The Pennsylvania State University

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THE INFLAMMATORY CONTROL VALVE: MODULATION OF RESPONSES TO LPS AND IFN-γ BY THE RON RECEPTOR TYROSINE KINASE *IN VITRO* AND *IN VIVO*

A Thesis in

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ABSTRACT

We have shown previously that activation of the *receptor d-origine nantais* (RON) receptor tyrosine kinase by macrophage stimulating protein (MSP) inhibits macrophage production of nitric oxide (NO) induced by interferon-gamma (IFN-γ) and lipopolysaccharide (LPS), *in vitro*, through the inhibition of inducible nitric oxide synthase (iNOS) expression. $RON^{-/-}$ mice exhibit elevated delayed-type hypersensitivity (DTH) responses and increased susceptibility to endotoxic shock *in vivo*. Here, we demonstrate that treatment of primary peritoneal macrophages with MSP followed by IFN-γ and LPS inhibits the production of interleukin-12 (IL-12) through the inhibition of IL-12p40 expression. IL-10 also inhibits IL-12p40 expression as part of a negative feedback response through the induction of signal transducer and activator of transcription 3 (STAT3) phosphorylation and suppressor of cytokine signaling (SOCS) gene expression, we have shown that the inhibition of IL-12 by MSP occurs independently of IL-10.

LPS-induced IL-12 production by macrophages initiates the production of IFN-γ by NK, NKT and memory T cells during an innate immune response which forms a positive feedback loop by enhancing the production of IL-12 by activated macrophages. Our data demonstrate that splenocytes from RON knockout mice express elevated levels of IL-12p40 within three hours following LPS administration when compared with control animals. Furthermore, we observe enhanced IFN-γ levels in the serum of these

animals at six and twelve hours following injection of endotoxin. By crossing RON knockout mice with IFN-γR knockout animals, we show that the enhanced susceptibility of RON knockout mice to endotoxemia is mediated, at least in part, through IFN-γ. Taken together, these data suggest that RON regulates IFN-γ production *in vivo* through its ability to regulate IL-12p40 expression in response to LPS, and that the de-regulation of IFN-γ production contributes to the increased sensitivity of RON knockout mice to septic shock.

IFN-γ secreted during the innate immune response supports the development of Th1 cells by up-regulating major histo-compatibility complex (MHC) class II expression and subsequent antigen presentation. Furthermore, enhanced IL-12 secretion by macrophages in response to IFN-γ results in a further elevation of IFN-γ production during the acquired phase of the immune response by promoting Th1 differentiation. Data described herein demonstrate that, in addition to regulating IFN-γ production *in vivo,* MSP inhibits IFN-γ-mediated responses in macrophages including expression of class II *trans*-activator (CIITA), resulting in decreased MHC class II expression. Additionally, MSP stimulation of primary peritoneal macrophages induces the expression of SOCS1 and 3, and reduces cell surface expression of the IFN-γ receptor (IFN-γR) in a proteasome-dependent manner. However, unlike IL-10 which also induces SOCS expression in macrophages, MSP does not result in the up-regulation of STAT3 phosphorylation in these cells. These data indicate that MSP activation of the RON

receptor negatively regulates IFN-γ-induced gene expression in macrophages via a signaling pathway that is distinct from IL-10 and STAT3.

In the initial stages of an immune response, LPS induces the production of IFN-β by activated macrophages via a MyD88-independent signaling pathway which feeds back to cooperate with LPS in the induction of a subset of LPS-responsive genes, which are also dependent on IFN-induced STAT1 phosphorylation. This occurs 2-4 hours following LPS stimulation resulting in a delayed induction of the expression of this subset of genes, suggesting that this pathway may be a physiologically relevant target of RON signaling. Here we demonstrate that MSP inhibits LPS-induced IL-12p40 production, even in the absence of IFN-γ priming. However we find that MSP specifically inhibits IFN-γ-induced STAT1 tyrosine phosphorylation, but not LPS-induced STAT1 activation. Furthermore, we demonstrate that LPS-induced IRF3 phosphorylation and IFN-β expression are not diminished in the presence of MSP. Based on our previous studies demonstrating that RON inhibits LPS-induced NFκB activation, we conclude that RON targets TLR4 signaling via the MyD88-dependent, but not the MyD88-independent, pathway.

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LIST OF ABBREVIATIONS

Ab……...…….Antibody

APC……Antigen Presenting Cells

ARGI…….Arginase I

ARGII……..Arginase II

BTK……..……Bruton's Tyrosine Kinase

CFU…….Colony Forming Unit

CFU-G……...CFU-Granulocyte

CFU-GM…….….CFU-Granulocyte/Macrophage

CFU-M……..CFU-Macrophage

CSF1…….…….Colony-stimulating factor 1

CSF1R……...….Colony-stimulating factor 1 Receptor

CIITA……...……Class II *Trans*-activator

COX2……Cyclooxygenase 2

ELISA……...Enzyme-Linked Immunosorbet Assay

ERK……..Extracellular Response Kinase

FBS……Fetal Bovine Serum

FITC……Fluorescein Isothiocyanate

GAB……..GRB2 Associated Binder

GAS…….Gamma Activating Sequence

γδT cells……...…….gamma-delta T cells

GAF……gamma-IFN Activated Factor

GM-CSF……Granulocyte Macrophage-Colony Stimulating Factor

GRB2……..……...……..Growth-Factor-Receptor Bound Protein 2

HGF…….…Hepatocyte Growth Factor

HGFL…….....…….Hepatocyte Growth Factor-Like

ICSBP……Interferon Consensus Sequence Binding Protein

IFN-α…….……..Interferon-alpha

IFN-β……..Interferon-beta

IFN-γ…….Interferon-gamma

IL-1……Interleukin 1

IL-1RA……Interleukin 1 Receptor Antagonist

IL-10……....Interleukin 10

IL-6……....Interleukin 6

IL-12……....Interleukin 12

IL-2……....Interleukin 2

iNOS…….Induciable Nitric Oxide Synthase

IRF1…….…Interferon Response Factor 1

IRF2…….…Interferon Response Factor 2

IRF3…….Interferon Response Factor 3

JAK…….*Janus* Activating Kinase

JNK……....*Jun* N-terminal Kinase

LPS…….…Lipopolysaccharide

MAL…….……..MyD88 Adaptor-Like

MAPK…….Mitogen Activated Protein Kinase

M-CSF……Macrophage Colony Stimulating Factor

MEK……..Mitogen Activated ERK-activating Kinase

MHC…….….Major Histocompatibility Complex

mRON…….. murine RON

MSP……...Macrophage Stimulating Protein

MyD88……Myeloid Differentiation Primary Response Gene 88

NCS…….….Newborn Calf Serum

NF-κB……..Nuclear Factor-kappa B

NK cells……...Natural Killer cells

NO……..Nitric Oxide

TNFα……..….Tumor Necrosis Factor alpha

PBS……..Phosphate Buffered Saline

PCR……Polymerase Chain Reaction

PI3K……Phosphatidylinsitol-3-Kinase

PIAS……....Protein Inhibitor of Activated STAT

PP1……..... Protein Phosphatase-1

RT-PCR…….Reverse Transcription-PCR

RON……...……..*Recepteur d-Origine Nantais*

S……..….Serine

SH……...Src Homology

SOCS1……..Suppressor of Cytokine Signaling 1

SOCS3……..……Suppressor of Cytokine Signaling 3

STK……...……Stem-cell Derived Tyrosine Kinase

Th1……T-helper 1

Th2……....T-helper 2

Y…….....……Tyrosine

vs……....…Versus

WT……...….Wild-Type

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Chapter 1

Literature Review

1.1 Macrophages

"Big eaters", better known as macrophages, are a diverse lineage of phagocytic cells of hematopoetic origin. In the late 19th century Ilia Metchnikokk performed two experiments in starfish larva and *Daphnia*. In these studies he observed phagocyte migration, attachment and microbial elimination by what became known as macrophages. The earliest sources of macrophages during development are the yolk sac and fetal liver [1]. In the yolk sac, cells with macrophage morphology were identified as macrophages by the expression of markers such as cFMS/ colony-stimulating factor 1 receptor (CSF1R), CD11b and the mannose receptor (MR) [2]. As development progresses the fetal liver increasingly becomes the source of hematopoesis, in general, and macrophages, in particular [3, 4].

Hematopoietic development of macrophages in the adult bone marrow begins with a common hematopoietic stem cell (HSC) which develops into the myeloid lineage and differentiates into a CFU-GM, CFU-M, monoblast, pro-monocyte, monocyte and macrophage after exposure to growth factors. Colony-stimulating factor 1 (CSF1) has

been show to be critical in macrophage development in mice, as well as tissue remodeling and organogenesis during fetal/neonatal life [5, 6]. The absence of CSF1 in osteopetrotic (*op/op*) mice, results in defects in macrophage development, differentiation and proliferation, resulting in osteopetrosis due to the lack of osteoclastic bone resorption [7, 8]. Additionally, granulocyte-macrophage CSF (GM-CSF) assists in the maintenance and population regulation of macrophages. During bacterial infection, GM-SF is produced and promotes increased hematopoiesis and macrophage development. GM-$CSF^{-/-}$ mice do not have defects in macrophage development; however, they do exhibit defects in macrophage mediated responses [9].

Tissue resident macrophages are primarily derived from circulating monocytes that migrate to tissues and become "resident" phagocytes. Migration of this population from circulating monocytes is mediated by soluble CX3C-chemokine ligand or CXC-chemokine ligand 12 [10]. Recognition of a plethora of endogenous and exogenous ligands, and the ability to respond appropriately, is critical to macrophage function in homeostasis (removal of cellular debris/ senescent cells and tissue remodeling/repair after injury or infection) as well as host defense in innate and acquired immunity. The inability to appropriately control these responses can result in autoimmunity, inflammation, and other immunopathologies. Tissue resident populations of macrophages express CD14, CD16, F4/80 and high amounts of MHC class II [11, 12]. Kupffer cells of the liver, alveolar macrophages of the lungs, microglia, Langerhans cells of the epidermis and peritoneal macrophages are all examples of tissue resident populations of macrophages. Additionally, tissue resident macrophages are also found

within the lamina propria of the gut and the interstitium of organs such as the heart, pancreas, and kidney.

Macrophages exposed to IFN-γ with or without microbial constituents, TNFα and/or GM-CSF are considered to be classically activated. In response, these cells produce substantial amounts of IL-12, reactive oxygen species, NO, IL-1β, TNFα, IL-6 and IL-23 [13]. The resulting cytokine profile supports activation of NK cells and multiple T lymphocyte populations. IL-1 and TNFα produced during endotoxin exposure are associated with septic shock, antigen specific immune responses, hepatitis and pancreatitis, and suppression of IFN-γ production by IL-6 has been linked to the shift of Th1 to Th2 in acquired immunity [14-16].

1.2 Interferon-gamma/Interferon-gamma receptor (IFN-γ/IFN-γR)

Interferon-gamma (IFN-γ) and its receptor (IFN-γR) have been shown to be very close to the crux of many immunological activities in the nearly 50 years since it was discovered [Reviewed in [17-19]] In the family of IFNs, named for their ability to exhibit viral interference [20, 21], there are two known classes, types I and II. The former class, type I, includes a wide range of IFNs, some of which are species specific. IFNs -α and -β, the most widely studied of the type I IFNs, are notably recognized for their potent anti-viral activity. Both are expressed within a wide range of cell types in response to viral infection. IFN-β also plays a major role in innate immunity by promoting antibacterial activities following induction by pathogen-associated molecular

patterns (PAMPs) of bacteria. Up-regulation of IFN-α/β activates a positive-feedback loop resulting in amplification of IFN-α/β expression [22-25]. Expression of type II IFN, IFN-γ, is induced by a wide range of stimuli, yet production of IFN-γ is limited in cell type. Natural killer (NK) cells, gamma-delta T cells (γδT cells), NKT cells, $CD8^+$ memory T cells and activated T-helper 1 (Th1) cells release IFN-γ in response to interleukin 12 (IL-12) stimulation [26-29]. Dendritic cells (DC) and macrophages have also been reported to produce IFN-γ [30-32]. Although IFN-γ has antiviral activities, it primarily promotes immunity to unicellular pathogens by activating macrophages and other cells of the myeloid lineage [Reviewed in [17]].

Figure **1-1**

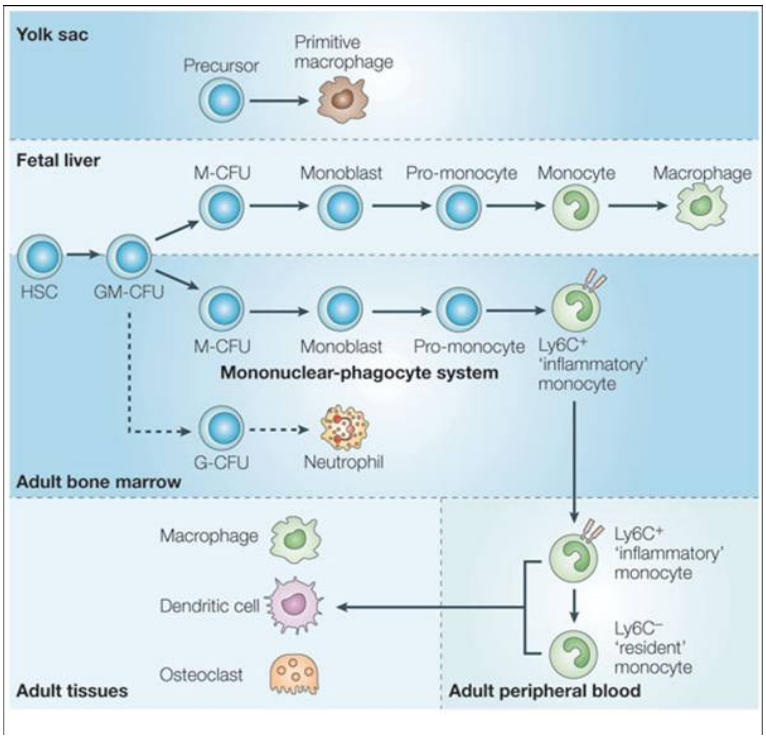

Figure **1-1**: Macrophage Development.

Macrophages are present at multiple stages of maturation of mammals from embryo to adulthood. Primitive macrophages are found in the yolk sac during embryo genesis. As a fetus HSC in the fetal liver progress from CFU-GM → CFU-M → monoblast → pro-monocyte → macrophages. In adult bone marrow macrophage development evolves with comparable stages as in the fetal liver with a divergence of monocytes circulating in the bloodstream into tissues where the terminally differentiate into tissue resident macrophage after reaching adult tissues. Image adapted from [2] and reprinted with permission.

Figure **1-2**

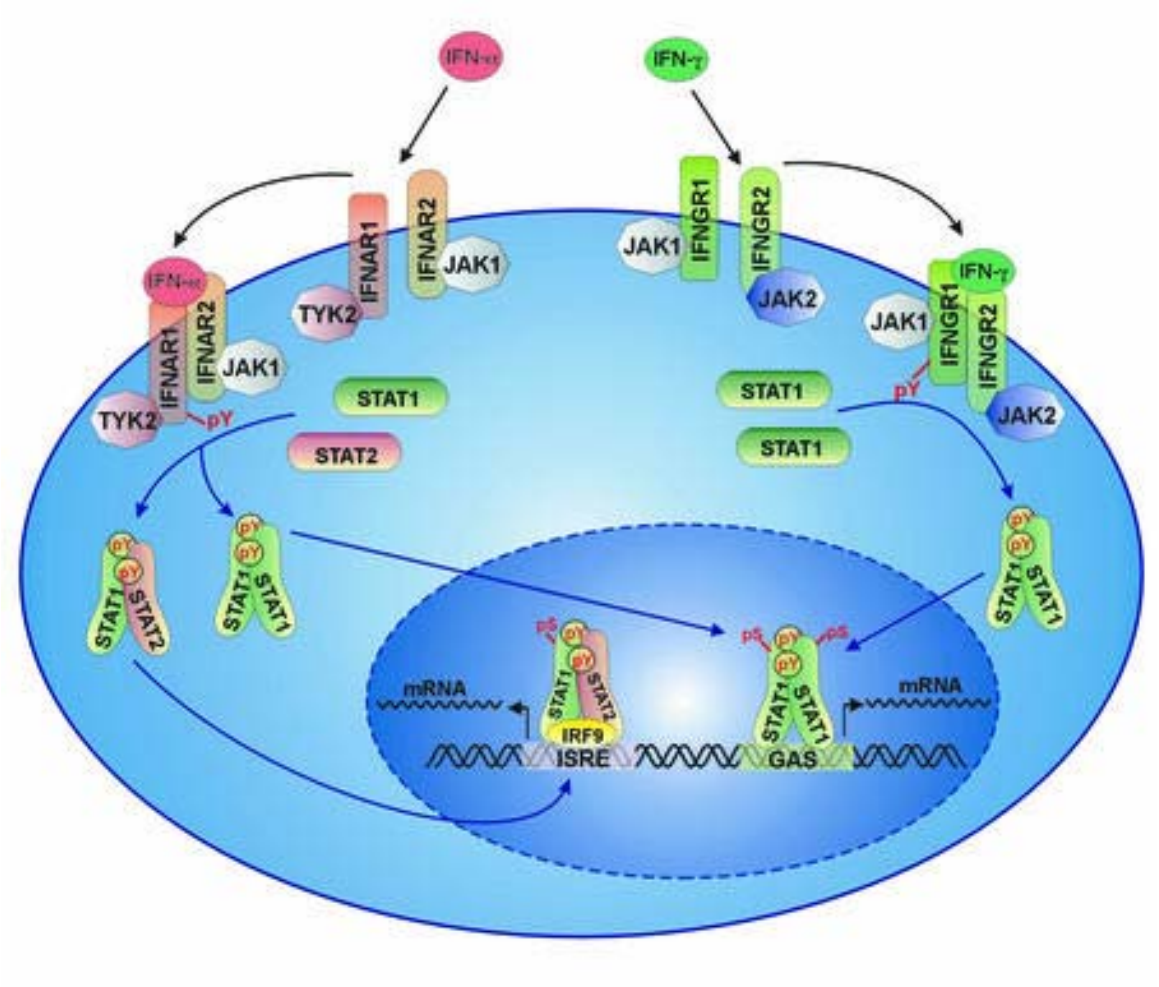

Figure **1-2**: Type I and II IFN Receptor Activation.

Upon binding of ligand, IFN receptor–associated JAKs are activated and phosphorylate receptor chains on tyrosine. Cytoplasmic STATs bind to the phosphorylated receptors via their SH2 domains. JAKs associated with the type I IFN receptor (IFNAR) then phosphorylate STAT1 and STAT2 on tyrosine, causing the formation of predominantly STAT1/STAT2 hetero-dimers. IFN-γR–associated JAKs phosphorylate STAT1, leading to the formation of STAT1 homo-dimers. STAT dimers translocate to the cell nucleus. Thereafter, STAT1/STAT2 hetero-dimers associate with a third protein, IRF9, and bind the ISRE, whereas STAT1 homo-dimers or GAFs activate gene expression by binding the GAS. Image adapted from [33] and reprinted with permission.

Figure **1-3**

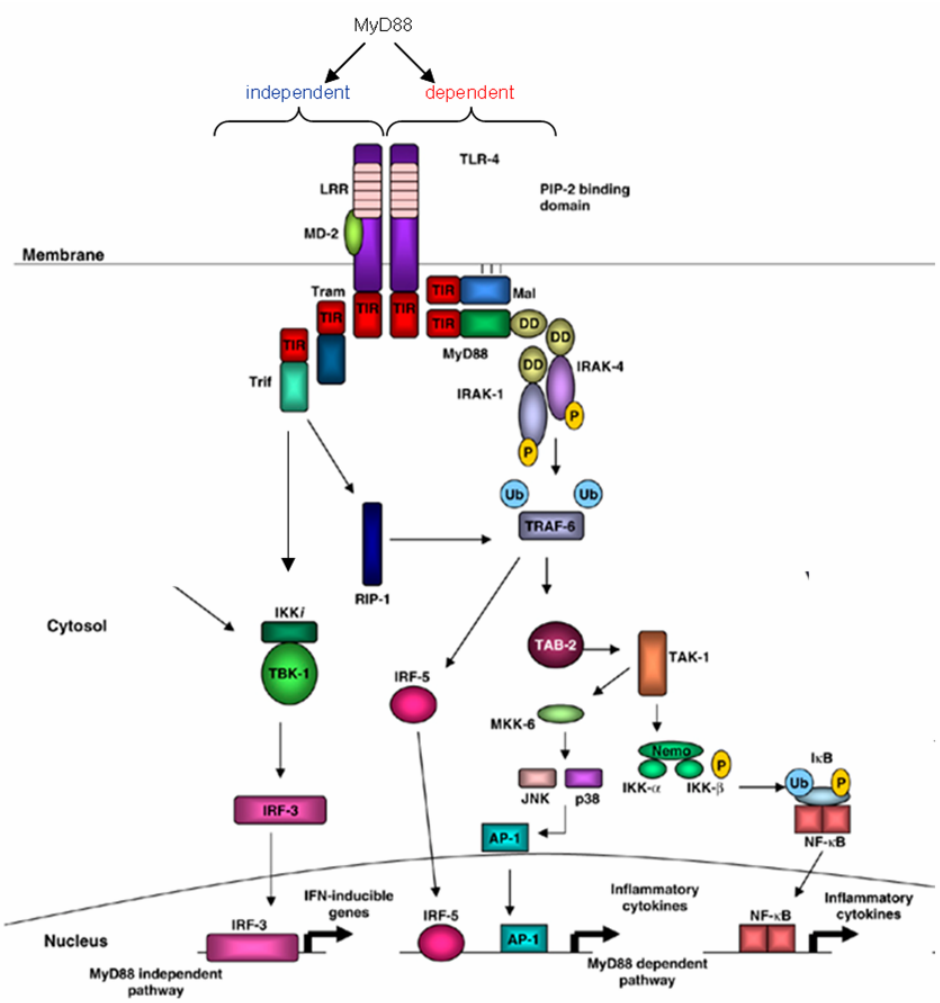

Figure **1-3**: TLR4 Signal Transduction.

The MyD88-dependent pathway is initiated by binding of the TIR domain of Mal to the TIR domain of TLR4, which has dimerized after ligand binding. Mal recruits MyD88, which binds IRAK4 and -1. IRAK1 is phosphorylated by itself and IRAK4 and leaves the membrane to activate TRAF6. After TRAF6 is ubiquitinated, it interacts with TAB2 to activate TAK1. TAK1 activates the IKK complex and IκB is phosphorylated, ubiquitinated and degraded allowing NFκB to translocate to the nucleus to produce proinflammatory cytokines. TAK1 also activates MKK6 which in turn activates JNK and p38 leading to AP-1 activation and the production of proinflammatory cytokines. TRAF6 can also activate IRF5. The MyD88-independent pathway is activated by binding of TLR4 and TRAM at the cell membrane. TBK-1 is activated, thus leading to the activation of IRF3, a transcription factor that translocates to the nucleus to produce IFN-inducible genes. Adapted from [34] and reprinted with permission.

Figure **1-4**

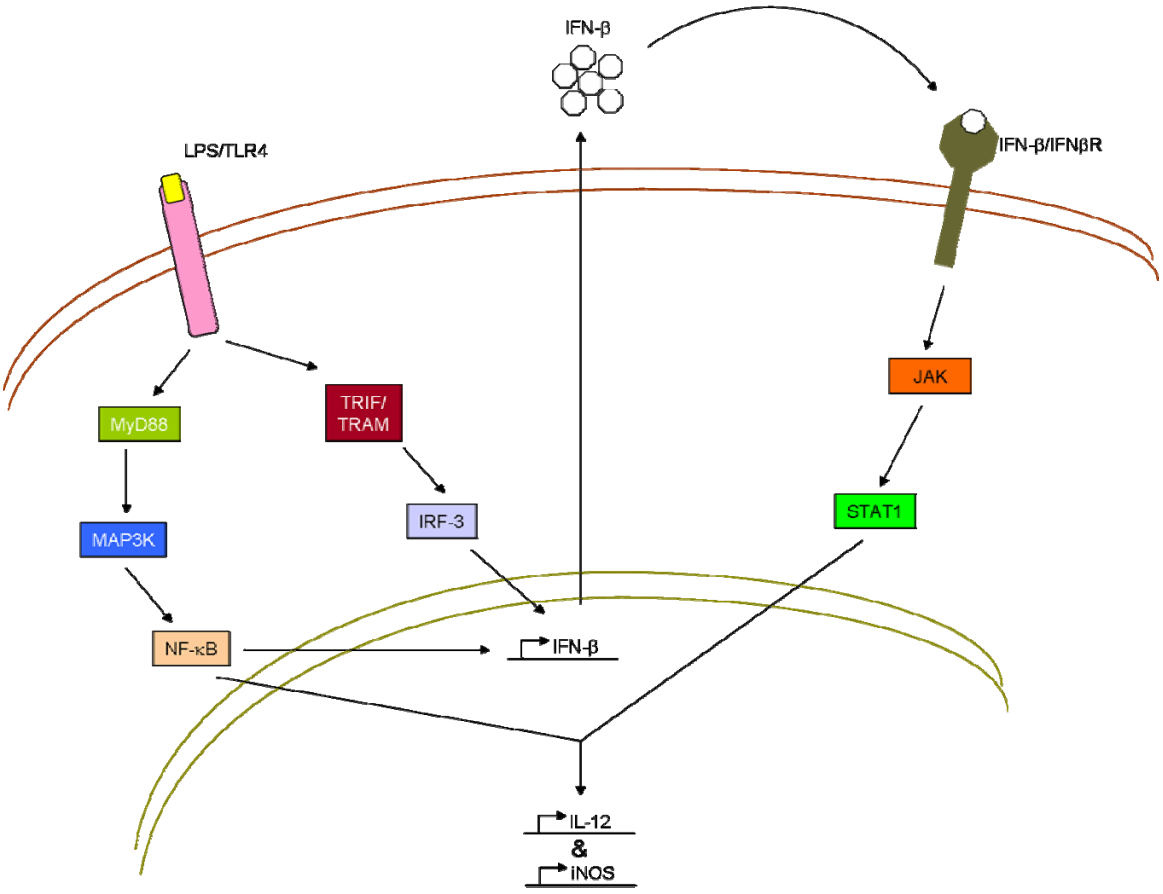

Figure **1-4**: IFN-β positively feeds forward to enhance IL-12 and iNOS induction by LPS.

An alternate pathway of TL4 signaling cascade independent of MyD88 is guided by IRF-3, which induces the expression of IFN-β as part of a positive feed by mechanism that results in IL-12 and iNOS transcription.

IFN-γ exists as a 143 amino acid homo-dimer that is intercalated, and differs from type I IFNs due to the lack of disulfide bonds and C-terminal helical configuration [35-37]. Functional IFN-γRs exist as linked hetero-dimers of IFN-γR1 and IFN-γR2 prior to IFN-γ ligation [38, 39]. Addition of ligand to the receptor complex results in the formation of a symmetrical quatrimer of IFN-γR1 and IFN-γR2 with IFN-γ binding sites located on IFN-γR1. Several tyrosine (Y) residues allow for adequate signal transduction upon phosphorylation by the constitutively associated Janus-kinases (JAKs) 1 and 2, which cross phosphorylate both γ-receptors. JAK2, phosphorylates tyrosine 457 (TYR457) on IFN-γR1, the binding site of the Src-homology 2 (SH2) containing signaling transducer and activator of transcription 1 (STAT1) [40-44]. Post docking of STAT1 to IFN-γR1, TYR701 of STAT1 is phosphorylated, leading to receptor disassociation and homo-dimerization of STAT1 [42, 45]. Activated STAT1 homo-dimers translocate to the nucleus and bind the gamma activated site (GAS) element (TTNCNNNAA), resulting in the primary IFN-γ response.

The type I and II IFN signaling pathways consist of common components, yet result in the activation of distinctive sets of genes [Reviewed in [46]]. Both classes of IFNs incorporate the JAK family of tyrosine kinases associated with their respective receptors. IFN-αR1 and IFN-αR2 constitutively associate with tyrosine kinase 2 (TYK2) and JAK1, respectively [47, 48]. Although both type I and II IFN receptors utilize STAT1 as a transcription factor, IFN-αR2 binds STAT2 via its SH2 domain which hetero-dimerizes with STAT1 upon phosphorylation [45, 49]. This hetero-dimer

associates with interferon-stimulated gene factor 3-γ (ISGF3γ)/p48 forming ISGF3/interferon response factor (IRF)9 which then translocates to the nucleus [50, 51]. DNA binding of this complex occurs at the interferon sequence response element (ISRE) site (AGTTTCNNTTTCNC/T) and promotes gene transcription (Figure **1-2**).

Mice with targeted disruptions of IFN-γ, the IFN-γR or STAT1 are defective in their response to pathogens and pathogen particulates. Targeted deletions in IFN-γ prevents MHC Class II surface expression, splenocyte proliferation, and NK cell mediated killing [52]. Lymphocytic choriomeningitis virus (LCMV) induces increased footpad swelling in IFN-γR1$^{-/-}$ [53]. STAT1$^{-/-}$ mice produce reduced levels of nitric oxide (NO) in response to both type I and II IFNs [54]. These mice are also more susceptible to *Listera monocytogenes*, vesicular stomatitis virus (VSV) and murine herpes virus (mHSV) [54, 55]. These observations are attributed to the fact that STAT1$^{-/-}$ cells do not up-regulate IRF1 [54, 55]. Additionally, many of the phenotypes exhibited by STAT1$^{-/-}$ mice have also been reported in IFN-γ$^{-/-}$ and IFN-γR1$^{-/-}$ animals, including reduced levels of NO and increased susceptiblity to *Listeria monocytogenes*.

The primary response of cells to IFN-γ/IFN-γR1 signaling promotes transcription implementing binding of the DNA GAS element by STAT1. Most GAS responsive genes are forward acting transcription factors, while others are antagonists of IFN-γ. The family of IRFs comprise the secondary response of cells to IFN-γ [56, 57]. IRF1 and IRF2 exhibit 75% homology and have antagonistic effects [58]. This class of

transcription factors interacts with the ISRE. IRF1 drives the expression of pro-inflammatory genes including inducible NO synthase (iNOS)/NOS2, IL-12p40 and cycloxygenase 2 (COX2), as well as the MHC class II master transcriptional regulator, class II trans-activator (CIITA). IRF2 binds to the same relative site of the ISRE and inhibits binding of IRF1 to the site, thus inhibiting the transcription of IRF1 responsive genes [58-60]. $IRF1^{-/-}$ mice exhibit defects in type I IFN gene induction [61, 62]. IL-12 production in $IRF1^{-/-}$ mice is reduced post IL-12 treatment due to defects in IFN-γ gene induction [63]. CIITA mRNA expression is also negatively affected by the absence of $IRF1^{-/-}$ [64].

The IFN consensus binding protein (ICSBP)/IRF8 is related to (ISGF3γ)/p48 and induced by IFN-γ [65-67]. Interactions between ICSBP, PU.1 and IRF1 result in cooperative DNA binding and activation of gene transcription [68]. In addition to serving as a GAS element gene activator, ICSBP combines with IRF2 to suppress ISRE-mediated transcription [69, 70].

While IFN-γ up-regulates gene expression, in order to mediate immunity, it also activates genes that serve to control cellular responses. Suppressor of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS) are two of the most studied negative regulators of cytokine signal transduction and gene activation. SOCS proteins inhibit cytokine signaling and are up-regulated by cytokine receptor activation [71-76]. SOCS proteins are characterized by a conserved carboxyl-terminal SOCS box and an

SH2 domain. Interruption of the JAK/STAT pathway is the main function of SOCS proteins. SOCS1 binds activated cytokine receptors via its SH2 domain, which inhibits recruitment of STAT proteins and subsequent signal transduction [77, 78]. Cytokine gene induction is also regulated by SOCS1 through the control of cytokine receptor degradation [79-81].

Phosphorylated STAT proteins are also bound by PIAS preventing them from binding DNA and thus preventing gene expression [82-84]. PIAS has also been reported to possibly target STAT proteins for proteosomal degradation [85, 86]. In addition, dephosphorylation of cytokine receptors, associated JAK kinases and both cytoplasmic and nuclear STATs by receptor and non-receptor tyrosine phosphatases including SHP1, SHP2 and CD45 plays a critical role in the regulation of cytokine signaling [77, 87-89].

1.3 Toll-like Receptor 4 (TLR4)

During an immune challenge from intracellular pathogens, the highly evolutionarily conserved pattern recognition receptors (PRRs) on the surface of tissues at the site of infection bind pathogen associated molecular patterns (PAMPs) of pathogens such as lipopolysaccharide (LPS) from Gram-negative bacteria, flagellin, double-stranded RNA (dsRNA), peptidoglycan, CpG DNA and lipotechoic acid (LTA) from Gram-positive bacteria [Reviewed in [90] and [91]]. A subclass of the PRRs were first identified as Toll in *Drosophila melanogaster* for conferring antimicrobial defense to fungus infection [92-97]. *Drosophila melanogaster*'s Toll structure consists of an extra-

cellular domain of leucine-rich repeats with a cytoplasmic domain that is homologous to the mammalian interleukin 1 receptor (IL1R), referred to as the Toll/IL-1R (TIR) domain. Signal transduction through Toll in *Drosophila melanogaster* results in the nuclear translocation of Dorsal, a Rel transcription family member [Reviewed in [98]].

TLR4, which was originally known as human Toll (hToll), was the first mammalian homolog of *Drosophila melanogaster* Toll identified [99, 100]. Subsequently, the mammalian receptors were coined as Toll-like receptors (TLR) by Rock et al [100]. Interestingly, a constitutively active form of TLR4 was shown to activate NFκB and induce pro-inflammatory mediators, thus creating a link between TLRs and immunity [99].

Although the list of ligands to TLR4 continues to grow, endotoxin or LPS, is the most widely studied ligand and was identified as such due to the tempered response of C3H/HeJ and C57BL10/ScCr mice to endotoxin [101, 102]. Activation of TLR4 by LPS is not achieved by direct binding to the receptor in isolation. LPS-binding protein (LBP) circulates in the serum and presents LPS to TLR4 by binding to cluster of differentiation 14 (CD14), which is a glycosylphosphatidylinositol anchored molecule preferentially expressed in monocytes/macrophages and neutrophils [103-108]. Surface expression of CD14 is usually in close proximity to TLR4 thus presenting optimal conditions for recognition and initiation of signal transduction [109]. After CD14 links to LPS, MD2, associated with TLR4, confers ligation and activation of TLR4. Depletion of MD2 in

animal models, or targeted deletion of CD14 and TLR4 conveys resistance to endotoxin [110-117].

The TIR domain is the site of initiation of cytoplasmic signal transduction down-stream of TLR4; however two distinct pathways, the myeloid differentiation primary response gene 88 (MyD88) -dependent and –independent pathways, are engaged to activate LPS responsive genes. MyD88 is an important adaptor protein that was previously linked to IL-1R signal transduction [118, 119]. Prior to engagement of MyD88, the TLR4 TIR domain co-adaptor MyD88-adaptor like (MAL)/ TIR-domain-containing adaptor protein (TIRAP), which is associated with the cell membrane by attaching to phosphatidylinositol 4,5-bisphosphate (PIP2), assists in recruiting MyD88 to TLR4 [120]. As signal transduction begins, the TIR domain of MyD88 associates with the TIR domain of TLR4 leading to the activation of a series of kinases that results in the nuclear translocation of multiple transcription factors such as NFκB, AP-1 and IRF5, as well as the induction of pro-inflammatory mediators including IL-1β, IL-6, TNFα and IL-8 [Reviewed in [34, 121-123]. Mice deficient in MyD88 are protected from endotoxin mediated death and vesicular stomatitis virus (VSV) infection [124, 125]. In response to LPS, MyD88$^{-/-}$ mice fail to express IL-6, TNFα and IL-1β [125].

MyD88-independent signaling incorporates TIR domain interactions between the TRIF-related adaptor molecule (TRAM) and TLR4. This pathway is responsible for inducing TIR-domain-containing adaptor inducing IFN-β (TRIF), also know as TIR-

containing adaptor molecule 1 (TICAM1) [126, 127]. IRF3 is the major transcription factor activated by the MyD88-independent signal transduction pathway downstream of TLR4 (Figure **1-4**), which converges with the MyD88-dependent pathway to up-regulate IFN-β in an autocrine-paracrine loop [22-25, 128]. IFN-β then positively feeds back to cooperate with LPS in the induction of a subset of LPS responsive genes, including iNOS and IL-12p40, that are dependent on IFN-induced STAT1 phosphorylation (Figure **1-4**) [46, 129, 130].

The pro-inflammatory cytokines produced by exposure to LPS of bacterial pathogens also induce negative feedback genes, such as IL-10. IL-10 was first described due to its ability to inhibit synthesis of the Th1 cytokine IFN-γ [131, 132]. In addition to inhibiting the induction of the pro-inflammatory cytokines IL-12, IL-6 and TNFα [133] just to name a few, IL-10 also up-regulates inflammatory antagonist like IL-1 receptor antagonist (IL-1RA) and soluble tumor necrosis-factor receptor (sTNFR) in macrophages [134, 135]. Mice deficient in IL-10 develop inflammatory bowel disease (IBD) and exhibit increased immune responses to *Listeria monocytogenes* [136, 137]. IL-10 induces gene expression primarily through the activation of STAT3. Mice with tissue specific deletions of STAT3 in macrophages and neutrophils also develop spontaneous IBD due to the dysfunction of the anti-inflammatory actions of STAT3 [138].

SOCS1 expression is also induced in response to LPS mediated TLR4 activation. Phosphorylated MAL is prevented from mediating TLR4 MyD88-dependant signaling after binding of SOCS1 [139] . Phosphoinositide Kinase-3 (PI3K) is also up-regulated

and perturbs IL-12 transcription in DCs after exposure to LPS [140, 141]. Additionally, LPS treatment reduces the expression of TLR4 which is attributed to the development of endotoxin tolerance.

1.4 RON/STK Receptor Tyrosine Kinase and Its Ligand MSP

The *recepteur d-origine nantais* (RON) receptor tyrosine kinase (RTK) is a member of the proto-oncogene *c*-MET family of receptor tyrosine kinases (RTKs) [142-145]. Screening of a human keratinocyte cDNA library resulted in the isolation of RON while the murine homolog, stem cell-derived tyrosine kinase (STK), was isolated from murine hematopoietic stem cells in an unrelated study [142, 143]. RON exists as a disulfide-linked 190 kD hetero-dimer composed of a 40 kD extra-cellular α-chain and a 150 kD trans-membrane β-chain that is formed after cleavage of a single precursor [143, 146]. Although the N-terminal α-chain and extra-cellular portion of the β-chain forms the ligand binding domain of RON, only the β-chain has kinase activity [146]. Additionally, the C-terminal tail of the β-chain has tyrosine residues that serve as docking sites that mediate the binding of down-stream signal transduction molecules that contain SH2 domains [147].

The earliest expression of RON is observed in the embryonic trophoblast where it is involved in embryonic implantation [148, 149]. During murine embryonic development and in the adult animal, RON is widely expressed in the epithelium of the digestive tract [146, 150]. Moreover, RON is overexpressed in a number of malignant

epithelial cancers including breast, pancreatic, ovarian and colon carcinomas [Reviewed in [151]]. RON is also expressed on hematopoietic progenitor cells and is down-regulated as these cells differentiate [152]. Circulating monocytes do not express RON; however when they migrate into tissues and differentiate into tissue resident macrophages RON expression is up-regulated [153]. Tissue resident macrophage populations that are RON positive include Kupffer cells, osteoclasts, resident peritoneal macrophages , Langerhan's cells, mesangial cells, splenic marginal zone macrophages and microglia [153-157].

Macrophage stimulating protein (MSP), which was originally referred to as hepatocyte growth factor like (HGFL), is the ligand for RON. Identified almost 20 years prior to RON, MSP was observed to induce morphological changes and chemoattractant responsiveness in resident macrophages [158, 159]. MSP is related to a family of serum proteins with kringle domains and disulfide loop structures conserved among family members such as prothrombin urokinase, hepatocyte growth factor (HGF) and plasminogen [160]. The active form of MSP is an 80 kDa serum protein, which is produced in the liver and exists in an inactive form prior to being processed by proteolytic cleavage by proteases in the coagulation cascade and an MSP convertase found on the surface of macrophages [161, 162].

Activation of RON, by MSP, induces auto-phosphorylation of two tyrosine residues in the activation loop of the kinase domain, resulting in up-regulation of its tyrosine kinase activity [146]. In addition, upon phosphorylation, two tyrosine residues

in the cytoplasmic tail of RON recruit Src homology 2 (SH) domain containing signaling molecules including growth-factor-receptor bound protein 2 (GRB2), PI3K, GRB2-associated binding protein 1 (GAB1), SHC, SHP2, SH2 domain-containing inositol 5'-phosphatases (SHIP1) and phospholipase C-gamma (PLCγ) [163-166]. Recent studies from our group have demonstrated that GRB2 binding to the second docking site tyrosine results in the recruitment of GAB2 to short-form RON (sf-RON), an N-terminally truncated from of RON [166], following sf-RON activation by Friend virus. Futhermore, Friend virus infection promotes STAT3 recruitment to the sf-RON/GRB2/GAB2 complex [167], and our group has shown that both GAB2 and STAT3 are required for Friend virus-induced erythroleukemia *in vivo.*

In addition to the multifunctional docking site tyrosines, serine phosphorylation of RON has been shown to mediate 14-3-3 binding, resulting in keratinocyte migration. 14-3-3 binding to the receptor is negatively regulated by protein phosphatase-1 (PP1) [168]. RON has also been shown to associate with a number of other transmembrane molecules including adhesion receptors, integrins, cadherins, the IL-3 receptor common beta chain, the EpoR and MET [169]. MSP activation of full-length RON cooperates with erythropoietin (EPO) to induce the growth of primary erythroblasts *in vitro* [170]. Downregulation of RON is mediated, in part, by binding of c-Cbl to the docking site tyrosines as well as a tyrosine in the juxtamembrane domain.

Comparison of the murine and human RON receptors revealed a deletion in the juxtamembrane domain of murine RON, resulting in enhanced constitutive activity and

altered substrate specificity of the murine receptor, suggesting that the juxtamembrane domain plays a critical role in the regulation of receptor signaling [171]. However, deletion of the c-Cbl binding site in human RON did not recapitulate the differences observed in murine RON, suggesting that these changes in signaling capacity in murine RON were not due to decreased receptor turnover mediated by c-Cbl [171]. In addition, studies of the ligand-independent signaling by murine RON uncovered three tyrosines in the kinase domain that appear to activate the Ras/MAP kinase pathway through recruitment of c-Src to the receptor complex [172].

1.5 The RON Receptor and Macrophage Activation

C3bi-mediated phagocytosis, chemotaxis and shape change of peritoneal macrophages is increased by MSP [165, 173, 174]. Our previous studies have demonstrated that RON physically associates with CR3, and that C3bi-mediated phagocytosis induced by MSP requires by the activation of PI3K and PKCζ by RON [165]. $RON^{-/-}$ mice are more susceptible to infection with *Listeria monocytogenes* [175]. *Listeria monocytogenes* that are phagocytosed by macrophages via the CR3 receptor are readily killed, even in the absence of inflammatory mediators [165]. Thus it is possible that RON regulates susceptibility to *Listeria monocytogenes* by regulating the uptake of *Listeria monocytogenes* by resident macrophages.

MSP stimulation of primary peritoneal macrophages inhibits the up-regulation of iNOS [176, 177] and COX2 [178] in response to IFN-γ and LPS stimulation, resulting in

reduced NO and prostaglandin E2 (PGE2) production by these cells. The inhibition of iNOS by RON requires activation of the PI3K pathway [163]. Mice with targeted deletions in the RON gene exhibit increased susceptibility to endotoxic shock and delayed-type hypersensitivity (DTH) responses due to increased NO production and Th1 mediated inflammation, respectively [179], suggesting a critical role for the RON receptor in protecting host tissues from inflammatory damage during an immune response to infection. $MSP^{-/-}$ mice, on the other hand, are not more susceptible to septic shock, suggesting the possible existence of additional ligands for RON, constitutive receptor activation or hetero-dimerization of RON with other receptors in the regulation of macrophage activation. Our recent observations that murine RON exhibits enhanced ligand-independent activity due to a deletion in the juxtamembrane domain may provide an explanation for the apparent contrast between the phenotypes of the $MSP^{-/-}$ and $RON^{-/-}$ mice [171].

One mechanism by which RON may regulate classical macrophage activation is through the regulation of NFκB. NFκB is a central regulator of pro-inflammatory gene expression, including iNOS and COX-2, in activated macrophages [180-183]. RON has been shown to inhibit the nuclear translocation and DNA binding of the p65 subunit of NFκB in macrophages in response to LPS and cytokine stimulation [184, 185]. RON is also reported to inhibit the phosphorylation and subsequent degradation of IκB, a central regulator of NFκB activity [184]. In addition to pro-inflammatory gene expression, the inhibition of NFκB activity by RON results in the negative regulation of human

immunodeficiency virus (HIV) transcription from the viral long terminal repeat (LTR) [184].

Our more recent studies have indicated that, in addition to inhibiting classical macrophage activation, stimulation of RON by MSP induces hallmarks of alternative or M2 macrophage activation [186]. We have shown that stimulation of primary peritoneal macrophages with MSP induces expression of arginase I, an enzyme that competes with iNOS for the substrate L-arginine. However, arginase metabolizes L-arginine to ornithine, a precursor for proline and polyamine synthesis which promote cell proliferation and matrix synthesis. Arginine can also decrease NO production by reducing the availability of L-arginine to iNOS [187]. MSP also induces expression of IL-1 receptor antagonist and Scavenger Receptor-A, which are associated with M2 macrophage activation [186]. Thus, the RON receptor may play an important role in the resolution of inflammation and promoting the healing process. Moreover, in the ensuing studies will seek to elucidate the role that modulation of IL-12 by MSP/RON has on *in vitro* and *in vivo* pathogenesis orchestrated by IFN-γ and LPS.

Chapter 2

The RON Receptor Tyrosine Kinase Regulates the Production of IL-12 by Macrophages Stimulated with LPS and IFN-γ

2.1 Introduction

Macrophages can be activated down distinct developmental pathways in response to immune challenge, which direct the host response to the invading pathogen [Reviewed in [188]]. This activation is regulated by signals present in the microenvironment of the macrophage. IL-12, produced by macrophages during the early stages of infection in response to bacterial cell components, induces the production of IFN-γ by NKT and $CD8^+$ T cells and promotes Th1 differentiation [189]. The production of IL-12 by macrophages and other APCs is regulated by a variety of mechanisms. IL-12 can be produced in a T cell-independent manner in response to bacterial products such as LPS, where IFN-γ is thought to have a priming effect, as well as in a T cell-dependent manner via CD40/CD40 ligand interaction of APCs with naive $CD4^+$ T cells [190-193]. The subsequent IFN-γ produced in response to IL-12 serves as a positive feedback regulator of IL-12 to maintain IL-12-induced Th-1 responses. To control IFN-γ-mediated augmentation of IL-12, there are also effective mechanisms to down-regulate IL-12 production. Cytokines such as IL-4, IL-10, and transforming growth factor β (TGFβ); chemoattractants; activators of complement; and phagocytic receptors have all been shown to be inhibitors of IL-12 production [Reviewed in [194]].

IFN-γ, in turn, induces classical macrophage activation by priming these cells for the production of cytokines, prostaglandins and free radicals, such as NO. Production of NO by classically activated macrophages following the up-regulation of iNOS expression plays a key role in the killing of invading micro-organisms. However, production of NO by macrophages can also be cytotoxic to host tissues [195].

The RON receptor is expressed on a subpopulation of tissue-resident macrophage/dendritic cell populations including resident peritoneal macrophages, osteoclasts, Kupffer cells, Langerhans cells, mesangial cells, splenic marginal zone macrophages and microglia [153, 196-198]. RON is a member of the MET family of receptor tyrosine kinases, composed of an extracellular α chain linked to a transmembrane β chain via a disulfide bond [199]. Ligand-induced activation of RON results in the up-regulation of intrinsic kinase activity regulated by two tandem tyrosines in the activation loop of the kinase domain, and the phosphorylation of two tyrosines in the c-terminal tail responsible for recruitment of a number of downstream signaling molecules such as, GRB2, PI3K, GAB1, SHC, SHP2, SHIP1 and PLCγ [163-166].

MSP, the ligand for RON, is a serum protein that induces chemotaxis of resident peritoneal macrophages [200, 201] and enhances C3bi-mediated phagocytosis and intercellular adhesion molecule 1 (ICAM1) binding through PI3K, protein kinase C zeta (PKCζ) -dependent activation of the αMβ2 integrin, CR3 [202]. Hepatocytes primarily express MSP, which circulates in the serum in an inactive form, and is cleaved to its

active form by multiple proteases including those of the coagulation cascade [203, 204]. MSP stimulation of primary peritoneal macrophages inhibits the induction of NFκB and the up-regulation of iNOS and COX2 in response to IFN-γ and LPS stimulation, resulting in reduced NO and PGE2 production by these cells [163, 185, 205]. Mice with a targeted deletion in RON exhibit increased susceptibility to endotoxic shock, associated with enhanced serum NO levels, and are more susceptible to infection with *Listeria monocytogenes* [179, 206, 207]. Taken together, these data suggest that the expression of RON on tissue-resident macrophages limits the tissue damage associated with an innate immune response by inhibiting the production of cytotoxic mediators in close proximity to host tissues.

2.2 Materials and Methods

2.2.1 *Cells and Animals*

The mouse strains used for these experiments were wild-type (WT) C57/BL6 mice (The Jackson Laboratory), IL-10$^{-/-}$ mice (kindly provided by Dr. Margherita Cantorna from the Pennsylvania State University), C57/BL6 mice with a targeted mutation in the RON gene, which were generated by backcrossing with CD-1 mice with a targeted mutation in the RON gene [179] for 12 generations, IFN-γR$^{-/-}$ mice (The Jackson Laboratory) and RON$^{-/-}$/IFN-γR$^{-/-}$ mice generated by crossing RON$^{-/-}$ and IFN-γR$^{-/-}$mice, both on C57/BL6 backgrounds. Murine resident peritoneal macrophages were obtained by peritoneal lavage with 10 ml of Roswell Park Memorial Institute (RPMI) containing

10% fetal bovine serum (FBS). Cells were incubated for 5-7 hours and then washed with PBS to eliminate non-adherent cells. All cell cultures were maintained in RPMI + 10% FBS at 37°C in a humidified incubator containing 5% CO_2. The Institutional Animal Care and Use Committee (IACUC) of The Pennsylvania State University granted approval for all experimental procedures.

2.2.2 *Reagents and Antibodies*

The following reagents were obtained from the indicated sources: DMEM, FBS, trypsin TRIzol® and PBS (Invitrogen); MSP, IL-12p70 ELISA kit and IL-12p40 ELISA kit (R&D Systems); IFN-γ (Pepro Tech); lipopolysaccharide (LPS), RPMI-1640, glutamine and β2-mercaptoethanol (Sigma-Aldrich); and LINCOPlex® (Millipore). Antibodies for Western blotting against pY^{701}STAT1, pS^{727} and STAT1 were purchased from Cell Signaling.

2.2.3 *RNA Extraction and RT-PCR*

Total RNA from WT murine peritoneal macrophages was isolated with the RNeasy mini column kit (Qiagen). Total RNA from WT and $RON^{-/-}$ murine spleens was isolated with TRIzol (Invitrogen). Reverse transcription was carried out for 40 minutes at 42°C using Oligo d(T)16 primers (Applied Biosystems) and murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems) on 0.1μg of total RNA. Conditions for PCR are as follows: IL-12 p40 [208], 95°C for 5 min, followed by 35 cycles of 95°C

for 1 min, 54°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min (IL-12p40 sense, 5'-ATC GTT TTG CTG GTG TCT CC-3'; IL-12 p40 anti-sense, 5'-AGT CCC TTT GGT CCA GTG TG-3'); IL-12p35, 95°C for 5 min, followed by up to 35 cycles of 95°C for 15s, 58°C for 20s and 72°C for 30s, with a final extension at 72°C for 6 min (IL-12p35 sense, 5'- ACC TGC TGA AGA CCA CAG AT-3'; IL-12p35 anti-sense, 5'- GAT TCT GAA GTG CTG CGT TG-3'). β-actin was amplified utilizing the same conditions as the genes of interest (β-actin sense, 5'-TGC TGC GTG ACA TCA AAG AG-3', β-actin antisense, 5'-TGG ACA TGA GGC CAG GAT G-3').

2.2.4 *Western Blot Analysis*

$5X10^6$ cells were incubated with the indicated cytokines. After incubation cells were washed with PBS and lysed for 15 min on ice in 500μl lysis buffer containing 150mM NaCl, 10mM Tris-HCl (pH 7.5), 0.5% Triton X-100, and protease inhibitors 1 μg/ml Leupeptin, 1μg/ml Pepstain A, and 2 μg/ml aprotinin. The protein contents were determined using the DC protein assay kit (Bio-Rad). Absorbance was measured at 750nm with a Beckman DU530 spectrophotometer. Proteins were mixed with 5X SDS sample buffer. SDS-PAGE, using 12.5% bis-acrylamide gel for separation, was performed with a MiniProtean II Cell (Bio-Rad) at 65V for 15 min, then at 150-160V for 1h. Immobilon P PVDF membranes (Millipore) were washed briefly in methanol, then ddH_2O, then equilibrated in trans-blotting buffer (48mM Tris 39mM glycine, and 20% methanol) for 5 min. The gels were washed in ddH20, then in trans-blotting buffer for 15min. Transblotting was performed using the Mini Trans-Blot Electrophoretic Transfer

Cell (Bio-Rad) for 1h at 100V. The blots were then placed in blocking buffer containing 5% non-fat dry milk for 1h at room temperature. The blots were then incubated over night with primary Ab, according to manufacturer's instructions. Anti-rabbit Abs conjugated with HRP were added at 1:8000 for 1 hour. Protein was detected by incubating the blots in chemiluminescense substrates (GE Healthcare) and exposing to x-ray film.

2.2.5 *Determination of Cytokine Concentrations*

Cells (1 x 10^5) were incubated with the cytokines indicated in a 96-well plate, and the cytokines in the supernatant were detected by standard sandwich ELISA or serum samples were analyzed with LINCOPlex®. IL-12 p70, IL-12p40 and IFN-γ levels were quantified according to the manufacturer's instructions. Colorimetric changes in enzyme substrates were detected at 430 nm using a HTS 7000 Bio Assay Reader (PerkinElmer).

2.2.6 *Endotoxin Challenges*

IFN-γR$^{-/-}$ and RON$^{-/-}$/IFN-γR$^{-/-}$ mice were challenged with 20 mg/kg, 35 mg/kg or 50 mg/kg of LPS via the intraperitoneal (i.p.) route post weighing of individual mouse. Mice were monitored every 6 hours over a total period of 60 hours with survival recorded at each interval.

2.3 Results

2.3.1 *IL-12 production in response to IFN-γ and LPS stimulation is inhibited by MSP*

Macrophages play an important role in initiating production of IFN-γ and directing cell mediated immunity through the production of IL-12 following exposure to pro-inflammatory pathogen components and cytokines, such as LPS and IFN-γ. In order to elucidate the potential role of MSP in the regulation of IL-12 production, we isolated primary peritoneal macrophages and treated them for 10 hours with or without MSP (100ng/mL) prior to activation with IFN-γ (10U/mL) and LPS (0.1μM). IL-12 production was quantified by ELISA. Treatment with MSP at the same time as IFN-γ and LPS does not affect the production of IL-12; however exposure to MSP for 10 hours before stimulation with IFN-γ and LPS results in the prevention of IL-12p70 production (Figure **2-1A**).

Biologically active IL-12p70 is comprised of a hetero-dimer of the p35 and p40 subunits. Constitutive expression of the IL-12p35 subunit exists in many cell types; whereas generation of the biologically active form is reliant on stimulus specific production of the p40 subunit [209]. Gene induction of IL-12p35 and IL-12p40 is activated by, but not limited to, LPS [209, 210]. Although IL-12 induction by IFN-γ in isolation has not been observed, low levels of IFN-γ is a well known and effective primer for IL-12p40 elicited by LPS in macrophages [190]. We determined the effect of MSP on the expression of the p35 and p40 subunits of IL-12 by performing RT-PCR on RNA

from primary peritoneal macrophages stimulated with IFN-γ and LPS after 10 hours of exposure to MSP. IL-12p40 induction was entirely ameliorated in the presence of MSP, while densitometry revealed a ~2 fold reduction in IL-12p35 transcripts (Figure **2-1B**). The down-stream effects of IL-12p40 inhibition are also manifested in the suppression of IL-12p40 protein production as assed by ELISA (Figure **2-1C**).

2.3.2 *Suppression of IL-12 production by MSP/RON is independent of IL-10*

Stimulation of macrophages with a combination of LPS and IFN-γ results in production of IL-12 at high levels; however regulation of this response is achieved via the negative-feedback of IL-10, which is a strong inhibitor of IL-12. Therefore we evaluated the potential role of IL-10 in IL-12 inhibition by MSP by stimulating primary peritoneal macrophages from IL-$10^{-/-}$ mice with MSP for 10 hours then treating them with LPS and IFN-γ. In the absence of IL-10, MSP retained the capacity to prevent IL-12 up-regulation by LPS and IFN-γ (Figure **2-2**), indicating that the inhibition of IL-12 by MSP does not require IL-10. Additionally, IL-10 is not induced by MSP (Figure **2-3**). Not only does MSP not induce IL-10, it does not induce STAT3 phosphorylation, a known mediator of IL-10 signaling (Figure **3-6**).

2.3.3 *RON Regulates IL-12p40 Expression and IFN-γ Production in vivo Following LPS Administration*

Our *in vitro* studies have demonstrated the ability of the RON receptor tyrosine kinase in conjunction with MSP to inhibit IL-12p40 production by macrophages in

reponse to LPS and IFN-γ. Here, we set out to determine whether the RON receptor tyrosine kinase retains these modulatory effects following *in vivo* administration of LPS. To determine whether RON regulates IL-12p40 expression *in vivo*, WT and $RON^{-/-}$ mice were injected with LPS (20mg/kg) via the intra peritoneal (i.p.) route. Spleens were then harvested and total splenic RNA was collected. At 1 hour post injection of LPS, we failed to detect any significant induction of IL-12p40 in either WT or $RON^{-/-}$ splenocytes. However, at 3 hours, RT-PCR analysis revealed that splenocytes from multiple $RON^{-/-}$ mice expressed significantly elevated levels of IL-12p40 transcript when compared with their WT counterparts (Figure **2-4**).

The up-regulation of IL-12p40 in response to LPS results in production of the active IL-12p70. Local tissue exposure to IL-12, leads to the activation of NK cells, $CD8^{+}$ memory T cells, NKT cells and γδT cells, which in turn release IFN-γ. In order to determine whether $RON^{-/-}$ mice exhibit differential production of IFN-γ as a result of LPS treatment, we injected $RON^{-/-}$ and WT mice with LPS (20 mg/kg) i.p. and monitored them for 0, 1, 3, 6 and 12 hours. Serum samples were collected and IFN-γ quantification was achieved using the LINCO*Plex*® cytokine assay. $RON^{-/-}$ mice began to produce higher levels of IFN-γ after 3 hours of LPS exposure (Figure **2-5**). The levels of IFN-γ continued to climb through 12 hours in $RON^{-/-}$ mice while WT levels peaked at 6 hours and declined by 12 hours. These data suggest that the ability of RON to inhibit IL-12p40 expression *in vivo* results in decreased production of IFN-γ by IL-12-responsive cells.

2.3.4 *Enhanced Susceptibility to Septic Shock of RON$^{-/-}$ Mice is Mediated by IFN-γ*

Previous studies indicate that the absence of regulators to quell pro-inflammatory responses mediated by LPS has an outcome that ends in severe morbidity or mortality in mice. We have shown previously that RON$^{-/-}$ mice are more susceptible to endotoxic shock than their WT counterparts. Here we sought to elucidate the physiological consequence of increased IFN-γ levels in RON$^{-/-}$ mice injected with LPS, by crossing the RON$^{-/-}$ mice with IFN-γR$^{-/-}$ animals. RON$^{-/-}$ and RON$^{-/-}$/IFN-γR$^{-/-}$ mice were treated with 20, 35 and 50 mg/kg of LPS, and post injection monitoring of these animals was carried out at 6 hour intervals. While RON$^{-/-}$ mice exhibited increased susceptibility to endotoxin compared with WT controls as described previously (Figure **2-6A**), RON$^{-/-}$ and RON$^{-/-}$/IFN-γR$^{-/-}$ mice had a comparable mortality rate with mortality increasing in a dose dependent manner within both groups of mice (Figure **2-6B**). These results suggest that IFN-γ is at the crux of the increased susceptibility of RON$^{-/-}$ mice to endotoxin and that RON regulates the physiological activities of IFN-γ *in vivo*.

Figure **2-1**

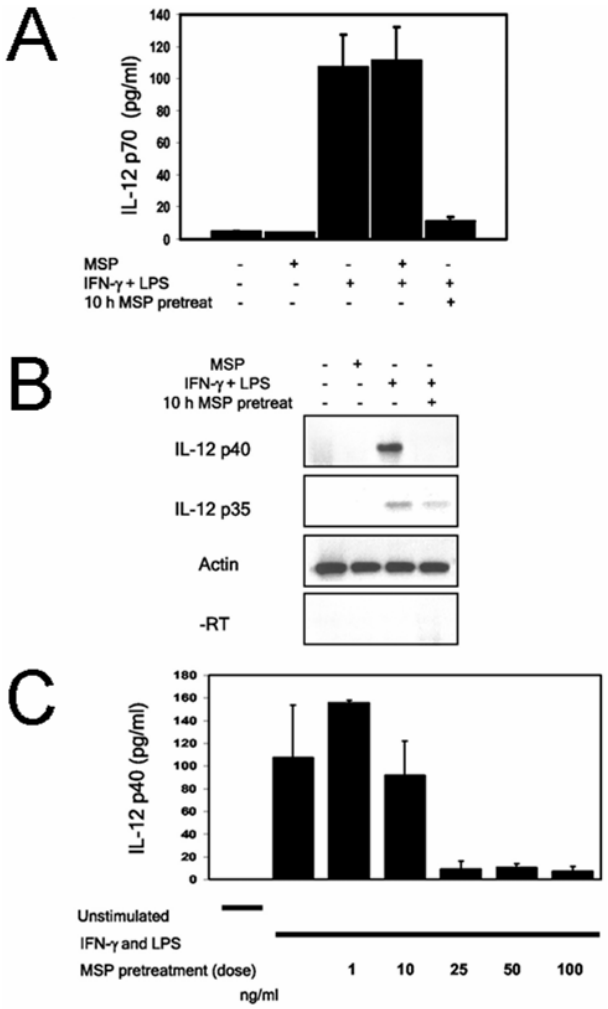

Figure **2-1**: MSP inhibits IL-12 production by primary peritoneal macrophages.

A, Peritoneal macrophages were treated with or without 100 ng/ml MSP for 10 h before stimulation with 10 U/ml IFN-γ, 0.1μM LPS, and/or 100 ng/ml MSP for 24 h. Supernatants were collected, and IL-12 p70 production was measured by ELISA. *B*, Peritoneal macrophages were treated with or without 100 ng/ml MSP for 10 h. RNA was collected from macrophages 6 h after stimulation with either 10 U/ml IFN-γ and 0.1μM LPS or MSP, and IL-12 p40 and p35 subunit expression was determined by RT-PCR. *C*, Supernatants from macrophages were pretreated with the indicated dose of MSP for 10 h before stimulation with IFN-γ and LPS for 24 h, and IL-12 p40 production was measured by ELISA. These data were adapted from [211].

Figure 2-2

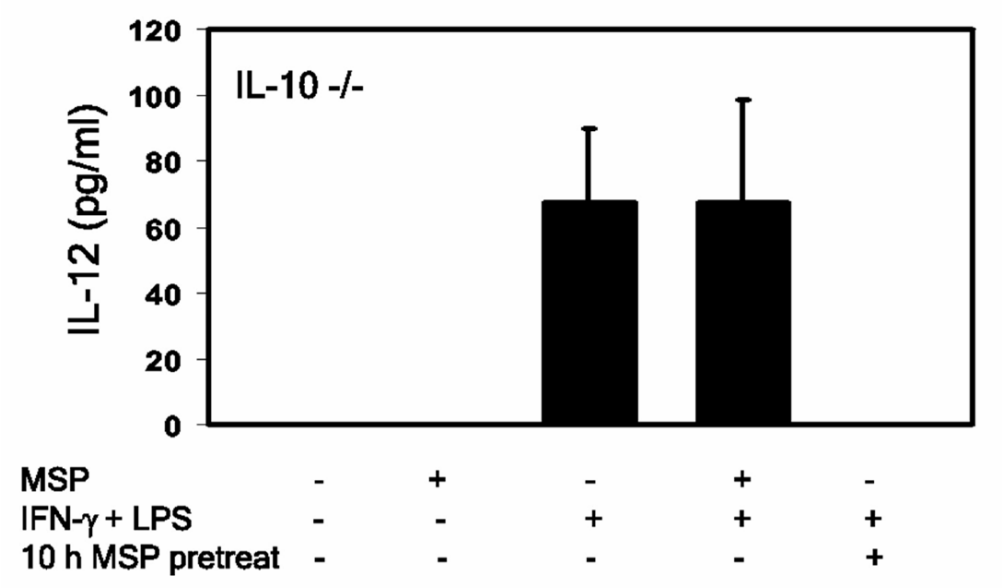

Figure **2-2**: Inhibition of IL-12 by MSP is independent of IL-10.

MSP inhibition of IL-12 production is independent of IL-10. *A*, Peritoneal macrophages from IL-$10^{-/-}$ were treated with or without 100 ng/ml MSP for 10 h before stimulation with 0.1 μM LPS plus IFN-γ and/or 100 ng/ml MSP for 24 h. Supernatants were collected, and IL-12p70 production was measured by ELISA. These data were adapted from [211].

Figure 2-3

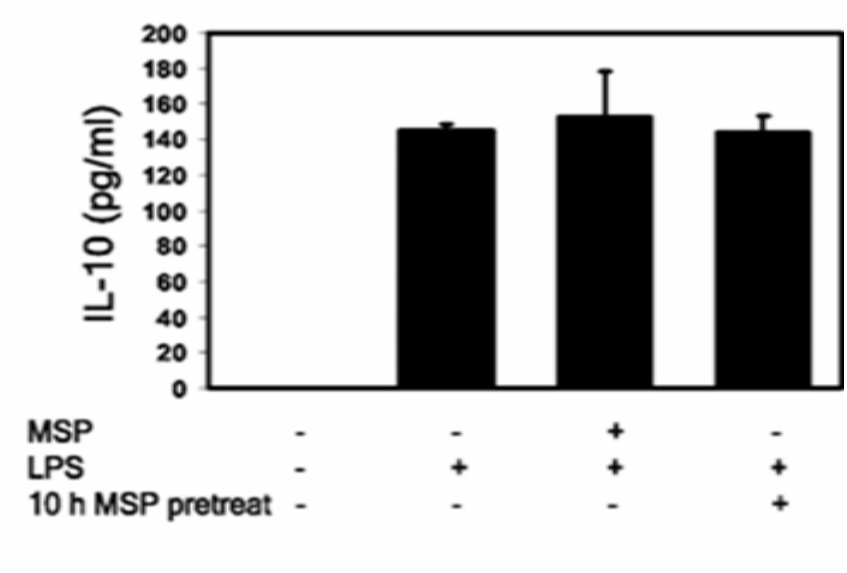

Figure **2-3**: IL-10 production is not induced by MSP.

Peritoneal macrophages were treated with or without 100 ng/ml MSP for 10h before stimulation with 0.1 μM LPS and/or 100 ng/ml MSP for 24h. Supernatants were collected, and IL-10 production was measured by ELISA. These data were adapted from [211].

Figure **2-4**

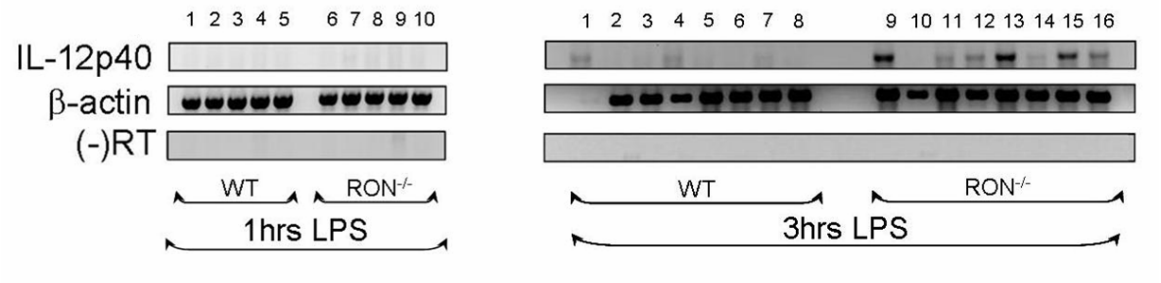

Figure **2-4:** RON$^{-/-}$ mice express more IL-12p40 *in vivo* after expose to LPS.

WT or RON$^{-/-}$ mice were treated with 20mg/kg of LPS i.p. for 1 and 3 hrs. Total splenic RNA was collected and IL-12p40 expression was examined by RT-PCR.

Figure **2-5**

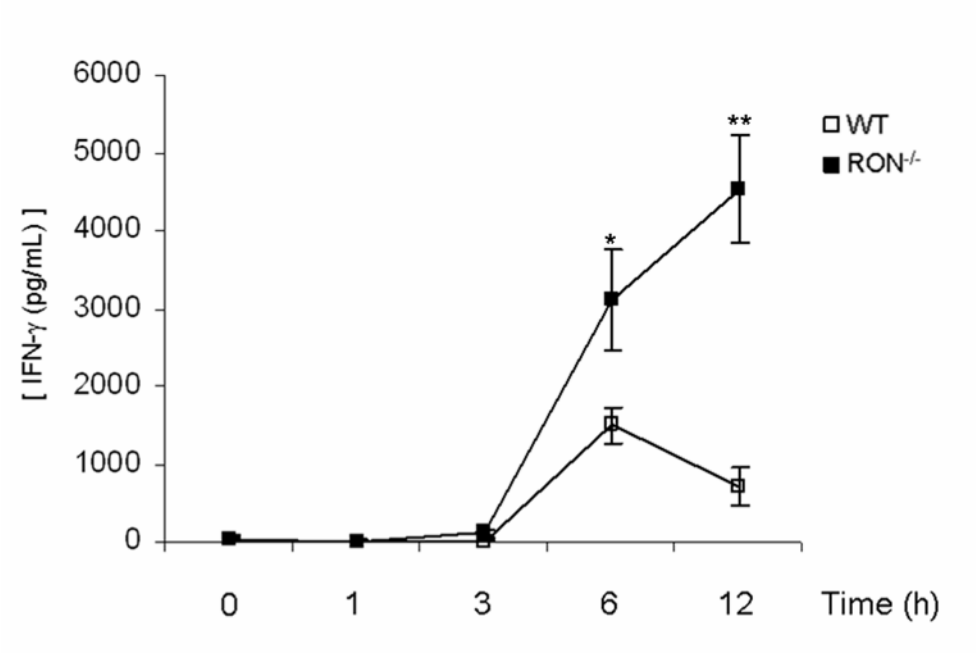

Figure **2-5**: IFN-γ production in RON$^{-/-}$ in response to LPS is induced at higher levels than in WT.

WT or RON$^{-/-}$ mice were treated with 20mg/kg of LPS i.p. for 0, 1, 3, 6 and 12 h. Serum levels of IFN-γ were measured by LINCOPlex®. These data were prepared in collaboration with Manujendra Ray and Daniel Sharda.

Figure **2-6**

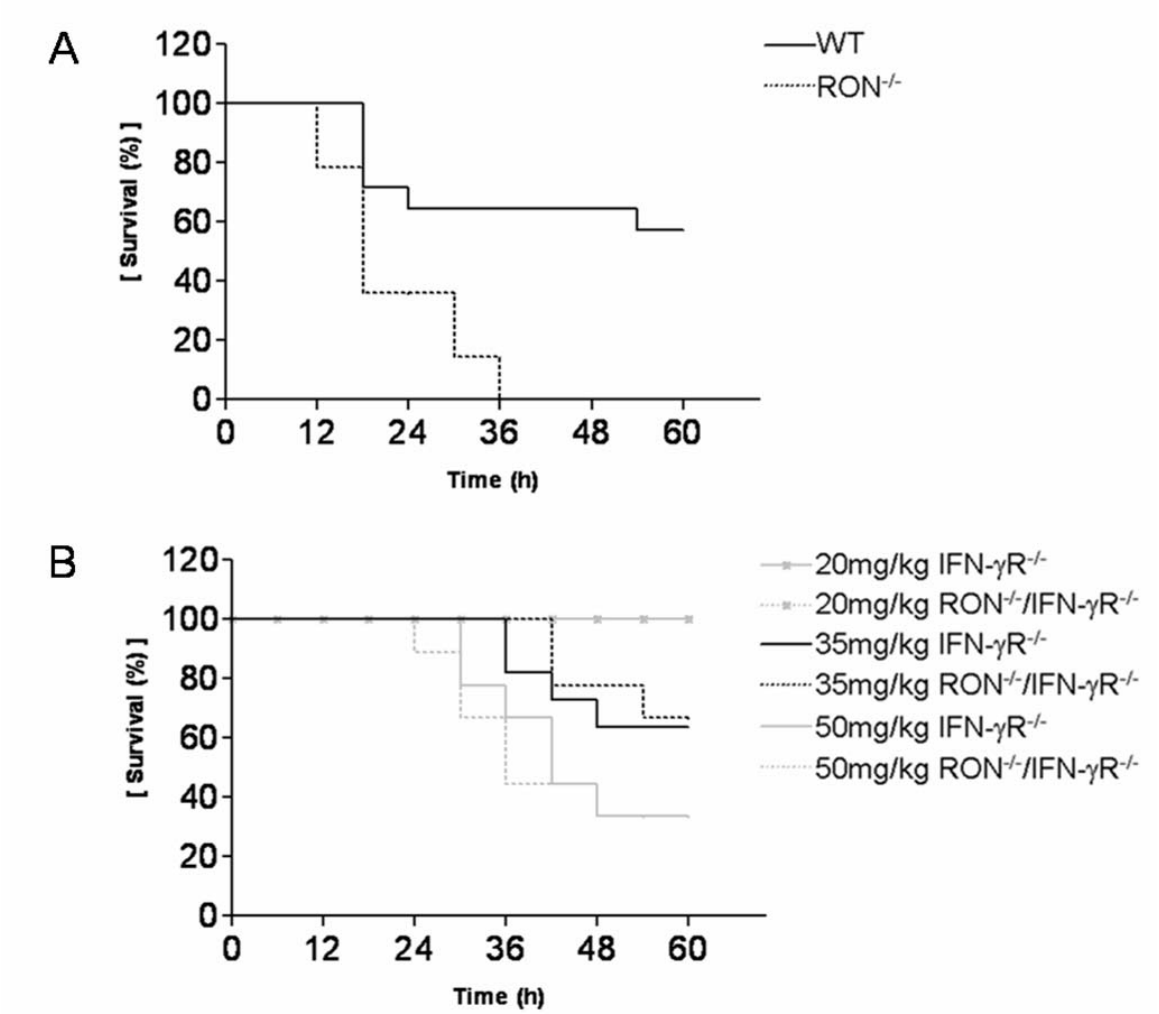

Figure **2-6**: $RON^{-/-}$ mice are more susceptible to LPS exposure than WT mice and IFN-γR signaling plays a role in increased susceptibility.

IFN-$\gamma R^{-/-}$ or $RON^{-/-}$/IFN-$\gamma R^{-/-}$ mice were treated with 20, 35 and 50mg/kg of LPS via the i.p. route. Viability was observed at 6 h intervals. These data were prepared in collaboration with Manujendra Ray and Daniel Sharda.

2.4 Discussion

The relationship of IL-12 and IFN-γ in innate and cell mediated immunity has proven to be pivotal in mounting a competent response to pathogens. In the presence of LPS, macrophages and dendritic cells produce IL-12 following up-regulation of the p40 subunit of the biologically active IL-12p70. IL-12p35, the other component of IL-12p70, is also up regulated. Exposure of NK cells and γδT cells during innate immunity to IL-12, which is produced after PRR ligation by PAMPs, induces local secretion of IFN-γ. The presence of IFN-γ augments the production of IL-12, as well as other innate immunity mediators such as NO, TNFα, and IL-6.

In the current studies we identify a critical role for the receptor tyrosine kinase RON in regulating IFN-γ production through the modulation of IL-12. Synergistic signaling of LPS and IFN-γ through TLR4 and the IFN-γR, respectively, is a potent inducer of IL-12. Our observations demonstrate that MSP prevents *in vitro* production of IL-12 in response to LPS and IFN-γ stimulation of macrophages. These findings suggest that MSP/RON regulates the threshold of signal required for the induction of pro-inflammation mediators. Further analysis revealed that the level of inhibition of IL-12 by MSP is at the IL-12p40 subunit , which is directly responsible for the up-regulation of IL-12p70 in response to inflammatory stimuli.

Alternatively, LPS/TLR4 signaling induces negative-feedback mechanisms, such as the transcriptional up-regulation of IL-10. IL-12, IL-6 and TNFα are inhibited by IL-10, while IL-10 upregulates anti-inflammatory genes including IL-1RA and sTNFR [133-135]. Development of inflammatory bowel disease (IBD) and increased immune responses to *Listeria monocytogenes* are hallmarks of IL-$10^{-/-}$ mice [136, 137]. Other signals that inhibit production of IL-12 by activated macrophages, including ligation of Fc receptors, also induce the up-regulation of IL-10 [212]. Here we clearly demonstrate that MSP/RON does not utilize IL-10 to obstruct IL-12 induced by LPS and IFN-γ.

Revelation of the *in vitro* negative-regulation of LPS and IFN-γ gene activation by MSP provides a framework to elucidate a role for RON in the *in vivo* modulation of LPS responses. Here, we have shown that RON inhibits IL12-p40 expression *in vivo* as well as *in vitro* following endotoxin administration, and that this inhibition correlates with reduced serum levels of IFN-γ. While RON is not expressed on most cell types that produce IFN-γ during an innate immune response including NK, NKT and $CD8^{+}$ memory T cells, we propose that the regulation of IFN-γ production *in vivo* in response to LPS challenge is indirect, due to the regulation of IL-12p40 production by RON (Figure **2-4**). The correlation of the *in vitro* and *in vivo* inhibition of LPS induced IL-12 firmly establishes a direct role for MSP/RON in the regulation of these pro-inflammatory responses

Consistent with our previous studies on the outbred CD-1 background, we show here that the absence of RON leads to increased susceptibility of mice to endotoxin challenge. Previous studies indicate that a lack of regulation of IFN-γ/IFN-γR signaling is critical to endotoxin susceptibility *in vivo*. IFN-γR1$^{-/-}$ mice are resistant to endotoxin induced death [53, 213]. In mice that lack both RON and IFN-γR signaling, we observe a reversal of the vulnerability of RON$^{-/-}$ animals to endotoxin challenge. These results indicate that the increased susceptibility of RON$^{-/-}$ mice to septic shock is mediated by the ability of RON to regulate IFN-γ-mediated responses. In all, the data in this chapter provide clear evidence for a role for the RON receptor in the regulation of the IL-12p40/IFN-γ axis *in vivo*.

Chapter 3

MHC Class II Expression is Regulated by MSP/RON by Inhibition of IFN-γ/IFN-γR Signal Transduction in Primary Peritoneal Macrophages *In Vitro*

3.1 Introduction

Macrophages are amongst the first responders to immune challenge due to their proximity to areas of potential antigen exposure in animal tissues. IFN-γ is a critical mediator of immunity by macrophages. NKT cells and $CD8^+$ T cells are directed by IL-12 to produce IFN-γ and thus propagate inflammation. One integral gene targeted by IFN-γ is MHC class II, the up-regulation of which prepares macrophages for antigen presentation to T lymphocytes. IFN-γ utilizes the JAK/STAT pathway to direct up-regulation of genes vital to the innate and Th1 immune response. STAT1 and IRF1 are necessary for the expression of CIITA. The gateway to MHC class II expression and subsequent antigen presentation by macrophages is mediated by CIITA.

The RON receptor tyrosine kinase, a member of the MET family of proto-oncogene RTKs, is a disulfide linked hetero-dimer with an extra-cellular α-chain and a trans-membrane β-chain. Isolation of RON was accomplished by screening of a human keratinocyte cDNA library, and murine RON (mRON) was cloned from hematopoietic stem cells [142, 143]. Activation of RON by its ligand MSP initiates dimerization and induces intrinsic kinase activity [146]. Phosphorylation of C-terminal tyrosine residues

results in the activation of various signaling cascades via recruitment through SH2 domains that include, but are not limited to GRB2, PI3K, GAB1, SHC, SHIP1 and PLC-γ [163-166]. MSP is a serum protein related to hepatocyte growth factor (HGF), which is produced by hepatocytes, circulates as an inactive pro-MSP form and is activated by proteases under pro-inflammatory conditions. MSP/RON inhibits IFN-γ and LPS induced NF-κB, iNOS and COX2 expression and subsequent NO and PGE2 production by primary peritoneal macrophages. Additionally, chemotaxis, morphological changes and increased C3bi mediated phagocytosis are induced by MSP/RON. Alternately, MSP/RON up-regulates factors that are anti-inflammatory such as IL-1R antagonist and SR-A, as well as genes involved in wound healing including ARG1.

Adequate responses of macrophages to pro-inflammatory mediators are critical to mounting an effective innate immune response. Previous studies from our group and others have demonstrated that the absence of RON *in vivo* increases susceptibility of mice to LPS mediated endotoxic shock [179]. IL-12p40 and IFN-γ is up-regulated at a faster rate in $RON^{-/-}$ vs. WT mice post exposure to LPS (Figures **2-4** and Figure **2-5**). *In vitro* investigations have elucidated a role of MSP/RON in negatively regulating the response of macrophages to LPS and IFN-γ [185, 186, 211, 214]. MHC class II and CIITA the central regulator of MHC class II expression, are down-regulated by activation of RON by MSP after stimulation with IFN-γ of primary peritoneal macrophages *in vitro*. MSP/RON up-regulate expression of SOCS1 and SOCS3 independently of STAT3. Furthermore, IFN-γR surface expression is reduced post treatment of primary peritoneal

macrophage with MSP without activation of the IFN-γR. In the current studies we seek to evaluate the effects of MSP/RON on the signaling pathway and target genes induced by IFN-γ *in vitro*.

3.2 Materials and Methods

3.2.1 *Cells and Animals*

The mouse strains used for these experiments were wild-type (WT) C57/BL6 (The Jackson Laboratory) and C57/BL6 with a targeted mutation in the RON gene, which were generated by backcrossing with CD-1 mice with a targeted mutation in the RON gene [179] for 12 generations. Murine resident peritoneal macrophages were obtained by peritoneal lavage with 10 ml of RPMI containing 10% fetal bovine serum (FBS). Cells were incubated for 5-7 hours and then washed with PBS to eliminate non-adherent cells. All cell cultures were maintained in RPMI + 10% FBS at 37^{o}C in a humidified incubator containing 5% CO_2. The Institutional Animal Care and Use Committee (IACUC) of The Pennsylvania State University granted approval for all experimental procedures.

3.2.2 *Reagents and Antibodies*

The following reagents were obtained from the indicated sources: DMEM, RPMI-1640, FBS, trypsin, and PBS (Invitrogen); MSP and IL-10 (R&D Systems); IFN-γ (Pepro Tech); lipopolysaccharide (LPS), glutamine and β2-mercaptoethanol (Sigma-

Aldrich). Antibodies for Western blotting against pY^{701}STAT1, pS^{727}STAT1, STAT1, pY^{705}STAT3 and STAT3 were purchased from Cell Signaling.

3.2.3 *Flow Cytometry*

Cells were harvested, incubated in the presence or absence of 100ng/ml MSP for 24 hours, and washed 3X with PBS. $5X10^5$ cells/100 μl were resuspended in PBS + 2% newborn calf serum (NCS) on ice. Fc receptors were blocked using 1 μl of anti-mouse CD32/16(FcγIII/II receptor; BD Biosciences) for 5 min on ice. MHC Class II and IFN-γR cell surface expression was determined by staining with fluorescein isothiocyanate (FITC) anti-mouse $I\text{-}A^b$ (BD Biosciences) and purified CD119 (BD Biosciences) plus FITC anti-CD119 (BD Biosciences) then analyzed by flow cytometry (XL; Coulter, Hialeah, FL).

3.2.4 *RNA Extraction and RT-PCR*

Total RNA from WT murine peritoneal macrophages was isolated with RNeasy mini column kit (Qiagen). Reverse transcription was carried out for 40 minutes at 42°C using Oligo d(T)16 primers (Applied Biosystems) and murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems) on 0.1μg of total RNA. Conditions for PCR are as follows: CIITA [215], 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 10 min (CIITA sense, 5'- TGC AGG CGA CCA GGA GAG ACA -3'; CIITA antisense, 5'-GAA GCT

GGG CAC CTC AAA GAT-3' and β-actin under the same conditions (β-actin sense, 5'-TGC TGC GTG ACA TCA AAG AG-3', β-actin antisense,5'-TGG ACA TGA GGC CAG GATG-3'). For real-time PCR total RNA from murine peritoneal macrophages was isolated as described above and reverse transcription was achieved using the Applied Biosystems High Capacity RT Kit at 37^{o}C for 2 hours. Amplification was carried out at 50^{o}C for 2 minutes, 95^{o}C for 10 minutes, 40 cycles of 95^{o}C for 15 seconds and 60^{o}C for 1 minute. Primer sequences and probes are as follow: mSOCS1 sense 5'-CCG TGG GTC CGC AGA AC-3', mSOCS1 anti-sense 5'-AAG GAA CTC AGG TAG TCA CGG AGT A-3' and probe 5'6-FAM d(TGG CGC GCA TCC CTC TTA ACC C) BHQ-1 3', mSOCS3 sense 5'-GCC ACC TGG ACT CCT ATG AGA A-3', mSOCS3 anti-sense 5'-GGA GCA TCA TAC TGA TCC AGG AA-3' and probe 5'6-FAM d(AGC TGC CT GC TG GA CC CA TT CG GG) BHQ-1 3'.

3.2.5 *Western Blot Analysis*

5X10^{6} cells were incubated with the indicated cytokines. After incubation cells were washed with PBS and lysed for 15 min on ice in 500μl lysis buffer containing 150mM NaCl, 10mM Tris-HCl (pH 7.5), 0.5% Triton X-100, and protease inhibitors 1 μg/ml Leupeptin, 1μg/ml Pepstain A, and 2 μg/ml aprotinin. The protein contents were determined using the DC protein assay kit (Bio-Rad). Absorbance was measured at 750nm with a Beckman DU530 spectrophotometer. Proteins were mixed with 5X SDS sample buffer. SDS-PAGE, using 12.5% bis-acrylamide gel for separation which was performed with a MiniProtean II Cell (Bio-Rad) at 65V for 15 min, then at 150-160V for

1h. Immobilon P PVDF membranes (Millipore) were washed briefly in methanol, then ddH_2O, then equilibrated in trans-blotting buffer (48mM Tris 39mM glycine, and 20% methanol) for 5 min. The gels were washed in ddH20, then in trans-blotting buffer for 15min. Transblotting was performed using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 1h at 100V. The blots were then placed in blocking buffer containing 5% non-fat dry milk for 1h at room temperature. The blots were then incubated over night with primary Ab, according to manufacturer's instructions. Anti-rabbit Abs conjugated with HRP were added at 1:8000 for 1hour. Protein was then detected by incubated the blots in chemiluminescense substrates (GE Healthcare) and exposing to x-ray film.

3.3 Results

3.3.1 *MSP inhibits IFN-γ induced MHC class II surface expression and the transcriptional regulator CIITA*

In Chapter 2 (Figure **2-1**) we demonstrated that MSP stimulation of the RON receptor tyrosine kinase on resident peritoneal macrophages inhibits the production of IL-12p40 in response to low dose IFN-γ priming in the presence of LPS. To elucidate whether MSP stimulation of RON regulates IFN-γ-dependent gene expression in response to high doses of IFN-γ, which are the conditions required for the induction of MHC Class II, we stimulated primary peritoneal macrophages from WT and RON-/- mice with MSP for 12 hours followed by treatment with 200 U/mL IFN-γ and examined

MHC class II expression 24 hours later by flow cytometry (Figure **3-1**). The resulting data suggest that MSP inhibits the induction of MHC class II surface expression in response to high doses of IFN-γ. Furthermore, utilizing $RON^{-/-}$ mice we show that this inhibition of MHC class II expression is dependent on the RON receptor tyrosine kinase. MHC class II expression is regulated by the master transcriptional regulator class II *trans*-activator (CIITA) which is in turn induced by activated STAT1 and IRF1. Therefore, we examined CIITA expression in primary peritoneal macrophages from WT and $RON^{-/-}$ mice by RT-PCR in response to IFN-γ in the presence or absence of MSP for 12 hours followed by 6 hours of IFN-γ exposure. Stimulation of these cells with MSP markedly decreased the induction of CIITA expression by IFN-γ (Figure **3-2**).

3.3.2 *STAT1 activation by IFN-γ is suppressed by MSP*

STAT1 is a key signal transducer, activated down-stream of IFN-γ in the induction of IL-12p40. IFN-γ activates STAT1 by inducing phosphorylation of Y701 and S727 via kinases associated with the IFN-γR signaling pathway thus facilitating optimal transcription [216]. Based on our observations that MSP down-regulates IFN-γ-induced MHC class II and CIITA expression, we sought to determine at what level MSP affects IFN-γ signal transduction by examining STAT1 activation in response to high-doses of IFN-γ. Therefore, we pre-stimulated primary peritoneal macrophages with MSP for 12 hours followed by stimulation with IFN-γ. Data from these experiments clearly

demonstrate that MSP inhibits IFN-γ-induced STAT1 phosphorylation at 30 and 60 minutes following IFN-γ stimulation (Figure **3-3**).

3.3.3 *Exposure to MSP down-regulates IFN-γR surface expression*

To determine whether the MSP/RON signaling pathway regulates surface expression of the IFN-γR, we stimulated primary peritoneal macrophages with MSP for 12 hours and examined surface expression of the IFN-γR by flow cytometry. MSP treatment resulted in the down-regulation of surface IFN-γR expression at levels comparable to that seen with IL-10 stimulation (Figure **3-4**). This suggests the possibility that MSP is regulating IFN-γ signaling in activated macrophages, in part, by promoting turnover of the IFN-γR.

3.3.4 *MSP up-regulates Expression of SOCS1 and 3; however, STAT3 is not activated by MSP/RON*

SOCS1, the expression of which is induced by IFN-γ, is a classic negative feedback regulator of IFN-γ signaling. SOCS1, which contains an SH2 domain through which it is recruited to the IFN-γR as well as receptor associated JAK1/2 kinases, targets the IFN-γR signaling complex for proteasome-dependent degradation. Therefore, we set out to determine whether MSP induces expression of SOCS1 and 3. IL-10 which has been shown to induce SOCS1 and 3 expression, was utilized as a positive control. Real-time PCR analysis demonstrated that SOCS1 and 3 expression is induced by MSP at one

and three hours following stimulation and returns to basal levels within six hours (Figure **3-5**). SOCS1 is a critical regulator of IFN-γ signaling *in vivo*, suggesting the possibility that MSP/RON regulates IFN-γ-dependent responses through up-regulation of SOCS1.

STAT3 is a critical regulator of macrophage activation, and IL-10-induced STAT3 activity is required for the majority of IL-10-mediated responses [217-219]. Receptor tyrosine kinases have also been shown to induce STAT3 tyrosine phosphorylation in several systems. Therefore, we set out to determine whether MSP induces tyrosine phosphorylation of STAT3 in primary peritoneal macrophages. Macrophages were treated with MSP for the indicated times, whole cell lysates were extracted and STAT3 phosphorylation was examined using anti-phospho-STAT3 antibodies (Figure **3-6**). IL-10 stimulation was utilized as a positive control. Over the time course tested, we failed to detect significant up-regulation of STAT3 tyrosine phosphorylation when compared with the induction by IL-10. We have shown previously that the RON receptor has some constitutive activity that is independent of ligand binding. Therefore, we compared the levels of STAT3 tyrosine phosphorylation in primary peritoneal macrophages from WT and $RON^{-/-}$ mice. Again, we failed to detect any significant differences in tyrosine phosphorylation either in the presence or absence of MSP or IL-10 signals. We conclude that MSP induces SOCS expression, the down-regulation of IFN-γR and the inhibition of IFN-γ-dependent gene expression by a mechanism that is independent of STAT3.

Figure **3-1**

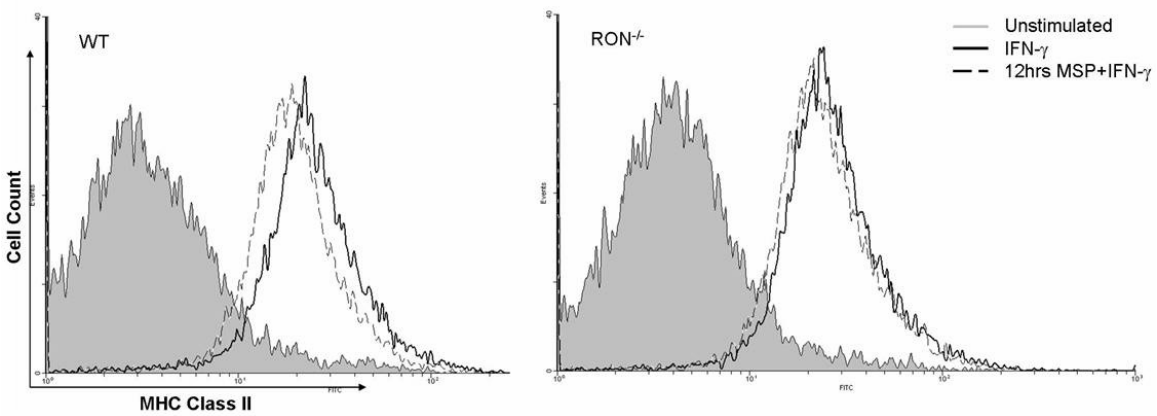

Figure **3-1**: MSP inhibits IFN-γ induced up-regulation of MHC Class II surface expression.

Primary peritoneal macrophages from WT and $RON^{-/-}$ mice were harvested by lavage and isolated by adherence. Stimulations were carried out with or without 100 ng/mL MSP overnight followed by 200U/mL IFN-γ. 24 hours later, the cells were harvested and MHC Class II surface expression was examined by flow cytometry using a FITC-labeled anti-IA^b.

Figure **3-2**

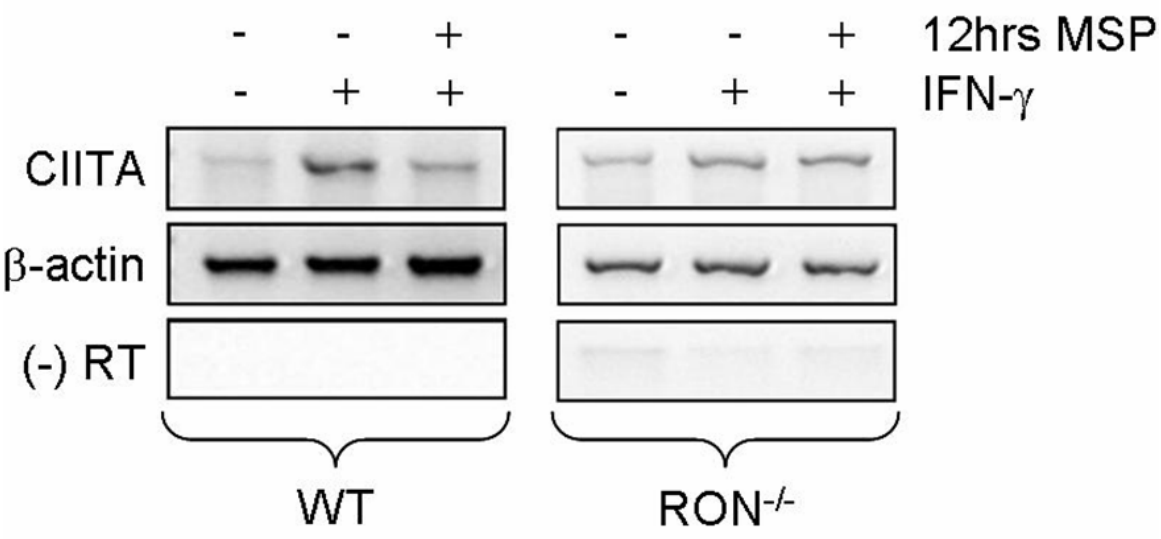

Figure **3-2**: CIITA expression is reduced by MSP.

Primary peritoneal macrophages from WT and RON$^{-/-}$ mice were harvested by lavage and isolated by adherence. Stimulations were administered with or without 100 ng/mL MSP for 12 hours followed by 200 U/mL IFN-γ. Six hours later, total mRNA was isolated. Expression of CIITA was assessed by RT-PCR analysis. Expression of β-actin from the same samples was used as a loading control.

Figure **3-3**

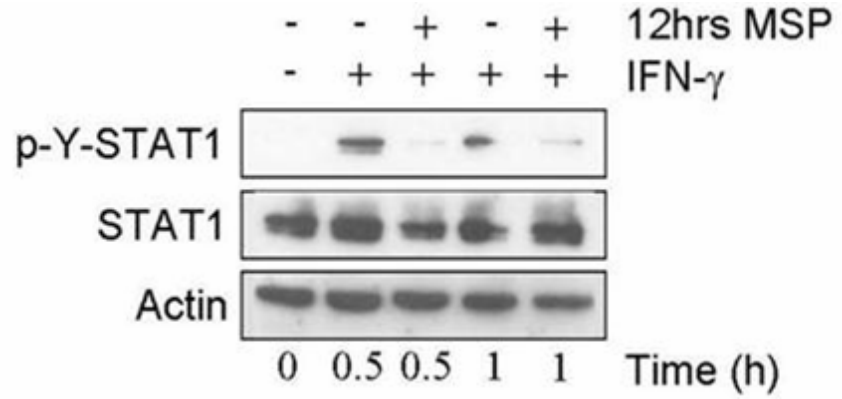

Figure **3-3**: Stimulation of the RON receptor tyrosine kinase with MSP inhibits IFN-γ induced STAT1 phosphorylation.

Peritoneal macrophages were treated with or without 100 ng/mL MSP and 200U/mL of IFN-γ for the indicated times. Whole cell lysates were harvested and tyrosine phosphorylation of STAT1 was assessed by Western blot analysis, and then the membrane was stripped and reprobed for total STAT1 and Actin.

Figure **3-4**

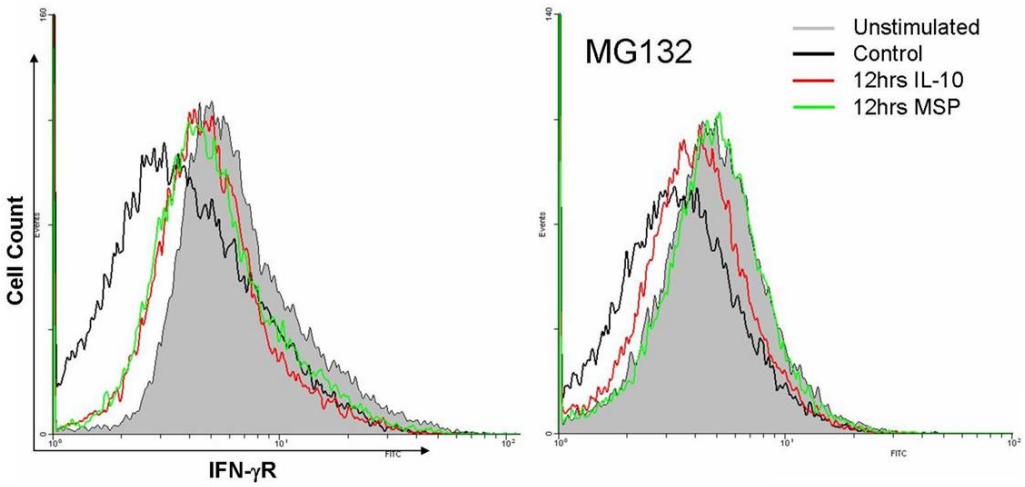

Figure **3-4**: MSP reduces IFN-γR surface expression.

MSP reduces IFN-γR expression. Primary peritoneal macrophages from were harvested by lavage and isolated by adherence. Stimulations were carried out with or without 100 ng/mL MSP or 10ng/mL IL-10 for the indicated times. IFN-γR surface expression was examined by flow cytometry using a primary anti-CD119 and FITC-labeled anti-CD119 secondary.

Figure **3-5**

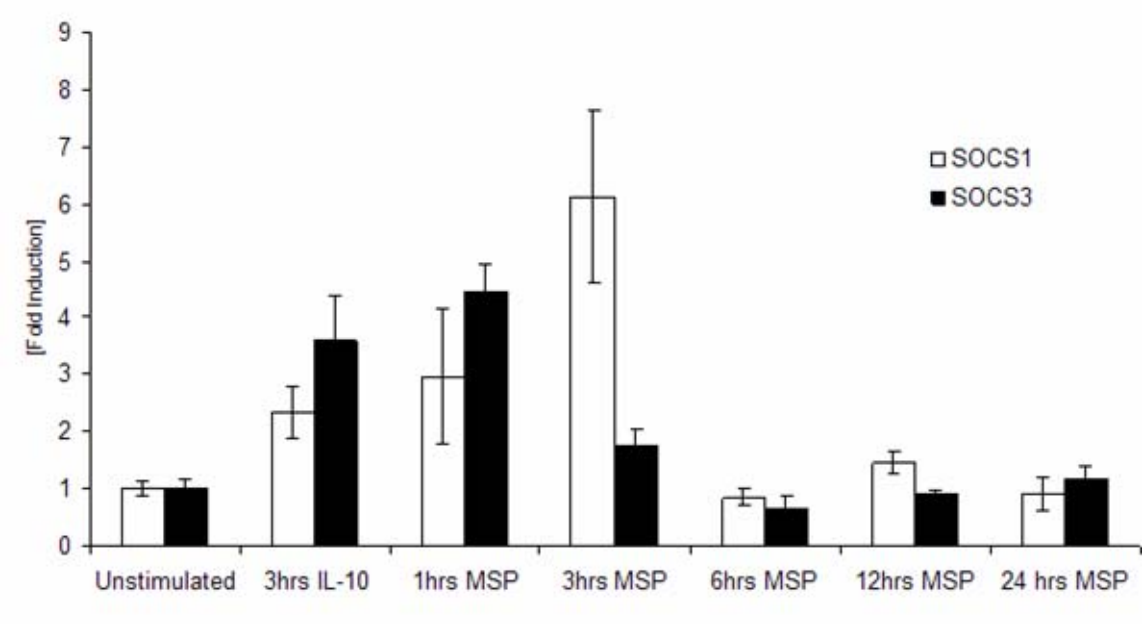

Figure **3-5**: MSP up-regulates SOCS1 and 3 transcripts.

Peritoneal macrophages were treated with or without 100 ng/mL MSP for 0, 1, 3, 6, 14 and 24 hrs and 10ng/mL of IL-10 for 3 hrs. Total mRNA was then collected and expression of SOCS1 & 3 was accessed by real time RT-PCR.

Figure **3-6**

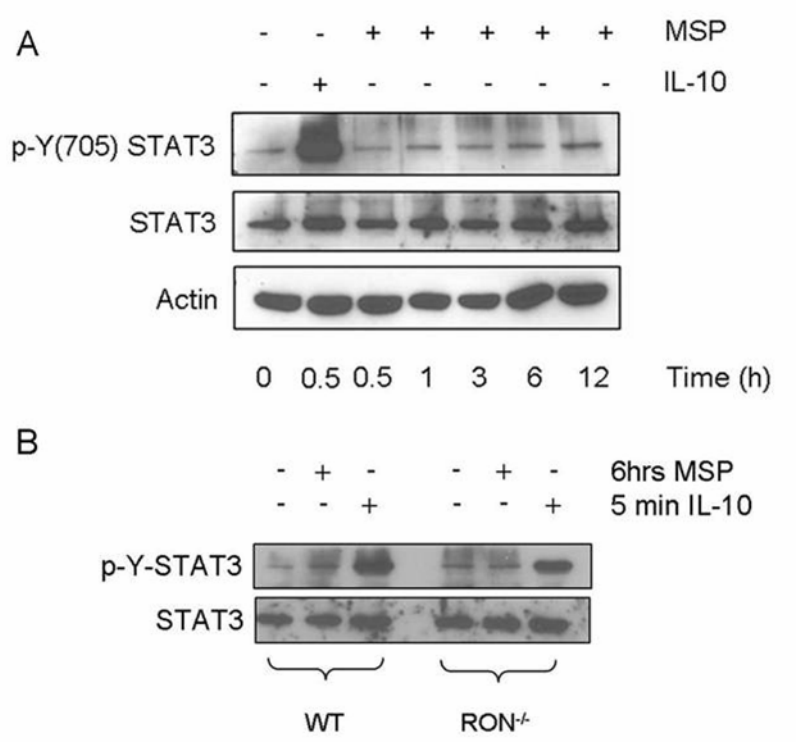

Figure **3-6**: MSP induces moderate STAT3 tyrosine phosphorylation and RON$^{-/-}$ have constant ability to activate STAT3 as WT.

A. Peritoneal macrophages were treated with or without 100 ng/mL MSP and 10ng/mL of IL-10 for the indicated times. Whole cell lysates were harvested and tyrosine phosphorylation of STAT3 was assessed by Western blot analysis, and then stripped and reprobed for STAT3 and Actin. *B*. Western blot analysis for phosphorylated STAT3 was performed on whole cell lysates from peritoneal macrophages from WT and RON$^{-/-}$ mice stimulated as prescribed in *A* for the indicated times.

3.4 Discussion

The data described herein indicate a role for MSP/RON in regulating macrophage responses to IFN-γ. Modulation of GAF/GAS activated genes not only directs local activities of macrophages but also regulates the potential magnitude of the global immune response to specific pathogens.

Signal transduction in macrophages down-stream of IFN-γ/IFN-γR incorporates activation of the JAK/STAT pathway through tyrosine phosphorylation of JAK1 and JAK2 followed by tyrosine and serine phosphorylation of STAT1. Homo-dimers of STAT1, also known as GAF are achieved by SH2 mediated binding of tyrosine phosphorylated monomers, resulting in nuclear translocation, while serine phosphorylation regulates transcriptional activation. As a transcription factor GAF promotes expression of IFN-γ responsive genes, such as IRF1, ICSBP/IRF8, iNOS, IL-12p40, COX2 and CIITA at the GAS DNA element.

A primary target of IFN-γ/IFN-γR signaling is MHC class II, and expression of MHC class II is tightly regulated by the transcription factor CIITA. Surface expression of MHC class II induced by IFN-γ is down-regulated by MSP/RON (Figure **3-1**). CIITA, which is upstream of MHC class II, is also reduced by MSP/RON (Figure **3-2**). Modulation of proteins such as MHC class II in macrophages suggests MSP/RON may

play a role in regulating antigen presentation and have a net effect on cell mediated immunity *in vivo*.

Activation of the IFN-γR by IFN-γ also results in activation of negative feedback pathways, including the induction of SOCS1. Mice with a targeted deletion in the SOCS1 gene are highly susceptible to IFN-γ mediated disease within in a few weeks of birth [220]. Data presented here indicate that MSP/RON signaling likewise results in an increase in SOCS1 and 3 transcripts in primary peritoneal macrophages (Figure **3-5**).

SOCS proteins act to disrupt cytokine receptor signaling at multiple levels. The inhibition of intrinsic auto-phosphorylation events are achieved when SOCS proteins bind to receptor associated phosphorylated tyrosine residues via their SH2 domains. SOCS is also an E3 ubiquitin ligase that targets the receptor complex for degradation. In addition, SOCS proteins can bind to phosphorylated JAK kinases and act as a pseudo-substrate, thus inhibiting JAK kinase activities. SOCS1 can also bind to the phosphorylated IFN-γR and inhibit recruitment of STAT1. The kinetics of SOCS up-regulation is transient. In the current studies, we investigated the potential relationship of SOCS1 up-regulation to the negative regulation of IFN-γ signals by MSP/RON by examining the ability of RON signaling to reduce the surface expression of IFN-γR. The results indicate that MSP/RON has an effect on available IFN-γR on the surface of primary macrophages (Figure **3-4**). It is therefore possible that MSP/RON inhibitory

effects are directly linked to a combination of reduction of IFN-γR availability to ligand and inhibition of STAT1 tyrosine phosphorylation downstream of the IFN-γR.

Due to the similarities of the anti-inflammatory properties of MSP/RON and IL-10/IL-10R, we wanted to determine if both receptors shared a common signaling cascade that incorporated the activation of STAT3, which is well know constituent of the IL-10R signal transduction pathway. We found that stimulation of macrophages with MSP does not induce phosphorylation of STAT3 to the levels observed for IL-10 (Figure **3-6A**). These observations were also consistent in a comparison of MSP stimulated macrophages from WT and $RON^{-/-}$ mice (Figure **3-6B**). IL-10 induces sustained STAT3 activation and expression of a constitutively active form of STAT3 (STAT3c) in macrophages stimulated with LPS results in suppression of TNFα and IL-6 production [221]. While MSP/RON does not appear to activate STAT3 in primary peritoneal macrophages, this does not rule out a requirement for STAT3 in the inhibition of macrophage activation by RON. Studies in STAT3 floxed mice will determine whether STAT3 plays a role in mediating the inhibition of IFN-γ-induced signals in macrophages by RON.

Other known pathways that are associated with RON signaling will have to be investigated in order, to determine the mechanism by which MSP/RON inhibits IL-12p40, STAT1 activation, CIITA up-regulation and MHC class II surface expression. The data presented here strongly indicates that the inhibitory affects of and the up-regulation of SOCS genes by MSP/RON maybe independent of STAT3. The *Src* family

of kinases (SFKs), PI3K, ERK, JNK and p38 MAPK have been identified as constituents of the down-stream signaling pathway of RON and other MET family RTKs. We have shown that mRON interacts with c-Src in a ligand-independent manner through three tyrosines in the kinase domain [171]. Further, we have shown that inhibition of IL-12p40 production in primary macrophages by RON is dependent on SFKs (Unpublished data). PI3K is part of a negative feedback loop that regulates TLR4 signals. $PI3K^{-/-}$ mice exhibit enhanced production of IL-12 in response to LPS and IFN-γ in dendritic cells (DC) [140, 141]. Previous studies from our group demonstrate that stimulation of RON by MSP activates the PI3K pathway in primary peritoneal macrophages [165]. Additionally, a role for p38 MAPK in the induction of SOCS3 in B cells has been identified [222].

Chapter 4

MSP/RON Preferentially Inhibits the MyD88-dependent Pathway of TLR4

4.1 Introduction

Macrophages are amongst the first responders to immune challenge due to their proximity to areas of potential antigen exposure in animal tissues. PRRs on the surface of macrophages allow them to identify antigenic factors such as LPS from Gram-negative bacteria and other PAMPs. LPS is a critical inducer of innate immunity by macrophages. Upon challenge with LPS, macrophages produce pro-inflammatory mediators such as IL-12, TNF-α, IL-1 and IL-6. These cytokines direct the macrophages and other constituents of the innate immune system to become activated and mobilize. NKT cells and $CD8^+$ T cells are directed by IL-12 to produce IFN-γ, thus propagating inflammation.

IFN-γ primes macrophages to respond to LPS through the induction of STAT1 phosphorylation and the up-regulation of IRF-1, which cooperate with LPS-induced NFkB to up-regulate the expression of several inflammatory genes. However, the JAK/STAT pathway can also be activated by LPS activation of TLR4 via the adaptor TRIF/TRAM, which is independent of the MyD88/TIR and NFκB pathway [126, 127, 223, 224]. Central to the activities of TRIF/TRAM is the activation of IRF3. This branch of the LPS/TLR4 pathway results in the positive feed-back production of IFN-β.

Ligation of the type I IFNR by IFN-β induces tyrosine phosphorylation of STAT1 resulting in the up-regulation of IFN responsive genes, such as IL-12p40 and iNOS. Furthermore, in response to LPS stimulation IFN-β activation of STAT1 has been recently shown to be critical for induction of IL-10 [225].

The RON receptor tyrosine kinase, a member of the MET family of proto-oncogene RTKs, is a disulfide linked hetero-dimer with an extra-cellular α-chain and a trans-membrane β-chain [142, 143, 226]. Isolation of RON was accomplished by screening of a human keratinocyte cDNA library, and murine RON was cloned from hematopoietic stem cells [142, 143]. Activation of RON by its ligand MSP initiates dimerization and results in enhanced intrinsic kinase activity. Phosphorylation of C-terminal tyrosine residues results in the activation of various signaling cascades via recruitment through SH2 domains that include, GRB2, PI3K, GAB1, SHC, SHIP1 and PLC-γ [163-166]. MSP is a serum protein related to hepatocyte growth factor (HGF), which is produced by hepatocytes, circulates as an inactive pro-MSP form and is activated by proteases under pro-inflammatory conditions. MSP/RON inhibits IFN-γ and LPS induced NF-κB activity, iNOS and COX2 gene expression and subsequent NO and PGE2 production by primary peritoneal macrophages [163, 185, 205]. Additionally, chemotaxis, morphological changes and increased C3bi mediated phagocytosis are induced by MSP/RON. Alternately, MSP/RON up-regulates genes associated with alternative macrophage activation including IL-1R antagonist , SR-A, and arginase I [186].

Adequate responses of macrophages to pro-inflammatory mediators are critical to mounting an effective innate immune response. Previous studies from our group and others have demonstrated that the absence of RON *in vivo* increases susceptibility of mice to LPS mediated endotoxic shock. In vitro investigations have elucidated a role for MSP/RON in the negative regulation of gene responses to LPS and IFN-γ. In response to LPS alone MSP, starting as early as 4 hours, exposure dampens the amount of IL-12p40 secretion by primary peritoneal macrophages. IFN-β gene induction and STAT1 activation by LPS is not inhibited by MSP. In the current studies we seek to evaluate the independent effects of MSP/RON on the signaling pathways and targeted genes of LPS.

4.2 Materials and Methods

4.2.1 *Cells and Animals*

The mouse strains used for these experiments were wild-type (WT) C57/BL6 (The Jackson Laboratory) and C57/BL6 with a targeted mutation in the RON gene, which were generated by backcrossing with CD-1 mice with a targeted mutation in the RON gene [179] for 12 generations. Murine resident peritoneal macrophages were obtained by peritoneal lavage with 10 mL of RPMI containing 10% fetal bovine serum (FBS). Cells were incubated for 5-7 hours and then washed with PBS to eliminate non-adherent cells. All cell cultures were maintained in RPMI + 10% FBS at 37^{o}C in a humidified incubator

containing 5% CO_2. The Institutional Animal Care and Use Committee (IACUC) of The Pennsylvania State University granted approval for all experimental procedures.

4.2.2 *Reagents and Antibodies*

The following reagents were obtained from the indicated sources: DMEM, FBS, trypsin TRIzol® and PBS (Invitrogen); MSP and IL-12p40 ELISA kit (R&D Systems); IFN-γ (Pepro Tech); lipopolysaccharide (LPS), glutamine and β2-mercaptoethanol (Sigma-Aldrich). Antibodies for Western blotting against pY^{701}STAT1, pS^{727}STAT1, STAT1, pS^{396}IRF3 and IRF3 were purchased from Cell Signaling.

4.2.3 *RNA Extraction and RT-PCR*

Total RNA from WT murine peritoneal macrophages was isolated with RNeasy mini column kit (Qiagen). Reverse transcription was carried out for 40 minutes at 42°C using Oligo d(T)16 primers (Applied Biosystems) and murine leukemia virus (MuLV) reverse transcriptase (Applied Biosystems) on 0.1μg of total RNA. Conditions for PCR are as follows: IFN-β [227], 94°C for 3 min, followed by 30 cycles of 94°C for 30s min, 60°C for 30s min, and 72°C for 1 min, with a final extension at 72°C for 10 min (IFN-β sense, 5'-ACA CAA GCT TAA CCA CCA TGA ACA ACA GGT GGA TCC TCC ACG C-3'; IFN-β antisense, 5'- GTT AGG AAT TCT CAG TTT TGG AAG TTT CTG GTA AGT CTT CG -3') and β-actin under the same conditions (β-actin sense, 5'- TGC TGC GTG ACA TCA AAG AG-3', β-actin antisense, 5'- TGG ACA TGA GGC CAG GAT G-3').

4.2.4 *Western Blot Analysis*

$5X10^6$ cells were incubated with the indicated cytokines. After incubation cells were washed with PBS and lysed for 15 min on ice in 500µl lysis buffer containing 150mM NaCl, 10mM Tris-HCl (pH 7.5), 0.5% Triton X-100, and protease inhibitors 1 µg/ml Leupeptin, 1µg/ml Pepstain A, and 2 µg/ml aprotinin. The protein contents were determined using the DC protein assay kit (Bio-Rad). Absorbance was measured at 750nm with a Beckman DU530 spectrophotometer. Proteins were mixed with 5X SDS sample buffer. SDS-PAGE, using 12.5% bis-acrylamide gel for separation which was performed with a MiniProtean II Cell (Bio-Rad) at 65V for 15 min, then at 150-160V for 1h. Immobilon P PVDF membranes (Millipore) were washed briefly in methanol, then ddH_2O, then equilibrated in trans-blotting buffer (48mM Tris 39mM glycine, and 20% methanol) for 5 min. The gels were washed in ddH20, then in trans-blotting buffer for 15min. Transblotting was performed using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 1h at 100V. The blots were then placed in blocking buffer containing 5% non-fat dry milk for 1h at room temperature. The blots were then incubated over night with primary Ab, according to manufacturer's instructions. Anti-rabbit Abs conjugated with HRP were added at 1:8000 for 1hour. Protein was then detected by incubated the blots in chemiluminescense substrates (GE Healthcare) and exposing to x-ray film.

4.2.5 *Determination of Cytokine Concentrations*

Cells (1 x 10^5) were incubated with the cytokines indicated in a 96-well plate, and the cytokines in the supernatant were detected by standard sandwich ELISA. IL-12p40 levels were quantified according to the manufacturer's instructions. Colorimetric changes in enzyme substrates were detected at 430 nm using a HTS 7000 Bio Assay Reader (PerkinElmer).

4.3 Results

4.3.1 *Four hours of MSP exposure is adequate to prevent production of IL-12p40 by LPS alone or in combination with IFN-γ*

The data represented in Chapter 2 is a clear indication of how activation of the RON receptor tyrosine kinase by MSP modulates responses mediated by LPS and IFN-γ in combination. Here, we set out to determine whether MSP exposure inhibits IL-12p40 induced by LPS alone and the length of time required for this inhibition. Therefore, we stimulated primary peritoneal macrophages with various concentrations of LPS alone or LPS in combination with IFN-γ after exposure to MSP for 4, 8 or 12 hours. We found that the presence of MSP for as little as 4 hours prior to stimulation with varying doses (0.01μM or 0.1μM) of LPS inhibits production of IL-12p40 (Figure **4-1**).

4.3.2 *MSP does not prevent up-regulation of IFN-β or IRF3 activation in response to LPS stimulation*

As indicated in the previous studies (Figure **4-1**), stimulation with LPS alone can induce IL-12p40 production. This pathway incorporates the type I IFN, IFN-β. Expression of IFN-β provides the transcriptional enhancement required for the induction of IL-12p40 through activation of the ISRE by phosphorylated STAT1. In the current studies we sought to determine whether MSP has an effect on IFN-β production induced by LPS stimulation. We treated primary peritoneal macrophages with or without 100ng/mL MSP for 12 hours followed by 0.1μM of LPS and then assessed IFN-β expression by RT-PCR. Data from these experiments indicate that MSP/RON signal transduction does not prevent IFN-β expression in response to LPS (Figure **4-3**).

The signaling pathway down-stream of TLR4 that is responsible for the induction of IFN-β is independent of MyD88 and incorporates IRF3. We set out to determine whether MSP/RON plays a role in regulation of this pathway by stimulating peritoneal macrophages with or without 100 ng/mL MSP for 12 hours followed by 0.1μM LPS for 30 minutes. IRF3 phosphorylation was examined by Western blot analysis. We found that IRF3 activation by LPS is not affected by the presence of MSP (Figure **4-4**).

4.3.3 *Activation of STAT1 by LPS alone is not inhibited by MSP*

LPS signaling has also been shown to induce STAT1 phosphorylation with delayed kinetics due to the IRF3-dependent induction of IFN-β by LPS. Therefore, we

stimulated primary peritoneal macrophages with MSP for 12 hours followed by 30 minutes or 2 hours of LPS (Figure **4-5**) We find that MSP does not influence the tyrosine or serine phosphorylation of STAT1 following LPS stimulation.

Figure **4-1**

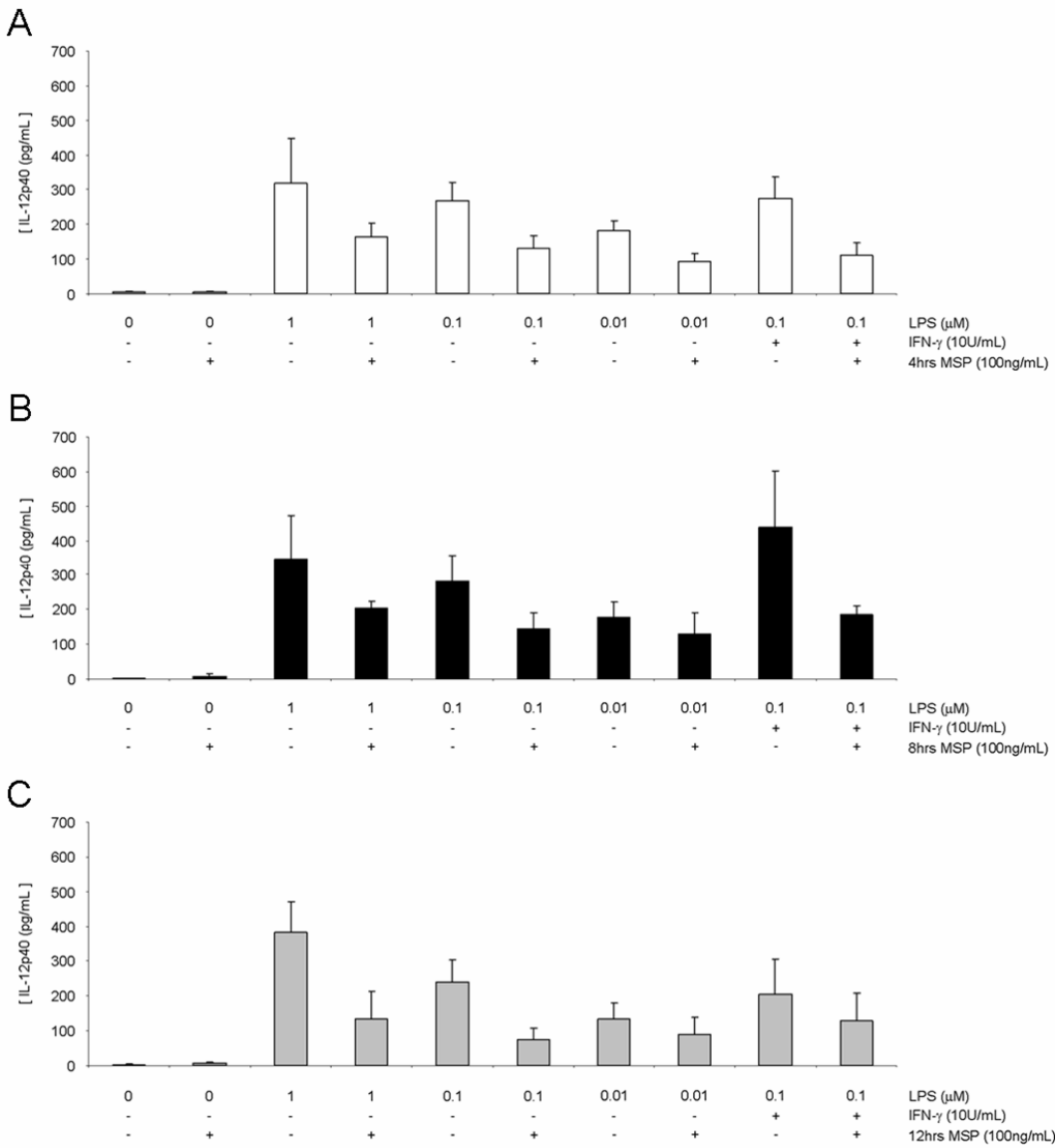

Figure **4-1**: Exposure to MSP as early as 4 hours inhibits IL-12p40 production by LPS with or without IFN-γ priming.

Peritoneal macrophages were treated with or without 100 ng/ml MSP for 4 (*A*), 8 (*B*) or 12 h (*C*) before stimulation with or without 10 U/ml IFN-γ, 0.1μM or 0.01μM LPS, and 100 ng/ml MSP for 24 h. Supernatants were collected, and IL-12p40 production was measured by ELISA. These data were prepared by Manujendra Ray.

Figure **4-2**

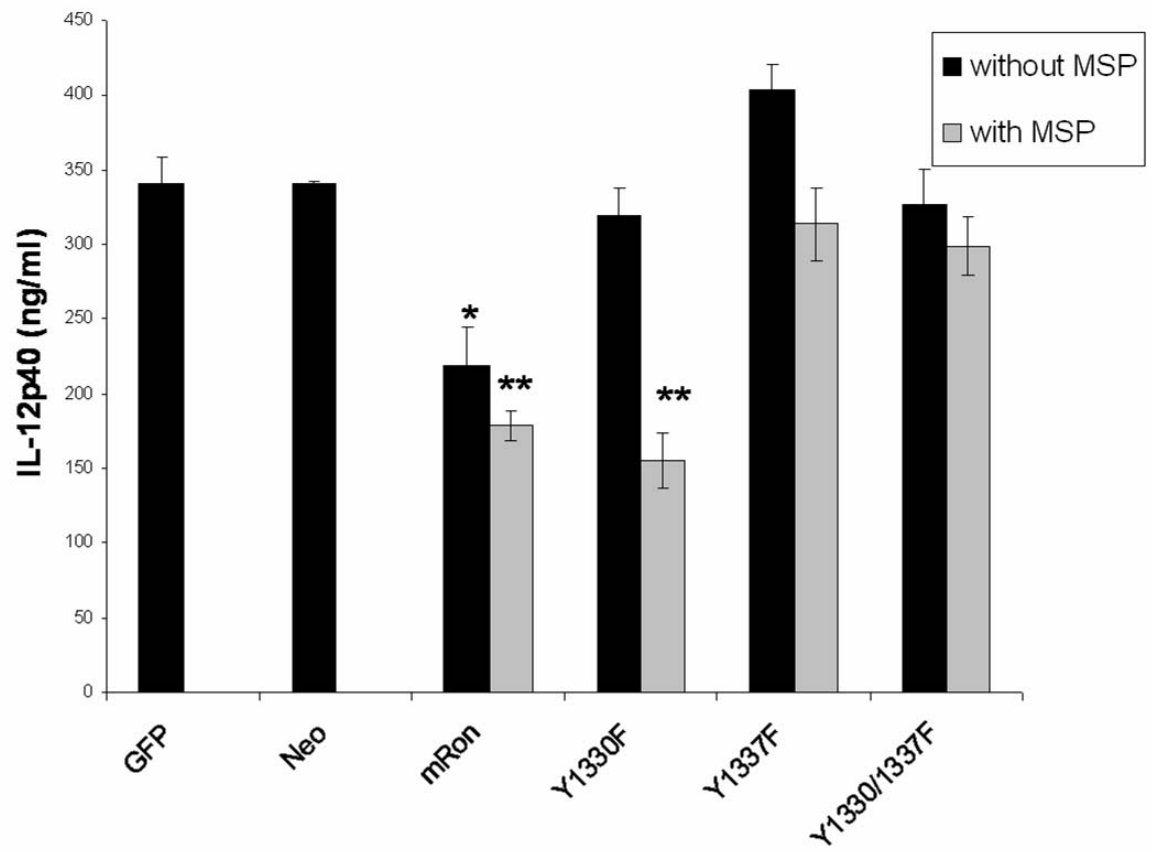

Figure **4-2**: The docking site tyrosines are required for ligand-dependent and –independent inhibition of IL-12p40 by RON.

Bone marrow-derived macrophages were cultured in DMEM with 20% FBS and 30% L929 conditioned media (source of MSCF) for three days then transduced in the presence of 8 mg/ml polybrene with retroviruses generated by packaging with pEco and VSVG in 293T cells. Bone marrow macrophages were transduced twice in a 24 hour period then stimulated with 0.1 mg/ml LPS for 24 hours in the presence or absence of 100 ng/ml MSP. Cell supernatants were collected and assayed for IL-21p40 production by ELISA. *$p<0.01$; **$p<0.001$. These data were prepared by Qing Ping Liu.

Figure 4-3

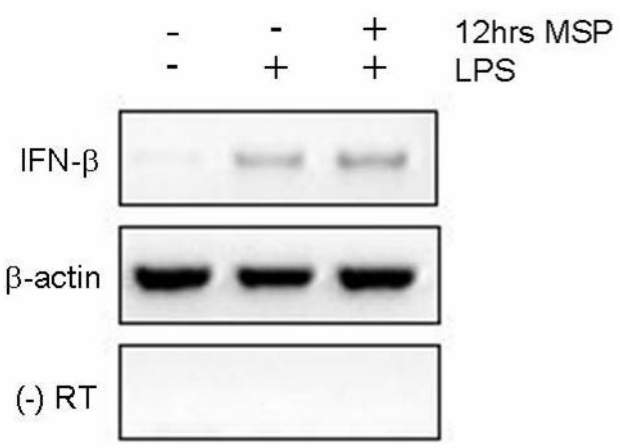

Figure **4-3**: MSP does not prevent the expression of IFN-β.

Primary peritoneal macrophages from WT mice were harvested by lavage and isolated by adherence. Stimulations were administered with or without 100 ng/mL MSP for 12 hours followed by 0.1μM of LPS. One hour later, total mRNA was isolated. Expression of IFN-β was assessed by RT-PCR analysis. Expression of β-actin from the same samples was used as a loading control.

Figure **4-4**

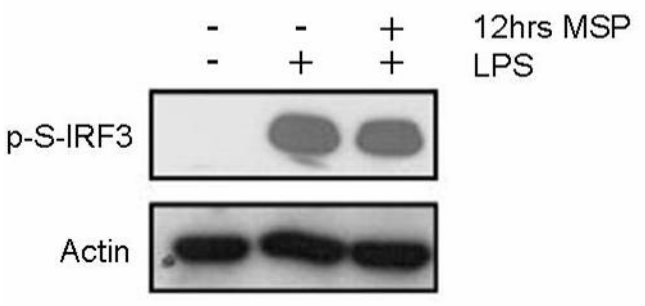

Figure **4-4**: Activation of IRF3 is not inhibited by MSP.

Peritoneal macrophages were treated with or without 100 ng/mL MSP and 0.1μM LPS for 30 minutes. Whole cell lysates were harvested and serine phosphorylation of IRF3 was assessed by Western blot analysis, and then the membrane was stripped and reprobed for Actin.

Figure **4-5**

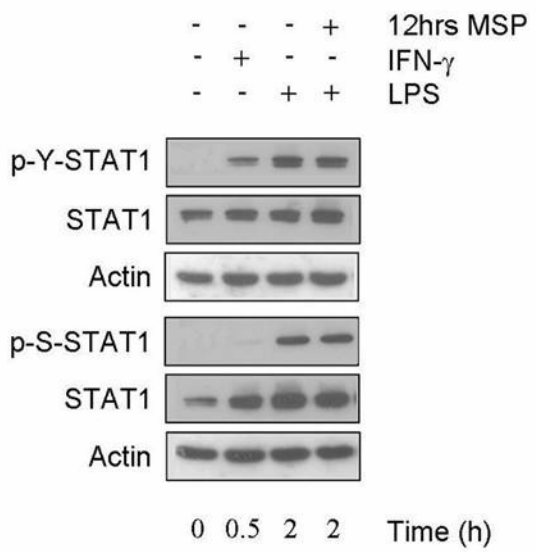

Figure **4-5**: MSP/RON does not inhibit STAT1 activation induced by LPS.

Peritoneal macrophages were treated with or without 100 ng/ml MSP, 200U/mL IFN-γ and 0.1μM LPS for the indicated times. Whole cell lysates were collected and tyrosine phosphorylation of STAT1 was assessed by Western blot analysis, and then the membrane was stripped and reprobed for total STAT1 and Actin.

4.4 Discussion

The data represented in the current studies addresses the ability of MSP/RON to regulate the activation of signaling pathways downstream of TLR4 in isolation. Prior studies from our group and others have demonstrated that the RON receptor tyrosine kinase inhibits pro-inflammatory responses of macrophages stimulated with LPS and low priming concentrations IFN-γ *in vitro* including IL-12 and NO [149, 179, 186, 207]. Along those same lines nuclear translocation and DNA binding of NFκB is decreased in RAW cells transfected with RON. We have also shown that MSP/RON inhibits IFN-γ signaling, resulting in inhibition of STAT1 phosphorylation (Figure **3-3**). Additionally, the presence and/or absence of RON has a direct effect on the viability of murine sensitivity to endotoxin and DTH responses *in vivo* [179].

It is well established that macrophages exposed to LPS alone will induce pro-inflammatory mediators in the absence of type II IFN. Stimulation of primary peritoneal macrophages with LPS induces activation of IRF3 by serine phosphorylation on multiple residues. IRF3 then induces expression of type I IFN which results in tyrosine phosphorylation of STAT1. We found that MSP does not prevents the induction of IFN-β, IRF3 or STAT1 following LPS stimulation (Figures **4-4** and **4-5**). Alternatively, we previously demonstrated that STAT1 activated by high doses of IFN-γ only is negatively regulated by MSP/RON (Figure **3-3**). This differential suppression of the STAT1

pathway by MSP/RON may be explained by the fact that LPS and IFN-γ utilized independent pathways that leads to the activation of the STAT1. There are two distinct pathways down-stream of LPS/TLR4 which are dependent and independent of MyD88, respectively. MyD88 dependent signaling leads to the induction of NFκB and MAPKs. IRF3 is a transcription factor targeted by the MyD88 independent pathway resulting in expression of IFN-β, and subsequent activation of the JAK/STAT pathway by the IFN-βR. Conversely, IFN-γ/IFN-γR activation of the JAK/STAT pathway is more direct. Resent studies have indicated that the IRF3 pathway is critical to the induction of IL-12 via the IL-12p35 subunit [228]. When compared to the prior studies from our group, signaling inducted by MSP/RON has directed effects on IL-12p35 induced by the combined stimulation of LPS and IFN-γ. These findings offer support to our belief that MSP/RON specifically targets genes down-stream of the IFN-γR.

We have previously shown that MSP induces SOCS1 expression in primary macrophages (Figure **3-5**). SOCS1 targets the MyD88-dependent pathway downstream of TLR4 through inducing degradation of MAL/TIRAP [139]. MAL is targeted to the plasma membrane through interactions with phosphatidylinositol bisphosphate (PIP2) after cleavage by caspase-1 and phosphorylation by Bruton's tyrosine kinase (BTK) and aids in the recruitment of MyD88 to activated TLR4 [120, 229, 230]. MAL-dependent signals also lead to the serine phosphorylation of the p65 subunit of NFκB activation by LPS [231, 232]. Thus, the mechanism by which MSP/RON inhibits LPS-induced IL-

12p40 could be through the SOCS1-dependent degradation of MAL. Future studies will determine whether RON regulates turnover of MAL [115].

Chapter 5

Conclusion

Although a competent immune response is characterized as the ability of the immune system to monitor, identify and resolve infections by pathogens, it is equally as important that the elicited response not exceed the magnitude required. Dys-regulation of the immune response leads to local tissue damage at a minimum and animal death as a worst case. Here we have outlined detailed studies that place the RON receptor tyrosine kinase at the managerial crux of cellular control to immune challenge by bacterial constituents.

The strategic placement of resident macrophages in tissues of the various areas of the body that will likely encounter microbial pathogens is evident in dermal macrophages, peritoneal macrophages, alveolar macrophages and Kupffer cells. Exposure to bacteria may result from various routes of infection; however, these cells are the first to interact with pathogen and as a result begin to "ring the alarm" to alert the immune system that a potential threat exists. These cells express the RON receptor tyrosine kinase at varying levels respective to their primary function.

Many research articles and reviews have focused on the importance of regulating the *in vivo* and *in vitro* response to LPS. Most studies have focused on cells that are directly responsive to LPS, such as macrophages, monocytes and DCs. Others have

identified indirect roles for other effector cells such as, NK cells and T lymphocytes in the response to LPS . With this in mind it would be advantageous for regulation to occur at both the direct and indirect levels.

In vivo and *in vitro* studies have clearly demonstrated that RTKs have an essential role in tempering the response to LPS, thus providing protection against LPS induced endotoxemia. LPS induces IL-12p70 by up-regulating expression of IL-12p35 and IL-12p40. IL-12 is able to self amplify by driving production of IFN-γ in NK cells and T lymphocytes. The combination of IFN-γ and TNFα will lead to endotoxemia if left unchecked, and RON appears to be involved in regulating endotoxemia at the earliest stages of disease. By restraining IL-12, RON essentially ensures limited production of down-stream IFN-γ with the net result of promoting effective immunity and animal recovery.

Although IL-12 production by macrophages is regulated by RON, it is also important to know if additional checks are present in macrophages in the event of defects in IL-12 modulation. Therefore, we set out to examine the role of RON in the response of macrophages to IFN-γ. We found that RON up-regulates SOCS genes which are critical in negatively regulating gene induction by IFN-γ. In the presence of microbes IFN-γ is not only responsible for increasing IL-12 production; it also prepares macrophages and other APC to present antigens to $CD4^+$ T cells. The ability of RON to modulate expression of MHC class II suggests that RON may also play a role in the

regulation of acquired immunity. If the innate responses are adequate to alleviate microbes, valuable energy resources are retained; therefore, allowing efficient use of metabolic materials. However, in the event that acquired immunity is required, similar checks and balances need to be employed in order to protect the host from tissue destruction. The ability of RON to regulate the response of macrophages to IFN-γ suggests there may also be links between RON and Th1 mediated immunity and disease.

Unpublished results from our lab indicate that activation of RON on macrophages inhibits Th1 cell development *in vitro* in the presence of exogenous IL-12. One potential explanation for this result is that the inhibition of MHC Class II expression by RON limits the antigen presenting capability of these cells to the developing Th1 cells. In addition, we have demonstrated increased T cell proliferation and IFN-γ production by splenocytes isolated from $RON^{-/-}$ mice following induction of experimental autoimmune encephalitis (EAE), a murine model of multiple sclerosis. Taken together, these results corroborate our *in vitro* findings suggesting that RON may regulate acquired, as well as innate, immune responses.

One way to test this question would be to utilize IL-$12p40^{-/-}$ mice crossed with $RON^{-/-}$ mice. A survival curve of these mice post exogenous administration of physiologically relevant levels IFN-γ with or without LPS would provide more evidence of the exact level of inhibition/protection that RON provides *in vivo*. Of almost equal importance is the relevance of the source of increased IFN-γ production observed in

$RON^{-/-}$ over there WT counterparts. To address this question, recombination-activating genes 2 (RAG2) deficient could be backcrossed to $RON^{-/-}$ mice. These animals would then be subjected to endotoxin exposure and observed for there ability to survive. This strategy would limit the contribution of IFN-γ in this system to NK cells because these recipient mice will not have T lymphocytes.

MSP/RON quells macrophage activation in response to LPS and IFN-γ combined; however, what is even more interesting is the fact that when either stimulus is given individually, MSP/RON induces partial or complete arrest of the resulting signal transduction and/or gene induction. These events seem to be very specific to the source of stimulation as evidenced by the fact that STAT1 tyrosine phosphorylation is only inhibited when activated by IFN-γ, and not LPS following induction of IFN-β. SOCS1 is up-regulated by both type I and II IFNs [233]. JAKs of both classes of receptors are bound by via the kinase inhibitory region (KIR) of SOCS1 which prevents phosphorylation of the STAT1 docking site of the cytoplasmic domain of IFN-βR and -γR [234]. If the docking site is activated prior to SOCS1 protein production, SOCS1 engages the receptor in a second process through its SH2 domain [77]. The net result of each event is that signal transduction is disrupted proximal to receptor. Thus, the difference in susceptibility of IFN-γ-induced vs. LPS-induced STAT1 tyrosine phosphorylation to inhibition by RON may be one of timing. In order for MSP/RON to inhibit IFN-γ-induced STAT1 tyrosine phosphorylation, several hours of pretreatment with MSP is required. This allows time for RON to induce expression of SOCS1.

However, following LPS stimulation, IFN-β expression is up-regulated and 2-4 hours elapse before IFN-β induces tyrosine phosphorylation of STAT1. Since LPS alone can also induce SOCS1 which presumably limits STAT1 phosphorylation by IFN-β, any additional induction by RON of SOCS1 may not further regulate this event.

Although, LPS/TLR4 mediated IFN-β signal transduction is not regulated by MSP/RON, MyD88-dependent activation of NFκB is down-regulated by MSP/RON. The inhibition of NFkB could be responsible for the down-regulation of NO, IL-12 and TNFα production by RON. MyD88 is recruited to TLR4 through interaction with the adaptor, Mal. Furthermore, phosphorylated Mal interacts with SOCS1 resulting in the inhibition of this pathway. Thus, SOCS1 up-regulation prior to LPS stimulation could result in enhanced degradation of MAL and subsequent inhibition of MyD88-dependent signaling by TLR4. The resulting reduction in availability of MyD88 to TLR4 in tissue resident macrophages that interact with microbial pathogens as they invade; would lead to the modulation of subsequent immunological events

Even though the RON receptor tyrosine kinase controls IFN-γ production in response to endotoxin, we would ultimately like to test whether other models of infectious disease are regulated by RON. Parasitic infection models in mouse with *Trypanosoma cruzi* and *Trypanosoma brucei rhodesiens* require IL-12 and IFN-γ to mediate protection [235-238]. Local immunity in the liver prevents colonization of *Trypanosoma cruzi* due to the production of IFN-γ by NK, NK T cells , γδT cells and

αβT cells [239]. IFN-$\gamma^{-/-}$ mice are more susceptible to *Trypanosoma brucei rhodesiens* infection [236]. These models of trypanosome infection would provide additional insight into the role of the RON receptor tyrosine kinase in the progression of parasitic disease. It is reasonable to presume that the increased expression of IL-12 and IFN-γ might confer increased protection in $RON^{-/-}$ mice to disease progression.

Species specific homologs of RON have been discovered in many organisms, from *Caenorhabditis elegans* to humans. Although the specific tissues that express RON in the individual species may not be exclusive to macrophages, it is apparent that the expression of RON has a very early evolutionary genesis. Furthermore, it is feasible to assert that as complex organisms developed and organized a repertoire of host responses to microbes, a regulatory role for RON evolved as well.

Figure **5-1**

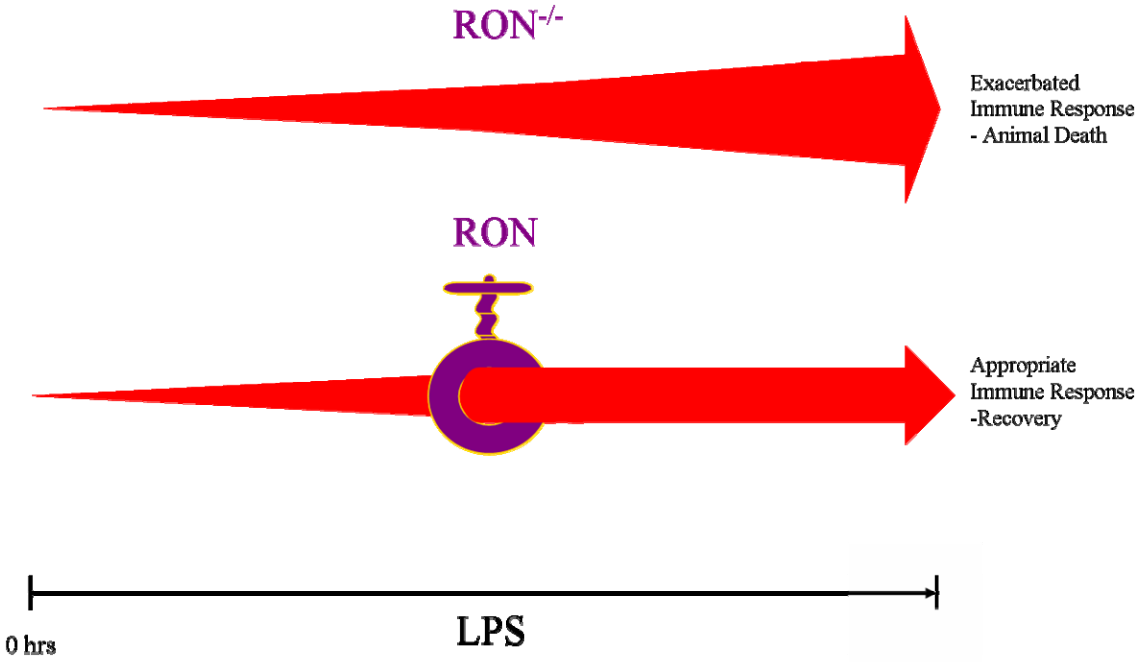

Figure **5-1**: The RON Receptor Serves as an Inflammatory Control Valve.

During the initiation of an inflammatory response by LPS, recognition of the endotoxin is recognized and induction of the appropriate response is activated. The presence of the RON receptor tyrosine kinase limits the magnitude of the response to LPS which results in an appropriate immune response and recovery.

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