th2 cells inhibits Th1 cell differentiation (56). Th1 cells activate the cell-mediated immune response to kill pathogens by secreting IFN $\gamma$  which promotes killing of phagocytized bacteria in macrophages or by helping in the induction of cytotoxic T lymphocytes (CTL) (56). In contrast, Th2 cells are thought to combat pathogens by activating the humoral immune response to produce antibody to these pathogens. These antibodies mediate complement-dependent killing or opsonize pathogens which target them for phagocytosis. SLE was once thought to be a Th2-mediated disease, however, an increased expression of Th1 cytokines IFN $\gamma$  and TNF $\alpha$  are higher in SLE patients than controls (106, 107). Similarly, in mouse models, both Th1 and Th2 cytokines are involved as, SLE prone MRL/lpr mice lacking IFN $\gamma$  and IL-4 separately, have less disease than mice with intact cytokines (108)

The Th1-related cytokines, IFN $\gamma$ , TNF $\alpha$ , IL-2, IL-12, and IL-18 are dysregulated in murine or human SLE. IFN- $\gamma$  was discussed previously, however more evidence for involvement of this cytokine is that SLE patients with high IFN $\gamma$  also have greater disease(109). In addition, NZB/W F<sub>1</sub> mice receiving IFN $\gamma$  had increased disease incidence and NZB/W F<sub>1</sub> mice lacking IFN $\gamma$  show reduction in disease (110, 111). Paralleling these studies, TNF $\alpha$  levels are elevated in the kidneys of SLE patients, and TNF $\alpha$  administration to lupus-prone NZB/W F<sub>1</sub> mice increases disease severity and mortality (112, 113). Interestingly, T cells from both murine and human SLE produce less IL-2, and consequently are less proliferative (114-116). IL-12 has been found to be increased in serum from SLE patients (117). In addition, DC from MRL/lpr mice have higher expression of IL-12 and consistent with these findings, disease-associated

pathology is increased in lupus-prone mice injected with IL-12 (118, 119).

Correspondingly, MRL/lpr mice lacking IL-12 have reduced disease (120). In both murine models of IL-12 dysregulation, modulation of IFN $\gamma$  is also observed. Lastly, IL-18 is known to act synergistically with IL-12. As such, MRL/lpr mice injected with IL-18 have increased disease pathology (121). In humans, an increased level of IL-18 correlates with increases in active SLE (122). Taken together, this data suggests Th1-associated cytokines are dysregulated in SLE and appear to exacerbate pathology.

The Th2-associated cytokine IL-10 is involved in SLE. Normally IL-10 acts to inhibit Th1 cytokines, promotes humoral responses and is produced by a variety of cells including APC and lymphocytes. Evidence to support the involvement of IL-10 in SLE is shown in studies using anti-IL-10 antibody treatment which delays the onset of disease in lupus-prone mice. These mice have lower anti-dsDNA antibody titers and increased survival (123). In addition, reports show correlation between increased IL-10 in lupus patients and disease severity as well as dsDNA antibody titers in a mouse model (124, 125). The Th2 cytokine IL-4 is also elevated in SLE patients (126).

Another set of cytokines involved in SLE are the Th17-associated cytokines, IL-6, IL-17 and TGF- $\beta$ . Th17 cells are distinct from Th1 and Th2 and are characterized by the production of IL-17 as well as IL-1 and IL-6. Both IL-6 and TGF- $\beta$  produced by APC are involved in Th17 differentiation (107). IL-17 is elevated in serum from SLE patients (127). The murine model of lupus, BXD2 mice, also has elevated levels of serum IL-17(128). In addition to IL-17, the cytokine IL-6, which supports Th17 cells, is also elevated in SLE patients and murine models of SLE (129-132). In addition, NZB/W F1 mice treated with IL-6 show increases in disease severity (133). In accordance, NZB/W

F1 mice treated with anti-IL-6 antibody show reduction in disease and increased survival (134, 135). In addition, since IL-6 inhibits T regulatory cells (Treg), it is possible that an increase in IL-6 activity correlates to a decrease Treg activity whereby leading to dysregulation of autoimmune T cells and a break in tolerance.

Two other cytokines are of importance to the discussion on SLE and *Mertk*. Those cytokines are BAFF and the type I IFN, IFN $\alpha$ . Evidence for the involvement of BAFF (also known as BLyS, TALL-1, THANK, and zTNF4) in SLE is found in studies where BAFF is elevated in some SLE patients and overexpression of BAFF in transgenic mice results in autoimmune disease similar to SLE (136-138). In addition, blockade of BAFF signaling using antibodies to its receptors shows a decline in mortality of lupus-prone NZB/W F1 mice (139). Of importance to the work presented in this manuscript, our lab also observes an increase in splenic BAFF mRNA from mice lacking *Mertk*, another murine model of SLE described previously (37). An increase in BAFF mRNA expression is also found in spleens of TAM $^{-/-}$  mice (40). A similar increase in BAFF production is found in *mertk* $^{-/-}$  DC, however this does not correlate to a increase in B cell survival when co-cultured with DC lacking *Mertk*(37). It is plausible that marginal zone B cells which are dependent on BAFF may have increased survival by *mertk* $^{-/-}$  DCs.

The type I IFNs were first identified as anti-viral mechanism produced by infected cells. However, there is mounting evidence for involvement of IFN $\alpha$  in SLE. A number of studies have shown patients undergoing IFN treatment have an increased risk of developing autoantibodies (140, 141) and multiple studies have identified increase levels of IFN $\alpha$  in serum of SLE patients (142-144). Serum IFN $\alpha$  levels in SLE patients also correlate with antibody production (145). IFN $\alpha$  may be regulating SLE directly or

indirectly through the upregulation of IFN responsive proteins. TLR7 and TLR9 are expressed in peripheral blood monocytes and are upregulated in response to IFN, thus may increase the sensitivity and response to nucleic acids by B cells or dendritic cells (146, 147). Another IFN-responsive protein family is the HIN-200 proteins. This family is known to be located within a lupus susceptibility region in both mice and humans (148). An in depth discussion of this family is found in 1.4.

The most active IFN $\alpha$ -producing cell is the pDC. pDC can be activated to secrete IFN $\alpha$  by a variety of insults(149). Interestingly and important for the involvement in SLE, pDC can produce IFN $\alpha$  in response to immune complexes (150). Immune complexes are formed by antibody and self proteins or nucleic acids and are found often in both murine and human SLE. Also important for the work discussed in this manuscript, TAM<sup>-/-</sup> and *merck*<sup>-/-</sup> mice have elevated splenic DC (37, 40). While neither group determined if these DC were plasmacytoid, our studies find CD11b<sup>+</sup> DC to be significantly elevated while CD8<sup>+</sup> DC are not (37). A synopsis of cytokines involved in SLE can be found in Table1.

**Table 1.** Cytokines dysregulated in SLE

Cytokine	Human	Mouse	Cell
IFN- $\gamma$	increased	KO NZB/W F1 less disease	Th1
TNF- $\alpha$	increased	administration leads to increased disease	Th1
IL-2	decreased	decreased	Th1
IL-12	increased	administration leads to increased disease; KO MRL/lpr less disease	Th1
IL-18	increased	administration leads to increased disease	Th1
IL-10	increased	anti-IL-10 delays disease	Th2
IL-4	increased	KO MRL/lpr less disease	Th2
IL-17	increased	increased in BXD2 lupus mice	Th17
IL-6	increased	administration leads to increased disease; anti-IL-6 decreases disease	Th17
BAFF	increased	overexpression leads to disease in non lupus prone mice; anti-BAFF decreases disease in NZB/W F1	B cell
IFN- $\alpha$	increased	KO NZB/W F1 for receptor leads to decreased disease; KO MRL/lpr has increased disease	DC and others?

### 1.3d Chemokines involved in SLE

A wide variety of chemokines and their receptors are implicated in SLE pathogenesis. Expression of CXCL10, a chemokine that targets activated T cells and binds to the CXCR3 receptor, correlates with SLE and disease severity by multiple groups (56, 151, 152). Another chemokine which binds CXCR3, CXCL9 is also elevated in serum of SLE patients(152). Serum concentrations of CXCL13, which acts on naïve B cells and activated CD4 T cells, correlates with disease index, and anti-dsDNA titers (153). CXCL13 is recognized by CXCR5 and is also critical for B-1 cell migration to the peritoneal cavity (154).

Another study investigates the association of serum MIP-1 $\alpha$ , MIP-1 $\beta$ , and RANTES with SLE. MIP-1 $\alpha$  (CCL3), MIP-1 $\beta$  (CCL4), and RANTES (CCL5) all target a variety of cells expressing CCR1 and CCR5 including M $\Phi$ , T cells, NK cells and immature DC (56). The study observes that SLE patients have higher concentrations of



all three chemokines(155). Another study finds RANTES promoter polymorphisms that correlate to SLE patients in Chinese children(156). Thus, these studies indicate that elevated chemokines and their receptors may be controlling a greater migration or abnormal accumulation of key immune cells at sites that may be conducive for autoimmunity.

Levels of a chemokine, CCL17 that binds CCR4 and acts on T cells, immature DC and NK cells, are significantly different in untreated and treated SLE patients. Those SLE patients that are treated with immunosuppressive therapy have significantly lower plasma levels of CCL17 than patients who are not treated (157). This study also examines MCP-1(CCL2) and finds no correlation between CCL17 and CCL2 levels. However, CCL2, which binds CCR2 on T cells and monocytes (56), is investigated by a number of groups in relation to SLE. Two different groups associate CCL2 secreted in urine of SLE patients with disease severity (158, 159). One of these groups suggests that CCL-2 secretion could be used as a biomarker for future disease flare (158). In addition promoter polymorphisms of CCL2 are evident in a Spanish cohort with SLE (160). A synopsis of chemokines involved in SLE can be found in Table 2.

Several chemokine receptors are also associated with SLE. First, CCR4, the receptor for CCL22 and CCL17 (56), has increased expression on peripheral blood T cells from SLE patients compared to healthy controls (161). This expression correlates with disease index and could be reduced with corticosteroid therapy(161). Another study examines CCR1, CCR2, CCR5, CXCR3, CXCR4, and CXCR3R1 for differences in whole blood samples (162). This study concludes that CCR2 and CXCR3 positive T cells were lower in blood of patients with active disease flares and the authors suggest that low

levels of circulating CCR2 and CXCR3 positive T cells can be used as a biomarker for SLE flare (162). CCR2 is found on T cells and monocytes and is the receptor for MCP1-4 or (CCL2, 8, 7, 13)(56). CXCR3 is found on T cells, B cells and pDC and is the receptor for CXCL9, 10, and 11 (56, 163-165). In support of the involvement of CXCR3 in SLE, MRL/lpr mice lacking CXCR3 also have decreased kidney disease (166). A synopsis of chemokine receptors involved in SLE can be found in Table 3. Taken together, these data show that many chemokines and their receptors are markers for SLE disease.

**Table 2:** Chemokines and their receptors dysregulated in SLE

Chemokine	Human	Target cell	Receptor
CXCL10	increased	T	CXCR3
CXCL9	increased	T	CXCR3
CXCL13	increased	B, B1, T	CXCR5
CCL2(MCP-1)	increased	MΦ, T	CCR2
CCL3(MIP1-α)	increased	MΦ, DC, T, NK	CCR1,5
CCL4(MIP1-β)	increased	MΦ, DC, T, NK	CCR1,5
CCL5(RANTES)	increased	MΦ, DC, T, NK	CCR1,5
CCL17(TARC)	increased	T, NK, DC	CCR4

Receptor	Human	Found on	Chemokine ligand
CCR4	increased on T	T, NK, DC	CCL17, 22
CCR2	decreased	MΦ, T cell	CCL2, 8, 7, 13
CXCR3	decreased	DC, T, B	CXCL9,10,11

### 1.3e Involvement of Peritoneal cells in SLE

Many types of cells are found in the peritoneal cavity including DC, MΦ, T cells, B cells, NK cells, and Mast cells. However, little is known of the regulation of the peritoneal environment in the pathogenesis of SLE. Since the majority of low affinity autoantibody producing B-1 cells found in the body are found in this location (167), the peritoneal cavity is an attractive location for the initiation of autoantibody production.

B-1 cells are known to produce antibodies to PAMPs that can cross react with self-antigens and these cells do not require T cell help for this process (167). However, T cell help promotes class switching of B-1 cell antibody production. In addition, B-1 cells are known to contribute virtually all circulating IgM. These cells are generally characterized by the following expression pattern  $CD19^+$ ,  $B220^{low}$ ,  $IgM^{hi}$ ,  $CD23^-$ ,  $CD43^+$ , and  $IgD^{low}$ . In addition the T cell activation marker CD5 can be found on B-1 cells as well. These cells are termed B1a, and  $CD5^-$  cells are termed B1b (167).

The contribution of B-1 cells to the development of autoimmunity is controversial. One study shows through hypotonic ablation, that B-1 cells contribute to disease in the lupus-prone NZB and NZB/W F1 mice (168). In addition, another group shows that NZB/W F1 *xid* mice, which have an X-linked mutation that results in no B-1 cells, had lower antibody production (169). More recent data has proven that high affinity autoantibody to DNA is produced exclusively by conventional B2 cells (170). However as discussed above, B-1 cells make low affinity autoantibody and there is potential for a break in tolerance at this level resulting in B-1 cell response to T cell help and subsequent class switching to high affinity IgG molecules(167). Whether the autoantibody production in the found the *mertk*<sup>-/-</sup> mice is due to the contribution of B-1 cells remains to be investigated. Although all the immune components necessary for a functional innate and adaptive immune response are found in this location, the relationship between autoimmune disease and other cells found in the peritoneal cavity is not clear.

### **1.3f TAM involvement in SLE**

Previously published data establishes that mice lacking *Mertk* as well as mice lacking all three TAM receptors have autoimmune like syndromes. Data from the Lemke lab has shown the propensity for the *TAM<sup>-/-</sup>* mice to develop a variety of autoimmune symptoms similar to those found in the human diseases Rheumatoid Arthritis, Pemphigus vulgaris and SLE(43). This study shows increased splenic weight from *TAM<sup>-/-</sup>* mice, *AM<sup>-/-</sup>*, and *TM<sup>-/-</sup>* as well as mice that were null for *Tyro3* and *Mertk* and heterozygote for *Axl*. In addition, they show increases in peripheral lymphocyte populations, as well as lymph invasion of liver and kidney among other organs. Not only were the lymphocyte populations increased, they also have higher expression of activation markers. These *TAM<sup>-/-</sup>* mice also have the ability to induce division of adoptively-transferred WT lymphocytes, suggesting these mice provide a hyper-proliferative environment. Most relevant to the discussion on SLE, autoantibody production to dsDNA, collagen, cardiolipin, and phosphatidylinositol are all increased in *TAM<sup>-/-</sup>* mice. Mice lacking other family members also show autoantibody production in this study, though not to the extent of the *TAM<sup>-/-</sup>* mice (43).

While the *TAM<sup>-/-</sup>* mice have a variety of autoimmune syndrome symptoms, the *mertk<sup>-/-</sup>* mice manifest symptoms similar only to that of SLE. Three different studies show autoantibody production in these mice. The first documentation of autoantibody production to dsDNA is by our lab in 2001. We show that over 50% of *mertk<sup>-/-</sup>* mice tested are sera positive for anti-DNA antibodies(28). In 2002, another group published that there are statistically significant increases in the amount of anti-chromatin antibodies found the serum of mice lacking *Mertk* (29). The same study finds *mertk<sup>-/-</sup>* mice positive for antibodies to Rheumatoid factor and ssDNA as well as mild renal pathology. Lastly,

the third study from our lab documented elevated anti-nucleosome antibody in the serum of *merlk*<sup>-/-</sup> mice in addition to anti-dsDNA antibodies (37). While SLE in humans has a wider variety of symptom indicators, the lack of Mertk is only sufficient to display autoantibody production and mild kidney pathology.

Three other studies of mice lacking Mertk are relevant to the discussion of autoimmunity. While one study shows Mertk expression on B cells in a model of chronic graft-versus-host disease is critical for direct maintenance of B cell tolerance (171). Two other studies focus on the ability of mice lacking Mertk to contribute to autoimmunity through the APC-mediated activation of lymphocytes. In this chronic graft-versus-host disease model, wild-type B cells upregulate Mertk upon activation. Surprisingly, this leads to autoantibody production and higher expression of activation markers. Contrary to other studies, this study is also remarkable in that a lack of Mertk was protective from autoimmune chronic graft-versus-host disease (171).

A second study from the Clarke lab showed that 2-12H transgenic mice which constitutively express anti-Sm antibody that were also lacking Mertk have greater anti-Sm antibody levels in the serum when compared to 2-12H transgenic animals with intact Mertk (172). In fact, a few *merlk*<sup>-/-</sup> mice that were not expressing the 2-12H transgene show elevated serum anti-Sm antibodies (172). 2-12H mice lacking Mertk also have elevated numbers of antibody-secreting B cells in the bone marrow, spleen, mesenteric lymph node and lamina propria (172). This study provides direct evidence that the defect in clearance of apoptotic cells contributes to the autoimmune potential of the *merlk*<sup>-/-</sup> mice.

The third study is from the Tisch lab showing that Mertk on APC is important for the maintenance of T cell tolerance (19). In this study, nonobese diabetic (NOD) mice, a model of autoimmune induced diabetes, which lack Mertk have exacerbated disease when injected with T cells specific for  $\beta$  cell antigens (19). In addition, T cell proliferation in response to induction of  $\beta$  cell apoptosis in NOD mice lacking Mertk was also elevated. This study also shows DC lacking Mertk have increased production of T cell stimulatory cell surface receptors and cytokines (19). Taken together, these last two studies show the potential for the APC to be involved in maintenance of tolerance towards self antigen.

#### **1.4 HIN-200 family- SLE susceptibility genes**

The HIN-200 proteins are termed HIN-200 for the hematopoietic expression, interferon induction, and nuclear localization with a characteristic 200 amino acid domain(173). This family currently contains 6 genes termed *ifi-202*, *ifi-203*, *ifi-204*, *ifi-205*, *aim2* (absent in melanoma), and *pyhin1* in mice, encoding the proteins p202, p203, p204, p205, AIM2, and Pyhin1 respectively. Four human homologs have been identified including, *ifi-16*, *aim2*, *mnda*(myeloid nuclear differentiation antigen), and *ifix*, encoding the proteins IFI-16, AIM2, MNDA, and IFIX respectively (173). These genes are all located on chromosome 1 in both mice and humans (173). Notably, this region is found within the Nba2 (New Zealand Black autoimmunity 2) locus and is a known susceptibility site for SLE in mice (174).

The conserved 200 amino acid domain of HIN200 proteins contains a conserved motif of MF/LHATVAT/S (173). This domain is important not only for self association

of p202 but also for its association with a p53 binding protein (175). Two other conserved sequences are found in this family though their location differs in each protein. LXCXE is important for pRb-binding, and a CDK2 phosphorylation site is also found (176, 177). The HIN-200 family also contains a conserved nuclear localization signal and a DNA-binding motif. All but p202 also contain a PYRIN domain (178). Proteins containing PYRIN domains are known to be involved in induction of apoptosis and processing of IL-1 $\beta$ , through their interaction with the inflammasome (179). Thus, p202 may be unique among the family members and may not induce pyroptosis or IL-1 processing. Our current understanding of these proteins is relegated to experiments investigating AIM2 and p202 which are described below.

#### **1.4a AIM2 expression and function**

AIM2 is first described as one of a set of genes that was not expressed in malignant melanoma cell lines (180). The gene *aim2* encodes a 1456 base pair RNA sequence that is predicted to lead to a 485 amino acid protein (NCBI database). AIM2 is expressed in spleen, small intestine and peripheral leukocytes (180). Similar to all the other HIN-200 family members, it has been shown to be induced with IFN treatment (180). AIM2 is localized to the cytoplasm in overexpressing cells (181). Overexpression of AIM2 also suppresses proliferation in a variety of cell types (181, 182).

More recently, AIM2 functions as a major binder of cytoplasmic dsDNA. Sensing of cytoplasmic dsDNA is important for identification of viral pathogens as well host DNA that has escaped regulated compartments. Three different groups simultaneously published that AIM2 can bind dsDNA (183-185). Burckstummer and

colleges use a genomic screen approach in which dsDNA known to be interferon stimulatory is immobilized and used to affinity purify nucleic acid-binding proteins from RAW264.7, L929, and NIH3T3 cell lines (183). Separately, they use the same cell lines to screen for genes regulated by IFN $\beta$  by microarray. These screens pull out a total of 7 proteins including Trex1 which is known to remove retroviral DNA. Among the 6 other proteins are three HIN-200 family members, p204, p205, and AIM2 (183). However, since only AIM2 is predicted to be localized to the cytoplasm, this group focused on AIM2. They reconfirm the ability of AIM2 to bind dsDNA, and went on to show that AIM2 can associate with apoptotic spec-associated protein containing a card domain(ASC) to activate the inflammasome leading to IL-1 $\beta$  cleavage (183).

A second group, Fernandes-Alnemri and colleges, identifies the HIN-200 family using a NCBI database screen for proteins that contained a PYRIN domain as well as an oligonucleotide binding domain (184). This group narrows the HIN-200 family down to AIM2 by over-expressing the human HIN-200 family proteins and finding only AIM2 could activate caspase cleavage. This group also finds that overexpressing AIM2 could lead to pyroptosome formation even greater than the known inducer PYRIN (184). Pyroptosomes are visual specs found when ASC aggregates in the cytoplasm. They also show that addition of dsDNA to the cytoplasm of cells can cause pyroptosome formation in an AIM2 dependent manner. In addition, they show that both AIM2 and ASC oligomerize in pyroptosomes containing dsDNA (184).

A third group, Hornung et. al., identifies the HIN-200 family using the PFAM database again screening for proteins containing PYRIN domains and known DNA-binding domains (185). They find that of the human HIN-200 proteins, only AIM2 is



primarily located in the cytosol, and overlaps microscopically with ASC in co-overexpression of fluorescently-tagged proteins. In addition, the pyroptosome formation is greatest with AIM2 overexpression, and immunoprecipitation for ASC could only pull down AIM2. Cell viability is also monitored and knockdown of AIM2 results in less dsDNA-induced cell death, using vaccinia virus (a dsDNA virus) and poly dA:dT transfection (185). Finally, they show that AIM2 uses its HIN domain to bind dsDNA (185). Taken together these studies provide overwhelming evidence that AIM2 is critical for detecting cytoplasmic dsDNA and inducing inflammasome and pyroptosome formation, caspase-mediated cell death, and primed IL-1 $\beta$  cleavage.

#### **1.4b p202 expression and function**

p202 was first described as a protein that was upregulated after IFN stimulation (186). The gene encoding p202, *ifi-202*, is about 1338 base pairs long and encodes a protein of 445 amino acids (NCBI database). By Northern blot analysis, p202 is expressed in heart, brain, spleen, liver, skeletal muscle, and kidney, but is absent from lung and testes (187). p202 is also expressed in fibroblast cell lines, M $\Phi$ , and DC (188-190). As with all of the HIN-200 family members, p202 is upregulated by IFN; however it has also been shown in various types of cells to be upregulated with IL-6 and LPS (190, 191).

Interestingly, serum growth factors appear to negatively regulate p202 expression (188). When in reduced serum conditions, cells upregulated p202, arrest the in G<sub>1</sub> phase, and activation of the transcription factor AP-1 is increased. As there is an AP-1 binding

site in the p202 promoter, thus, it makes sense that reduced serum growth factors would upregulate p202.

In addition to AP-1, two other putative binding domains have been identified in the p202 promoter. These include Stat3 and E2F1 binding sites. IL-6 is a known activator of Stat3 phosphorylation and subsequent translocation to the nucleus (192). Binding of Stat3 to the *ifi-202* promoter is increased upon IL-6 stimulation, and mutation of this site resulted in a decrease in *ifi-202* promoter induced luciferase expression (191). E2F1 is identified as a transcriptional regulator of *ifi-202* as p202 is found to be elevated in E2F1 knock-out mouse embryonic fibroblasts (193). Interestingly, E2F1 appears to be negatively regulated by p202 because increased expression of p202 results in decreased expression of E2F1 and its target genes (193).

Like all of the HIN-200 family members, p202 has a DNA-binding domain. Although, direct binding of p202 to promoters has not been established, p202 modulates a number of transcription factors (194, 195). For example, p202 binds AP1, cFos and cJun and inhibits their transcriptional activity (195). In addition to its interaction with AP-1 transcription factors, p202 binds NF- $\kappa$ B p65 and p50 and inhibits transcriptional activity of NF- $\kappa$ B (195). Two subsequent studies investigate the modulation of NF- $\kappa$ B activity by p202. Using fibroblasts overexpressing p202, p202 inhibits the transactivation complex of p65/p50 NF- $\kappa$ B binding while enhancing inhibitory p50/p50 NF- $\kappa$ B binding to DNA (194). A subsequent study investigates p202 in DC from the SLE mouse model NZB/W F1. This study shows that expression of p202 correlated to enhanced LPS stimulated IL-12, IL-6, IL-10, and TNF $\alpha$  production (190). In addition, knock down of p202 in a M $\Phi$  cell line showed decreased expression of these cytokines

(190). Taken together these studies show that p202 has transcriptional modulation capabilities that may be cell type-specific.

In addition to its ability to bind and modulate transcription factors, p202 also modulates cell cycle and cell death (188, 189, 196, 197). The first identified binding partner of p202 was the retinoblastoma protein(pRb)(176). p202 has a pRb consensus-binding sequence of LXCXE and p202 can bind pRb at two distinct locations. As pRb negatively controls cell cycle and proliferation, the authors hypothesize that p202 may be binding pRb to modulate cell cycle (176). Another cell cycle control protein that binds to p202 was the p53 binding protein 1(53BP1). This study uses yeast two-hybrid technology to identify p202 and 53BP1 as binding partners and shows that this binding was dependent on a histidine in the conserved 200 amino acid region found in a HIN-200 proteins(187). As its name states, 53BP1 binds to p53 and thus it is not surprising that overexpression of p202 would modulate expression of p53 dependent genes (187).

Subsequently, p202 binds to p53's proline rich region using its C-terminal conserved 200 amino acid region (197). Interestingly UV treatment increases p202 expression in multiple cell types (196, 197). Because UV-induced apoptosis is mediated by p53, and p202 can bind p53, it is likely that cells expressing high levels of p202 would be more resistant to UV-induced apoptosis (196, 197).

In addition to the role of p202 in the suppression of p53-mediated apoptosis, a recent study implicates p202 in suppression of intracellular dsDNA-induced apoptosis. Roberts et. al. first demonstrates that intracellular dsDNA could induce apoptosis in a concentration and fragment size dependent manner. p202 binds and co-localizes to dsDNA in a manner that was dependent on DNA fragments being larger than 100 bp. In

addition, using siRNA knock-down of p202, they show that p202 could prevent the activation of the inflammasome, thus preventing caspase1 and 3 cleavage(189). This study also shows that p202 co-localizes with dsDNA in the cytoplasm in small aggregates unlike the large specs(pyroptosomes) formed by dsDNA and AIM2 (183-185, 189). In addition, when p202 is highly expressed as in NZB MΦ, there is less caspase1 and 3 cleavage (a measure of cell death) as compared to C57BL/6 MΦ which p202 expression is low (189). Taken together, the studies provide a model in which the ratio of p202 and AIM2 in a cell dictates whether the cell will live or die in response to intracellular dsDNA.

## **Summary**

The literature presented above provides a detailed introduction to the TAM family, responses to infectious agents and apoptotic cells, as well as SLE. Since Mertk is expressed on APC and mice lacking Mertk manifest an SLE-like autoimmune disease, these mice provide a unique opportunity to investigate SLE disease in a model in which only one gene is mutated, *mertk*, and that gene is not normally expressed on T or B cells. Subsequent chapters of this dissertation investigate 1) the role of the TAM family in the phagocytosis and killing of bacteria, 2) the increased peritoneal cells found in mice lacking Mertk, and 3) the ability of Mertk to regulate cytoplasmic dsDNA-induced cell death through the p202. The overall hypothesis is that Mertk regulates innate immune responses by MΦ. Application of knowledge gained from this dissertation may provide novel insights towards mechanisms of autoimmunity and may advance treatments for SLE.

## **Chapter 2:**

# **TAM Receptors are Dispensable in the Phagocytosis and Killing of Bacteria**

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## Abstract

Many receptors that are employed for the engulfment of apoptotic cells are also used for the recognition and phagocytosis of bacteria. Tyro3, Axl, and Mertk (TAM) are important in the phagocytosis of apoptotic cells by macrophages. Animals lacking these receptors are hypersensitive to bacterial products. In this report, we examine whether the TAM receptors are involved in the phagocytosis of bacteria. We found that macrophages lacking Mertk, Axl, Tyro3 or all three receptors were equally efficient in the phagocytosis of Gram-negative *E. coli*. Similarly, the phagocytosis of *E. coli* and Gram-positive *S. aureus* bioparticles by macrophages lacking TAM receptors was equal to wild-type. In addition, we found that Mertk did not play a role in killing of extracellular *E. coli* or the replication status of intracellular *F. tularensis*. Thus, while TAM receptors may regulate signal transduction to bacterial components, they are not essential for the phagocytosis and killing of bacteria.

Keywords: Macrophage, *E. coli*, Mertk, Axl, Tyro3, phagocytosis

## Introduction

During the course of a bacterial infection, innate immune cells such as neutrophils and macrophages sense bacteria and bacterial products via pathogen-associated molecular patterns (PAMPS) such as lipopolysaccharide (LPS) or peptidoglycan. PAMPs are recognized by several pattern recognition receptors on innate immune cells, including the Toll-like receptors (TLR)(57). Following recognition, the resident cells release pro-inflammatory cytokines and chemokines to recruit neutrophils and additional macrophages to the lesion. These responding neutrophils and monocytes arrive equipped as professional phagocytes to quickly ingest and kill the invading bacteria. This rapid immune response and phagocytosis of bacteria is critical to the resolution of a bacterial infection.

Many other receptors are implicated in the recognition and phagocytosis of bacteria including: Scavenger receptor A, Mannose receptor, TLRs, integrins, as well as Fc receptors and complement receptors(70, 72). Upon binding of bacteria, these receptors signal a rearrangement of the actin cytoskeleton that is mediated by the Rho family of GTPases, Rac, Rho, and Cdc42, ultimately leading to engulfment of the bacteria(63). One indication of the importance of these molecules is evident in that some bacteria attempt to evade phagocytosis by producing molecules that modulate the signaling to and from the Rho GTPases (198, 199).

Phagocytosis is not only important for controlling infection, it is also key in down-regulating inflammation and limiting tissue damage by returning the infected area to proper homeostasis. This is partly accomplished by the phagocytosis of apoptotic cells. As bacteria grow and/or infect cells, these cells often succumb to the infection and

die, either indirectly by toxic bacterial by-products or directly by intracellular pathogens. For example, neutrophils have been shown to become apoptotic after treatment with *E. coli*(200). Apoptosis is a form of cell death that has been well characterized and includes exposure of phosphatidylserine from the inner membrane to the outer surface of the dying cell. Subsequently, these apoptotic cells are cleared from the infected area typically by macrophages. Interestingly, many of the same receptors that facilitate phagocytosis of bacteria are also important for the phagocytosis of apoptotic cells including CD14, Scavenger receptor A, Fc Receptor, complement, and integrins(74, 75).

The Tyro3/Axl/Mertk (TAM) receptors have been shown to be required for efficient phagocytosis of apoptotic cells (18, 28). All three receptors are expressed by macrophage and dendritic cells(DC), however, Mertk is also expressed by NK cells, but is not expressed on neutrophils or non-malignant lymphocytes(7, 18, 201). These proteins are receptor tyrosine kinases that are similar in structure and function. Each family member has two Ig-like and two fibronectin III-like extracellular domains, as well as a characteristic KWAIAES sequence in the intracellular domain(7, 8). In macrophages, Mertk is essential for rapid ingestion of apoptotic cells(28); though Axl and Tyro3 are partly involved(18). In contrast, Axl and Tyro3 appear to function in DC and Mertk has no role in phagocytosis although the process is much slower(18, 29). Knock-out mice have been generated for all three family members(35, 39). The inability to properly clear apoptotic cells in these knock-out mice results in the development of a lupus-like autoimmunity characterized by autoantibody production as well as splenomegaly(28, 29, 43). Gas6, a ligand for the TAM family members, binds these receptors with a relative affinity of Axl>Tyro3>>Mertk(20). Gas6 is produced by



several cell types including macrophages, DC and apoptotic thymocytes(18, 19).

Another ligand for the family member Tyro3 is the serum protein, Protein S (15). Both Gas6 and Protein S bind phosphatidylserine on apoptotic cells and it is through this molecular bridging interaction that the TAM family members bind apoptotic cells(202).

In addition, to their role in phagocytosis of apoptotic cells, the TAM family members have also been shown to have a role in the innate immune response. Mice lacking functional Mertk (*mertk*<sup>-/-</sup>) are hypersensitive to Gram-negative bacterial LPS and are susceptible to endotoxic shock that is mediated by prolonged NF-κB activity resulting in over production of TNFα(35). This regulation of NF-κB by Mertk shown in macrophages has been corroborated in DC and the phagocytosis of apoptotic cells by DC appears to be important for self tolerance in a model of diabetes(36). Furthermore, knock-out mice lacking all three TAM family members (TAM<sup>-/-</sup>) mice also displayed an increase in serum TNFα levels following LPS injection(43) and DC from TAM<sup>-/-</sup> mice have been shown to have hyper-immune responses to TLR ligands(40). Interestingly Axl and Interferon receptor appear to cooperate to modulate STAT inhibiting the innate immune response to TLR mediated activation by the induction of SOCS1(40). However in these studies, the response to whole bacteria and importantly, phagocytosis of bacteria was not thoroughly examined.

Previous studies that have investigated the involvement of TAM receptors in phagocytosis of bacteria have provided conflicting results(28, 43). Macrophages isolated from *mertk*<sup>-/-</sup> mice were shown to phagocytize the Gram-positive bacterium *L. monocytogenes* similarly to wild-type(28). However, the TAM<sup>-/-</sup> macrophages displayed an increased ability to phagocytize Gram-negative *E. coli*(43).

Based on the importance of TLRs for phagocytosis, the ability of the Mertk to regulate LPS signaling, and the increase in phagocytosis of *E. coli* by the TAM<sup>-/-</sup> macrophages, we sought to determine whether Mertk regulated phagocytosis of the Gram-negative bacterium, *E. coli*. In this study we demonstrate, by multiple methods, that individually or collectively the TAM family members are not essential for phagocytosis of *E. coli* by thioglycollate-elicited macrophages. In addition, we show that individual TAM receptors are also not important for phagocytosis of bacteria by resident and bone marrow-derived macrophages. Complementary to the lack of a role in phagocytosis, we show that Mertk is not essential for killing of *E. coli* or modulation of growth of the intracellular pathogen *F. tularensis*.

## Materials and Methods

### *Animals*

Mice were housed in a specific pathogen-free facility and maintained according to the UNC-Chapel Hill Institutional Animal Use and Care Committee guidelines. *merTk*<sup>-/-</sup> mice were developed here at UNC. *merTk*<sup>-/-</sup> is the updated nomenclature which was described previously as *mer*<sup>kd</sup> or *merTk*<sup>kd</sup> (18, 28). Although the cytoplasmic kinase domain was targeted, the lack of protein expression makes these mice null for Mertk. *axl*<sup>-/-</sup>, and *tyro3*<sup>-/-</sup> mice were kindly provided by Dr. Stephen P. Goff (Columbia University, New York) and Dr. Greg Lemke (Salk Institute for Biological Studies, San Diego) respectively. TAM<sup>-/-</sup> mice were generated by breeding of backcrossed single knock-outs. All genotypes were backcrossed at least 6 generations to the C57BL/6J background. Male mice 8-12 weeks old were used in all studies. Wild-type mice used were strain C57BL/6J.

### *Macrophages*

Thioglycollate-elicited macrophages were obtained as previously described (28). Briefly, 3 mL of 3% thioglycollate was injected intraperitoneally (I.P.) 3 days prior to harvest by peritoneal wash in PBS. Cells were washed 3 times in PBS and allowed to adhere for 3 hours in media (RPMI 1640 (Gibco) supplemented with 5% fetal bovine serum, 1 mM sodium pyruvate, 2 x 10<sup>-5</sup> 2-ME, 10 mM HEPES, 50 units penicillin G and 50 µg/mL streptomycin sulphate). Non-adherent cells were washed off and fresh media was added. In order to eliminate activity due to stimulation by thioglycollate, macrophages were allowed to rest at 37°C 5% CO<sub>2</sub> for seven days prior to experimental use.

Resident peritoneal macrophages were obtained by 60 seconds of gentle peritoneal lavage in Versene. Cells were removed, washed and allowed to adhere onto glass coverslips in 24 well plates or plastic tissue culture dishes. Non-adherent cells were washed off which resulted in a highly enriched population of resident macrophages.

Bone marrow-derived macrophages were obtained by harvesting bone marrow from mouse femurs. Cells were flushed from femurs, washed and incubated overnight in DMEM-H (Gibco) supplemented with 50 units penicillin G, 50 µg/mL streptomycin sulphate and 10 mM HEPES. Non-adherent cells were removed and plated in media containing 10 ng/mL recombinant mouse M-CSF (PeproTech). Fresh media was added on day 4. Macrophages were harvested on day 8 and re-plated at appropriate density for experimental use on day 9. Bone marrow-derived macrophages were confirmed to be 100% CD11b-positive and 100% CD11c-negative by flow cytometry (data not shown).

#### *Phagocytosis Assays*

Phagocytosis assays were performed using a MOI of 10 *E. coli* O111:B4 (ATCC) transformed with green fluorescent protein plasmid (pEGFP; Clontech). Green fluorescent protein (GFP)-containing *E. coli* (*E. coli* O111:B4-GFP) were added to antibiotic-free, serum-free media and aliquoted into wells containing macrophages on glass coverslips. Macrophages were allowed to phagocytize bacteria for the indicated time periods. Macrophages were then washed 5 times in PBS and 0.2% trypan blue was added to wells to quench fluorescence from extracellular bacteria. Cells were then fixed in 2% paraformaldehyde and coverslips were mounted on slides with Vectamount mounting media for fluorescence (Vector). Phagocytosis was determined by overlays of bright field and GFP images captured using an Olympus fluorescent microscope

equipped with a DP70 digital camera and Image Pro Plus software (Opelco). At least 100 macrophages were counted per time point with triplicate samples per genotype.

Fluorimetric phagocytosis assays were also preformed. Briefly, cells were plated in 96 well plates and a MOI of 10 bacteria in serum-free and antibiotic-free media was added to each well. After indicated time periods, cells were washed as described above and trypan blue was added to quench any extracellular fluorescence. Plates were then read on a Fluoroskan Ascent FL fluorimeter (Thermo Labsystems) according to manufacturers instructions. *E. coli* and *S. aureus* Bioparticles used for fluorimetric assays were obtained from Molecular Probes. Normalized fluorescent intensity was calculated by obtaining a ratio of fluorescent cell tracker green-labeled wild-type macrophages to *mertk*<sup>-/-</sup> macrophages (measured in separate wells) and multiplying this ratio to the ingested bacterial bioparticle fluorescence.

In addition, phagocytosis assays were performed and analyzed by flow cytometry. For these experiments, cells were plated in 60 mM dishes and a MOI of 10 bacteria in serum-free, antibiotic-free media was added for indicated time periods. Cells were then washed and trypan blue was added as previously described. Cells were then fixed and analyzed using a BD FACScan flow cytometer.

#### *Apoptotic Cells*

Apoptotic cells were harvested as previously described (18). Briefly, thymi from 4-6 week old wild-type mice were harvested and tissues were mechanically dissociated using forceps. Cells were then washed and incubated in media containing 2  $\mu$ M Dexamethasone. After 5 hours, Cell tracker Orange (Molecular Probes) was added to thymocytes for 30 minutes according to manufacturer's instructions. Cells were then

washed in 1x PBS and then allowed to incubate in media for 30 minutes prior to experimentation.

#### *Bactericidal Assays*

Thioglycollate-elicited macrophages were plated and rested as described above. Cells were washed 3 times with PBS to remove media containing antibiotics. MOI of 10 *E. coli* O111:B4-GFP was added to wells. After 30 minutes 5 µg/mL gentamicin was added to each well to kill extracellular bacteria. After the allotted times, cells were harvested by scraping in PBS and plated in serial dilutions on LB agar containing ampicillin to select only *E. coli* O111:B4-GFP.

#### *Gentamicin protection assays*

All *Francisella tularensis* Live Vaccine Strain (LVS) gentamicin protection assays were performed under appropriate bio safety precautions. Approximately  $10^5$  bone marrow-derived macrophages were plated per well in a 96-well tissue culture dish. Bacteria were added at a multiplicity of infection of 10:1 or 100:1 in a volume of 100 µL per well. Supernatants were aspirated and replaced with media containing gentamicin (25 µg/mL) to kill extracellular bacteria at 4 hours post-inoculation. Six and 24 hours post-inoculation, cells were rinsed with 1x PBS and scraped from the wells with sterile wooden applicator sticks, vortexed, diluted, and plated onto chocolate agar for bacterial recovery. All gentamicin protection experiments were performed in triplicate and the mean and standard deviations were calculated.

#### *Statistics*

Statistics were performed using Graph Pad Prism Software. Where appropriate students t-test or 2-way ANOVA with Bonferroni's post-tests were performed. A p value of less than 0.05 was considered significant.

## Results

While a role for the TAM receptors in phagocytosis of apoptotic cells has been well documented(18, 28), their participation in phagocytosis of bacteria remains controversial. To determine if the TAM receptors play a role in the phagocytosis of bacteria, thioglycollate-elicited macrophages were harvested from wild-type, *merlk*<sup>-/-</sup>, *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup>, *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> double knock-outs and TAM<sup>-/-</sup> triple knock-out mice and exposed to *E. coli* O111:B4-GFP for one hour. No significant differences were found in terms of the number of cells that had phagocytized at least one bacterium (percent phagocytosis, Figure 2.1A). In contrast, macrophages from *merlk*<sup>-/-</sup> and *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> mice harvested at the same time were deficient in phagocytosis of apoptotic cells similar to previous publications, and documented for the first time, this deficiency was most apparent when all three receptors were lacking (Figure 2.1B). To determine whether one strain was phagocytizing more bacteria on a per cell basis, we also performed a phagocytic index. The phagocytic index was calculated as the total number of bacteria ingested by 100 cells. In corroboration of the percent phagocytosis results, the total number of bacteria ingested was similar between strains tested across the time course (Figure 2.1C). Thus, despite the importance of Mertk, and to a lesser extent Axl and Tyro3, in the phagocytosis of apoptotic cells, neither Mertk nor the other family members appear to play a prominent role in bacterial engulfment.

To determine whether Mertk could affect phagocytosis of *E. coli* at earlier time points, we performed a similar experiment using wild-type and *merlk*<sup>-/-</sup> thioglycollate-elicited macrophages and bone marrow-derived macrophages (BMMΦ) from 15 minutes to 1 hour. While phagocytosis proceeded in a time dependent manner for both types of



macrophages, there were no statistically significant differences between wild-type and *merck*<sup>-/-</sup> strains in percent phagocytosis (Figure 2.2A) or phagocytic index (Figure 2.2B). Interestingly, though independent of genotype, the thioglycollate-elicited macrophages were found to be significantly more active in phagocytosis of bacteria compared to BMMΦ by 2- way ANOVA in percent phagocytosis and phagocytic index (Figure 2.2A and B).

Since the macrophages used in the previous study were either thioglycollate-elicited or derived *in vitro* with cytokines, we wanted to confirm our results using resident peritoneal macrophages. Unlike BMMΦ, resident peritoneal macrophages phagocytize similarly to thioglycollate-elicited macrophages. However, there still remained no differences in phagocytosis of *E. coli* between wild-type and *merck*<sup>-/-</sup> macrophages. In addition to *merck*<sup>-/-</sup>, macrophages from *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> single knock-out mice also showed no statistical differences in percent phagocytosis when compared to wild-type resident macrophages (Figure 2.3).

To confirm that phagocytosis was indeed similar between wild-type and *merck*<sup>-/-</sup> macrophages, we also assessed the phagocytosis of *E. coli* O111:B4 GFP by thioglycollate-elicited macrophages using a flow cytometry-based approach. This approach allows for a large scale analysis of the amount of phagocytosis that is taking place. As the cells phagocytize bacteria, they accumulate GFP, become fluorescent and are therefore detectable by flow cytometry. As shown in Figure 2.4, the number of fluorescent thioglycollate-elicited macrophages increased rapidly with time; however, similar to our previous findings, wild-type, *merck*<sup>-/-</sup>, and TAM<sup>-/-</sup> macrophages showed similar numbers of cells containing fluorescence at all time points tested. In addition,

even at 120 minutes a few cells did not become fluorescent indicating that not all macrophages will phagocytize a bacterium (Figure 2.4).

*E. coli* O111:B4 is known to be an extracellular bacteria that deliberately prevents phagocytosis and forms pedestals on the gut epithelial cells which it normally encounters(203). To rule out a role for this process and lack of an effect by Mertk on phagocytosis of bacteria, we performed a phagocytosis assay using *E. coli* bioparticles. Bioparticles are chemically or heat-killed bacteria that are fluorescently labeled and thus easily detected by a fluorimeter. *E. coli* bioparticles were phagocytized similarly by both wild-type and *merck*<sup>-/-</sup> thioglycollate-elicited macrophages (Figure 2.5A). Thus, regardless of whether the *E. coli* were alive or dead; their phagocytosis was unimpaired in wild-type or *merck*<sup>-/-</sup> macrophages. Since components of Gram-positive and Gram-negative bacteria are recognized by different TLRs, we wanted to demonstrate that our results were not specific to Gram-negative bacteria. Therefore, we performed the same phagocytosis assay using Gram-positive *S. aureus* bioparticles. As with the *E. coli*, wild-type and *merck*<sup>-/-</sup> macrophages phagocytized *S. aureus* bioparticles equally (Figure 2.5B). Therefore, Mertk is not essential for phagocytosis of bacterial bioparticles regardless of Gram status.

Previous reports indicate *merck*<sup>-/-</sup> macrophages produce greater amounts of TNF $\alpha$  in response to *E. coli* LPS(35). Thus, we hypothesized that after phagocytosis these hyper-activated macrophages may be more efficient at bacterial killing than wild-type. To determine if the lack of Mertk affected the ability of the macrophages to kill bacteria, we performed a bactericidal assay using the naturally extracellular *E. coli* O111:B4 GFP used in previous experiments. As shown in Figure 2.6A, *merck*<sup>-/-</sup> macrophages ingested

and killed the bacteria equally to wild-type macrophages over a 6 hour period. Thus, Mertk does not inhibit or enhance the ability of the macrophage to kill this strain of bacteria.

We further examined whether the presence or absence of Mertk would affect the killing of intracellular pathogens that replicate in the cytoplasm. *Francisella tularensis* is a Gram-negative facultative bacterium that is able to escape the phagolysosome and replicates in the cytosol of the host cells such as macrophages(204). *F. tularensis* is able to down-regulate phagocytosis and TLR signaling and inhibits apoptosis of macrophages which appears to provide an advantage in propagation of the bacterium. As shown in Figure 2.6B and C, *F. tularensis* Live Vaccine Strain (LVS) had similar replication kinetics in *mertk*<sup>-/-</sup> and wild-type macrophages at two different MOIs. These data suggest that Mertk does not affect the ability of the macrophage to slow intracellular replication or kill bacteria.

## Discussion

Many receptors including CD14, scavenger receptor, integrins, complement receptors and Fc receptors recognize and participate in the phagocytosis of apoptotic cells as well as bacteria(72-74). The Tyro3, Axl, and Mertk (TAM) family of receptors has been shown to be important for the phagocytosis of apoptotic cells(18, 28). Previous studies have found conflicting evidence for the role of the TAM receptors in the phagocytosis of bacteria(28, 43). Our past findings suggested that the Mertk receptor did not play a critical role in the phagocytosis of the Gram-positive bacteria *L. monocytogenes* by macrophages(28). This study used live *L. monocytogenes* and this intracellular bacteria can invade host cells independent of phagocytosis(205). As such, it is possible that the results from this study were merely a consequence of the bacteria being equally able to invade cells in the presence or absence Mertk. A second study used only the TAM triple knock-out macrophages and examined phagocytosis of the Gram-negative bacteria *E. coli* (43). In this study, the investigators found that phagocytosis of bacteria was increased over 3-fold in the TAM<sup>-/-</sup> cells; however, these cells were derived from mice with a heterogeneous background of founder genes that may have influenced the phenotype.

In this report, we find that macrophages from *mertk*<sup>-/-</sup>, *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup>, *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> double knock-outs and TAM<sup>-/-</sup> triple knock-out mice on the C57BL/6 background ingest *E. coli* bacteria equally to wild-type macrophages (Figure 2.1, 2.2, 2.3, 2.4). Our current findings that TAM family members do not play a role in phagocytosis of *E. coli* is in contrast to a previously published report(43). Furthermore, we examined the phagocytosis of *S. Aureus* and found Mertk does not affect the engulfment of Gram-

positive bacteria, corroborating our past finding with *L. monocytogenes*(28).

Interestingly prior exposure of macrophages to apoptotic cells significantly reduced the phagocytosis of bacteria; however this process too was independent of the TAM family members as knock out macrophages also could not ingest bacteria after treatment with apoptotic cells (data not shown). It is plausible that apoptotic cells bind multiple receptors that share recognition of bacterial components such as complement receptors, Scavenger Receptor or CD14 and these could be acting as competitive ligands that prevent macrophages from recognizing the bacteria. Nonetheless, our results strongly suggest that *Mertk* and the family members, *Axl* and *Tyro3* are not essential for the engulfment of Gram-positive or Gram-negative bacteria. It is likely that other receptors present on macrophages that recognize microbial molecular components may compensate for the lack of TAM receptors.

We also examined the role of *Mertk* on three populations of macrophages in the event that different populations might use the TAM receptors preferentially. We show fewer bone marrow-derived macrophages (BMM $\Phi$ ) were phagocytic for bacteria compared to thioglycollate-elicited macrophages (Figure 2.2). Even though the thioglycollate-elicited macrophages were rested prior to phagocytosis for 7 days, the data suggests that they phagocytize in greater numbers than BMM $\Phi$ . For example, at the one hour time point, about 65% of wild-type thioglycollate-elicited macrophages had engulfed at least one bacterium as compared to only about 45% of the wild-type BMM $\Phi$  (Figure 2.2). Interestingly resident unstimulated macrophages ingest *E. coli* similar to thioglycollate macrophages (Figure 2.3). These results point to the possibility that different macrophage inducing agents produce different phagocytic activities among

macrophages. Those collected after thioglycollate exposure tend to be more phagocytic whereas BMMΦ may be more differentiated and less phagocytic.

Since *merlk*<sup>-/-</sup> macrophages produce elevated TNFα in response to LPS (8), we hypothesized that macrophages from these mice may be able to kill bacteria more efficiently. As we showed in Figure 2.6A, the lack of Mertk had no effect on the ability of the macrophage to kill the naturally extracellular pathogen *E. coli*. In addition, we examined a similar scenario using an intracellular pathogen, *F. tularensis* LVS. *F. tularensis* is Gram-negative bacterium that is able to escape the phagolysosome and replicate in the cytosol of macrophages (204). We examined the ability of the macrophages lacking Mertk to inhibit this replication. As with the *E. coli*, we saw no difference in the replication of *F. tularensis* LVS between the *merlk*<sup>-/-</sup> and wild-type macrophages (Figure 2.6B and C). Thus, Mertk also does not play an essential role in inhibition of replication or killing of bacteria.

In summary, while previously published data document a role for TAM receptors in response to bacterial by products, we demonstrate that Mertk, Axl, and Tyro3 appear to have no essential role in the phagocytosis of Gram-negative or Gram-positive bacteria or in the killing of extracellular or intracellular bacteria. This is in contrast to our previous understanding of TAM receptors in bacterial clearance.

## Acknowledgements

The authors would like to thank Stephen Goff for the *axl*<sup>-/-</sup> and Greg Lemke for the *tyro3*<sup>-/-</sup> mice. We are also grateful to Heather Seitz and Paul Gholke for technical

assistance and Andrea Portbury, Lorelei Taylor, and Kasturi Puranam for critical review of the manuscript.

**Figure 2.1.** *TAM receptors are not required for the phagocytosis of E. coli O111:B4 GFP by macrophages.* A, Phagocytosis assay using MOI of 10 *E. coli* O111:B4 GFP by thioglycollate-elicited macrophages from wild-type (WT), *merck<sup>-/-</sup>* (*M<sup>-/-</sup>*), *axl<sup>-/-</sup>* (*A<sup>-/-</sup>*), and *tyro3<sup>-/-</sup>* (*T<sup>-/-</sup>*), *axl<sup>-/-</sup>/tyro3<sup>-/-</sup>* (*AT<sup>-/-</sup>*), and TAM<sup>-/-</sup> mice. B, *In vitro* phagocytosis assay of apoptotic cells 10:1 to thioglycollate-elicited macrophages from wild-type (WT), *merck<sup>-/-</sup>* (*M<sup>-/-</sup>*), *axl<sup>-/-</sup>/tyro3<sup>-/-</sup>* (*AT<sup>-/-</sup>*), and TAM<sup>-/-</sup> mice. C, Phagocytic index calculated as number of bacteria phagocytized by 100 cells from samples in 1A. Figures are mean and standard error, n=3 and representative of at least 2 separate experiments. \* p<0.01 by students t test vs. wild-type. A and C are not statistically significant by one-way ANOVA or post test.

**Figure 2.2.** *Phagocytosis of E. coli O111:B4 is time dependent and Mertk independent in thioglycollate-elicited and bone marrow-derived macrophages*

A, *In vitro* phagocytosis assay using a MOI of 10 *E. coli* O111:B4 GFP by thioglycollate and BMMΦ from wild-type and *merck<sup>-/-</sup>* mice, over a time course of 15 to 60 minutes. B, Phagocytic index was calculated as number of bacteria phagocytized by 100 cells from samples in A. Figures are mean and standard error, n=3 and representative of at least 2 separate experiments. No statistically significant differences were found between genotypes of either cell type by 2 way ANOVA for both panel A and panel B. Statistically significant differences were found between BMMΦ and thioglycollate-elicited macrophages by 2 way ANOVA in both genotypes p<0.0001 for 2. 2A. Bonferroni's post tests also show significance between wild type BMMΦ and thioglycollate-elicited macrophages at 30, 45, and 60 minutes p<0.01 for figure 2.2A.



Statistical significance was also found by Bonferroni's post test between *merlk*<sup>-/-</sup> BMMΦ and thioglycollate-elicited macrophages at 30 and 60 mins p<0.05 and 0.001 respectively for Figure 2.2A. For Figure 2.2B statistically significant differences were found between BMMΦ and thioglycollate-elicited macrophages by 2-way ANOVA in wild-type and *merlk*<sup>-/-</sup> p<0.0001, p<0.05 respectively. Post tests indicate significance at the 45 and 60 minute time points for wild-type, p<0.001.

**Figure 2.3** *Phagocytosis of E. coli O111:B4 is similar in thioglycollate and resident peritoneal macrophages. In vitro* phagocytosis assay of MOI of 10 *E. coli* O111:B4 GFP and thioglycollate (□) and resident peritoneal (■) macrophages from wild-type (WT), *merlk*<sup>-/-</sup> (M<sup>-/-</sup>), *axl*<sup>-/-</sup> (A<sup>-/-</sup>), and *tyro3*<sup>-/-</sup> (T<sup>-/-</sup>). Figures are mean and standard error, n=3 and representative of at least 2 separate experiments. No statistically significant differences found between genotypes or cell types by one-way ANOVA.

**Figure 2.4.** *In vitro* phagocytosis assay by flow cytometry. A time course of 30 to 120 minutes was used to examine the phagocytosis of MOI of 10 *E. coli* O111:B4 GFP by wild-type, *merlk*<sup>-/-</sup>, and TAM<sup>-/-</sup> thioglycollate-elicited macrophages. Data is representative of 2 separate experiments

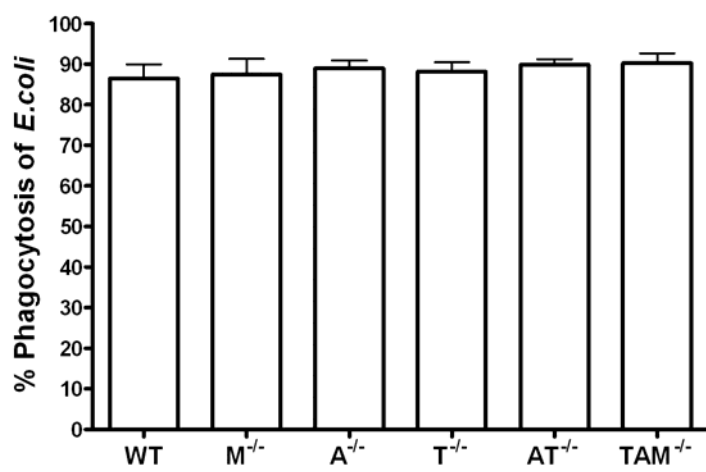
**Figure 2.5.** *Lack of affect in merlk*<sup>-/-</sup> macrophages is not dependent on Gram status. *In vitro* phagocytosis assay of *E. coli* (A) and *S. aureus* (B) bioparticles at a ratio of 10:1 by wild-type (□) and *merlk*<sup>-/-</sup> (■) thioglycollate-elicited macrophages. Bioparticles were incubated over a time course of 15-120 minutes. Groups at each time point are mean and

standard error of n=4, and is representative of 3 experiments. No statistically significant differences were found between genotypes by 2-way ANOVA.

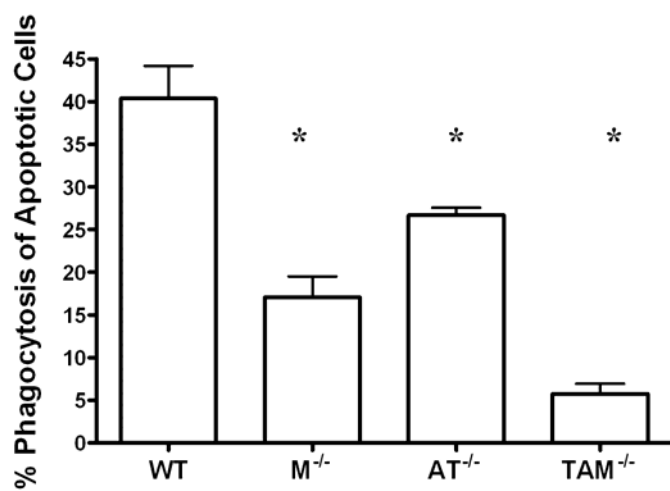
**Figure 2.6.** *Killing and replication of bacteria are not Mertk dependent.* A, *In vitro* bactericidal assay of *E. coli* O111:B4 GFP by thioglycollate-elicited wild-type (■) and *mertk*<sup>-/-</sup> (▲) macrophages was conducted over a 6 hour time course and at a MOI of 10:1. B and C, *In vitro* gentamicin protection assay was used to examine the killing of the intracellular pathogen *F. tularensis* at a MOI of 10 (B) or MOI of 100 (C) by wild-type (□) and *mertk*<sup>-/-</sup> (■) BMMΦ. Each bar is the mean and standard deviation of triplicate wells, n=3 and is representative of 3 separate experiments. No statistically significant differences were between genotypes found by 2-way ANOVA.

**Figure 2.1**

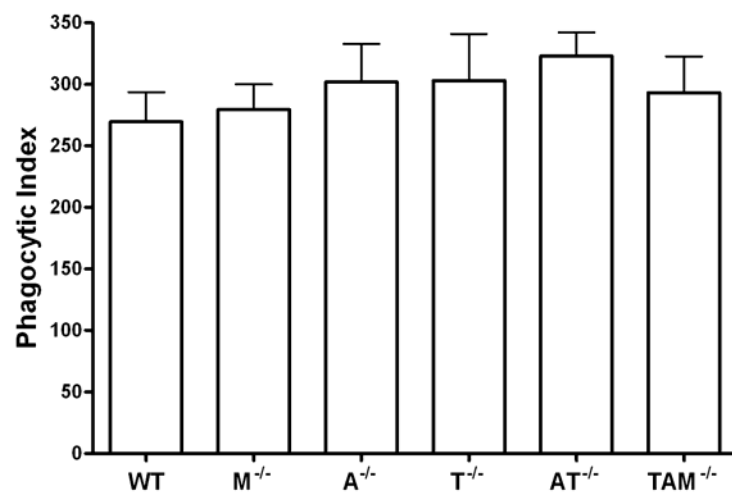
**A**



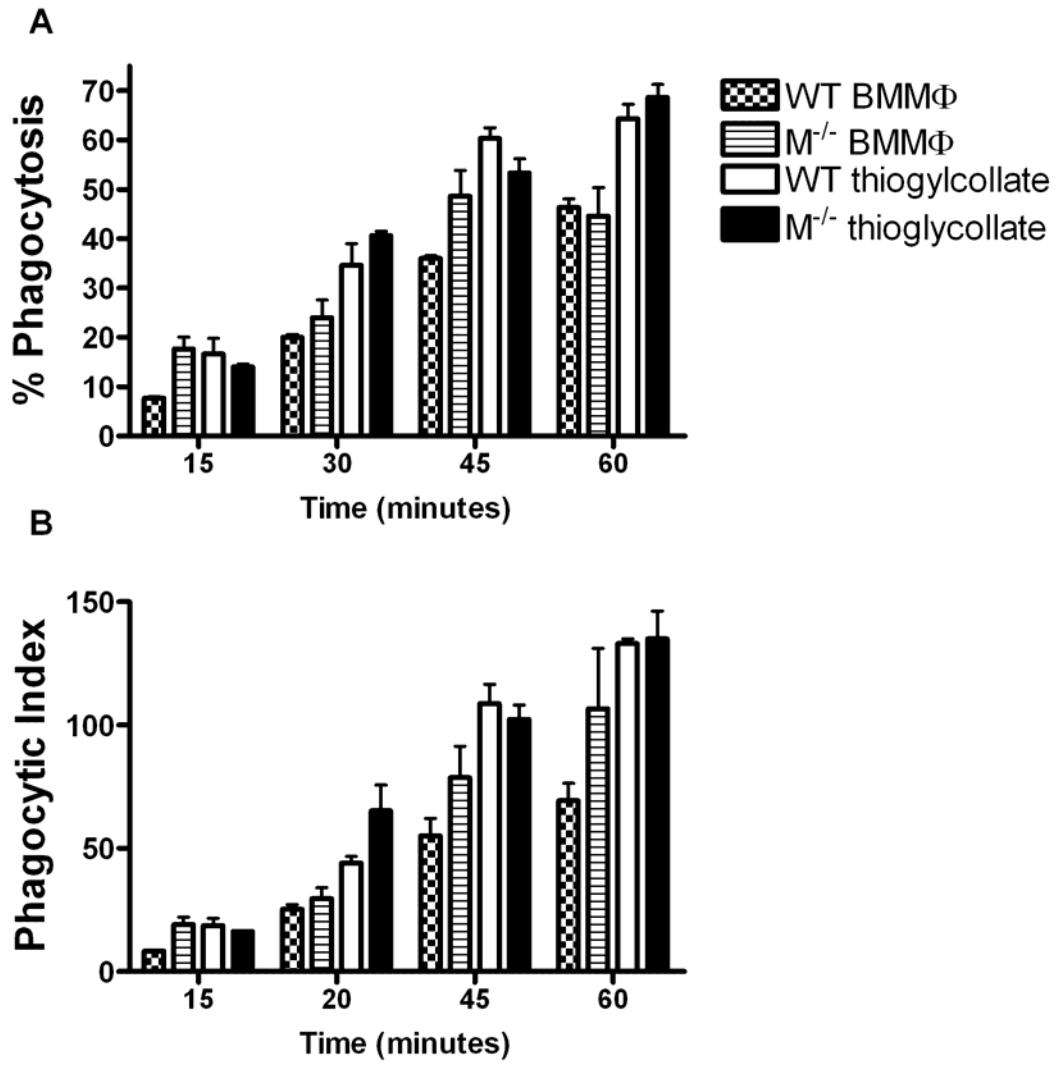
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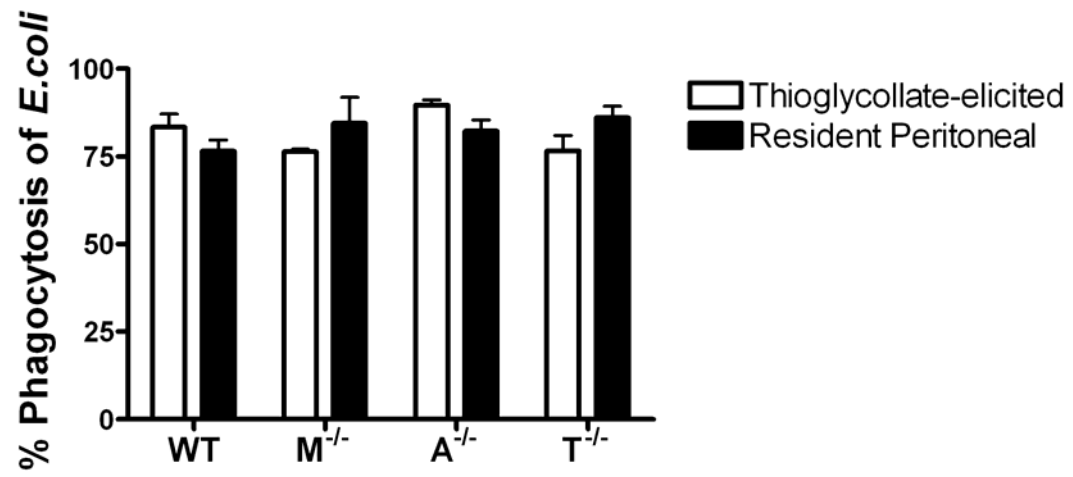
**C**



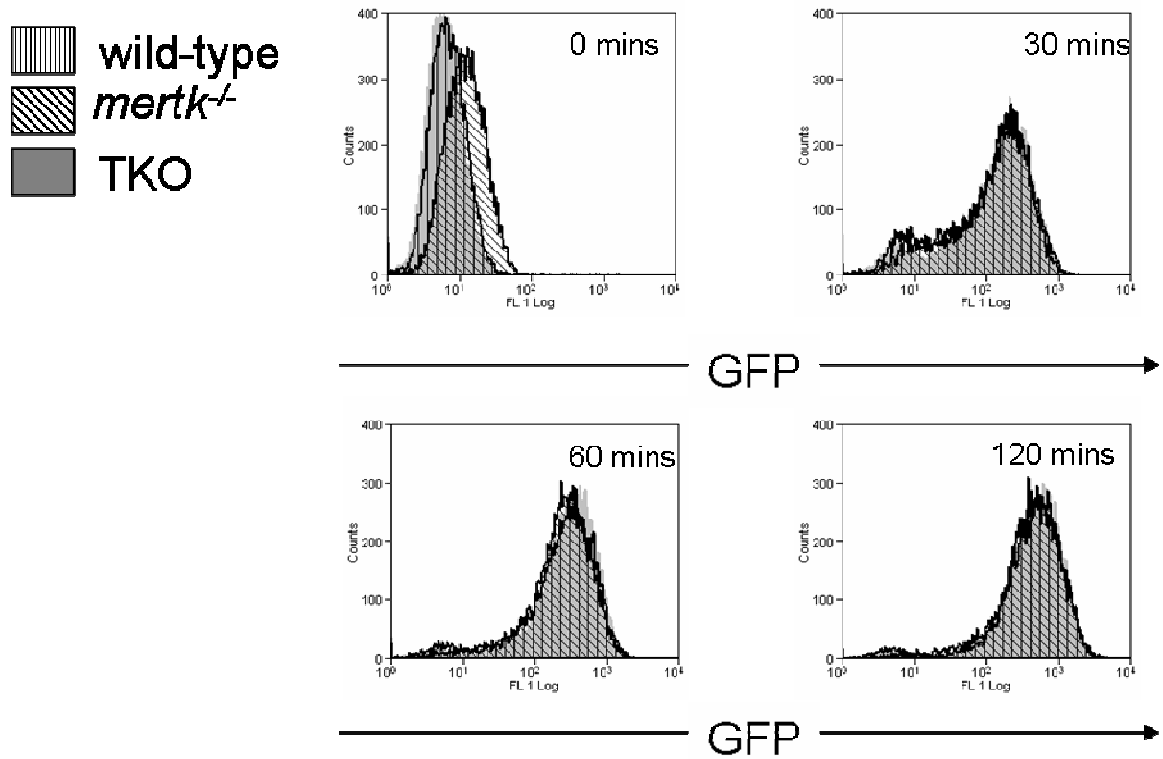
**Figure 2.2**



**Figure 2.3**

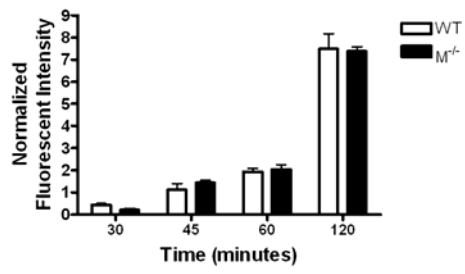


**Figure 2.4**

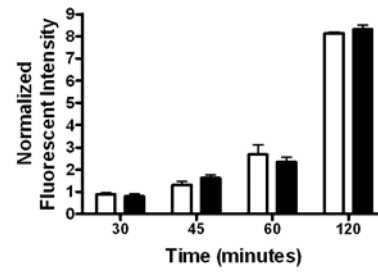


# Figure 2.5

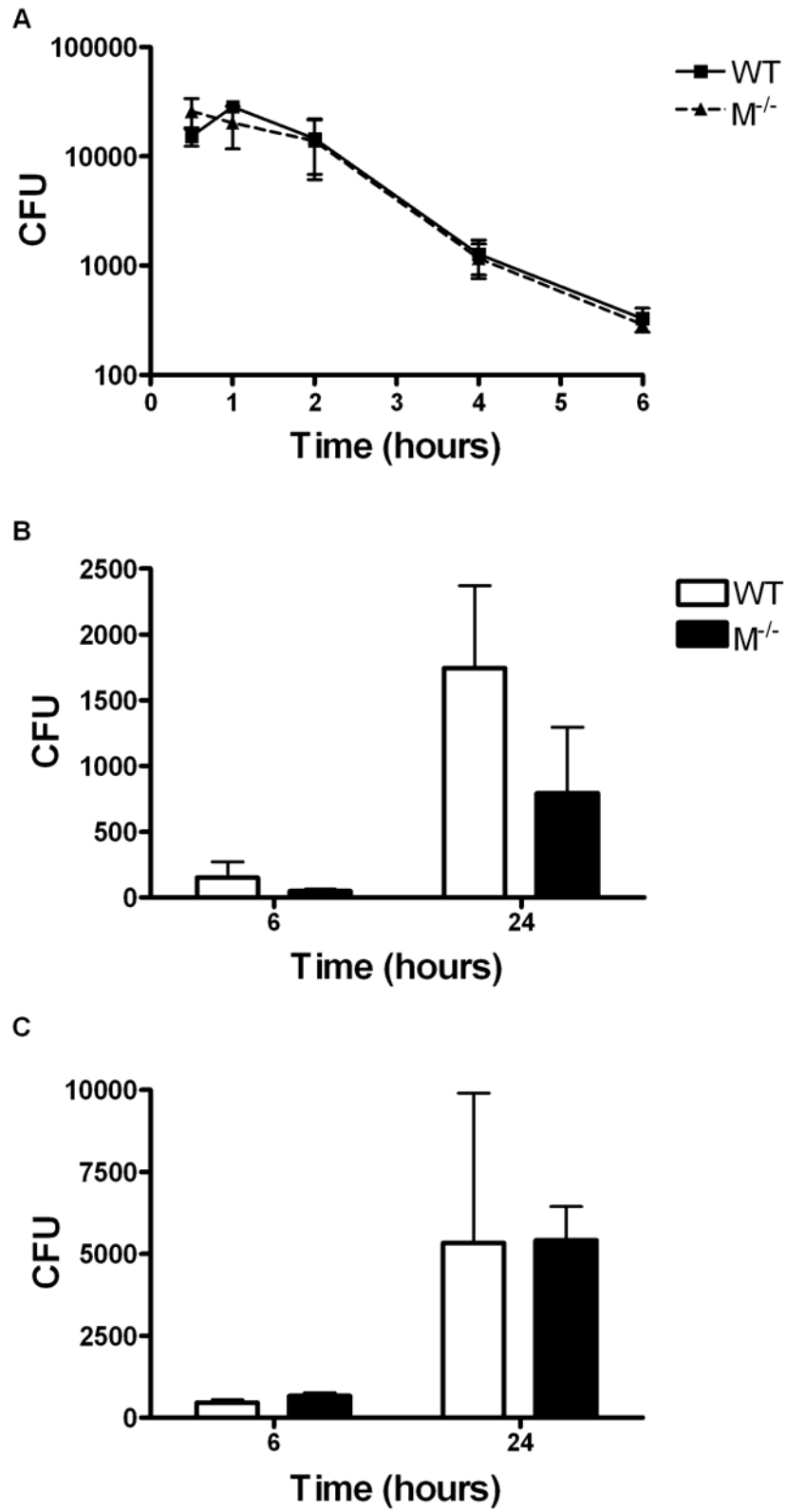
A



B



**Figure 2.6**





## **Chapter 3:**

### **Increased hematopoietic cells in the *merlk*<sup>-/-</sup> mouse peritoneal cavity: a result of augmented migration**

Submitted as: Increased hematopoietic cells in the *merlk*<sup>-/-</sup> mouse peritoneal cavity: a  
result of augmented migration. Williams, J.C., Wagner, N.J., Vilen, B.J., Matsushima,  
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## Abstract

The peritoneal cavity is recognized as an important site for autoreactive B cells prior to their transit to other immune tissues; however, little is known of the genes that may regulate this process. Mice lacking the receptor tyrosine kinase *Mertk* display a lupus-like autoimmune phenotype with splenomegaly and high autoantibodies titers. Here, we investigate whether *Mertk* regulates the composition of peritoneal cells that favor an autoimmune phenotype. We found an increase in the number of macrophages, DC, plasmacytoid DC, T cells and B cells in the peritoneal cavity of *mertk*<sup>-/-</sup> mice when compared to wild type mice. This disparity in cell numbers was not due to changes in cell proliferation or cell death. In adoptive transfer experiments, we showed an increase in migration of labeled donor cells into the *mertk*<sup>-/-</sup> peritoneal cavity. In addition, bone marrow chimeric mice showed hematopoietic-derived factors were also critical for T cell migration. Consistent with this migration and the increase in the number of cells, we identified elevated expression of CXCL9, its receptor CXCR3, and IL-7 receptor on peritoneal cells from *mertk*<sup>-/-</sup> mice. This control of peritoneal cells numbers correlated with autoantibody production and was exclusively attributed to *Mertk* since mice lacking other family members, *Axl* or *Tyro 3*, did not display dysregulation in peritoneal cell numbers or the autoimmune phenotype.

## Introduction

The peritoneal cavity is a site of leukocyte colonization in which the development and regulation of immune cells is poorly understood. The peritoneal cavity is similar to a lymph node in that some cells reside in the cavity while others are transient, passing through enroute to other targeted tissues. Immune cells found in the peritoneal cavity include, macrophages, T cells, B cells, NK cells, mast cells and dendritic cells (DC)(206, 207). This heterogeneous population of cells is involved in both innate and adaptive immunity. While macrophages may be the predominant cell type of the peritoneal cavity, the B1-B cell is the largest lymphocyte population in this location. Peritoneal B1-B cells are the primary secretor of serum IgM, which targets pathogen associated molecular patterns (PAMPs)(167, 208). B1-B cells can also produce low affinity autoantibody and their development and regulation has been the focus of autoimmune disease models (167, 209). Increases in B1-B cells are found in human autoimmune diseases as well as the NZB/NZW mouse model of lupus (167, 210).

More recently, an additional murine model of autoantibody production and lupus is found in the *merlk*<sup>-/-</sup> mice. Mice lacking Mertk as well as mice lacking all three receptor family members, Tyro3, Axl, and Mertk, (TAM<sup>-/-</sup>) display a lupus-like autoimmune phenotype characterized by splenomegaly and dysregulated lymphocyte activation as well as high auto-antibody titers to dsDNA, ssDNA, nucleosomes, chromatin and Smith antigen (Sm) (28, 29, 37, 43). Also, 2-12H/*merlk*<sup>-/-</sup> transgenic mice display increased numbers of peritoneal B1-B cells as compared to 2-12H transgenic mice with wild-type Mertk (172). Furthermore, Sm-specific B cells injected into the peritoneal cavity of *merlk*<sup>-/-</sup> mice become activated, and are selectively lost. This loss is

most likely due to differentiation and migration to the lamina propria and mesenteric lymph node (172). Lastly, ablation of peritoneal B-1 cells abrogated autoantibodies in autoimmune-prone mice (168). These studies suggest the peritoneal cavity is an important transit site as autoreactive B cells migrate through prior to entering terminal immune sites.

Mertk is a receptor tyrosine kinase found on macrophages, DCs, and natural killer cells as well as other non-hematopoietic cells, however T and B lymphocytes lack expression under normal conditions (1, 7, 23, 201). Mertk is a member of the Tyro3/Axl/Mertk (TAM) family of receptors. This family of receptors is characterized by an extracellular domain containing two Ig like and two fibronectin III like motifs and by an intracellular kinase domain with a characteristic KWIAIES motif (7, 8). Gas6 is a ligand for all three family members with a relative binding affinity of Tyro3>Axl>>Mertk (20). Gas6 is produced by macrophages and can also be found pre-bound to apoptotic thymocytes (18, 19). In addition to Gas6, Protein S, has also been identified as a ligand for this family (15, 18). These ligands bind phosphatidylserine which is exposed on the outer-membrane of apoptotic cells and provide a molecular bridge to the TAM family members. This subsequently triggers the phagocytosis of apoptotic cells by macrophages and dendritic cells, presumably through integrin-mediated, Rac1-activated signaling (18, 28, 32).

In addition to a defect in phagocytosis of apoptotic cells, macrophages and DCs lacking Mertk are hypersensitive to LPS stimulation, resulting in hyper-activation of NF- $\kappa$ B and subsequent over-production of TNF- $\alpha$  (35, 36, 43). Recently, Rothlin et. al. presented a mechanism by which the TAM family members cooperate with the type I

IFN receptor to induce expression of the suppressor of cytokine signaling 1(SOCS1), which regulates TLR signaling and cytokine production (40). The TNF- $\alpha$  family member BAFF is also produced in greater amounts by DCs lacking Mertk or all three TAM receptors (37, 40). This is of particular interest to the autoimmune phenotype of the *mertk*<sup>-/-</sup> mice as excess BAFF can lead to an increased number of B cells and a propensity for autoimmune disease (211-213). However, DC from *mertk*<sup>-/-</sup> mice expressing high levels of BAFF did not increase survival of B cells, indicating other factors may be contributing (37).

There has been much speculation as to the reason for the autoimmune phenotype of mice lacking the TAM receptors. It is presumed that the lack of ability to phagocytize apoptotic cells results in higher availability of self-antigen and greater priming of autoreactive B cells. However, as in humans, it appears that the autoimmunity in these animals is more likely a multi-factorial development of molecular and cellular events that lead to a break in tolerance.

In this report, we hypothesized that the cell populations in the peritoneal cavity maybe different in the autoimmune prone *mertk*<sup>-/-</sup> mice. We characterized peritoneal exudate cells (PEC) in both wild-type and *mertk*<sup>-/-</sup> mice, and found an increase in all immune cell types in *mertk*<sup>-/-</sup> mice. We also characterize the activation status of the B cell population as well as the subpopulations of the T cells. We determined that the increase in cells was not due to aberrant division or defects in apoptosis, but rather an increased propensity for cells to migrate to the peritoneal cavity. We also determined that this migration was independent of cellular development in the *mertk*<sup>-/-</sup> mouse and show that neither hematopoietic nor non-hematopoietic factors exclusively contribute to

this recruitment/migration. However, T cells are recruited by hematopoietic factors. We identify one chemokine and two receptors that are elevated in the *mertk*<sup>-/-</sup> peritoneal cells and they may contribute to increases in the migration of cells. Finally, we show that mice lacking Mertk family members, *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup> and *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> showed no differences in resident peritoneal cell numbers compared to wild-type. Correspondingly, splenomegaly and autoantibody production were found only in *mertk*<sup>-/-</sup> mice and not other mutant mice.

## Materials and Methods

### *Animals*

All animals were housed in Specific Pathogen Free facilities according to the guidelines of the UNC-Chapel Hill Institutional Animal Use and Care Committee. *mer*<sup>kd</sup> (also known as *mer*<sup>kd</sup> or *mer*<sup>kd</sup> mice) were generated as previously described(35). *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> were generously provided by Dr. Stephen P. Goff (Columbia U., New York) and Dr. Greg Lemke (Salk Institute for Biological Studies, San Diego) respectively. *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> mice were bred by crossing *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> mice. All animals are backcrossed at least 6 generations to C57BL/6J which serves as wild-type in all experiments. Three month old male mice were used unless otherwise indicated. Actin-green fluorescent protein (GFP) C57BL/6 transgenic mice were purchased from Jackson Labs.

### *Resident Peritoneal Exudate Cells (PEC)*

Mice at indicated ages were euthanized with isofluorane. Mice were injected i.p. with 3mL of Versene, after 60 seconds of peritoneal massage cells were harvested. Cells were washed three times with 1X PBS prior to experimentation. Total number of cells was counted using a hemocytometer and Trypan Blue.

### *Flow Cytometry*

Before antibody labeling, cells were incubated with Fc Block (anti-CD16/CD32) from BD biosciences. Cells were then stained for surface expression using the following antibodies, anti-CD19-PE-Cy5, anti-CD5-PE, anti-B220-PE, anti-CD11b-PE-Cy5, anti-PDCA-1-FITC, anti-CD44-APC, anti-CD62L-PE, and anti-IL7R-PE antibodies are from ebioscience. Anti-CD8-PE, anti-CD11b-FITC, and anti-CD4-FITC are from Caltag.

Anti-CD11c-PE or PE-Cy7, anti-CD3-FITC, and anti-I-A<sup>b</sup>-FITC are from BD/Pharmingen. Anti-F480-PE-Cy5 was obtained from Serotec. Anti-CXCR3 antibody was obtained from Zymed. Secondary antibody to detect primary anti-CXCR3 antibody was anti-Rabbit-Alexa 405 obtained from Invitrogen. All washes and staining were done with 2% FCS in PBS and samples were analyzed using a Dako-Cyan and Summit 4.3 software. At least 15,000 cells were analyzed from each sample. Total number was calculated using percent cells positive for each stain.

#### *Migration to Peritoneal Cavity*

Resident PECs were obtained as described previously. Cells were washed with PBS and stained with Cell-Tracker green (Molecular Probes) according to manufacturers instructions. Three million cells were injected in 0.5mL 1X PBS via tail vein of naïve recipient mice. 24 hours after injection, cells were harvested from the peritoneal cavity using Versene to lavage out cells. PECs were analyzed by flow cytometry to identify percent of Cell Tracker green-positive cells in the collected. Number of cells migrated was then calculated by multiplying the percent Cell Tracker green-positive cells by total number harvested.

#### *Bone Marrow Transplant Chimeras*

Recipient male mice at 4 weeks of age were lethally irradiated with 800 rads using either <sup>137</sup>Cs gamma irradiator or X-ray irradiator. Twenty-four hours post irradiation, bone marrow cells were obtained from femurs and tibia of donor mice. Bone marrow cells were treated with red blood cell lysis buffer to remove red cells and  $8 \times 10^6$  white cells were injected i.v. into irradiated recipient mice. Each irradiation experiment contained at least one actin-driven eGFP control donor mouse to assess hematopoietic



reconstitution of chimeric mice. Percent reconstitution of chimeric mice at three months of age was assessed by quantifying the % GFP<sup>+</sup> cells from the bone marrow and the peritoneum using flow cytometry. Reconstitution of chimeric mice with GFP<sup>+</sup> cells was approximately 90% as expected.

#### *BrdU Injection and Staining*

Mice at the indicated ages were injected 24 and 48 hrs prior to harvest with 0.75mL of 10mg/mL BrdU (Sigma) in 0.9% NaCl to quantify proliferating cells. Cells were harvested and identified by staining with antibodies against cell surface markers. Cells were then permabilized using Cytofix/Cytoperm buffer system (BD) and DNase treated to expose the BrdU. BrdU incorporation was visualized by using anti-BrdU-FITC antibody from BD. Cells were analyzed by flow cytometry using a Dako-Cyan and Summit 4.3 software.

#### *Cell Death analysis*

Resident PECs were harvested as described. Apoptosis was detected using TACS AnnexinV-FITC/PI Apoptosis Detection Kit (Trevigen) according to manufacturer's instructions. For VAD-FMK staining, VAD-FMK-FITC (Promega) was diluted 1:1000 in PBS and 0.5mL was injected intraperitoneally 1 hour prior to harvest of resident PEC. Both assays were analyzed by flow cytometry using a Dako-Cyan and Summit 4.3 software.

#### *Real-Time PCR*

Resident PEC from at least 4 mice of each genotype were pooled and RNA was purified according to manufacturer's instructions using the RNeasy Mini Plus Kit (Qiagen). RNA was quantified and equal amounts were converted to cDNA using

Superscript II reverse transcriptase (Invitrogen) using random hexamer primers. Equal amounts of cDNA were used for the Real-Time PCR reaction in 2x Sybr Green buffer (Applied Biosystems). Reactions were conducted in 96 well plates and were performed on the ABI 7500 Realtime PCR machine in the UNC Neuroscience Center Expression Profiling and SNP Genotyping Core Facility. Primers were designed using FASTA sequences and primer express software3.0 (Applied Biosystems). GAPDH was used as the internal control. Primers set used are as follows: GAPDH, Forward TGTGTCCGTCGTGGATCTGA, Reverse CCTGCTTCACCACCTTCTTGA IL-5, Forward AGCACAGTGGTGAAAGAGACCTT, Reverse CATCGTCTCATTGCTTGTCACA. IL-6, Forward CCACGGCCTTCCCTACTTC, Reverse TTGGGAGTGGTATCCTCTGTGA. IL-7, Forward GCCATGCCTTCTTGCTTCTC, Reverse AGCTATCTGTACCACTGAGTAGAGCATT. IL-9, Forward TTGCCTCTGTTTTGCTCTTCAG, Reverse CCCCATGTGGTGCTGCAT. IL-10, Forward GATGCCCCAGGCAGAGAA, Reverse CACCCAGGGAATTCAAATGC. BAFF, Forward CCCAAAACACTGCCCAACA, Reverse CTCATCTCCTTCTTCCAGCCTC. CXCL9, Forward AAGTCGTCGTCGTTCAAGGAA, Reverse AAGATTCAGGGTGCTTGTTGGT. CXCL13, Forward CCATCATGAGGTGGTGCAAAG, Reverse GGACTGTTTCCCCCAGGAA. CXCL14, Forward GGCCCAAGATCCGCTACA, Reverse CAGGCATTGTACCACTTGATGAAG. LFA, Forward CCTGAGGGTGGGCTGGAT,

Reverse GCCAATTCCTCCGGACAT. PECAM, Forward  
TGACCCAGCAACATTCACAGAT, Reverse CCGCTCTGCACTGGTATTCC. L-  
Selectin, Forward TGACGCCTGTCACAAACGA, Reverse  
GGCTGGCAAGAGGCTGTGT. CXCR5, Forward CCTTCGCTGGCGTAAAGTTC,  
Reverse CAGCCCAGCTTGGTCAGAA. CXCR3, Forward  
TCGGACTTTGCCTTTCTTCTG, Reverse CTCTCGTTTTCCCATAATCGT. IL-7  
Receptor, Forward GCCGCTGTACACAGTGCAA, Reverse  
AACTGTTTCTGGTGGGCTGACT.

#### *Anti-nucleosome ELISA*

ELISA plates (Dynatech) coated with 100 $\mu$ L of 40 $\mu$ g/mL histones  
(Immunovision) were incubated overnight at 4°C. Plates were then washed with boric-  
acid buffered saline (BBS) and wells were coated with 100 $\mu$ L of 10 $\mu$ g/mL dsDNA  
(Sigma) in BBS with 0.5%BSA and 0.4% Tween for 3 hrs at room temperature. Wells  
were then washed with BBS. Samples and standards were loaded and incubated  
overnight at 4°C. Samples were detected with anti-mouse Ig-alkaline phosphatase and  
developed with p-nitrophenyl phosphate (Sigma). Wells were read at 405 nM.

#### *Statistical analysis*

Statistical analysis was performed using Graph Pad Prism software. Statistical  
tests used were one way or two-way ANOVA with Bonferroni's post-tests, or Students *t*-  
test. The appropriate test was applied to each data set, and the particular test used is  
indicated in the figure legends.

## Results

In *merlk*<sup>-/-</sup> mice, there is an age-related increase in the production of auto-antibodies. In addition, activated B1-B cells are known to migrate more quickly out of the *merlk*<sup>-/-</sup> peritoneal cavity to become antibody secreting cells in the lamina propria and mesenteric lymph nodes (28, 29, 37, 43, 172). As cells from the peritoneal cavity are important for autoantibody production in other autoimmune mouse models (168), we first assessed whether the absence of *Merk* affected the overall resident peritoneal cell population. We harvested cells from the peritoneal cavity, and found an increase in the total peritoneal cells (PEC) found in the *merlk*<sup>-/-</sup> mice as compared to wild-type mice (Figure 3.1A) at 1.5, 3, and 6 months of age. Flow cytometry identified the different cell types as F4/80<sup>+</sup> macrophages, CD19<sup>+</sup> B cells, and CD3<sup>+</sup> T cells which were all found to have statistically significant increases when comparing the *merlk*<sup>-/-</sup> mice to wild-type mice across a time course of 1.5 to 6 months of age (Figure 3.1B, C, D).

Because the increase in immune cells occurs most abundantly at 3 months of age, our subsequent experiments focus on that time point. Our previous analysis of the spleen found an increase in the number of CD11c<sup>+</sup> DC (37). We examined whether there is a similar increase in these cells in the peritoneal cavity of the *merlk*<sup>-/-</sup> mice. Indeed, we did find a statistically significant increase in the number of CD11c<sup>+</sup> DC found in the peritoneal cavity of the *merlk*<sup>-/-</sup> mice (Figure 3.2A). Similarly, the number of plasmacytoid DCs, as identified by positive staining for CD11c, B220, and PDCA1, were also statistically increased in the peritoneum of *merlk*<sup>-/-</sup> mice as compared to wild-type mice (Figure 3.2B). The abundance of these cell types suggests the environment is conducive for the activation of the B1-B cells.

The increase in peritoneal lymphocyte populations shown in Figure 3.1 is particularly interesting because *Mertk* is not known to be expressed on either of the cell types under normal conditions. In addition, the increase in these lymphoid cell types was not found in other lymphatic organs (37). Therefore, we characterized these lymphocytes further to determine whether one subpopulation could account for the increase in total number of cells. As shown in Figure 3.2C, both CD4<sup>+</sup> and CD8<sup>+</sup> T cells were present in higher numbers in the peritoneal cavity of *mertk*<sup>-/-</sup> mice compared wild-type mice. We then used CD44 and CD62L staining to determine the activation state of the T cells from the peritoneal cavity of both wild-type and *mertk*<sup>-/-</sup> mice. We found that there were statistically significant increases in effector (CD62L<sup>lo</sup>, CD44<sup>hi</sup>), naive (CD62L<sup>hi</sup>, CD44<sup>lo</sup>), and central memory T (CD62L<sup>hi</sup>, CD44<sup>hi</sup>) cells. The largest increase was in the naive T cell population (Figure 3.2D). Thus, the absence of *Mertk* prevents proper regulation of the T cell population within the peritoneal cavity.

We next analyzed the B cell populations which were also expanded in the *mertk*<sup>-/-</sup> mice (Figure 3.1C). Since the peritoneal cavity is known to contain a large number of B1-B cells, we used CD19, CD11b, and CD5 staining to analyze the B1a (CD19<sup>+</sup>, CD11b<sup>+</sup>, CD5<sup>+</sup>) and B1b (CD19<sup>+</sup>, CD11b<sup>+</sup>, CD5<sup>-</sup>) subsets of the B1 (Cd19<sup>+</sup>, CD11b<sup>+</sup>) cells. As shown in Figure 3.3A, we found a statistically significant three-fold increase in the overall B1 population. Furthermore, in the absence of *Mertk*, there were over a four-fold increase in the number of B1b cells and about a three-fold increase in the B1a cell population. Thus, the increase in B1-B cells was due to an increase in both populations with a greater fold increase in B1b B cells. The number of CD19<sup>+</sup> Cd11b<sup>-</sup> B2 cells was negligible in comparison to the number of B1 cells; nonetheless, there was an increase of

B2 cells in *merlk*<sup>-/-</sup> mice compared to wild-type mice (Data not shown). Furthermore, we wanted to determine whether the absence of Mertk affected the activation of the B cell population in the peritoneal cavity. Using the activation markers, MHC II, CD80, and CD86, we found that there were statistically significant decreases in the mean fluorescent intensity of MHC II and CD80 on CD19<sup>+</sup> cells from *merlk*<sup>-/-</sup> mice (Figure 3.3B, C, D, E). Thus, while there was an increase in the number of B cells in the peritoneal cavity of *merlk*<sup>-/-</sup> mice, the B cells were less activated than those of wild-type mice.

We hypothesized three reasons that could account for the increase in the number of cells in the peritoneal cavity of the *merlk*<sup>-/-</sup> mice. These included an increase in cell division, a decrease in cell death, and an increase in migration of cells into the peritoneal cavity of the *merlk*<sup>-/-</sup> mice. We first used BrdU incorporation to delineate cells undergoing proliferation within the peritoneal cavity of the *merlk*<sup>-/-</sup> mice. As shown in Figure 3.4A, an overall statistically significant decrease in the percent of dividing cells is evident when comparing cells collected from *merlk*<sup>-/-</sup> to wild-type mice at three different ages. However, the total number of cells dividing was not different when comparing the genotypes (Figure 3.4B). To assess whether excessive division was occurring elsewhere, we examined the bone marrow and found that there was no difference in cell division in the bone marrow (Figure 3.4C). While the spleen is a location for division of lymphocytes and leukocytes upon infection, we observed very few dividing cells in the spleen of both genotypes of mice under unstimulated conditions (unpublished observations). Thus, the large number of cells in the peritoneal cavity of *merlk*<sup>-/-</sup> mice was not due to local proliferation.

We next examined the possibility that the large number of cells in the peritoneal cavity of *mer1k*<sup>-/-</sup> mice was attributed to decreased in cell death. We used several methods for measuring cell death at different stages, to provide a comprehensive profile. We first used VAD-FMK to analyze the number and percent of cells that were undergoing apoptosis. VAD-FMK-FITC binds to active caspase 3, a key component for the induction of apoptosis. We found the percent of cells that were VAD-FMK-FITC-positive were not statistically different (Figure 3.5A). However, while not reaching statistical significance, we found a trend toward an increase in the number of VAD-FMK<sup>+</sup> cells in the peritoneal cavity of *mer1k*<sup>-/-</sup> mice (Figure 3.5B). In addition to VAD-FMK-FITC staining, we also used Annexin V and PI staining to identify cells that were undergoing early apoptosis (Annexin V<sup>+</sup>) as well as those cells that were already in late apoptosis (PI<sup>+</sup>). We found no statistically significant differences in the percentage of cells that were undergoing apoptosis via Annexin V/PI staining (Figure 3.5C). However, there was a significant increase in the number of cells undergoing cell death at all stages of apoptosis in the peritoneal cavity of the *mer1k*<sup>-/-</sup> mice (Figure 3.5D). We speculate this increase is likely due to an increase in total number of cells found in the peritoneal cavity of the *mer1k*<sup>-/-</sup> mice. Taken together, these data suggest that a difference in cell death could not explain the increase in peritoneal cells found in the *mer1k*<sup>-/-</sup> mice.

Our third hypothesis for the large number of cells was an increase in migration of cells into the peritoneal cavity *mer1k*<sup>-/-</sup> mice. To investigate this hypothesis, we labeled resident PEC from *mer1k*<sup>-/-</sup> mice and adoptively transferred them into wild-type mice as well as in reciprocal combination to track migration into the peritoneal cavity. As controls, we injected wild-type PEC into wild-type mice and *mer1k*<sup>-/-</sup> PEC into *mer1k*<sup>-/-</sup>

mice. We found that there was indeed an increase in the number of labeled cells recovered from the peritoneal cavity of *mer1k<sup>-/-</sup>* mice (Figure 3.6). Interestingly the origin of the donor cells was not a factor since wild-type and *mer1k<sup>-/-</sup>* donor cells migrated similarly to the peritoneal cavity of wild-type mice. Correspondingly, both donors migrated similarly to the *mer1k<sup>-/-</sup>* peritoneal cavity. This data not only suggests that an increase in migration is the reason for the increase in cells found in the peritoneal cavity of the *mer1k<sup>-/-</sup>* mice; but also that the environment of the *mer1k<sup>-/-</sup>* mice influences the migration of cells.

In order to differentiate between hematopoietic and non-hematopoietic sources for the increase in cells migrating to the peritoneal cavity of *mer1k<sup>-/-</sup>* mice, we performed bone marrow transplants of *mer1k<sup>-/-</sup>* bone marrow into wild-type mice and visa versa. If the hematopoietic cells from *mer1k<sup>-/-</sup>* mice were responsible for the greater migration, we would expect that the wild-type mice reconstituted with *mer1k<sup>-/-</sup>* bone marrow would have an increase in PEC. Conversely, we would expect that the *mer1k<sup>-/-</sup>* mice reconstituted with bone marrow from wild-type mice would show a decrease in PEC. Control *mer1k<sup>-/-</sup>* recipients reconstituted with bone marrow from *mer1k<sup>-/-</sup>* donors showed the expected increase in numbers of cells in the peritoneal cavity compared to control wild-type mice reconstituted with wild-type bone marrow (Figure 3.7A). However wild-type and *mer1k<sup>-/-</sup>* mice reconstituted with heterologous bone marrow showed similar number of peritoneal cells. This indicates that factors from the immune cells and the host environment may be influencing migration into the peritoneal cavity.

We next analyzed the composition of the peritoneal cells and found no significant increases in F4/80<sup>+</sup> macrophages, CD11c<sup>+</sup> DCs, or CD19<sup>+</sup> B cells (Figure 3.7 B, C, D).



However, a significant increase was found in CD3<sup>+</sup> T cell number when wild-type recipients were given bone marrow from *mertk*<sup>-/-</sup> mice as compared to donor wild-type mice (Figure 3.7E). Correspondingly, there was a significant decrease in the number of T cells recovered from the peritoneal cavity of *mertk*<sup>-/-</sup> mice receiving wild-type donor cells as compared to the peritoneal cavity of *mertk*<sup>-/-</sup> mice receiving *mertk*<sup>-/-</sup> bone marrow. Concurrent with previously published data, the number of B cells recovered from the peritoneal cavity after bone marrow transplantation was dramatically reduced most likely due to the lack of B1 cells (167). B1 cells are not able to repopulate because their precursors are only available from fetal tissue and not adult bone marrow (167). Taken together, these data suggest that neither hematopoietic nor non-hematopoietic cells contribute solely to the overall increase in cells found in the peritoneum of the *mertk*<sup>-/-</sup> mice. However, the increase in T cells is most likely due to signals exclusively from the hematopoietic compartment.

The increase in migration of immune cells into the *mertk*<sup>-/-</sup> peritoneal cavity may be due to several candidate chemokines and cytokines. We hypothesized that the chemokine CXCL13 would be increased in *mertk*<sup>-/-</sup> cells since this chemokine is required for migration of B1-B cells to the peritoneal cavity (154). However as shown in Figure 3.8A, real-time PCR of mRNA for CXCL13 was similar in both the *mertk*<sup>-/-</sup> and wild-type resident PEC. Likewise, IL-5, IL-6, IL-7, IL-9, IL-10, and BAFF were not different between the strains (Figure 3.8A or data not shown). However, a marginal increase in mRNA of the chemokine CXCL14 and a 3 fold increase in CXCL9 were observed in the *mertk*<sup>-/-</sup> population. In addition to the soluble factors, we also examined receptors present on migrating cells to determine if a certain receptor was up-regulated and contributing to

the increase in number of cells found in the peritoneal cavity of *mer1k*<sup>-/-</sup> mice (Figure 3.8B). We found the migration-associated receptors LFA, PECAM, and L-selectin were expressed similarly in cells from both genotypes (data not shown). Also, mRNA for CXCR5, the receptor for CXCL13, was similar in *mer1k*<sup>-/-</sup> and wild-type cells (Figure 3.8B). Therefore this combination of ligand-receptor pairing is unlikely to explain the large numbers of cells in the peritoneal cavity of *mer1k*<sup>-/-</sup> mice.

The receptor for CXCL9, CXCR3 is important to lymphocyte development and homing of T cells and B cells and its upregulation would be consistent with the increase of T and B cells within the peritoneal cavity of *mer1k*<sup>-/-</sup> mice. In fact, the CXCR3 transcript was increased greater than 2-fold in cells from *mer1k*<sup>-/-</sup> mice (Figure 3.8B). In addition to CXCR3, IL-7 receptor mRNA was also found to be expressed over 4-fold greater in the cells harvested from *mer1k*<sup>-/-</sup> mice. This increase in mRNA for CXCR3 and IL7R was confirmed by flow cytometry (Figure 3.8C and F). Most of the expression of CXCR3 was on the cell surface of lymphocytes with the largest population on T cells in both wild-type and *mer1k*<sup>-/-</sup> mice (Figure 3.8C). However, there was a significantly greater number of B cells (two-fold) and T cells (nearly four-fold) expressing CXCR3 in the peritoneal cavity from *mer1k*<sup>-/-</sup> mice. Representative analysis of CXCR3 on wild-type and *mer1k*<sup>-/-</sup> T cells demonstrates similar high expression levels (Figure 3.8D). Interestingly, percent of B cells that express CXCR3 are similar on wild-type and *mer1k*<sup>-/-</sup> cells; however, the expression is very low (Figure 3.8E). Thus, the greater numbers of lymphocyte subpopulations found in *mer1k*<sup>-/-</sup> mice is not explained by expression levels of CXCR3. Furthermore, a similar trend was observed for the number of B and T cells expressing IL-7R, however only T cells were significantly increased (Figure 3.8F).

Similar to CXCR3, T cells from wild-type and *merlk*<sup>-/-</sup> mice showed similar expression levels of IL-7R (Figure 3.8G). There were very few macrophages expressing either CXCR3 or IL-7R receptor (Figure 3.8C and F). Thus, we have identified potential ligands and receptors that appear to mark lymphocytes that are in greater numbers in *merlk*<sup>-/-</sup> mice.

After finding the absence of Mertk led to an increase in PECs, we wanted to determine if knock out mice lacking the other TAM family members, Axl or Tyro3, had a similar increase in PEC number. Interestingly, we found that there were no statistically significant differences in the total number of cells collected from the peritoneal cavity of the other knock-out mice when compared to wild-type mice (Figure 3.9A). Along these lines, there were no differences compared to wild-type mice in the number of CD19<sup>+</sup> B cells, CD3<sup>+</sup> T cells, or F4/80<sup>+</sup> macrophages found in the *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup> and *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> double knock-out mice (Figure 3.9B). Previously, we and others have shown *merlk*<sup>-/-</sup> mice produce autoantibodies to several self-antigens including dsDNA and nucleosomes (28, 29, 37, 43). Since the peritoneal cavity is an important reservoir for the B1-B cell population which is known to play a role in autoantibody formation, we assessed anti-nucleosome antibody production of these mice. Although autoantibodies had been attributed to the absence of all three family members(43), *merlk*<sup>-/-</sup> mice were the only mutant strain to show elevated levels of anti-nucleosome antibody (Figure 3.9C). In addition, increased splenic weights were only associated with *merlk*<sup>-/-</sup> mice whereas *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> mice were similar to wild-type mice (Figure 3.9D). This suggests that Mertk is the critical TAM family receptor that dictates the composition of cells in the peritoneal cavity and is important in regulating the production of autoantibody.

## Discussion

Mice lacking Mertk manifest a lupus like syndrome including splenomegaly, nephropathy, and autoantibody production to many different auto-antigens (28, 29, 37, 43, 172). Autoimmune diseases in mouse models are often correlated with enlarged lymphocyte compartments. We previously demonstrated that splenic weight was increased in *mertk*<sup>-/-</sup> mice, however only the DC populations were significantly elevated(37). In this study, we characterized an increase in total cell number found in the peritoneal cavity of *mertk*<sup>-/-</sup> mice, and found this increase to be in multiple cell types including cells involved in both innate and adaptive immunity. Our analyses demonstrate that only Mertk regulated the peritoneal cell numbers and the other family members Axl or Tyro3 were not important. This increase in peritoneal immune cells correlated well with the increased production of autoantibodies since only *mertk*<sup>-/-</sup> showed excessive levels. Our data suggests that in the absence of Mertk, migration of cells into the peritoneal cavity is dysregulated and this may be due to an elevation of specific chemokines and receptors associated with leukocyte homing and survival. These findings provide insights for the propensity of *mertk*<sup>-/-</sup> mice to develop a lupus like syndrome highlighted by the inability to clear apoptotic cells normally and by the increased production of auto-antibodies.

Interestingly, we found that *mertk*<sup>-/-</sup> mice had an age dependent increase in PECs. This difference between wild-type and *mertk*<sup>-/-</sup> mice was most evident at 3 months of age (Figure 3.1). In the absence of Mertk, we found an increase in the lymphocyte compartment of both B cells and T cells from the peritoneal cavity (Figure 3.1). In the *mertk*<sup>-/-</sup> lymphocyte population, CD19<sup>+</sup> B cells were over 5-fold greater in number than

wild-type, and CD3<sup>+</sup> T cells were over 6-fold greater (Figure 3.1). Further analyses indicated that the B1-B cells, the B1a and B1b cells were elevated compared to wild type mice (Figure 3.3). An increase in B1 cell number was also found in the peritoneal cavity of the 2-12H-*merck*<sup>-/-</sup> mice (172). In the T cell population, the CD4<sup>+</sup> and CD8<sup>+</sup> T cell compartments were elevated in the *merck*<sup>-/-</sup> mice although at equal ratios to that of wild-type mice (Figure 3.2C). The largest T cell increase was found in the naive T cells, shown in Figure 3.2D. Lastly, there were elevated numbers of effector and memory T cells as indicated by the distribution of CD44 and CD62L (Figure 3.2D). Thus, it appears in the absence of Mertk, T lymphocytes accumulate in greater numbers in the peritoneal cavity and this accumulation appears to be dependent on hematopoietic derived factors (Figure 3.7). It is plausible that the increase in T cells may increase the frequency that a T cell may aid antigen activation of B cells.

While the peritoneum acts as a lymphoid organ, it lacks the architecture similar to that of a lymph node or spleen, thus presenting unconventional possibilities for antigen presentation and subsequent lymphocyte activation. This may be particularly true for an animal with an increased lymphocyte population and greater numbers of antigen-presenting cells within the peritoneal cavity. Although B1-B cells normally produce T independent, low-affinity auto antibodies (167); the *merck*<sup>-/-</sup> mice present a unique scenario with increased levels of B1-B cells as well as T cells in the peritoneum (Figure 3.1, 3.2, 3.3), thus creating an environment with greater likelihood that these B cells would receive T cell help, class switch and begin producing higher affinity auto antibodies.

B1-B cells are critical for the autoimmune hemolytic anemia found in the NZB and NZB/W mice, as hypotonic ablation of peritoneal B1-B cells decreases autoantibody titers and prevents disease development in aged animals (168). Interestingly these and other autoimmune mice display increased B1 cell levels in the peritoneal cavity prior to disease development (210, 214, 215).. Previously published data from our lab and others indicates that autoimmune disease in the *merck*<sup>-/-</sup> mice is not manifested until greater than 3 months of age(29, 37, 43). Similar to the NZB mice, we have found a dramatic elevation of peritoneal B cells by 3 months of age in *merck*<sup>-/-</sup> mice (Figure 3.1C) prior to an age when auto-antibodies are prevalent (Figure 3.9C)(28, 29, 37, 43). The influence of Mertk on the lymphocyte populations may be indirect as T cells normally do not express Mertk (1) and our analysis of the LPS-stimulated and unstimulated B cell population found no detectable expression of Mertk by flow cytometry (data not shown). Thus, the peritoneal cavity is an important site for autoreactive cells and it would be interesting to investigate if the B1-B cells were critical for autoantibody production in the *merck*<sup>-/-</sup> mice similar to the NZB strain.

At 3 months of age *merck*<sup>-/-</sup> mice have nearly four times the number of B1 cells compared to their wild-type counterparts (Figure 3.3A). Using the 2-12H Tg model to track anti-Sm B cells on the *merck*<sup>-/-</sup> background, the 2-12H auto-reactive B cells are antigen-activated in the peritoneum and migrate out to the lamina propria and mesenteric lymph nodes to become antibody-secreting cells, but not to the spleen or bone marrow (172). Thus, the peritoneal cavity appears to be a recruitment area for the migration of T and B cells, DC, and macrophages that optimizes the priming of auto-reactive B1-B cells. In the absence of Mertk, it is plausible that the presence of apoptotic cells may not be

adequately cleared by the Mertk-deficient macrophages (28), resulting in exposure of B cells and T cells to self antigens. Over time this constant activation of auto-reactive B cells accumulates and may contribute to the increasing proportion of autoantibody-secreting cells and the escalation of autoantibody titers associated with the Mertk defect (29, 37).

The large number of cells in the peritoneal cavity of *mertk*<sup>-/-</sup> mice is likely due to an increase in migration (Figure 3.6) of leukocytes rather than an increase in local proliferation (Figure 3.4) or an inhibition of cell death (Figure 3.5). In support of this, we examined a list of candidate cytokines and chemokines as well as receptors known to be involved in homing and migration of cells. While most of the molecules investigated did not show differences, we identified the chemokine CXCL14 as having a marginal increase in the *mertk*<sup>-/-</sup> mice (Figure 3.8A). CXCL14 is a chemokine responsible for migration of macrophages to adipose tissue and also affects insulin uptake (216). This is particularly interesting because the *mertk*<sup>-/-</sup> mice have a larger peritoneal fat pad as compared to wild-type mice (unpublished observations).

However, we identified a more dramatic over-expression of the chemokine CXCL9 in the PECs of the *mertk*<sup>-/-</sup> mice which coincided with an increased expression of the cognate receptor CXCR3 (Figure 3.8A, B). CXCL9 is a chemokine that can act as a chemoattractant for CXCR3 expressing T cells, B cells and plasmacytoid DCs (pDC)(163-165). Type I IFNs are known to induce CXCL9 expression (164) and one common finding in patients diagnosed with systemic lupus erythematosus is increased in production of type I IFN which maybe a result of DNA/immune complexes activating pDC (217, 218). Thus, it is plausible that the greater numbers of pDC (Figure 3.2B), T

cells and B cells (Figure 3.1, 3.2, 3.3) found in the peritoneal cavity of the *merck*<sup>-/-</sup> mice are a consequence of greater amounts of CXCL9. This amplification could result in greater expression of CXCL9, thus leading to an increase in CXCR3-expressing cells migrating to the peritoneal cavity. In fact, we found there are more T cells and B cells that express CXCR3 in *merck*<sup>-/-</sup> mice than in wild type mice (Figure 3.8C). It would be of interest to determine if the *merck*<sup>-/-</sup> pDC produce greater amounts of type I IFN in response to DNA/immune complexes, thereby leading to greater expression of CXCL9 and a subsequent increase in migration of cells. Clearly the role of Mertk on pDC warrants further investigation with regards to a role in autoimmune disease.

The other receptor that was notably increased in the *merck*<sup>-/-</sup> PEC was the IL-7 receptor. Mice lacking the IL-7 receptor have low peritoneal B cell levels (219); however mice lacking the ligand, IL-7, have normal numbers of peritoneal B cell suggesting the importance of another ligand for the IL-7 receptor in the peritoneal B cell population (220). In addition, the chemokine critical for B cell homing to the peritoneal cavity, CXCL13, has been shown to act synergistically with the IL-7 receptor in development of lymph nodes, particularly the mesenteric lymph node (221). This is of interest because while we did not find differences in expression levels of CXCL13, we did find an increase in expression of IL-7 receptor (Figure 3.8B and D), presenting the potential for the synergy of CXCL13 and IL-7 receptor in establishing the peritoneal cavity lymph system as well. Thus, although we have not detected Mertk on B cells (data not shown), Mertk may be indirectly influencing the B cell populations through the IL-7 receptor and perhaps account for the increase in total B cell and B1 cell numbers in the peritoneal cavity.



Equally intriguing is the expression of IL-7 receptor on T cells and we found a four-fold greater number in the absence of Mertk (Figure 3.8D). It is plausible that IL-7R may allow greater immigration of T cells into the peritoneal cavity similar to B cells, increase the survival of T cells within the peritoneal cavity or dampen their egress. This appears to be particularly true in the naïve T cell population since they show the greatest representation (Figure 3.2D). Furthermore, IL-7 receptor has been associated with an increase in survival of CD8<sup>+</sup> effector T cells that convert to memory T cells (222) and indeed, we find elevated numbers of effector T cells and central memory T cells in the peritoneal cavity of *mertk*<sup>-/-</sup> mice compared to wild-type mice (Figure 3.2C). Taken together, this data indicates that Mertk has a profound effect on the regulation of T cell populations within the peritoneal cavity.

Although Mertk is not normally expressed on lymphocytes, we observed increased numbers of T and B cells. We deduce that typically, Mertk-expressing cells may be regulating the expression of chemoattractants and cytokines to prevent excessive accumulation of peritoneal cells that may promote autoimmune disease. Work presented here identifies an increase in the cellular populations found in the peritoneal cavity of *mertk*<sup>-/-</sup> mice. We show that this increase is not due to one particular cell type but rather all cell types investigated. We also determined this increase in B cells, T cells and antigen-presenting cells is due to enhanced migration/recruitment into the peritoneal cavity, not a change in local proliferation or death of cells. Our data suggest that Mertk may be increasing the accumulation of immune cells by upregulating CXCL9 and its receptor CXCR3 as well as IL-7R. Consequently, by establishing an abnormally large number of these immune cells in the peritoneal cavity, the likelihood of interaction and

support of autoreactive B1-B cells is favored. We find only Mertk to be critical for the regulation of peritoneal cell recruitment as mice lacking Axl and Tyro3 do not show a similar increase (Figure 3.9). This is consistent with our current findings that the onset of high autoantibody titers was found only in *merlk*<sup>-/-</sup> mice and not *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> mice. Our report provides a platform to study additional mechanisms that are controlled by Mertk and it appears that Mertk is a primary regulator driving the autoimmune phenotype.

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**Figure 3.1.** *Increase in peritoneal cellular populations in the  $\text{mertk}^{-/-}$  mouse.* PEC were harvested from male mice at indicated ages and quantified by flow cytometry. A) Total number of PEC from wild-type (■) and  $\text{mertk}^{-/-}$  (▲) mice were counted. B) The total number of F4/80<sup>+</sup> macrophages, C) The total number of CD19<sup>+</sup> B cells, and D) The total number of CD3<sup>+</sup> T cells were also examined. \*\*\*  $p < 0.0001$  by 2-way ANOVA comparing wild-type to  $\text{mertk}^{-/-}$  mice.  $n \geq 6$  \*\*  $p < 0.001$  by 2-way ANOVA comparing wild-type to  $\text{mertk}^{-/-}$  mice.

**Figure 3.2.** *Increase in DC, pDC, and T cells by subset in  $\text{mertk}^{-/-}$  peritoneal cavity.* Cells were stained and quantified by flow cytometry. A) PEC were stained for CD11c<sup>+</sup> DC or B) CD11c<sup>+</sup> B220<sup>+</sup> PDCA1<sup>+</sup> plasmacytoid DC. C) PEC were stained for CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells. D) PEC were stained for Effector T cells (CD44<sup>hi</sup> CD62L<sup>lo</sup>), Naïve T cells (CD44<sup>lo</sup> CD62L<sup>hi</sup>), and Central Memory T cells (CD44<sup>hi</sup> CD62L<sup>hi</sup>) T cells (CD3<sup>+</sup>). The sample number is  $n \geq 4$ . Statistical significance was analyzed by Student's *t*-test comparing samples from wild-type and  $\text{mertk}^{-/-}$  mice. *p*-values are: \* $p < 0.05$ ; \*\* $p < 0.005$ ; and \*\*\* $p < 0.0001$ .

**Figure 3.3.** *Increase in B1 cell populations and lower B cell maturation status in  $\text{mertk}^{-/-}$  peritoneal cavity.* A) PEC were stained for B1a (CD19<sup>+</sup> CD11b<sup>+</sup> CD5<sup>+</sup>) and B1b (CD19<sup>+</sup> CD11b<sup>+</sup> CD5<sup>-</sup>) cells. B) PEC were stained with CD19 to identify B cells and then double labeled with MHC II, CD80 and CD86. The Mean Fluorescence Intensity on CD19<sup>+</sup> cells was graphed. The number of samples is  $n \geq 4$ . Statistical significance by Student's *t*-test was \* $p < 0.05$  and \*\* $p < 0.005$ .

**Figure 3.4.** *Increase in cells is not due to proliferation.* Proliferating PEC were identified by their incorporation of BrdU. PEC were collected after injection of BrdU i.p. and stained with anti-BrdU antibody. (A) Percent and (B) total number of BrdU<sup>+</sup> cells across aged mice from 1.5 months to 6 months were examined.  $n \geq 6$ . C) Bone marrow cells from wild-type (WT) or *mertk*<sup>-/-</sup> (*M*<sup>-/-</sup>) mice were stained for BrdU incorporation and the data is expressed as the percent of BrdU<sup>+</sup> cells,  $n \geq 3$  and representative of at least 2 separate experiments. Statistical significance is \*  $p < 0.05$  by 2-way ANOVA. \*  $p < 0.05$  by Bonferroni's post-test.

**Figure 3.5.** *Increase in cells is not due to cell death.* 3 month old mice were injected with VAD-FMK-FITC 1 hour prior to PEC harvest to detect caspase-positive cells. Cells were analyzed by flow cytometry. (A) The percent VAD-FMK<sup>+</sup> cells. (B) The total number of VAD-FMK<sup>+</sup> cells were examined. PEC were harvested from 3 month old mice, stained with Annexin V-FITC and PI, and subsequently analyzed by flow cytometry. Data is expressed as (C) percent or (D) total number of Annexin-V<sup>+</sup> or PI<sup>+</sup> PEC.  $n \geq 4$ . Statistical significance comparing samples from wild-type and *mertk*<sup>-/-</sup> mice is \*\*\*  $p < 0.0001$  by 2-way ANOVA. (\*\*)  $p < 0.001$  by Bonferroni's post-test. \*  $p < 0.05$  by Bonferroni's post-test.

**Figure 3.6.** *Enhanced migration to *mertk*<sup>-/-</sup> peritoneal cavity.* Indicated donor PECs were harvested, stained with cell tracker green and injected via tail vein into recipient mice. PEC were harvested from 24 hrs after injection and analyzed by flow cytometry.

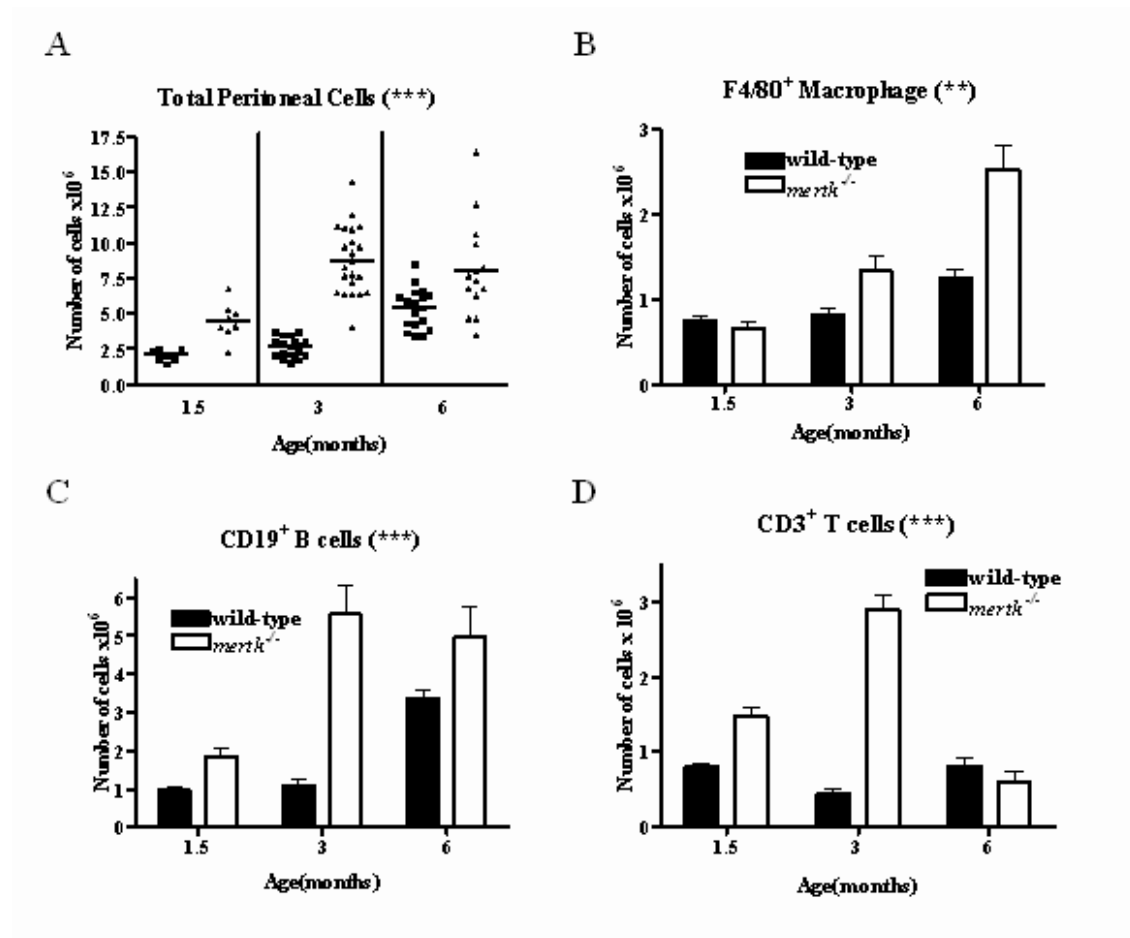
Data is expressed as number of cell tracker green positive cells recovered from recipient peritoneum.  $n \geq 10$ . Statistical significance is \*  $p < 0.01$  by Student's *t*-test.

**Figure 3.7.** *Both hematopoietic and non-hematopoietic sources are required for cellular increase in  $mer1k^{-/-}$  peritoneal cavity.* Recipient 4 week old mice were lethally irradiated and given indicated donor bone marrow 1 day later. At 3 months of age PEC were collected and (A) total cell number was counted. Cells were then stained for (B) F4/80<sup>+</sup> macrophage, (C) CD11c<sup>+</sup> DC, (D) CD19<sup>+</sup> B cells, and (E) CD3<sup>+</sup> T cells. WT = wild-type  $M^{-/-} = mer1k^{-/-}$  mice. Statistical significance using the Student's *t*-test \*  $p < 0.05$  and \*\*  $p < 0.0005$ .

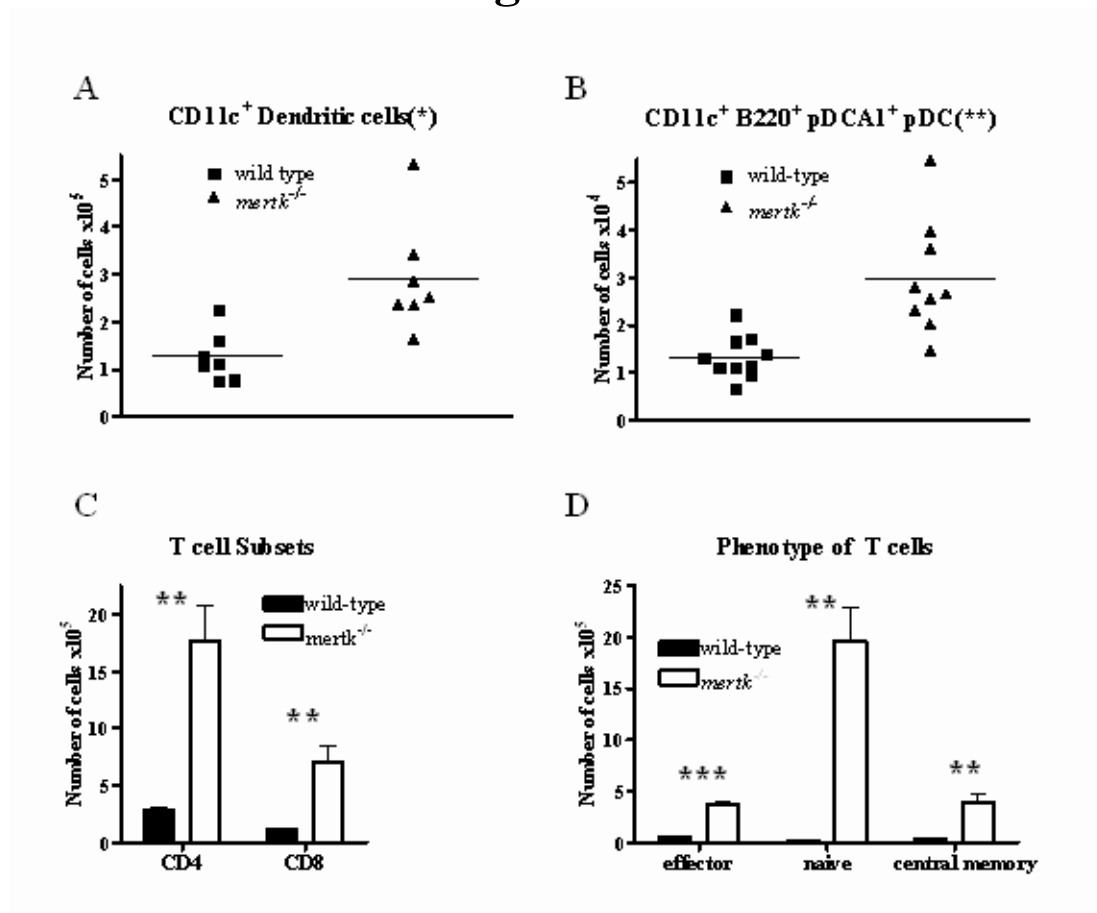
**Figure 3.8.** *Expression of chemokines, cytokines, and migration receptors in PEC.* PEC were pooled from at least 4 three month old male mice. RNA was harvested and reverse transcribed to cDNA. A) Real-time PCR was performed for soluble molecules, IL-7, BAFF, CXCL9, CXCL13, and CXCL14. B) Real-time PCR was performed for receptors, CXCR5, CXCR3, and IL-7R. C) PEC from wild-type and  $mer1k^{-/-}$  mice were double-stained for CD3, CD19, or F480 and CXCR3. Representative histograms of CXCR3 expression gated on CD3<sup>+</sup>(D) or CD19<sup>+</sup>(E). F) PEC from wild-type and  $mer1k^{-/-}$  mice were double stained for CD3, CD19, or F480 and IL-7R. G) Representative histograms of IL-7R expression CD3<sup>+</sup> cells. Mean and standard deviation are graphed.  $n \geq 3$ . Statistical significance are indicated, \* $p < 0.01$ , \*\* $p < 0.005$  by the Student's *t* test when comparing wild-type vs  $mer1k^{-/-}$  mice.

**Figure 3.9.** *Increase in peritoneal cells and autoantibody production is due to Mertk and not Axl or Tyro3.* PEC were harvested from 3 month old male mice from wild-type (WT), *merlk<sup>-/-</sup>* (*M<sup>-/-</sup>*), *axl<sup>-/-</sup>* (*A<sup>-/-</sup>*), *tyro3<sup>-/-</sup>* (*T<sup>-/-</sup>*), *axl<sup>-/-</sup>/tyro3<sup>-/-</sup>* (*AT<sup>-/-</sup>*). A) Total cells were counted and stained for B) F4/80<sup>+</sup> macrophage, CD19<sup>+</sup> B cells and CD3<sup>+</sup> T cells. C) Anti-nucleosome antibody titers were determined by ELISA. From serum of mice 6 months of age or older D) Splenic weight was measured of mice 6 months of age or older.  $n \geq 6$  No statistically significant differences were found by ANOVA with post-tests between WT, *A<sup>-/-</sup>*, *T<sup>-/-</sup>* and *AT<sup>-/-</sup>* peritoneal cells. Statistical significance is indicated in comparisons with samples from wild type and *merlk<sup>-/-</sup>* mice, \* $p < .01$  \*\* $p < .001$  by ANOVA post-test.

Figure 3.1



# Figure 3.2





# Figure 3.3

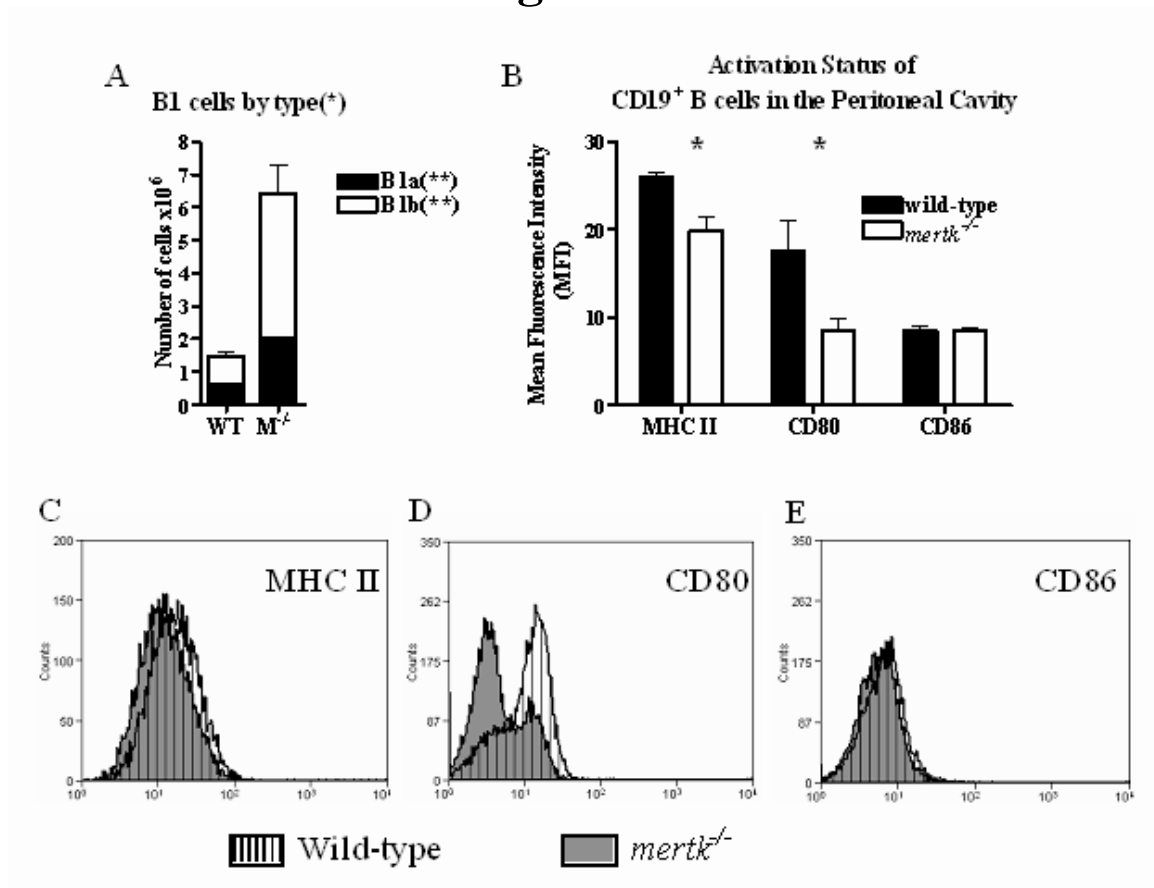
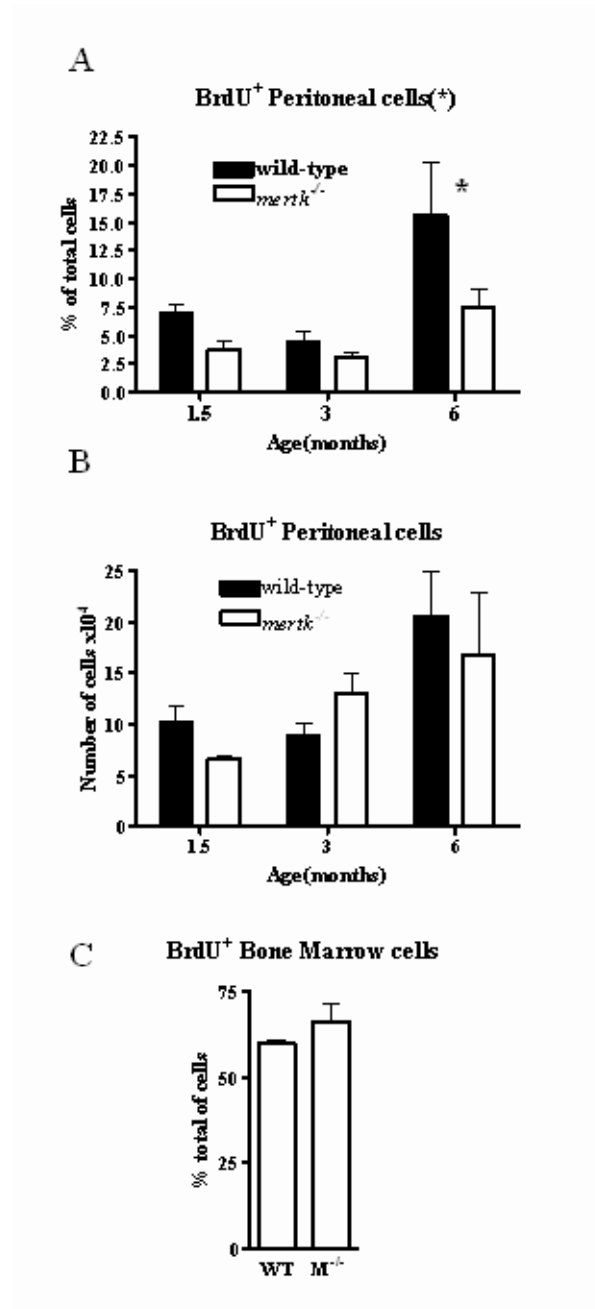
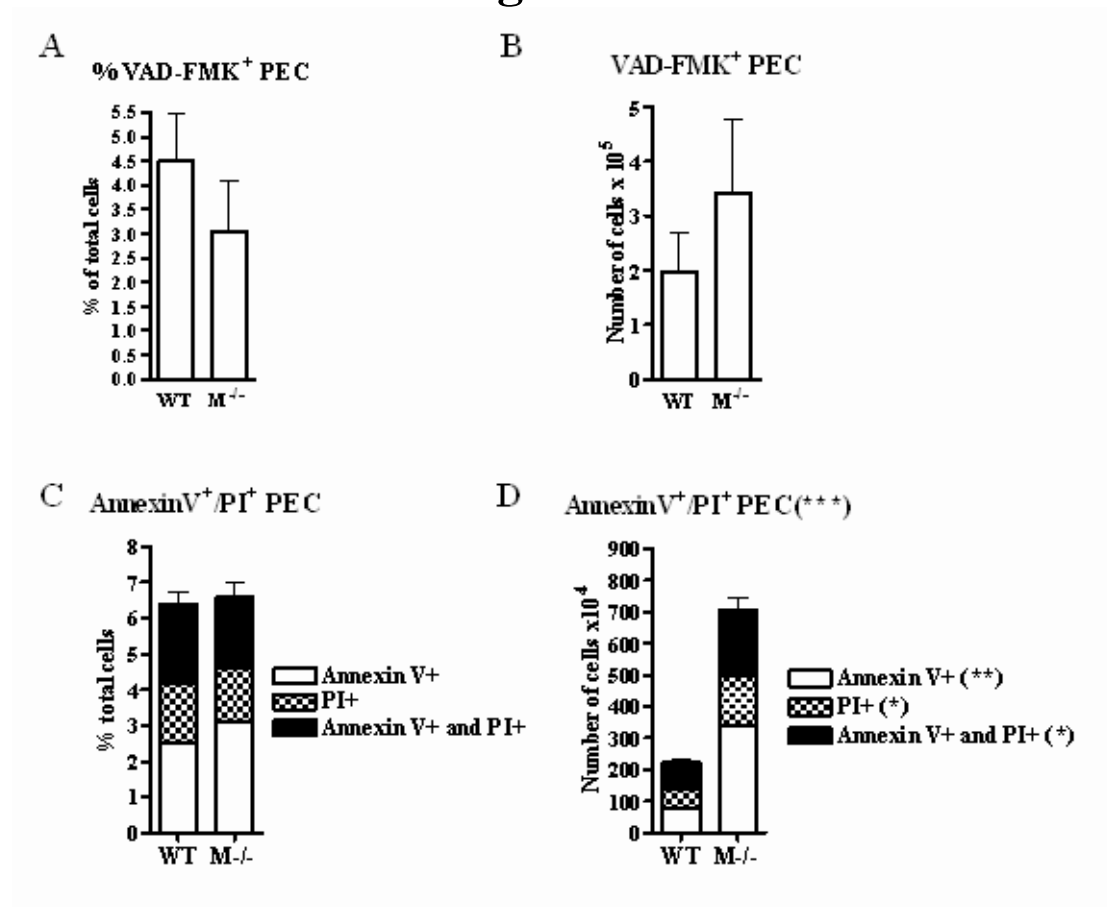


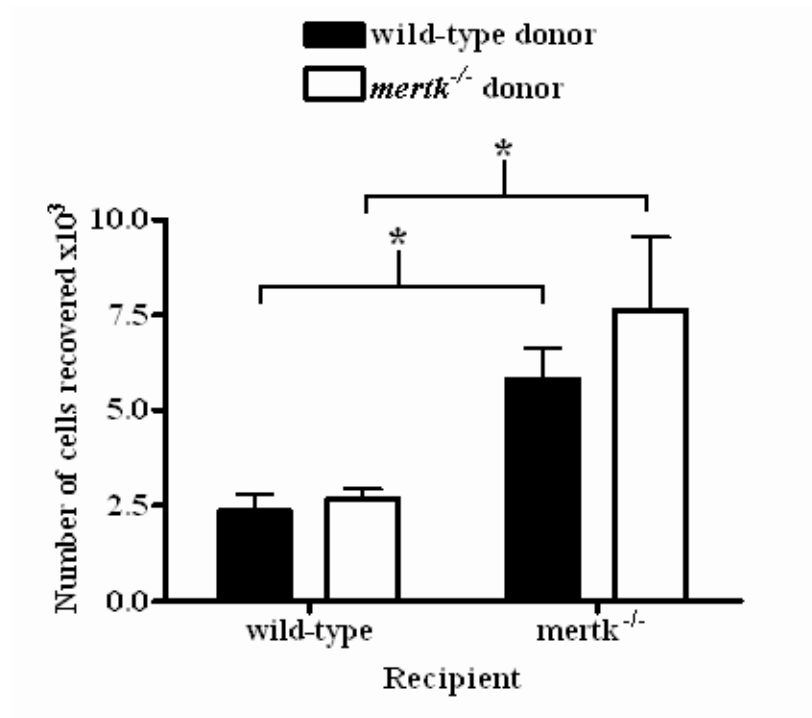
Figure 3.4



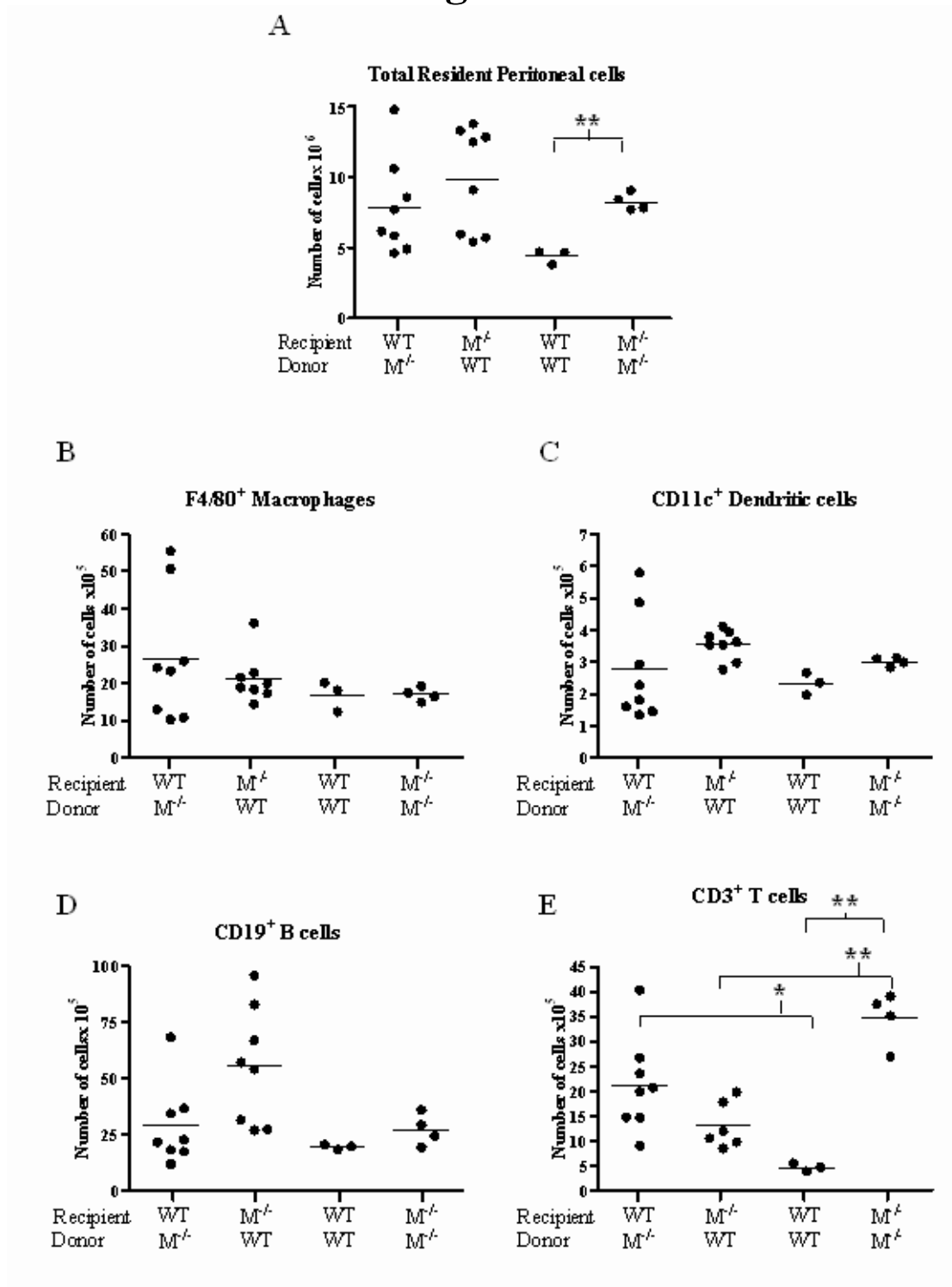
# Figure 3.5



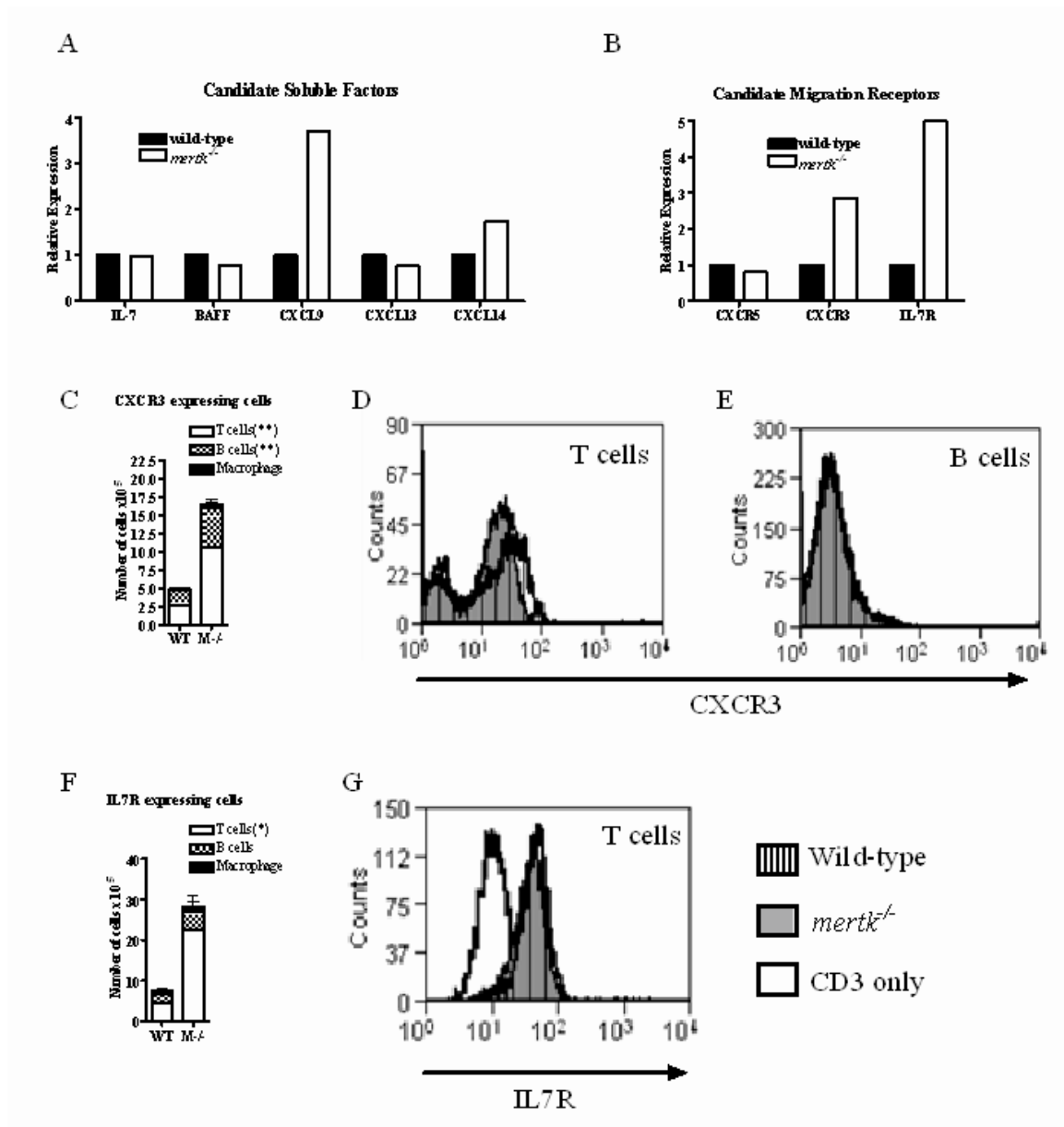
**Figure 3.6**



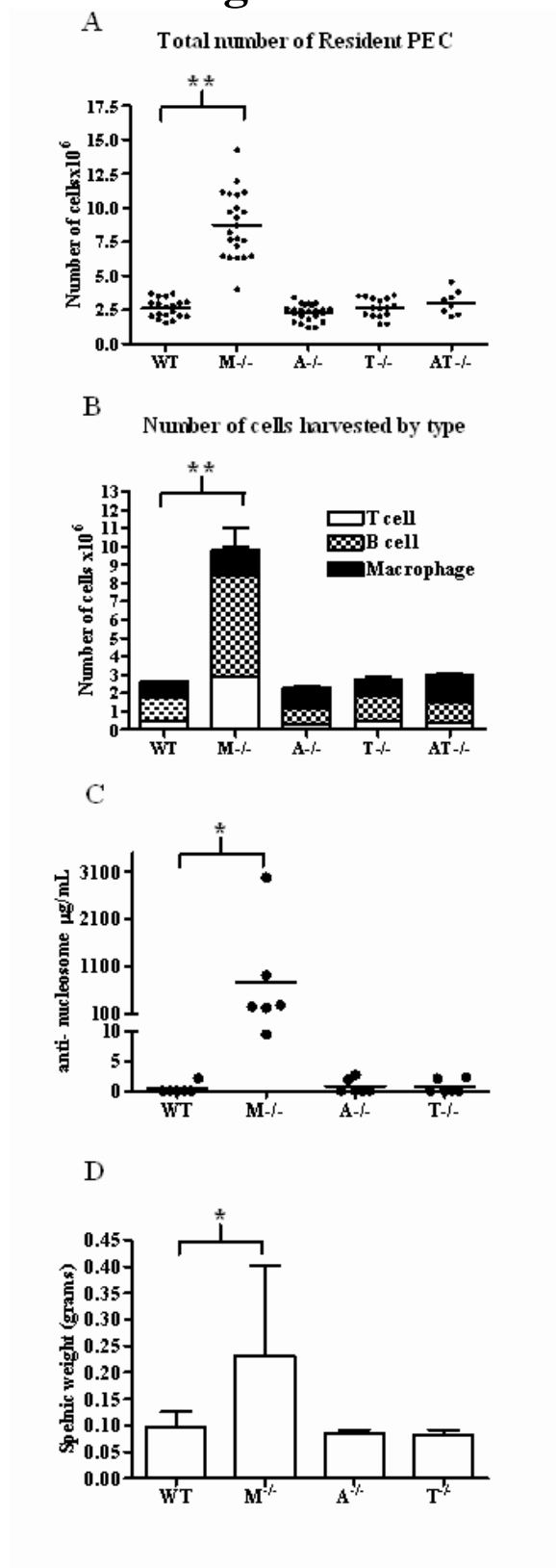
# Figure 3.7



**Figure 3.8**



# Figure 3.9



## **Chapter 4:**

**Mertk on macrophages is a critical regulator of p202 and DNA-induced  
cell death**



## Abstract

The regulation of cell death by cytoplasmic double-stranded DNA is governed by the HIN-200 family proteins AIM2 and p202 (183-185, 189). These proteins are known to be associated with Systemic Lupus Erythematosus (148, 174). However, the etiology of this multi-factorial disease is currently unknown. There is mounting evidence for a viral infection as a potential initiation point for autoimmunity (89, 223, 224). Here we show that Mertk regulates cytoplasmic DNA-induced cell death by controlling the expression of p202. We found cleavage of caspase 1 and 3, and cell death due to cytoplasmic DNA was greatly reduced in cells lacking Mertk. We screened each of the HIN-200 family members and found p202, which can bind DNA, to be upregulated in macrophages and dendritic cells lacking Mertk. The HIN-200 family member AIM2 which senses dsDNA and can trigger caspase cleavage was expressed at similar levels in wild type macrophages and *mertk*<sup>-/-</sup> macrophages suggesting variations in p202 expression may be critical for preventing AIM2-mediated cell death. This upregulation of p202 was not affected by Axl or Tyro3 which, the other members of the Tyro3/Axl/Mertk family of receptors. We also showed that type I IFN dramatically upregulated *ifi-202* mRNA and p202 protein expression in *mertk*<sup>-/-</sup> macrophages and this was correlated to reduced caspase cleavage induced by cytoplasmic dsDNA. Our results indicate that Mertk is a critical regulator of cytoplasmic DNA-induced cell death and high levels of p202 found in *mertk*<sup>-/-</sup> and other lupus-prone mice may allow greater survival of antigen-presenting cells which in turn may contribute to the hyperactive immune phenotype associated with autoimmunity.

## Introduction

Mertk is a receptor tyrosine kinase belonging to the Tyro3/Axl/Mertk (TAM) family of receptors. The TAM receptors are characterized by two extracellular fibronectin III like domains and two extracellular Ig like domains as well as an intracellular kinase domain containing a signature KWIAIES sequence (7, 8). These receptors are expressed on many cell types; however, Mertk is restricted primarily to cells of myeloid, epithelial and reproductive origin and is not detected on normal lymphocytes or neutrophils (7, 18).

Different combinations of these receptors have been shown to be required for efficient phagocytosis of apoptotic cells by macrophages(MΦ) and dendritic cells(DC)(18, 28). The serum proteins Gas 6 and Protein S are two ligands that have been identified for the TAM receptors. These ligands can bind phosphatidylserine on apoptotic cells and it is through this molecular bridging to the receptors that triggers the phagocytosis apoptotic cells. The inability to adequately clear apoptotic cells and to maintain B cell anergy are two common features associated with autoimmune disease. Mice lacking these receptors are deficient in phagocytosis of apoptotic cells and they display a lupus-like autoimmune disease characterized by autoantibody production and splenomegaly (28, 29, 37, 43). Furthermore, these receptors affect signal transduction, cell activation, and cell maturation pathways which regulate the function of antigen-presenting cells (APC). Hence, these mice lacking TAM receptors provide an opportunity to explore mechanisms that may link these receptors to dysregulation of APCs and lymphocytes.

Systemic Lupus Erythematosus (SLE) is a multifactoral autoimmune disease that is characterized by multiple clinical symptoms, including malar rash, lupus nephritis, oral ulcers, and high titers of autoantibodies. These autoantibodies are usually directed against double-stranded DNA (dsDNA), Smith antigen (Sm), as well as other nuclear antigens (42). The autoantibodies form immune complexes with self antigens and their deposition in blood vessels and various tissues promote autoimmunity and facilitate pathologies (42).

This debilitating disease is believed to be caused by both genetic and environmental components (42). Genetic correlations to SLE include polymorphisms that control expression of critical genes or mutations that affect cell function including phagocytosis of apoptotic cells, regulation of B cells, and production of type I interferon (IFN)(42). One current theory for an environmental cause is viruses that possess nucleic acids or proteins that act in molecular mimicry as self antigens. Furthermore, viral infection can trigger type I IFN or alter cell function and tolerance which may also be a potential initiation of SLE. In support of this notion, many diagnosed SLE patients have high Epstein Barr Virus (EBV) titers (88). Interestingly, one of the major EBV peptides that antibodies are raised against, Epstein Barr Virus Nuclear antigen-1, has the potential to induce autoantibodies to Ro and Sm through molecular mimicry (89). Furthermore, increased in serum levels of type I IFN is correlated both with viral infection and SLE (56, 225-227).

One family of IFN-inducible genes, the HIN-200 family, has been previously correlated with SLE (186). In particular, p202 has been found to be overexpressed in many mouse models of SLE including, MRL<sup>lpr/lpr</sup>, NZB, and B6Nba.2 (148). This family

of proteins is characterized by a conserved 200 amino acid sequence containing the highly conserved peptide motifs, MFHATVAT, proposed to be involved in protein-protein interactions and a pRb-binding motif, LXCXE (173). These genes are clustered in the q23 region of chromosome 1 in both mice and humans (148). In addition, most of the HIN-200 family members also possess a PYRIN domain known to be important for inflammasome caspase activation that mediates IL-1 $\beta$  cleavage and cell death (173, 178, 179). However, p202 is the sole family member that does not contain a PYRIN domain.

Recent publications have identified Absent in melanoma 2 (AIM2) and p202 of the HIN-200 family as important regulators of dsDNA-induced cell death (183-185, 189). These groups demonstrated that AIM2 can bind dsDNA as well as the inflammasome component apoptosis-associated speck-like protein containing a CARD (ASC) (183-185). This interaction between dsDNA, AIM2 and ASC results in cleavage of caspase 1 and 3 and subsequent cell death by apoptosis or pyroptosis. It is believed that this AIM2-mediated mechanism may be critical in sensing DNA from intracellular pathogens and it may be a means to remove infected cells that may harbor pathogens. However, p202 appears to compete with AIM2 for the binding of dsDNA and this balance between the two cytoplasmic molecules controls cell susceptibility to DNA-induced death (189). Because p202 cannot associate with ASC and cannot activate caspases, the high levels of p202 found in lupus-prone cells may prevent proper disposal of key APCs and autoreactive B cells. Therefore, it is plausible that high levels of p202 associated with lupus-prone mice prevent proper execution of virally-infected APCs or autoreactive B cells.

The fact that SLE maybe triggered by viral infections and that APCs such as MΦs and DCs are important for T cell-dependent autoantibody production, we hypothesized that MΦs lacking Mertk were less susceptible to a model of viral dsDNA-induced cell death. In this report, we show that cells from the *mertk*<sup>-/-</sup> mouse are less susceptible to cytoplasmic dsDNA-induced caspase 1 and 3 cleavage and subsequent cell death. Of the HIN-200 family of IFN-inducible proteins, we found p202 is negatively regulated by Mertk under resting or stimulated conditions in MΦs and DCs. More importantly, we also found that Mertk is the only TAM family member that negatively regulates p202. Interestingly, Stat3, a known *ifi-202* promoter element, is hyperactivated in cells lacking Mertk. Finally, we provide evidence that the stimulation of *mertk*<sup>-/-</sup> MΦs with IFN increased p202 expression and subsequently further decreased cleavage of caspase 1 and 3.

## Materials and Methods

### *Animals*

All animals were housed in Specific Pathogen-Free Facilities according to the University of North Carolina Institutional Animal use and Care committee (IACUC). *mertk*<sup>-/-</sup> mice were generated as previously described(35). *axl*<sup>-/-</sup> and *tyro3*<sup>-/-</sup> were generously provided by Dr. Stephen P. Goff (Columbia U., New York) and Dr. Greg Lemke (Salk Institute for Biological Studies, San Diego) respectively. All congenic strains were backcrossed at least 6 generations on the C57BL6/J background. *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> and TAM<sup>-/-</sup> triple knock out mice lacking all three receptors were derived by crossing congenic mutant mice. Male mice 8-12 weeks of age were used in experiments. C57BL6/J strain was used as a wild-type control to compare individual mutant mice above.

### *Antibodies*

Anti-Caspase 1, Anti-IFI-202(p202) antibodies were obtained from Santa Cruz. Anti-Caspase 3 antibody was obtained from Cell Signaling. Anti-actin antibody was obtained from Millipore.

### *Electroporation of Thioglycollate-Elicited Peritoneal Exudate Cells (Thio-PEC)*

Thio-PEC were obtained as previously described(228). Cells were plated  $2 \times 10^6$  in 6 well plates and washed after 3 hrs to remove non-adherent cells. The remaining cells were greater than 95% MΦs. At least 1 day after washing wells, cells were electroporated with Calf Thymus DNA (Sigma) or poly dA:dT (Sigma) at indicated doses. Electroporation was carried out using petri-pulser (BTX) and ECM 830 (BTX) with conditions of 2 pulses of 100V for 10msec each. Brightfield pictures were taken 3 days post electroporation. For Cy3<sup>+</sup> cell death counts, poly dA:dT was labeled with Cy3 using the

LabelIT DNA-labeling kit(Mirus) according to manufacturer's instructions. The membrane impermeable nuclear dye Sytox green(Invitrogen) was used as a vital dye marker. Pictures were obtained using an inverted fluorescent microscope (Leica DMIRE2) and Metamorph 5.0 software(Universal Imaging Corp). Overlays of pictures were compiled using IMAGE J, and cell counts were obtained using Image Pro Plus software. Caspase cleavage was detected by Western blots using whole cell lysates that were obtained 30 minutes after electroporation. Where indicated, cells were treated with 1000U/mL of IFN for 3 days prior to electroporation.

#### *Real-Time PCR*

Cells were plated and left untreated or treated for indicated time periods with either 1000 U/mL IFN (PBL), 200ng/mL LPS (Invivogen), 20ug/mL Poly I:C (Invivogen) or 150nM Gas6 (R and D Systems). After treatment, cells were harvested in RLT Plus lysis buffer (Qiagen) and subjected to clean up with RNeasy Plus kit (Qiagen) according to manufacturers instructions. RNA was quantified and converted to cDNA using Superscript II reverse transcriptase (Invitrogen) with random hexamers. Gene-specific primers were designed with primer express software (Applied Biosystems). Real-time PCR was carried-out using 2x Sybr Green (Applied Biosystems) solution and run in an ABI 7500 real-time PCR machine that is housed in the UNC Neuroscience Center Expression Profiling and SNP Genotyping Core. Primers used are the following.

AIM2-Forward: CACTGAGGGTATAAGTATGGCAGGAT

Reverse: CAGGCGTCTCTCCGACAAG

Pyhin1-Forward: GAGGTAAATGATGGCGAACCA

Reverse: TGTTCGATTGGCACTTTTCCT

IFI 202 Forward: TCTGCCTTGTTGGAGATCTAGGA

Reverse: CTTCAAGCCAACTTCTTC

IFI203 Forward: TGGCAACCTCAACACTTCCTT

Reverse: CTGGGCTCTGTTTTTGGAGACT

IFI 204 Forward: GGCTGCTCCTGACCAAATGA

Reverse: GCTGGCATTTCATTTCTGA

IFI205 Forward: GCATCCTGGAGATCAATGAGACT

Reverse: TGGGCACTTCAATCATTGTGTTT

GAPDH Forward: TGTGTCCGTCGTGGATCTGA

Reverse CCTGCTTCACCACCTTCTTGA

#### *Bone Marrow-derived DCs*

Bone marrow-derived DCs were obtained as previously described(37). Cells were harvested from low culture dishes on day 7 counted and re-plated at  $2 \times 10^6$  per well for treatment with IFN 1000U/mL at indicated time periods starting on day 9.

#### *Statistics*

Statistics were performed using Graph Pad Prism Software. Where indicated, Students *t*-test were performed. A p value of less than 0.05 was considered significant.



## Results

We first investigated whether MΦs lacking Mertk were more susceptible to cytoplasmic dsDNA-induced cell death. Shown in Figure 4.1A, MΦs lacking Mertk were less susceptible to electroporated dsDNA-induced cell death when compared to wild-type MΦs. There are many fewer phase bright cells 3 days post electroporation in the wild-type well that received DNA (top left panel Figure 4.1A) as compared to *mertk*<sup>-/-</sup> MΦs (bottom left panel). This cell death was not due to electroporation alone as cells not receiving DNA remained healthy and phase bright (right two panels). This reduction in cell death was confirmed for *mertk*<sup>-/-</sup> MΦs by MTT Assay (data not shown).

Some wild type MΦs did not undergo cell death after electroporation which may be reflective of a lack of dsDNA. To more accurately correlate dsDNA with MΦ cell death, we used Cy3-labeled DNA and the vital dye Sytox green to determine which cells received dsDNA and were dead. While similar numbers of wild-type and *mertk*<sup>-/-</sup> cells were transfected with the Cy3-labeled DNA, almost all of the wild-type cells that were Cy3-positive were also vital dye-positive cells, indicating that their membranes were compromised and they were dying. In contrast, very few of the *mertk*<sup>-/-</sup> cells that received dsDNA were also positive for the Sytox green vital dye (Figure 4.1B). These data are quantified as a percent of Cy3-positive cells that are dead (vital dye-positive) in Figure 4.1C; where greater than 90% of wild-type cells that received DNA are dead as compared to less than 15% of *mertk*<sup>-/-</sup> cells. Furthermore, caspase 3 and 1 cleavage in response to electroporated DNA was dramatically reduced in the *mertk*<sup>-/-</sup> cells as compared to their wild-type counter parts (Figure 4.1D).

Given that the cells lacking Mertk were less susceptible to dsDNA-induced cell death, we hypothesized that HIN-200 family members may be dysregulated in the *mertk*<sup>-/-</sup> cells. Since these molecules are known to be induced with type I IFN, we examined the HIN-200 family members by real-time PCR over a time course of 3 and 24 hours of IFN treatment in wild-type and *mertk*<sup>-/-</sup> MΦs. We found a 900-fold overexpression of *ifi-202* RNA in unstimulated *mertk*<sup>-/-</sup> cells as compared to wild-type MΦs, with a greater difference after 3 and 24 hours of IFN stimulation (Figure 4.2 top left panel). Upon stimulation with IFN, *mertk*<sup>-/-</sup> macrophages dramatically upregulated *ifi-202* RNA by over 7000-fold which is in stark contrast to wild-type macrophages. While RNA for the other HIN-200 family members (*ifi-203*, *ifi-204*, *ifi-205*, *aim2*, *pyhin-1*) increased after stimulation with IFN, only *ifi-203* had a difference greater than 3 fold. We found that *ifi-203* showed a greater than 20-fold decrease comparing *mertk*<sup>-/-</sup> MΦs to wild-type (Figure 4.2, top middle panel). Unfortunately, little is known about the function of this family member. In addition, *mertk*<sup>-/-</sup> cells show up to 3-fold less RNA for *ifi-205* compared to wild-type cells after 24 hour stimulation (Figure 4.2, lower left panel). RNA for AIM2 showed a slight reduction in *mertk*<sup>-/-</sup> MΦs compared to wild-type MΦs (Figure 4.2 bottom middle panel).

We focused on *ifi-202*(p202) because of extremely high expression in *mertk*<sup>-/-</sup> MΦs and the recent publications regarding p202 in the regulation of cytoplasmic dsDNA-induced cell death. Previous reports indicated that LPS and IL-6 could activate p202. We also wanted to examine whether Gas6, a ligand for Mertk, and Poly I:C, an RNA analog, could induce *ifi-202* expression. As shown in 4.3A, in addition to IFN stimulation, *ifi-202* RNA increased upon stimulation with LPS, Gas6, and Poly I:C.

However, IFN was the strongest inducer of *ifi-202* expression in the *mertk*<sup>-/-</sup> cells. We corroborated the RNA data with protein expression and found p202 was over-expressed as early as 12 hours after IFN stimulation (data not shown). Subsequent time points revealed even greater p202 expression after 48, 72, and 96 hours of IFN stimulation (Figure 4.3B). p202 expression was occasionally detectable in *mertk*<sup>-/-</sup> cells under unstimulated conditions (data not shown); however p202 was undetectable in all wild-type samples tested. This suggested that Mertk is dominant in restricting p202 expression and that this repression is tightly controlled. In addition, we could detect p202 protein in *mertk*<sup>-/-</sup> MΦs with as little as 250U/mL of IFN after 6 days of treatment (data not shown). Interestingly we also found that electroporated dsDNA could induce p202 protein expression in *mertk*<sup>-/-</sup> MΦs (Figure 4.3C).

After confirming over-expression of p202 by protein, we sought to determine if the other TAM family members would regulate *ifi-202* similarly to Mertk. However, we found that after IFN stimulation, MΦs lacking Axl (*axl*<sup>-/-</sup>) singly, or cells lacking Axl and Tyro3 (*axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup>), or cells lacking all three receptors, TAM<sup>-/-</sup>, did not show over-expression of *ifi-202* compared to wild-type (Figure 4.4A). In fact, we found MΦs from the TAM triple knock-out mice have lower *ifi-202* expression than *mertk*<sup>-/-</sup> MΦs, indicating that Axl and Tyro3 may be providing a positive signal for IFN-induced stimulation of *ifi-202* gene expression. This lack of over-expression of RNA by cells lacking Axl or Tyro3 singly, Axl and Tyro3, or all three family members was confirmed at the protein level as well (Figure 4.4B).

After ruling out Axl and Tyro3 receptors as being involved in suppression of *ifi-202* at the RNA level and p202 at the protein level, we wanted to determine if other cells

that normally express *Mertk* were regulating p202 similarly. While DCs also express *Mertk*, the role of this receptor is different from its role on MΦs. For example, DCs lacking *Mertk* are equally efficient at phagocytosis of apoptotic cells when compared to wild-type DCs (18, 29). We found that unstimulated bone marrow-derived DCs from *mertk*<sup>-/-</sup> mice had greater than 500-fold more *ifi-202* RNA by real-time PCR (Figure 4.5A). Upon type I IFN stimulation, this difference increased to greater than 20,000-fold as compared to wild-type DCs (Figure 4.5A). In addition, LPS stimulation also resulted in an upregulation of *ifi-202* in *mertk*<sup>-/-</sup> DCs similarly to IFN; however, IL-6 stimulation did not increase *ifi-202* above unstimulated in *mertk*<sup>-/-</sup> DCs (Figure 4.5B). IL-6 had been previously shown to induce p202 over-expression in another lupus mouse model (191). We again confirmed the RNA over-expression of *ifi-202* by assessing p202 protein expression using Western blot. In corroboration of the *ifi-202* RNA findings, we found p202 protein in *mertk*<sup>-/-</sup> bone marrow-derived DC after 48 hours of 1000U/mL of IFN; in contrast, p202 was undetectable in wild-type DC (Figure 4.5C).

We next wanted to determine the mechanism by which *Mertk* was regulating p202 expression. Since there is a known Stat3-binding element in the *ifi-202* promoter region (191), we first investigated whether activation of Stat3 was different in MΦs lacking *Mertk* compared to wild-type MΦs. We found that *mertk*<sup>-/-</sup> MΦs had greater IFN-stimulated phosphorylation of Stat3 compared to wild-type after 15 and 30 minutes (Figure 4.6A). Interestingly, pStat3 was detectible as early as 5 minutes after stimulation but was dramatically reduced by 60 minutes in both *mertk*<sup>-/-</sup> and wild-type MΦs (Figure 4.6A and data not shown). We also found this increase in phosphorylation to be dose dependent (Figure 4.6B). Since Stat1 had previously been shown to be regulated by the

TAM family member Axl (40), and to be sure that this regulation was specific to Stat3, we also investigated the phosphorylation of Stat1. We found that IFN-stimulated pStat1 was similar in both *merlk*<sup>-/-</sup> and wild-type MΦs (Figure 4.6C). Thus, Mertk appears to negatively regulate IFN-induced Stat3 phosphorylation.

After establishing that p202 was dramatically upregulated in *merlk*<sup>-/-</sup> cells after IFN stimulation, we wanted to demonstrate that these high p202 levels had functional consequences regarding caspase activation. IFN-stimulated or unstimulated MΦs from wild-type and *merlk*<sup>-/-</sup> mice were transfected with dsDNA and then harvested for protein lysates. As shown in Figure 4.7A, IFN treatment decreased the cleavage of both caspase 1 and 3 to almost undetectable levels in response to dsDNA in *merlk*<sup>-/-</sup> MΦs (Figure 4.6A). In addition, the wild-type MΦs had slightly more cleavage of caspase 1 or 3 after IFN treatment, which was anticipated given the increase in *aim2* expression and lack of increase in *ifn-202* expression shown in Figure 4.2. Figure 4.7B depicts lysates from Figure 4.7A showing an increase in p202 in *merlk*<sup>-/-</sup> MΦs that correlated to the decrease in caspase cleavage. Taken together these data suggest that the lack of dsDNA-induced cell death in *merlk*<sup>-/-</sup> cells is correlated to p202 over-expression. With IFN treatment, a further increase in p202 expression resulted in even less caspase cleavage in *merlk*<sup>-/-</sup> cells.

## Discussion

The data presented in this chapter provide a unique mechanism by which Mertk regulates dsDNA-induced cell death through the stringent regulation of p202. We have shown that intracellular dsDNA rapidly induces caspase cleavage and cell death in wild-type MΦs. This caspase cleavage and cell death was reduced in MΦs lacking Mertk (Figure 4.1). We subsequently identified the HIN-200 family member, *ifi-202*, as upregulated over 900-fold in unstimulated cells lacking Mertk and this upregulation can be dramatically increased to over 7000-fold following IFN stimulation (Figure 4.2). The restriction of p202 by Mertk is not a function of Axl nor Tyro3; however, Axl or Tyro3 may cooperate with the IFN receptor to induce *ifi-202* in the presence of IFN. Lastly, *aim2* expression did not differ dramatically, suggesting the varied levels of p202 may be effectively competing for dsDNA binding away from AIM2 and dictating protection from caspase 1 and 3-mediated cell death. Please see Figure 4.8 for a schematic description.

While nearly all HIN-200 family members were found to be upregulated by wild-type and *mertk*<sup>-/-</sup> MΦs, p202 is the only family member increased in *mertk*<sup>-/-</sup> MΦs (Figure 4.2). In contrast, *ifi-203* is regulated in a reciprocal fashion to *ifi-202* and is found to have 50-fold lower expression in MΦs lacking Mertk. Unfortunately, the literature is lacking with regard to p203 function. It contains a PYRIN domain similar to AIM2 and characteristic 200 amino acid domain found in all HIN-200 family members. However, *ifi-203* has been shown to have a similar reciprocal expression pattern in DBA/2 mice compared to C57BL/6J(229). While it is likely that p203 does bind DNA, the DNA-binding screens which identified AIM2 and p202 as regulators of dsDNA-induced cell death did not detect p203. However, one of these screens did identify p204 and p205,

and did not identify p203 (183). The predicted localization of p203 to the nucleus also suggests that p203 may regulate transcription and is unlikely to play a role in the cell death induced by intracellular dsDNA.

*ifi-202* expression was found to be induced by several pathways other than IFN stimulation. We found that stimulation with LPS, Gas6, and Poly I:C could induce *ifi-202* expression at least 200-fold in the absence of Mertk compared to wild-type MΦs. p202 protein was occasionally detectible by Western blot in unstimulated *mertk*<sup>-/-</sup> MΦs and upon IFN stimulation, p202 showed a dose and time-dependent increase in expression (Figure 4.3B). However, p202 protein levels were never detectible by Western blot in wild-type cells. This suggests that Mertk is able to repress p202 despite leverage from positive stimulants.

Interestingly, electroporated calf thymus (CT) DNA was also able to induce p202 protein expression in *mertk*<sup>-/-</sup> MΦs (Figure 4.3C). It is possible that intracellular CT DNA is activating the cells to produce IFN and not directly activating the p202 production; however, the levels of CT DNA-induced p202 were higher than the IFN-induced levels. If CT DNA was inducing IFN, CT DNA would have to induce IFN at levels greater than 1000U/mL. It is also possible that CT DNA may be binding p202 and inducing p202 to trigger signal transduction or act as a transcription factor for its own expression in a positive feedback loop. It makes sense that a cell surviving a viral insult would want to produce more of the protein that allows it to survive.

We also found that p202 levels were only dramatically upregulated in the absence of Mertk. In contrast, *ifi-202* expression was not up-regulated in the absences of the other TAM family members Axl or Tyro3. However, cells lacking all three receptors

were more similar to wild-type than *mertk*<sup>-/-</sup> MΦs in *ifi-202* expression (Figure 4.4A). This suggests that while Mertk is clearly a negative regulator of p202 expression, Axl and/or Tyro3 may be acting as positive regulators of IFN-induced *ifi-202* expression. In fact Axl has been shown previously to interact with the IFN receptor in DC upon Gas6 stimulation (40). It is plausible that Axl-IFNR may be mediating p202 induction upon IFN stimulation, yet this induction does not override the overwhelming dominant repression imparted by an intact Mertk.

The observation that Mertk may be repressing p202 expression is novel; however, the mechanism mediating the suppression is unknown. Stat family of transcription factors are well known to be activated after IFN stimulation. In addition, there is a Stat3 binding site in the *ifi-202* promoter region (191). We have shown that Stat3 but not Stat1 is hyper-phosphorylated in *mertk*<sup>-/-</sup> MΦs (Figure 4.6). In support of our findings, an over-expression system of constitutively active mutated Mertk led to hyper-activation of Stat3 as compared to wild-type Mertk (230). Conversely, in DCs, Stat3 has been shown to be activated by apoptotic cells and Gas6 (86). We did not find Stat3 activation by Gas6 in MΦs (data not shown). It is possible that similar to the differential roles of Mertk in phagocytosis of apoptotic cells by MΦs versus DCs, Mertk may be regulating Stat3 differently in different cell types.

We also show that in *mertk*<sup>-/-</sup> MΦs, IFN stimulation, which produces increased phosphorylation of Stat3 and increased expression of p202, also leads to a decrease in intracellular dsDNA-induced caspase 1 and 3 cleavage (Figure 4.7). Stat3 expression has been associated with cell survival in other cell types and is a concern with oncogenesis (231). Our data suggest that the high levels of activated Stat3 may be assisting activation



of *ifi-202* and this correlates Stat3 function to our observations here regarding cell survival. Moreover, E2F1, which has been shown to inhibit p202 expression and is known to inhibit cell cycle and cell survival, is not over-expressed in *merlk<sup>-/-</sup>* MΦs (193). The E2F1 expression in *merlk<sup>-/-</sup>* MΦs is similar to wild-type MΦs (data not shown). Therefore, the high amounts of Stat3 and the unremarkable expression of E2F1 is consistent with conditions that favor dominance of p202.

Although p202 levels have been shown to be correlated with SLE-like disease in mouse models, the regulation of p202 expression in these SLE models is not understood. These mouse models include MRL<sup>lpr/lpr</sup>, NZB, and B6Nba.2 (148). Because these models produce SLE-like disease due to multi-factoral genetic and environmental components, it would be difficult to determine the regulation of p202 expression. Here we present an SLE mouse model where only *Mertk* is knocked out allowing for identification of all subsequent alterations to be traced back to *Mertk*. These studies provide a link with *Mertk*, p202, and the autoimmune phenotype beyond the association of *Mertk* to phagocytosis of apoptotic cells. *Mertk* is a receptor with multiple effector functions that may be critical for providing check points that prevent autoimmune disease. Furthermore, *Mertk* has been associated with cytokine regulation, such as TNFα, and BAFF, that may be inhibiting or promoting B cell activation (35, 37, 40); therefore, *merlk<sup>-/-</sup>* mice may be an excellent model to define downstream effects of p202 and consequences to autoimmunity. Similarly, it may be interesting to determine if *Mertk* expression levels or function are altered in other SLE mouse models that show increased levels of p202.

This chapter also provides important insight into the autoimmune disease SLE. As SLE patients often have increased type I IFN levels and EBV titers, it is possible that Mertk on DC and MΦs may be a regulator of cell death in response to dsDNA viruses through its control of p202. Thus, a dsDNA virus may infect an APC and depending on the levels or functionality of Mertk and thus p202, the cell may die, or continue to live providing aberrant antigen presentation. If the APC survives and is hyper-activated as indicated by high levels of p202 and other factors such as BAFF (37), then aberrant stimulation of autoreactive T cells or autoreactive B cells may break anergy. Chronic presentation of antigens is especially important in a scenario in which molecular mimicking of self antigens by pathogens such as EBV infection or a scenario found in the *mertk*<sup>-/-</sup> mice where there is an abundance of autoantigens due to a defect in phagocytosis of apoptotic cells. This hyperactivity and perhaps excessive presentation of self antigens or foreign nucleic acids has the potential to jump start autoimmune disease resulting in the autoantibody production found in the *mertk*<sup>-/-</sup> mice and other lupus-prone mice.

**Figure 4.1.** *MΦs lacking Mertk are less susceptible to cytoplasmic DNA-induced cell death.* A) 3 days after calf thymus (CT) DNA electroporation, brightfield pictures were obtained of wild-type and *mertk*<sup>-/-</sup> MΦs. B) 4 hrs post electroporation, Cy3-labeled poly dA:dT and 30 Sytox Green-labeled cells were imaged and C) quantified. D) 30 minutes post electroporation cells were harvested and whole cell lysates were examined by Western blot with the indicated antibodies. Figures are representative of 3 independent experiments.  $p < 0.0001$ .

**Figure 4.2.** *Mertk negatively regulates ifi-202 expression.* Real-time PCR was performed on time course of IFN-treated wild-type and *mertk*<sup>-/-</sup> MΦs. Transcripts of genes analyzed were *ifi-202*, *ifi-203*, *ifi204*, *ifi-205*, *aim2*, and *pyhin-1*. Graphs are representative of at least 2 independent experiments.

**Figure 4.3.** *Mertk negatively regulates ifi-202 mRNA and p202 protein expression in response to a variety of stimulants.* A) Quantitative Real-Time PCR was performed for *ifi-202* expression on wild-type and *mertk*<sup>-/-</sup> MΦs stimulated with 150nM Gas6, 1000U/mL of IFN, 200ng/mL of LPS, and 20μg/mL Poly I:C for 3 hours. B) Whole cell lysates from wild-type and *mertk*<sup>-/-</sup> MΦs stimulated with IFN for 48, 72, and 96 hours were subjected to Western blot analysis for indicated proteins. C) Whole cell lysates from wild-type and *mertk*<sup>-/-</sup> MΦs that were electroporated with 50μg Calf Thymus DNA were subjected to Western blot analysis for the indicated proteins. Figures are representative of at least 2 separate experiments.

**Figure 4.4.** *Axl* and *Tyro3* do not negatively regulate *ifi-202* mRNA and *p202* protein expression. A) MΦs from indicated genotypes were treated with 1000U/mL IFN for 24hrs. mRNA was obtained and subjected to quantitative Real-Time PCR for *ifi-202* expression. B) Macrophages from the indicated TAM genotypes were treated with 1000U/mL of IFN for 48 hours and whole cell lysates were obtained and subjected to Western blot analysis for the indicated proteins. Figures are representative of at least 2 separate experiments.

**Figure 4.5.** *Mertk* regulates *ifi-202* mRNA and *p202* protein expression in bone marrow-derived DC. A) mRNA from wild-type and *mertk*<sup>-/-</sup> bone marrow-derived DC were treated with 1000U/mL of IFN for indicated time periods and subjected to quantitative Real-Time PCR for *ifi-202* expression. B) mRNA from wild-type and *mertk*<sup>-/-</sup> bone marrow-derived DC were treated with 1000U/mL of IFN, 200ng/mL of LPS, or 10ng/mL of IL-6 and subjected to quantitative Real-Time PCR for *ifi-202* expression. C) Whole cell lysates were obtained from wild-type and *mertk*<sup>-/-</sup> bone marrow-derived DC left untreated or treated with 1000U/mL of IFN for 48 hours. Lysates were then subjected to Western blot analysis for indicated proteins. Figures are representative of at least 2 separate experiments.

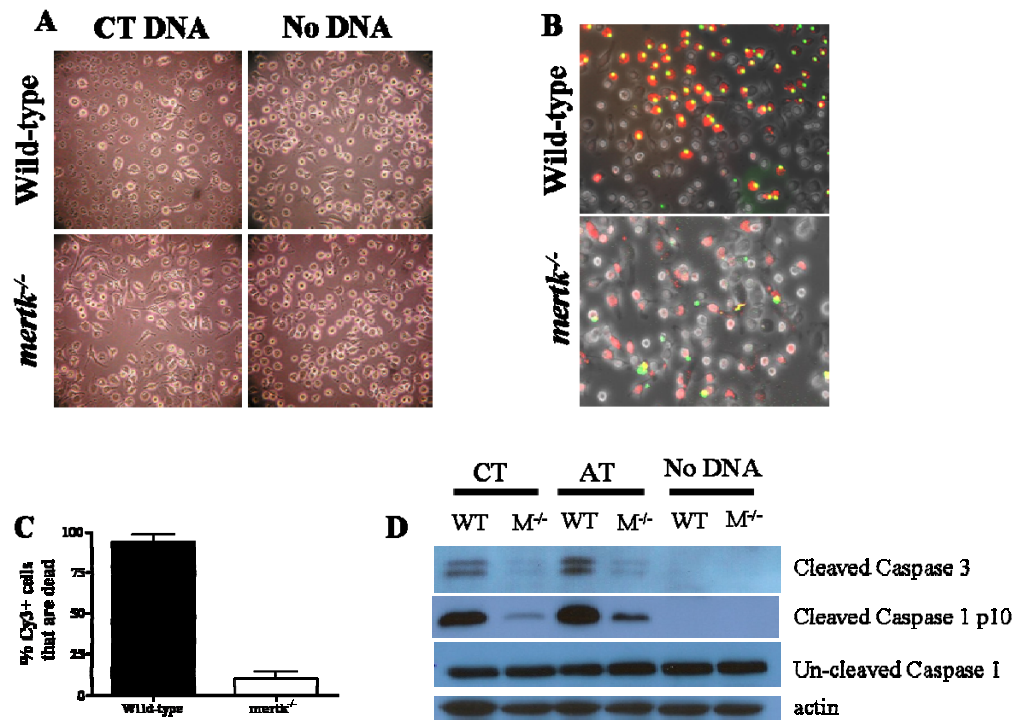
**Figure 4.6.** *Mertk* suppresses IFN-stimulated *Stat3* phosphorylation. A) MΦs were treated with 1000U/mL of IFN for 15, 30 and 60 minutes. Whole cell lysates were subjected to Western blot analysis for pStat3 and total Stat3. B) MΦs were treated with 500, 1000, or 2000U/mL of IFN for 30 minutes. Whole cell lysates were subjected to

Western blot analyses for pStat3 and total Stat3. C) Whole cell lysates treated as in A were subjected to Western blot analysis for pStat1 and total Stat1. Blots are representative of at least 3 separate experiments.

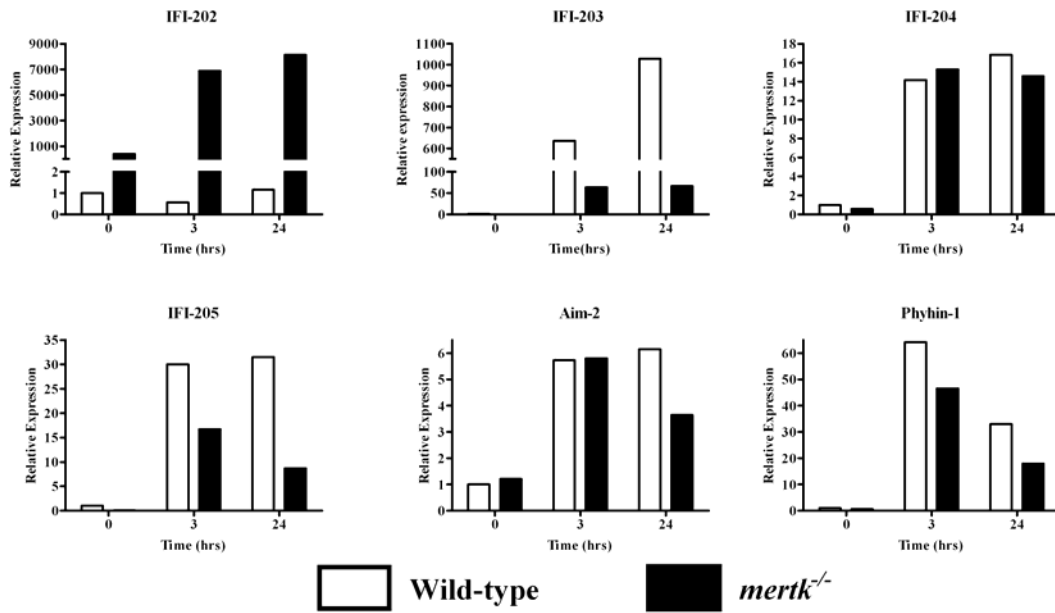
**Figure 4.7.** *IFN stimulation increases p202 expression and decreases response to cytoplasmic DNA in  $mer1k^{-/-}$  cells.* A) MΦs were either treated with IFN for 72 hrs or left untreated prior to Calf Thymus (CT) or poly dAdT (AT) electroporation. Whole cell lysates were obtained 30 minutes after electroporation and subject to Western blot analysis for the indicated proteins. Note that different exposure times were required for uncleaved caspase 1 (p45) and cleaved caspase 1(p10). B) Whole cell lysates from A were subjected to Western blot analysis for the indicated proteins. Figures are representative of 5 separate experiments.

**Figure 4.8.** *Schematic of AIM2 and p202 function in the context of  $Merk$ .* A) In wild-type cells AIM2 binds cytoplasmic dsDNA, interacts with ASC and forms the inflammasome to induce Caspase 1 and 3 mediated cell death. p202 is at low levels, suppressed by an unknown mechanism. B) In cells from autoimmune mice, p202 and AIM2 are present. p202 competes for binding of cytoplasmic dsDNA with AIM2. Binding of p202 to dsDNA prevents inflammasome activation and subsequent cell death. C) In  $mer1k^{-/-}$  cells is absent, levels of p202 are increased via overactivation of Stat3. Thus dsDNA induced capsase cleavage and cell death are prevented. The amount of p202 dictates the prevention of cell death.

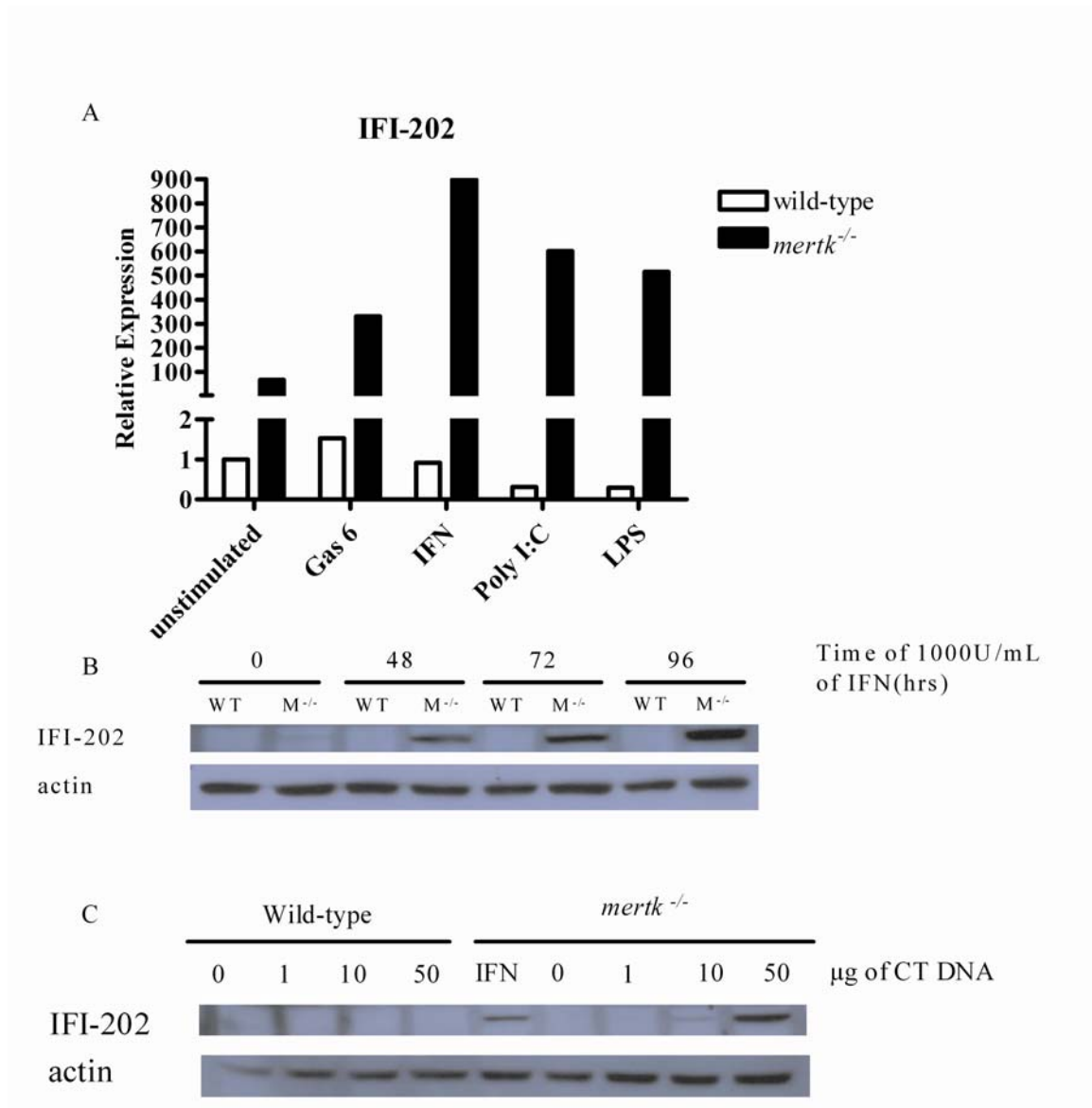
# Figure 4.1



# Figure 4.2

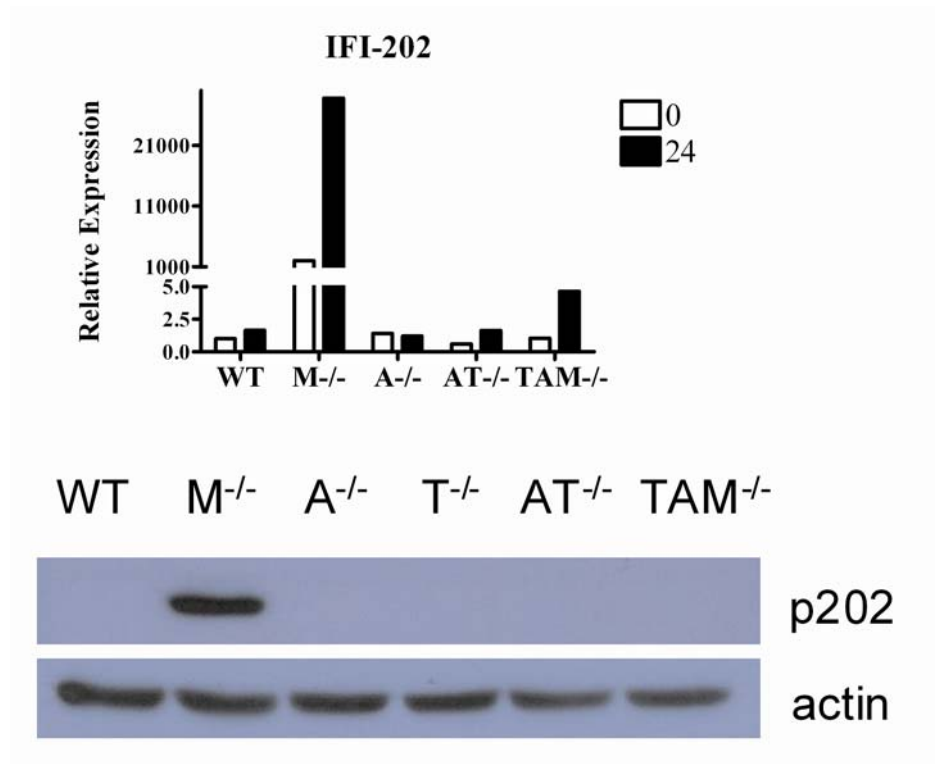


# Figure 4.3

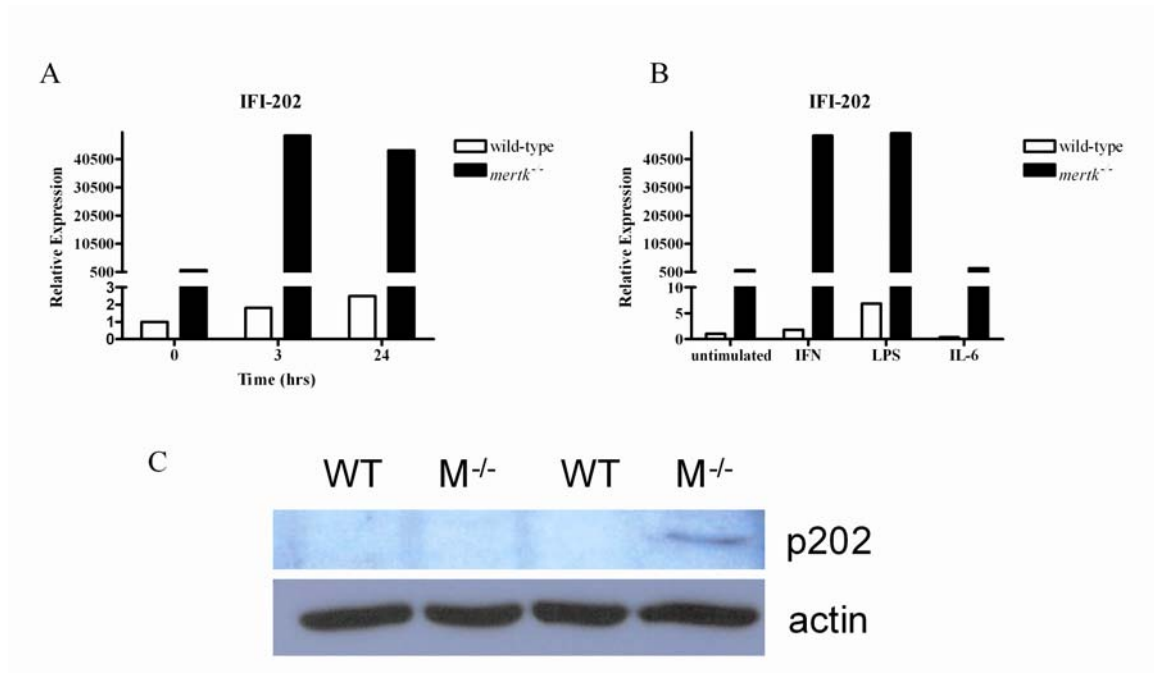




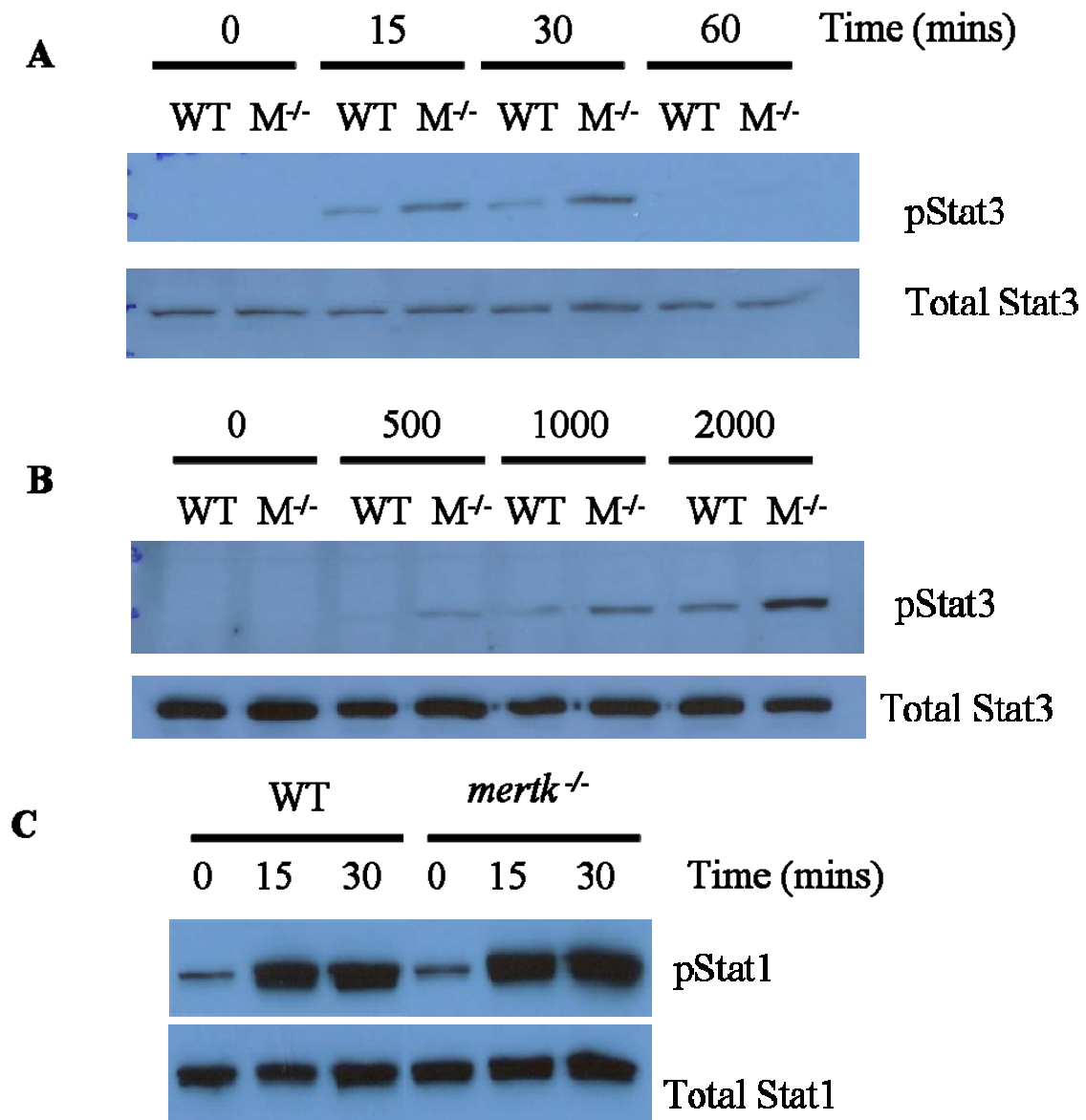
**Figure 4.4**



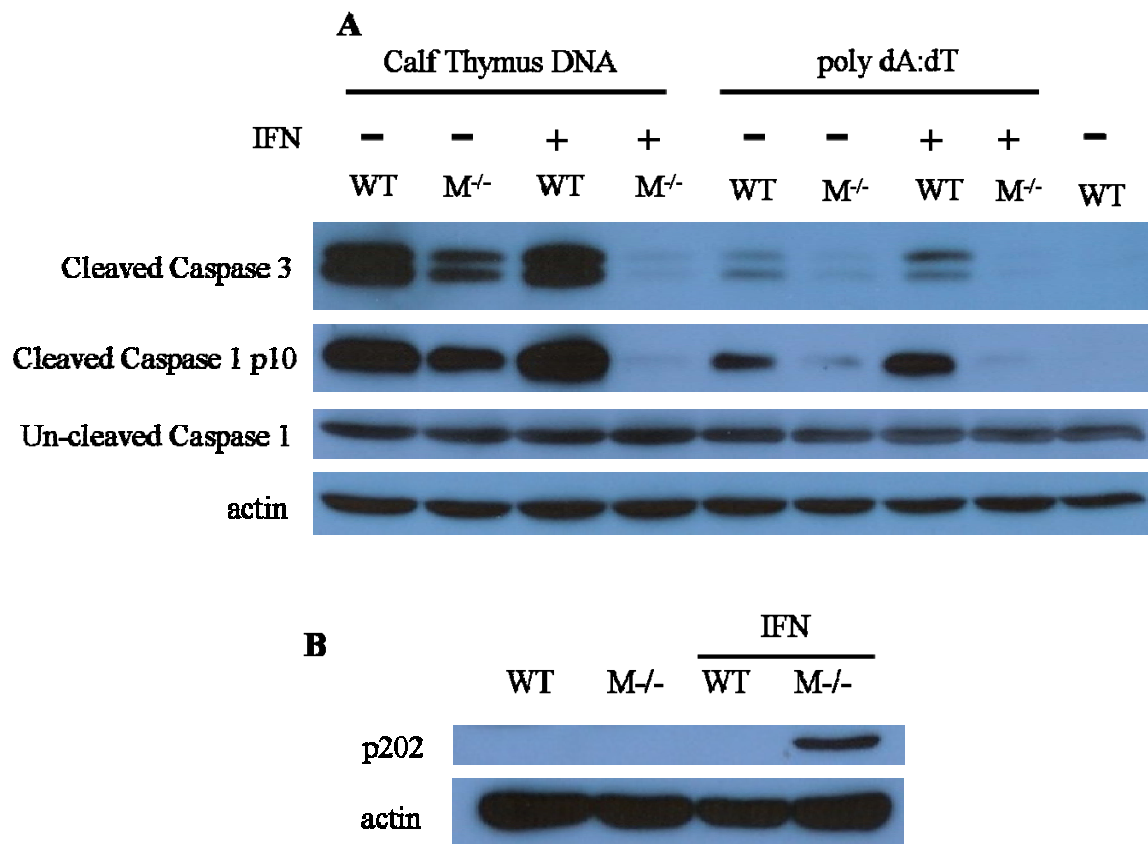
**Figure 4.5**



**Figure 4.6**

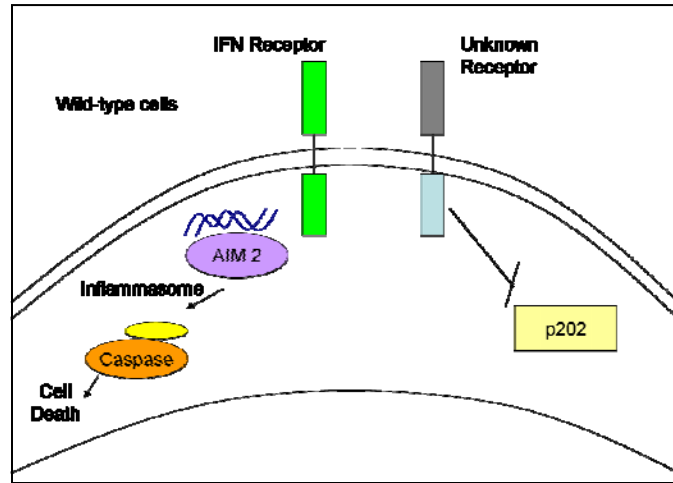


# Figure 4.7

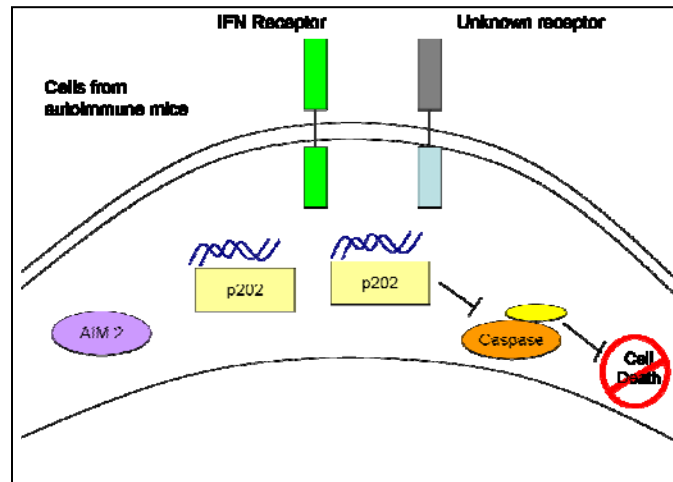


**Figure 4.8**

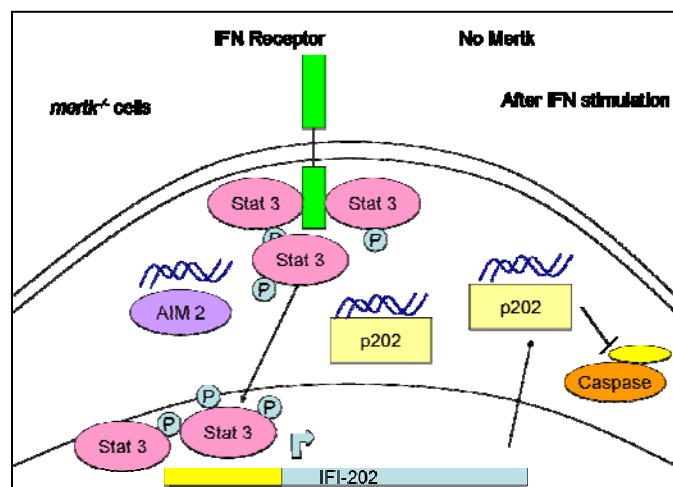
**A**



**B**



**C**



## **Chapter 5:**

### **Summary and Future Directions**

The studies undertaken here have greatly expanded our understanding of the role of TAM receptors on innate immune cells. This dissertation focuses on three major areas: innate immunity to bacteria, peritoneal cells in autoimmunity, and innate immune responses to viral cell death cues. These multiple facets are partly tied together by the fact that autoimmune disease may be triggered by pathogens, that dysregulation of innate immune cells may be contributing to immune deviation in the peritoneal cavity, and that proper cell death may be important in restricting APC stimulation of autoreactive lymphocytes. The hypothesis is that Mertk or Axl or Tyro3 may be central regulators in these processes and sought to answer these important questions. Mertk is likely the critical negative regulator that restricts the autoimmune phenotype. Thus, the studies here expand the role of TAM receptors in regulating immune populations and have provided insights to the autoimmune phenotype primarily controlled by Mertk.

### **The function of TAM receptors with regards to bacterial infection.**

Previous reports regarding the role of TAM receptors in clearing bacteria are controversial (28, 43). Scott et. al. do not see differences in ingestion of Gram-positive *Listeria monocytogenes* by macrophages from *mertk*<sup>-/-</sup> mice; however, *Escherichia coli* particles are phagocytized in greater numbers when TAM receptors are absent (28, 43). Chapter 2 conclusively demonstrates that phagocytosis and killing of Gram-negative and Gram-positive bacteria was not affected by the TAM family members. This is observed using multiple experimental protocols and definitively shows that the TAM receptors are not involved in phagocytosis of bacteria.

Although TAM receptors are not involved in phagocytosis or killing of bacteria, we speculate that they may be involved in clearance of systemic bacterial infections. Evidence to support this hypothesis includes both MΦs and mice lacking *Mertk* produce excess pro-inflammatory cytokines in response to bacterial byproducts (35). Thus, it would be interesting to determine if *mertk*<sup>-/-</sup> mice are less susceptible to bacterial infections due to the local and systemic increase in pro-inflammatory cytokines. One way a systemic increase in pro-inflammatory cytokines and chemokines would result in better clearance of an infection is by recruiting more cells in response to the infection. In addition to more cells in the area, pro-inflammatory cytokines can also induce other genes or anti-bacterial effects which may allow for better killing of cells. For example, TNFα can prime neutrophils to produce NADPH-oxidase, a potent anti-bacterial response (232). Perhaps NADPH-oxidase production by neutrophils in the *mertk*<sup>-/-</sup> mouse is elevated due to increased TNFα. Since the absence of *Mertk* does not affect killing or division of Gram-negative bacteria, production of pro-inflammatory cytokines is independent of the MΦ's ability to kill bacteria. Therefore, if *mertk*<sup>-/-</sup> mice are less susceptible to bacterial infection, it is most likely due to cooperation of the entire immune system rather than the ability of individual cells to kill bacteria.

The increase in production of pro-inflammatory cytokines by the *mertk*<sup>-/-</sup> MΦs may indirectly affect the ability of another cell, perhaps the neutrophil, to be better at killing bacteria. This brings up the idea of the age old question of inflammation “friend or foe”? A certain amount of inflammation in response to pathogens is necessary for a proper immune response (inflammation as a friend). However under certain circumstances, the inflammatory response is actually more detrimental than the



pathogenic insult, which can result in exacerbated pathology or in more severe circumstances, endotoxic shock. Furthermore, chronic stimulation of the immune cells may lead to inflammatory diseases or autoimmune disease (inflammation as a foe). Our lab previously shows that mice lacking *Mertk* are more susceptible to LPS-induced endotoxic shock and that this is prevented by anti-TNF $\alpha$  antibody administration (35). Under physiologic conditions, the amount of LPS produced by an infectious agent is much lower than experimentally administrated levels. Therefore it is conceivable that the increased pro-inflammatory response by *mertk*<sup>-/-</sup> mice may actually aid in the systemic response as opposed causing the animal to go into shock. Since *Mertk* is expressed on fibroblasts and other non-hematopoietic cells, the possibility of the resident infected cell also producing excess pro-inflammatory responses must also be considered in the resolution of infections.

Our lab also shows that mice lacking *Mertk* have increased intestinal inflammation upon LPS administration (35). These mice do not have spontaneous intestinal inflammation, thus this is an example of how an improper inflammatory response could lead to inflammatory bowel disease. Interestingly one of the top treatments for many patients with inflammatory bowel diseases such as Crohn's disease and Ulcerative Colitis is a humanized mouse anti-human TNF $\alpha$  antibody, Infliximab (87). This antibody blocks TNF $\alpha$  in the serum and on the cell surface, and can leave patients in remission for up to a year.

Another potential treatment for inflammatory diseases may be through the activation of *Mertk* to prevent pro-inflammatory cytokine production. Since *Mertk* is known to suppress inflammation, perhaps the activation of this receptor with antibodies

or Gas6 would aid in resolution of excess inflammation. In support of this, administration of apoptotic cells has been shown to protect mice from LPS-induced endotoxic shock (233). However, the exact mechanism of this protection is not known. Apoptotic cells have been shown to have Gas6 pre-bound to phosphatidylserine on their surface (19). Therefore, it is possible that the protection afforded by apoptotic cells maybe through the activation of Mertk.

### **Insights regarding the role of TAM receptors in the peritoneal immune populations.**

The role of the peritoneal cavity and innate immune cells in autoimmunity is not clear. The TAM receptors are implicated in autoimmunity due to their role in clearing apoptotic cells and the production of autoantibodies (18, 28, 29, 37, 43). In Chapter 3, we explore whether the TAM receptors dictated resident cells in the peritoneal cavity. In summary, Chapter 3 provides data characterizing the MΦ, T cell, B cell, and DC populations in both wild-type and *merlk*<sup>-/-</sup> mice. While the total number of peritoneal cells increases over time in wild-type animals (Figure 3.1), the *merlk*<sup>-/-</sup> animals have the greatest number of peritoneal cells at 3 months of age. At 3 months old, *merlk*<sup>-/-</sup> mice have 4-fold more cells in the peritoneal cavity than wild-type mice, and this is due to migration and not local division or cell death (Figure 3.4, 3.5, 3.6). In addition the migration of the T cell populations appears to be due to hematopoietic-derived factors (Figure 3.7). The overall increase in cells could be due to overproduction of CXCL9 by *merlk*<sup>-/-</sup> peritoneal cells or a greater number of cells expressing IL-7R or the receptor for CXCL9, CXCR3 (Figure 3.8). In addition, the increased number of cells in the peritoneal cavity was exclusive to *merlk*<sup>-/-</sup> mice, as *axl*<sup>-/-</sup>, *tyro3*<sup>-/-</sup>, or *axl*<sup>-/-</sup>/*tyro3*<sup>-/-</sup> mice had similar

numbers to wild-type mice (Figure 3.9). This increase also correlates to autoantibody production later in life and again was only found in *merck*<sup>-/-</sup> mice and not *axl*<sup>-/-</sup> or *tyro3*<sup>-/-</sup> mice.

There are B1 B cells in the peritoneal cavity that contribute to natural immunity and an earlier study suggests the peritoneal cavity is critical for the induction of autoantibody formation (168). Other studies that observe peritoneal B1 cells usually focus on their antibody production roles *ex-vivo* or in relationship to the marginal zone B cell characteristics. However, studies regarding what cells are important in the induction of autoimmunity and how this occurs in the peritoneal cavity are sparse. Our studies in Chapter 3 characterizing immune cells in the peritoneal cavity provide substantial insights on the dynamics of the resident peritoneal cell populations in lupus-prone *merck*<sup>-/-</sup> mice versus normal wild-type mice. To my knowledge, there has never been a thorough characterization of the cells found in the peritoneal cavity. In relationship to SLE, virtually all of the publications describing cells in the peritoneal cavity focus on macrophages and B1 cells.

The data presented in Chapter 3 poses many other questions to be answered with future experiments. First, hypotonic ablation of B1 cells from the peritoneal cavity of NZB and NZB/W F1 lupus-prone mice resulted in less disease (168). It would be extremely interesting to investigate if hypotonic ablation of B1 cells would decrease autoantibody production in the *merck*<sup>-/-</sup> mice as well. Given that both *merck*<sup>-/-</sup> and NZB mice also have an increase in peritoneal cells prior to the onset of autoantibody production, it would not be surprising if hypotonic ablation of these cells leads to similar reduction in autoantibody formation in both strains (234). Thus, if *merck*<sup>-/-</sup> mice act

similarly to NZB, without the B1 cells, we expect a decrease in the amount of autoantibody produced and/or number of mice that were making detectible autoantibodies. This also brings up an interesting option for treatment of SLE. If it were possible to selectively delete the B1 population from the peritoneal cavity, it may also be possible to decrease human disease.

It is also important to consider the role of B1 cells as APC. Peritoneal B1 cells express MHC II and are more efficient than peritoneal MΦ when acting as APC to T cells in response to CpG oligonucleotide stimulation (235). This study shows that B1 cells were efficient at processing and presenting antigen to T cells. These B1 cells also produce IL-10 and possess high levels of CD86 and MHC II (235). The idea of the B1 cell acting as an APC is often overlooked. However, given increases in B1 cells in murine models of SLE, it is possible that these cells may be acting as more than just low affinity autoantibody-producing cells. This could be addressed using a transgenic cells, stimulating B1 cells with antigen (OVA or HEL) and determining activation response of T cells.

With regard to macrophages, the previous studies usually remove the cells as a method of easily harvesting resident macrophages for *ex-vivo* experiments. There have been few reports examining the function of peritoneal macrophages in the context of inducing autoreactive B cells. One could deplete macrophages from wild-type mice and *mertk*<sup>-/-</sup> mice and determine whether there is a shift in the cell populations within the peritoneal cavity and if autoreactive B cells are diminished.

Surprisingly, the literature is particularly deficient in peritoneal T cell data, though it has been acknowledged that both B1 cells and T cells contribute to SLE, and

the peritoneal cavity is a location that harbors T cells and the majority of the B1 cells in the body. Thus, the abundance of T cells in the peritoneal cavity of the *mer1k*<sup>-/-</sup> mouse provides greater opportunity for T cell help to autoreactive B cells. Chapter 3 shows that T cells are being recruited to the peritoneal cavity by a hematopoietic-derived factor (Figure 3.7). Interestingly, bone-marrow transplant has been used as a therapy for catastrophic cases of SLE (236), however, as the disease is not normally immediately life threatening, both doctors and patients are reluctant to pursue this option. In addition, the majority of cells in the peritoneal cavity expressing CXCR3 and IL-7R were T cells. In order to determine if peritoneal T cells play a role autoantibody production in the *mer1k*<sup>-/-</sup> mice, it would be possible to remove T cells from the peritoneal cavity with repeated I.P. injections of anti-CD3 antibody and then measure autoantibody production.

Chapter 3 attempts to investigate the mechanism behind the increase in cells found in the *mer1k*<sup>-/-</sup> peritoneal cavity by identifying increased CXCL9, CXCR3 and IL7-R expression (Figure 3.8). First, it would be interesting to determine if other SLE mouse models had increased expression of these molecules. As NZB mice appear to follow a similar peritoneal infiltration and autoantibody time courses, investigation of these molecules would be especially interesting. In addition, analysis of the *mer1k*<sup>-/-</sup> peritoneal cavity after blockade of these molecules or crossing with knock out mice would determine definitively if these molecules are indeed involved in the increased migration to the peritoneal cavity. CXCR3<sup>-/-</sup> and IL-7R<sup>-/-</sup> mice are viable, however CXCL9<sup>-/-</sup> mice have not been produced. In addition, antibodies to all of the molecules are available commercially,

The PCR-based screen of potential receptors expressed by peritoneal cells in Chapter 3 highlights IL-7R. IL-7R knock-out mice have reduced peritoneal B cell numbers (219). As shown in Figure 3.8, we find an increase in the number of IL-7R-expressing peritoneal T cells as well as B cells; however, the number of peritoneal T cells found in the *il-7r<sup>-/-</sup>* mouse is not known. It would be important to determine if the peritoneal T cell levels are also low in mice lacking the IL-7R. A low level of T cells would suggest IL-7R maybe important in attracting or retaining T cells in the peritoneal cavity and if crossed to *mertk<sup>-/-</sup>* mice, autoantibody assessment might link these IL-7R<sup>+</sup> T cells as important components. Interestingly, mice lacking the known IL-7R ligand, IL-7, do not have decreased peritoneal B cell numbers (220). Thus, there is most likely another, as yet unidentified, ligand for the IL-7R. It is therefore possible that the unidentified ligand may be upregulated in the *mertk<sup>-/-</sup>* peritoneal cavity. Although this ligand is currently unknown, it may be possible to identify it through yeast two-hybrid studies. Currently, a role for IL-7R in SLE pathology has not been elucidated; however, our data suggests that elevated levels of IL-7R may correlate with disease.

Mice lacking CXCR3 and CXCL9 have been generated; however, the composition of the peritoneal cavity of these mice has not been investigated. Interestingly, CXCL9 has been implicated in the progression of immune-mediated kidney disease (237). Both CXCL9 and CXCL10, ligands for CXCR3, have been shown to be elevated in plasma from SLE patients and this correlated to disease index (152). In addition, MRL/lpr mice lacking CXCR3 have reduced kidney pathology and decreased T cell recruitment to the site of injury (166). It would be interesting to determine if these mice also had fewer cells in the peritoneal cavity and less autoantibody production.

Since, the CXCL9/CXCR3 chemokine-receptor combination is involved in recruitment of T cells and kidney pathology progression in SLE; it is possible that these molecules may also be involved in the migration of cells to the peritoneal cavity in SLE. Either way, blockade CXCL9 and/or CXCR3 is an attractive target for SLE therapies, whether to prevent onset by preventing migration to the peritoneal cavity, or preventing subsequent kidney pathology. Thus, a number of directions can be pursued based on work in this Chapter 3. The most timely and urgent is to determine the function of these cells and whether elimination or inhibition of a particular cell type abrogates autoimmune disease.

### **Role of Mertk and apoptosis of APCs**

Lastly, we show that Mertk is central for MΦs to ingest apoptotic cells; however, the death of these macrophages themselves is not known and studies show macrophages are critical in the formation of autoimmunity. In summary, Chapter 4 presents a set of experiments showing MΦ lacking Mertk are more susceptible to intracellular dsDNA-induced cell death. The mechanism behind this cell death appears to be due to an increase in p202 production (Figure 4.2, 4.3). p202 can bind dsDNA and prevent activation of the inflammasome and thus prevents subsequent caspase cleavage and cell death (189). MΦ lacking Mertk upregulate p202 in response to a variety of stimulants (Figure 4.3). DC lacking Mertk also produce greater amounts of p202 compared to wild-type, and this suggests other cell types which express Mertk may also be affected similarly. In addition, IFN stimulation to increase p202 levels decreases the amount of caspase cleavage found in MΦ lacking Mertk (Figure 4.6).

Chapter 4 exclusively uses electroporation as a method of transfecting the dsDNA into the cytoplasm. It would be interesting to determine if a dsDNA virus would act similarly to the dsDNA electroporation. Hornung et. al. showed using cells lacking AIM2 that were exposed vaccinia virus were less susceptible to cell death (185). Given the AIM2/p202 relationship proposed by Roberts et. al., it makes sense that when AIM2, which normally activates the inflammasome, is removed, p202 will be the major binder of dsDNA and prevent inflammasome activation (189). Thus, we would expect a similar outcome with the *mer1k*<sup>-/-</sup> MΦ, an infection with vaccinia virus would indeed be comparable to the electroporated dsDNA. In fact, given that viruses induce type I IFN responses by MΦ with more than just their nucleic acid, and IFN increases the amount of p202, it is possible that *mer1k*<sup>-/-</sup> MΦ treated with vaccinia virus are even less susceptible to cell death than dsDNA alone.

Along these lines, it would be important to investigate the infection of *mer1k*<sup>-/-</sup> mice with a dsDNA virus to examine systemic affects. In this case, there are multiple possible outcomes. Since the levels of p202 are dramatically higher in *mer1k*<sup>-/-</sup> MΦ, we would expect that *mer1k*<sup>-/-</sup> mice would have a greater number of viable APCs during dsDNA virus infection. The increased APC life span presents two scenarios. First, increased APC viability could result in greater production of viral particles compared to wild-type macrophages. In this case, the inability of the *mer1k*<sup>-/-</sup> cell to induce apoptosis would be detrimental to the animal. Second, an increase in APC life span would also result in greater antigen presentation to lymphocytes leading to greater activation of the adaptive immune response.

Which scenario is more important would depend on the particular virus.



If a virus replicates preferentially in APCs, increased *mertk*<sup>-/-</sup> APCs would serve as viral production factories, increasing viral load and facilitating viral spread. Thus, the virus would be more likely to kill the host, a clear disadvantage in host survival. However, if the APC is not the preferred cell for viral replication, then the increase in live APC and activation of adaptive immune response would be an advantage for the host.

Another caveat to consider when predicting the outcome of a viral infection in the *mertk*<sup>-/-</sup> mice, is whether APCs and non-APCs have similar death responses to dsDNA. This would be particularly important if the virus preferred replication in a non-APC. As the expression of p202 in *mertk*<sup>-/-</sup> non-APCs is not known, it is difficult to predict the outcome of an infection of non-APCs. Thus, it would be important to investigate the expression of p202 on non-APCs, especially those cells which normally express Mertk, including those of the epithelial and reproductive tissues.

The relationship of the HIN-200 family members to SLE is another exciting avenue of investigation. The data in Chapter 4 reinforces the correlation between the expression of p202 and SLE mouse models. It is also relevant that SLE patients have increased EBV titers (87, 88) and increased levels of the antiviral cytokine IFN $\alpha$  (142-144), which is a known inducer of the HIN-200 family of proteins. The serum levels of Type I IFNs in the *mertk*<sup>-/-</sup> mice have not been investigated. However, we have preliminary data showing M $\Phi$  from *mertk*<sup>-/-</sup> mice do not have elevated IFN under stimulated or unstimulated conditions. Currently there is no human homologue identified for p202, thus in humans the AIM2/p202 ratio may not be a relevant explanation of intracellular dsDNA-induced cell death. It would be quite interesting to

know if the levels of the HIN-200 family members are indeed altered in SLE patients and if this correlates to intracellular dsDNA-induced cell death.

Further investigation into the signaling pathway activating and suppressing p202 is also required. Chapter 4 shows that in wild-type MΦ, Mertk normally suppresses the expression of p202 (Figure 4.2, 4.3), thus allowing for intracellular dsDNA-induced cell death to proceed. However, the exact mechanism of this suppression is not yet fully understood. There is a known Stat3-binding element in the p202 promoter (191). Data from our lab suggests that in MΦ, Mertk may be normally suppressing Stat3 phosphorylation and activation in response to IFN stimulation. In contrast, the Tisch lab has shown that Mertk activates Stat3 in response to AC or Gas6 stimulation (86). It has previously been shown that Mertk is used differently by different cell types (18), and thus it is possible that the use of Mertk to activate or suppress Stat3 may also be different in different cell types.

The discovery of the AIM2/p202 dsDNA-binding paradigm is a relatively new addition to the literature. Our current understanding is based on four recently published papers, three of which focus on AIM2 with no mention of p202, and one which focuses on p202 and peripherally investigates AIM2. Some unanswered questions arise based on this literature and the work presented in Chapter 4. First, the cleavage and activation of caspase 3 has not been attributed to the inflammasome. Caspase 3 is known to be activated by caspase9 via a complex termed the apoptosome. The apoptosome is comprised cytochrome c, APAF1 and caspase 9, which in turn cleaves caspase 3 (238). APAF contains a CARD domain, however there are no known PYRIN domains in the apoptosome complex. None the less, perhaps AIM2 may also be binding and activating

the apoptosome through an adaptor molecule. Another possibility is that activation of caspase 1 may be required for the activation of caspase 3. It is known that the inflammasome comprised of ASC and one of a number PYRIN domain containing proteins activates caspase 1 through a cleavage event. However, the method of dsDNA-induced caspase 3 cleavage remains to be elucidated.

The literature describes inflammasome and caspase 1-dependent cell death as pyroptosis. Pyroptosis is unique from apoptosis and is thought of as an inflammatory host response, which is independent of caspase 3, 6, and 8 (239). Loss of mitochondrial integrity and cytochrome c release which are characteristics of apoptosis also do not occur in pyroptosis (240, 241). Pyroptosis is also characterized by loss of osmotic integrity resulting in cellular swelling and eventual plasma membrane rupture and spillage of inflammatory contents (239). A similar swelling of the plasma membrane is observed shortly after dsDNA electroporation (unpublished observations). The intracellular dsDNA-induced cell death appears has characteristics of both apoptosis (caspase 3 cleavage) and pyroptosis (caspase 1 cleavage). Clearly more investigation into the differences and similarities of these two processes is warranted.

### **Final thoughts**

In conclusion, this work advances the understanding of the function of MERTK on innate immune cells and whether these processes can be tied to the autoimmune phenotype displayed by mice lacking MERTK. We find here that MERTK is not essential for the phagocytosis of bacteria, but it appears to be central in regulation of peritoneal cell migration and autoantibody production, and MERTK is a critical regulator of intracellular

dsDNA-induced cell death. The later two observations are novel and add to the growing list of processes associated with the multi-functional Mertk receptor. While these topics are quite diverse, they each have valuable contributions to the role of Mertk. This work provides evidence that a correlation between expression and function of Mertk in other lupus models and SLE patients is warranted and may allow for the identification of novel drug targets.

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