INDUCTION OF MACROPHAGE ARGINASE I EXPRESSION BY RON AND THE
IMPLICATIONS FOR PROMOTING SYNGENEIC TUMOR GROWTH

A Dissertation in
Pathobiology

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2010
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In the innate immune response, macrophages play a role as the first line of defense against microbial infection through phagocytosis and secretion of inflammatory mediators such as nitric oxide (NO) and reactive oxygen species (ROS). The adaptive immune response is primed through antigen presentation by macrophages, which then respond to T cell cytokines that further aid in the clearance of pathogenic insult. Following clearance of pathogens, macrophages downregulate the inflammatory response of both the innate and adaptive branches of immunity, and this is coupled with the upregulation of genes that promote resolution of inflammation. Macrophage regulation of inducible nitric oxide synthase and arginase I expression have long been studied as prototypic markers of classical (inflammatory) versus alternative (anti-inflammatory) macrophage activation. Dampening of classical activation by the Ron receptor tyrosine kinase in macrophages has been well characterized in the murine model of septic shock where Ron-/-/ mice produce elevated levels of IFNγ and succumb to death while wild-type animals survive. Conversely, it has been demonstrated that the Ron receptor tips the balance of macrophage activation away from the classically activated phenotype and promotes hallmarks of alternative macrophage activation, including arginase I expression.

In this dissertation, we first set out to determine the mechanism by which arginase I is regulated by Ron. We demonstrate that, while IL-4 and the ligand for the Ron receptor, MSP, both enhance arginase I expression in macrophages, MSP induction of the arginase I promoter occurs at sites independent from those induced by IL-4. MSP, but not IL-4, induces potent MAPK activation in primary macrophages and, through systematic mutagenesis, we demonstrate that induction of the arginase I promoter in response to MSP is mediated by an AP-1 binding site located 433bp upstream of the transcription start site (TSS). In contrast, IL-4 induces arginase I expression through a Stat6 site located ~2.9kb upstream of the TSS. The role of these sites in
vivo in primary macrophages is supported by results from ChIP analysis demonstrating enhanced binding of Fos to the AP-1 site following MSP, but not IL-4 stimulation, and the enhanced binding of Stat6 primarily by IL-4 or to a lesser extent by MSP. These data suggest that MSP and IL-4 induce arginase I expression in macrophages by both shared and divergent mechanisms.

While alternative macrophage activation of arginase I is important in altering the inflammatory response to pathogens and in promoting wound healing, tumor development hijacks elevated expression of arginase I in macrophages as a means of enhancing tumor growth. Because Ron alters the balance between classically and alternatively activated macrophages, we hypothesized that activation of Ron on macrophages would promote tumor growth by enhancing arginase I expression. Here, we find that growth of the syngeneic tumors 3LL, B16-F10, and EG.7 is reduced in Ron−/− mice. Concurrently, Ron−/− mice exhibit reduced induction of myeloid derived suppressor cells (MDSCs) as well as cytokines produced upon tumor onset that promote the recruitment of MDSCs to the tumor microenvironment, where they differentiate into tumor associated macrophages (TAMs). While the overall percentage of TAMs associated with the tumors are not affected by the absence of Ron, expression of arginase I by these cells is severely reduced in Ron−/− mice. However, Ron expression was not detected on TAMs, suggesting an indirect mechanism by which Ron promotes enhanced arginase I expression in TAMs. MDSCs and TAMs inhibit T cell activity in a manner dependent on arginase I expression. Consistent with these observations, we demonstrate that Ron−/− tumor bearing mice (TBM) harbor increased numbers of CD4 and CD8 T cells. In addition, splenocytes from Ron−/− TBM produce increased levels of IFNγ and decreased levels of IL-10 in response to mitogenic challenge, and exhibit increased proliferation to allogeneic challenge in a mixed lymphocyte reaction. Together, these findings suggest that Ron modulates the immune response to tumor challenge such that the
absence of Ron in the tumor microenvironment enhances tumor immunity and reduces tumor growth.

Taken together, the work presented in this dissertation demonstrates that Ron induces the expression of arginase I in macrophages \textit{in vitro} and \textit{in vivo} via both direct and indirect mechanisms. The ability of Ron to promote arginase I expression \textit{in vivo} likely plays a protective role in response to inflammation and infection, but promotes tumor growth by inhibiting T cell activity. These results indicate that the activation or inhibition of Ron could have potential therapeutic effects in the treatment of chronic inflammation or tumorigenesis, respectively.
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ACKNOWLEDGEMENTS

The journey through graduate school has taken me longer and humbled me more than I had hoped or expected. Some will ask “what would you have done differently if you had to do it again”? The truth of the matter is I don’t think I would have changed a thing. It is true that I made a great number of mistakes, was less disciplined than I could have been, and there were a number of other faults, both mine and others, that could have been better, but, it was through these experiences where the bulk of the learning took place. Reflecting back on this journey, I realize the importance of various individuals in making the journey so enjoyable.

The Department of Veterinary and Biomedical Sciences in Henning Building has been a joy to work with from the front office to the surrounding laboratories. To the ladies in the flow lab, Elaine, Susan, and Nicole, troubleshooting has never been so enjoyable, nor has a work place to conduct experiments as a guest.

With quite remarkable amazement I can say I was truly glad to meet and work with all of the Hankey lab members, and likewise that I was truly sad to see each of them move on. In particular: Ping and Jie for their help in getting me started with a number of things and their good cheer. Mike and Amy who introduced me to the world of Penn State and State College intramurals. Xin Wei for all of your enthusiasm, your love of music -- including conducting along while reading papers, and of course all of the constructs that you made. Shuang who always made me laugh with her indelible sense of humor. Manu, a partner in macrophage troubles, thank you for teaching me about both research and friendship. Shihan, you were a quiet “mouse” at the beginning who became a great friend and someone to bounce ideas off of, both with science and life. All of the other members not mentioned here for making the lab so pleasant. Lastly, thank you Pam for your constant encouragement and enthusiasm, the directional nudges when needed, and the freedom to roam in scientific exploration.
I could not think of the word encouragement without thanking my family of champion cheer leaders. My sisters, their husbands, their children, my grandmother, aunts and uncles, friends, church, I have been blessed to call you all my family, friends, and siblings in Christ. I especially thank my parents for providing me with educational opportunities throughout my life, including the most important lessons that were learned in the home: that my value does not lie in a career, but as a child of God, that every person deserves respect, and that the pursuit of learning is a lifelong endeavor no matter the subject. It wasn’t so much what you said, but how you lived it. Thank you.

Isaiah, thank you for reaffirming that my worth does not lie in my ability as a scientist each and every day the moment I walk in through the door. I can’t wait to meet your sibling so that I will have a double dose of that reality. It is all for the better.

Jori, thank you for being my encourager, my companion, my shoulder to rest a weary head upon, my arms to rejoice with, my soul mate to pray with. Words are simply inadequate to describe my thanks to you. This wouldn’t have been completed without you!

Finally, if I may be so bold as to culminate the acknowledgement of this work with my love of science and God as to say:

To the Invisible, the Immortal. The Creator! No one is without excuse. No, not one, and certainly least of all me. “For since the creation of the world God's invisible qualities—his eternal power and divine nature—have been clearly seen, being understood from what has been made, so that men are without excuse.” Romans 1:20.

I have studied the heavens, the glory of your creation, and conclude, yes, definitively yes, that you exist. Though the fallibility and frailty of this soul are many, you oh Lord, have undertaken the task to present me as one who is blameless and without fault in your glorious presence. For that, I am eternally grateful.
Chapter 1

Ron is a homeostatic guardian against excessive inflammation

A literature review
Ron: receptor d’origine natasis

In 1976, a ~100kD protein was fractionated from serum by gel filtration and ion exchange chromatography and found to induce the migration of macrophages\(^1\). Two years later this molecule was named macrophage stimulating protein (MSP)\(^2\), and found to also increase phagocytosis by macrophages\(^3\). The study of MSP was largely curtailed for the next 15 years, awaiting the identification of its corresponding receptor.

In 1993, the receptor d’origine natasis (RON) was identified by Ronsin \textit{et al}. as a receptor tyrosine kinase (RTK)\(^4\), although the ligand for RON was unknown. Comparison of RON with other RTKs revealed that RON is related to the MET proto-oncogene. While the ligand recognition domains of RON and MET share less homology, RON and MET are 63\% homologous in the intracellular region. Shortly thereafter, human RON was simultaneously identified by two independent groups as the receptor for MSP\(^5,6\). Human monocytes were found to migrate in a chemoattractant manner to MSP, and MSP induced the phosphorylation of RON in these cells\(^5\). Moreover, MSP stimulation of epithelial cells expressing exogenous RON induced proliferation and migration of these cells in a RON-dependent manner\(^5,6\). These results confirmed the identification of RON as the receptor for MSP, and set in motion a number of studies which have lead to our current understanding of MSP/RON and its function.

A search for novel RTKs expressed on hematopoetic stem cells led to the discovery of the stem cell-derived tyrosine kinase (STK) receptor, the murine homolog of the RON receptor. STK is 73.6\% homologous to the RON receptor at the amino acid level on the whole, and more than 88\% homologous within the kinase domain\(^7\), and MSP was subsequently shown to be the ligand for STK\(^8\). MSP induced both the phosphorylation of the STK receptor and morphological
change in NIH 3T3/STK expressing cells, while the related protein, hepatocyte growth factor (HGF), the ligand for the closely related MET receptor, failed to induce these responses. Sea, cloned in chicken, is the avian homolog of RON. Because of their sequence homology and conserved mechanisms of activation, RON and STK are considered synonymous, and for simplicity, RON will be used throughout the remainder of this review, unless conflicting results occur in which human and murine will be appropriately noted.

MSP: macrophage stimulating protein

MSP was simultaneously discovered by two groups. Skeel et al. performed protein sequencing of MSP isolated from serum and concluded that MSP is related to human prothrombin, plasminogen, and HGF, and is secreted as a pro-protein. The sequence similarity between MSP and HGF is 80% which accounts for 50% amino acid homology, and as such, originally led to the nomenclature of MSP as HGF-like protein. The expression of MSP was most apparent in the liver, with some expression observed in lungs, kidney, and pancreas.

The prevailing view is that MSP is secreted predominantly by the liver as a pro-protein and must be cleaved at sites of activation. MSP is cleaved by members of the serine protease family including coagulation enzymes (kallikrein, factor XIIa, and factor Xa) and by nerve growth factor-γ (NGFγ) and epidermal growth factor-binding protein (EGF-BP). These studies were performed using purified protein preparations, however, blood clotting itself is insufficient to induce proteolytic cleavage of MSP. This could be explained by the subsequent observation that α1-antichymotrypsin can block the cleavage of pro-MSP, and exists within the blood at 7uM, a concentration sufficient to block cleavage. Alternatively, in extracellular regions, α1-antichymotrypsin is expected to exist at concentrations of 0.4uM, which is insufficient to block cleavage. Macrophages residing in tissues throughout the body also possess a pro-MSP
convertase that is capable of cleaving pro-MSP, as well as a second protease that promotes the degradation of MSP\textsuperscript{16}. The lower concentration of α1-antichymotrypsin is sufficient to block the degrading protease but not the pro-MSP convertase. Thus, it appears as if the activation of MSP is tightly regulated at local sites of action. Further support of this hypothesis is evidenced by the rapid degradation and clearance of injected I\textsuperscript{125}-pro-MSP within 180 minutes\textsuperscript{18}.

Upon cleavage, MSP is composed of a disulfide linked heterodimer. Like HGF, the α-chain of MSP contains 4 kringle domains, while the β-chain contains a serine protease-like domain\textsuperscript{11,12}. RON is translated as a 1400 amino acid single chain pro-protein, and cleaved to form a disulfide linked dimer consisting of a 150kDa β-chain and 50kDa α-chain\textsuperscript{4,7}. The α-chain is located entirely in the extracellular domain of RON, while the β-chain is a transmembrane protein that consists of both extracellular and intracellular domains, the latter of which includes the kinase domain and critical signaling residues (Figure 1.1).

There remains some debate over what portion of MSP is required for ligand stimulation of RON. In one report, the β-chain of MSP was found to be sufficient to bind to the receptor, but unable to induce phosphorylation of RON, whereas the α-chain, or the two N-terminal kringle domains, of MSP were unable to bind the receptor\textsuperscript{19}. An important role for the β-chain is supported by mutation of cysteine 672 to alanine, located within the serine protease domain. It is hypothesized that this mutation stabilizes intramolecular disulfide bonding, and improved MSP potency by as much as 20 fold\textsuperscript{20}. Conversely, using the same construct, other studies have shown that the two N-terminal kringle domains are sufficient to induce receptor phosphorylation\textsuperscript{5}. These results are consistent with studies of the closely related family member, HGF and its receptor MET, which support a central role for the kringle domains in receptor activation. While the crystal structure of the β-chain of MSP has been resolved\textsuperscript{21}, the α-chain of MSP, and RON itself have yet to be crystallized. Such analysis will be critical for a more complete understanding of the interaction of MSP with RON.
Figure 1.1: Diagram of the disulfide linked receptor d’origine natasis (Ron) receptor tyrosine kinase and its ligand, macrophage stimulating protein (MSP). Ron is displayed on the left with mRon features shown in black, and corresponding, or unique sites of human shown in blue. MSP is shown on the right in its cleaved and active form. Disulfide bonds are represented with a dotted line. Structure of MSP adapted from Wahl et al, JBC, 1997, 272(24):15053
Ron/MSP Signaling:

Murine and human RON are potent inducers of mitogen activate protein kinase (MAPK) signaling and cell migration. However, these species specific homologues exhibit some biological differences, including the ability of murine Ron, but not human RON, to promote transformation in an NIH3T3 assay. The most notable difference between the human and mouse receptors is found in the juxtamembrane (JM) domain. The JM domain of human RON includes an exon encoding 27 amino acids that is absent in the murine counterpart. This JM domain provides an additional level of signaling regulation in the human receptor that is absent in the murine receptor. Murine Ron exhibits constitutive activity in a number of overexpression systems, in which the activation of human RON requires ligand stimulation. In the absence of ligand, murine Ron co-immunoprecipitates with c-Src, and this interaction requires tyrosines 1175, 1265, and 1294 in the kinase domain. Similarly, deletion of the JM domain in human RON leads to constitutive activation of the receptor. Following ligand stimulation of human RON, phosphorylation of Y1360 in the c-terminal tail results in the recruitment of Cbl in a Grb2-dependent manner, and, subsequently polyubiquitination and degradation of RON are observed. Interestingly, ubiquitinization, but not Cbl binding, also requires Y1353 in the c-terminal tail and Y1017 in the JM domain. These results suggest the possibility that Y1017 the JM domain in human RON adds an additional level of receptor regulation by promoting receptor turnover upon activation. This additional layer of regulation could be an important mechanism for preventing the oncogenic function of RON.

The c-terminus of RON and MET share a conserved multifunctional docking site with the sequence of Y-hydrophobic-X-hydrophobic-(X3)-Y-hydrophobic-N-hydrophobic. Upon MSP stimulation of RON, these docking site tyrosines become phosphorylated, recruit SH2
domain containing proteins, and mediate strong MAPK activation\textsuperscript{25}. Phosphorylation of either tyrosine results in the recruitment of the p85 subunit of PI3K\textsuperscript{28,29}, while Grb2 is primarily recruited to the second docking site tyrosine\textsuperscript{25,28}. The recruitment of Grb2 to RON further propagates downstream signaling by recruiting the adaptor proteins, Gab2\textsuperscript{30} and Gab1, or the guanine nucleotide exchange factor, Sos\textsuperscript{31}. Alternatively, Gab1 can be directly recruited to the receptor through an intrinsic MET binding domain\textsuperscript{32}. Recruitment of Gab1 and Gab2 to the RON receptor amplifies MAPK and PI3K signaling by providing additional docking sites, including those for p85 and the tyrosine phosphatase, Shp2. Phosphorylation of the docking site tyrosines in Ron leads to cell migration in a PI3K dependent manner\textsuperscript{29}. Mutation of either of these sites individually reduced migration by approximately two fold, while mutation of both docking site tyrosines completely blocked migration. Expression of a dominant negative p85 in MSP stimulated cells also abolished migration\textsuperscript{29}.

The cellular environment has a profound effect on the activation of downstream signaling and the promotion of cellular responses by Ron. Ron stimulation in a murine erythroleukemia cell line, MEL, increased apoptosis, while Ron activation in a pro-B cell line, Ba/F3, promoted cell survival\textsuperscript{28}. In both of these cases, the multifunctional docking site was required for mediating these responses in a PI3K dependent manner. The difference in response likely involves cell specific partnering of Ron with downstream signaling mediators as Ron induced strong and sustained JNK activation in MEL cells, while in Ba/F3 cells JNK activation by Ron was low and transient.

Activation of the PI3K pathway by Ron results in the phosphorylation of S1394 in the c-terminal tail of Ron in an AKT-dependent manner\textsuperscript{33}. Phospho-S1394 binds the scaffold protein, 14-3-3, which then recruits the α6β4 integrin (the ligand for laminin). In the unstimulated state, α6β4 is found in the hemidesmosomes (structures supporting cell adhesion), but following MSP stimulation, α6β4 relocalizes to the lamellipodia where it associates with actin, and thereby
enhances migration. S1394 also functions as a site for negative feedback of Ron receptor activation as it can recruit protein phosphatase 1 (PP1). PP1 recruitment dephosphorylates S1394 and also inhibits Src activation.

**Development/Expression**

Two reports independently established the expression of Ron as early as embryonic day 12.5, but not prior to that point. On E12.5 Ron expression is restricted mostly to neuronal structures. Later during embryonic development, Ron is also expressed in the developing gut and bone; expression patterns that persist in the adult. The importance of Ron expression in early development of sympathetic neurons has been suggested due to their MSP responsiveness. In mammary glands, Ron is expressed in pubescent (5-8 weeks), pregnant, and lactating mice, but is absent in the mammary glands of 10 week old virgin adults. In pubescent mice harboring Ron with an inactive tyrosine kinase domain (Ron TK-/-), increased mammary gland development is observed, both in extension and branching of glands. Paradoxically, the Ron TK-/- mammary glands exhibited higher MAPK activity, and this activity was required for the increase in development.

Currently, the full extent of the developmental requirement for Ron expression is not known. Muraoka et al., demonstrated that Ron-/- mice are embryonic lethal at E6.5. However, Correll et al., found that deletion of Ron (those used in this dissertation) resulted in full developmental viability. Moreover, Ron TK-/- mice are also viable but have smaller ovaries with fewer corpus lutea. One possible reason for the discrepancy of Muraoka et al., is that in constructing their knockouts, they deleted exons 1-15 of Ron (of 19 total) eliminating the coding sequences for the extracellular domain, transmembrane domain, and a portion of the intracellular
domain. In contrast, Correll et al., replaced the first 850 base pairs of exon 1 with a β-gal cassette which leaves the promoter intact but removes the translation initiation codon. A second explanation may be related to a Ron transcript that encodes a truncated form of the Ron receptor (short form Ron, or Sf-Ron) that is found on a number of tissues and cells in a similar manner as full length Ron\(^7, 42\). This transcript arises from an internal promoter within intron 10, thus Sf-Ron lacks most of the extracellular domain while retaining the transmembrane and kinase domains. Sf-Ron is encoded by the Friend virus susceptibility 2 locus, Fv2, and is required for the development of erythroleukemia in mice in response to Friend virus infection\(^42\). Ron-/-, in which exon 1 was deleted, express low levels of Sf-Ron which renders these mice resistant to Friend virus induced erythroleukemia, however it remains possible that sufficient levels of sf-Ron expression are present at critical times during development. However, it should be noted that Sf-Ron-/- mice, that maintain expression of full length Ron, are completely viable\(^43\). Moreover, MSP-/- mice develop normally\(^44\), and thus on the whole, is seems as if Ron is not required for development.

Ron expression is found on a wide range of cells including hematopoietic progenitors, epithelium, and tissue macrophages (Table 1.1). It is striking that Ron expression appears to correlate with environments that encounter pathogens, or innate cells that participate in the clearance of pathogens. For example, in the lungs, RON is expressed on the apical surface, but not the basal surface of the epithelium\(^45\). Similarly, throughout the digestive tract, RON is expressed in the epithelial cells lining the gut\(^46\). Likewise, RON is expressed in alveolar macrophages, kupffer cells, and dermal macrophages cells in the skin, sites at which these cells would be poised to respond to pathogen insult.
### Table 1.1: Ron expression. Known expression profile of Ron (h is human, m is mouse)

<table>
<thead>
<tr>
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<td>m&lt;sup&gt;35&lt;/sup&gt;</td>
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(2). The role of RON in oncogenesis:

RTKs have long been studied for their oncogenic potential, and RON is no exception. RON is expressed in a large variety of epithelial cells, and is associated with a number of epithelial cancers (Table 1). Consistent with its ability to promote cell migration, RON may play a critical role in metastatic tumor development as metastatic tumors exhibit higher levels of RON expression. Additionally, RON also has the ability to regulate claudin expression. Claudins regulate tight junctions in epithelial cells, and an increase in claudin1 is associated with tighter junctions. RON decreases claudin1, and increases claudin3 and claudin4, thereby leading to enhanced migration in epithelial cells. In these cells, a normal epithelial monolayer was restored upon forced expression of claudin1, suggesting that RON may regulate metastasis via altered expression of claudins. Primarily though, RTKs are thought to promote oncogenesis through constitutively enhanced receptor activation leading to aberrantly high MAPK activation, and the manner by which RON gains oncogenic potential is discussed below.

**Overexpression:** Overexpression of the Ron receptor promotes the transformation of NIH3T3 cells, and targeted overexpression of Ron in mice induces oncogenesis in lung epithelial cells. Furthermore, targeted overexpression of Ron, or a constitutively active form of Ron (Ron M-T), in mammary epithelium led to an increase in tumor incidence and metastasis. Increased β-catenin, a protein associated with poor prognosis, was also observed within these mammary cells further implicating the oncogenic potential of Ron. Conversely, Ron TK-/- mice develop fewer spontaneous mammary tumors in a transgenic model of breast cancer.

**Mutation:** Point mutations in the kinase domain of the Ron receptor that result in constitutive activation of Ron promote transformation in NIH3T3 cells and tumor growth. Furthermore, isolation of RON cDNA from human colorectal carcinoma cells revealed additional
receptor activating mutations\textsuperscript{50}. Given the important role of the JM domain in preventing constitutive activation of human RON, it is conceivable that mutation of this domain would also lead to tumor growth\textsuperscript{23}. A role for alteration of the JM domain in promoting oncogenesis has been demonstrated for the Flt3 receptor in acute myeloid leukemia\textsuperscript{86}.

**Splice variants**: Alternative splicing of the RON receptor also leads to aberrantly high receptor activation. Wild-type RON is a 180kD protein. To date, RON\textDelta170, RON\textDelta165, RON\textDelta160, RON\textDelta155, RON\textDelta110, and RON\textDelta52 have all been identified, and are named for their resulting molecular weight\textsuperscript{77,87}. The first to be identified in colorectal cancer was RON\textDelta160, which results in an in-frame deletion of 109 amino acids in the extracellular region of the β-chain. This mutation leads to enhanced kinase activity\textsuperscript{50}. RON\textDelta165, RON\textDelta160, and RON\textDelta155 all contain partial deletions in the extracellular domain of the β-chain. Interestingly, RON\textDelta165 and RON\textDelta155 are not cleaved and remain as single chain proteins. In vitro analysis of RON\textDelta160 and RON\textDelta155 revealed an increase in cell scattering in MDCK cells and increased focus formation in NIH3T3 cells compared with wild-type RON, implicating these splice variants in promoting oncogenic transformation\textsuperscript{51}. In vivo evidence for the role of RON\textDelta165 and RON\textDelta155 are inferred from recent analysis revealing the absence of RON\textDelta165 and RON\textDelta155 in healthy brain tissue, but high incidence of these variants in glioblastomas\textsuperscript{55}.

A naturally occurring N-terminally truncated form of Ron in mice, sf-Ron promotes the development of erythroleukemia in response to Friend virus\textsuperscript{42}. This truncated form of the receptor is activated by a defective envelope protein, gp55, encoded by Friend virus. Gp55 interacts with Sf-Ron through cysteines in the extracellular domain of Sf-Ron and the ecotropic domain of Sf-Stk resulting in enhanced Sf-Ron phosphorylation and cell surface localization\textsuperscript{88}. The internal promoter that drives expression of Sf-Ron in mice is conserved in humans, and human SF-RON can also be activated by interacting with the viral protein, gp55. The observation
that SF-RON is highly expressed in a variety of tumors, suggests that this form of the receptor may also play a role in the development of human cancers\textsuperscript{89}.

**MSP activation:** The potential role of MSP in tumorigenesis was first demonstrated by Willet \textit{et al.}, who observed increased MSP expression in the lung following application of nitrosamine carcinogens\textsuperscript{53, 54}. As shown in Table 1, RON is expressed in lung cancer, and Willet \textit{et al.}, demonstrated co-expression of RON and MSP in lung cancer lines, suggesting a potential autocrine/paracrine loop involved in tumor formation and progression. In addition, overexpression of MSP in a murine model of breast cancer promoted increased metastasis to the bone, and expression of MSP in breast cancer patients correlates with poor prognosis\textsuperscript{90}.

**Blocking RON/tumor growth:** Blocking RTK induction of tumor growth is the focus of intense research, particularly within the pharmaceutical industry. Several agents have been identified for their blocking potential of RON induced tumor growth. A group from Amgen identified a small molecule inhibitor that specifically inhibits RON and MET phosphorylation including all known RON splice variants. This molecule also inhibited the migration potential of several tumor lines, and blocked their growth \textit{in vivo}\textsuperscript{91}. This is in line with findings by a group from ImClone, who developed a RON blocking antibody\textsuperscript{49}. The antibody blocked MSP binding to the RON receptor, and reduced RON expressing tumor growth in nude mice by approximately 50\%. Using pancreatic cancer cell line, BXPC-3, that expresses both RON and the EGFR, they found that while blocking RON or EGFR with individual antibodies resulted in reduced tumor growth, combined treatment completely abolished growth.

Recently, the RON promoter was discovered to contain two NF\textsubscript{κ}B sites that regulate its expression\textsuperscript{47}. Using curcumin, a natural NF\textsubscript{κ}B inhibitor from the root from \textit{curcuma longa}, RON expression was completely abolished, and this treatment eliminated the migration of cells in response to MSP. Curcumin has been identified as a potential therapeutic agent in treating
cancers that are non-responsive to chemotherapy\textsuperscript{92}, and it will be interesting to see if Curcumin acts upon RON expression in these instances to reduce tumor growth.

In addition to these exogenous agents that inhibit RON activation, there also exist endogenous mechanisms of inhibition that, when altered, may lead to oncogenesis. One such protein, hyaluronidase 2 (HYAL2), binds and negatively regulates RON in bronchial epithelial cells. However, the envelope protein (Env) of Jaagiekte sheep retrovirus, binds to HYAL2, which frees RON, and thereby promotes oncogenic development that is dependent on RON\textsuperscript{59}. Recently, a novel splice variant, RON\textDelta90, was identified in normal brain tissue, while reduced in some glioblastomas\textsuperscript{55}. RON\textDelta90 lacks the intracellular and transmembrane domains, and is thus a secreted protein that contains the MSP binding site. RON\textDelta90 has the ability to bind and sequester MSP, and thereby reduce migratory potential. This portion of the receptor was previously synthetically created as a potential inhibitor of RON activation\textsuperscript{93}, but identification of RON\textDelta90 provides endogenous evidence of negative regulation of the RON receptor.

(3). Function: Maintaining homeostasis and warding off excessive inflammation

Having established a role for RON in mediating oncogenic potential, and its conservation between species, one must ask: what is the normal function of RON? Most strains of Ron\text/-/- mice appear to develop normally. However, Ron\text/-/- mice challenged with inflammatory insult are more susceptible to cytotoxic insult, often resulting in death. The remainder of this chapter will focus on the protective function of RON, and proposes that RON is positioned at the gateway of homeostasis, warding off excessive inflammation.
A: Epithelium

**Integrins:** A prominent feature of epithelium is growth in uniform monolayers, and as such, integrins are ‘integral’ in this process. As discussed previously, Ron interacts with the α4β6 integrin via 14-3-3 and induces subsequent migration of keratinocytes, and RON regulates claudins to alter tight junctions. The importance of cell adhesion in regulating RON signaling is evidenced by the observation that, upon MSP stimulation, phosphorylated ERK is retained in the cytoplasm of cells in suspension, while nuclear localization of phosphorylated ERK is observed when cells are adherent. Interestingly, type IV collagen found in the extracellular matrix induces the phosphorylation of RON. Further analysis led to the observation that RON co-immunoprecipitates with β1 integrin, a receptor for type IV collagen. This process involves integrin activation of Src, which leads to RON phosphorylation, and ultimately PI3K activation and cell migration. This ability of Ron to crosstalk with other receptors to induce migration or morphological change is also observed in Ba/F3 pro-B cells, where Ron induces shape change via a mechanism independent of its docking site tyrosines. There, Ron interacts with the βc chain of the IL-3 receptor in a manner that utilizes the ligand binding capacity and kinase domain of Ron and the c-terminal signaling domain of the IL-3 βc chain to induce migration and ERK phosphorylation.

**Keratinocytes:** Ron is also expressed on keratinocytes, where it plays a role in the resolution of wound healing. The expression of Ron and MSP is upregulated in rat excisional wounds at days 7, 14, and 21. Additionally, active MSP is found at 24 hours in human burn wounds and in breast reduction surgery wounds. There, 50% of MSP is in the active form, compared with just 8% in plasma. Furthermore, wound fluid cleaves pro-MSP. In burn and post surgery wounds, RON is expressed on keratinocytes below the leading edge in the
proliferating region, on the spinous stratum of the dermas, and on differentiating cells of resurfacing epithelium. RON/MSP may provide several benefits to a healing wound including enhanced proliferation and migration. Furthermore, wounds are sites of inflammatory and pathogenic insult, and RON confers protection from apoptosis in epithelial cells. The importance of Ron in wound healing was further established by the use of a punch biopsy wound healing model, where topical application of MSP significantly improved wound closure.

**Kidney and Endometrium, models of autocrine/paracrine activation:** In addition to hepatocyte production of MSP, its expression is often observed in tandem with Ron expression, or neighboring Ron expressing cells, suggesting autocrine and/or paracrine activation respectively. For example, *in vivo* and *in vitro* MSP is expressed in tubular kidney cells, and RON expression is found on mesangial cells. *In vitro*, MSP expressed by tubular cells promotes both growth of the tubular cells in an autocrine manner, and stimulates migration, proliferation, and IL-6 production by mesangial cells in a paracrine manner. The physiological significance of this is not yet well understood, but given the normal development of Ron-/- mice, speculation that this expression is positioned to respond to glomerular inflammation seems reasonable.

Microarray analysis has revealed that MSP, RON, 14-3-3, and Sos are all upregulated in late stage endometriosis. Real-time PCR revealed that MSP is upregulated in ectopic endometrium compared with eutropic endometrium from the same patients. Conversely, RON is primarily expressed in the eutropic endometrium. The authors suggest that MSP/RON may be important in promoting the pathophysiology of endometriosis. It is interesting that the expression pattern of RON in endometriosis is similar to its expression in basal keratinocytes in the healing wound. As endometriosis is an event requiring the growth of underlying epithelium, MSP may act in an autocrine or paracrine manner, stimulating eutropic (underlying) endothelium to proliferate, prevent apoptosis, or simply to reduce inflammation.
**Gastro intestinal tract:** RON is expressed on the apical epithelium of both the small intestine and colon. The coding sequence for RON is located on chromosome 3p21\(^4,7\) as is MSP\(^13\). Chromosome 3p21 is linked to Crohn’s disease (CD) and ulcerative colitis (UC)\(^{100,101}\). Recently, two groups found linkage between RON and MSP with CD and UC. They analyzed single nucleotide polymorphisms (SNPs) in CD and UC patients and found association with RON\(^{102}\) and MSP\(^{103}\). In the case of RON, amino acid changing variants were identified that could affect function of the receptor. The SNP identified for MSP is predicted to reduce its ability to bind to RON. In both cases, it is possible that non-functional RON/MSP signaling in the GI tract could contribute to the development or progression of inflammatory bowel disease (IBD). This is supported by the findings of Wang *et al.*, who demonstrated that intestinal epithelial cells expressing RON exhibit reduced apoptosis in response to TGFβ1 when treated with MSP\(^{104}\). We have observed that Ron-/- mice display significant weight loss to dextran sodium sulfate challenge in a murine model of IBD (Hankey/Cantorna, unpublished observations). These observations lay the groundwork for a possible role for RON in the digestive system where MSP/RON signaling could hold inflammation in check.

**Lung:** In the lung, RON is expressed on the apical surface of ciliated epithelia. Application of MSP increases ciliary beat frequency, and thus suggests that MSP/RON may be involved in host defense and/or mucociliary function\(^60\). Indeed patients that have bronchiectasis, which is caused by bacterial induced inflammation, have twice as much MSP in their sputum, though not in the serum\(^45\). Together these findings suggest that MSP/RON may promote a compensatory mechanism for impaired mucociliary pathogen clearance.

Thus, preventing excessive epithelial inflammation appears to be a primary function of RON where RON could play a role as a negative feedback mechanism to downregulate inflammation. Attempts at negative feedback regulation through the upregulation of RON expression could lead to overexpression of RON and promote its oncogenic potential. For
example, as mentioned, the RON promoter has two NFκB sites that drive its expression. Excessive inflammation, ie constitutive NFκB activation, may lead to overexpression of RON and thereby promote its oncogenic potential. This is consistent with models of inflammatory driven carcinoma, and the location of many RON expressing tumors would be coherant with this hypothesis.

**B: Macrophages**

Macrophages are ubiquitous cells of the immune system and are capable of regulating and responding to a pleiotropic range of stimuli. In the innate immune response, macrophages play a role as the first line of defense against microbial infection through phagocytosis and secretion of inflammatory mediators such as nitric oxide (NO) and reactive oxygen species (ROS). The adaptive immune response is primed through antigen presentation by macrophages, which then respond to T cell cytokines that further aid in the clearance of pathogenic insult. Following clearance of pathogens, macrophages downregulate the inflammatory response of both the innate and adaptive branches of immunity, and this is coupled with the upregulation of genes that promote healing and angiogenesis. Like the anti-inflammatory responses initiated by RON in the epithelium, RON plays a similar role within macrophages.

*Alveolar macrophages:* Acute lung injury by intranasal administration of lipopolysaccharide (LPS) is more severe in Ron TK-/- mice, and is thought to be dependent on Ron expressing alveolar macrophages. In this model, MSP decreases TNFα production from alveolar macrophages in response to LPS and increases levels of IκB, thereby inhibiting NFκB activation. Furthermore, Ron was identified as a negative regulator of ADAM17, a metalloprotease involved in TNFα processing\(^4\). Moreover, Ron TK-/- mice succumb to death
more quickly and produce increased levels of the inflammatory cytokines IL-6, MCP-1, and NO in nickel induced acute lung injury\textsuperscript{105}. Microarray and IHC analysis revealed that resting alveolar macrophages in TK-/- mice have elevated levels of granzyme suggesting that, prior to injury, these macrophages are primed to promote a greater inflammatory response, and that Ron serves an important role as a negative regulator of inflammation\textsuperscript{106}.

Alternatively, normal healthy alveolar macrophages can induce respiratory burst and superoxide (O$_2^-$) production in response to MSP stimulation, a process that is dependent on MAPK activation\textsuperscript{65}. In smokers, O$_2^-$ production by alveolar macrophages is enhanced by MSP at greater levels. Moreover, the production of TNF$\alpha$ is further increased by MSP in smokers compared with non-smokers, whereas the opposite expression pattern is observed for the anti-inflammatory cytokine, IL-10. This result may be explained by the observation that MSP induced nuclear localization of the NF$\kappa$B subunits p50 and p65 in these cells. However, in unstimulated alveolar macrophages, the smoker group exhibited higher background levels of nuclear NF$\kappa$B\textsuperscript{66}. The ‘switch’ from IL-10 to TNF$\alpha$ production by MSP in alveolar macrophages from chronically inflamed lungs suggests that the homeostatic function of RON, under these conditions, may not only be lost, but also that RON could contribute to further inflammation.

**Glia:** Ron is expressed on microglia and astrocytes, and while MSP doesn’t affect proliferation or survival of these cells cultured in vitro, it induces the migration of these cells\textsuperscript{70}. RON also inhibits the transcription of HIV in macrophages by decreasing NF$\kappa$B binding to the long terminal repeat (LTR) of HIV\textsuperscript{69,107}. In a manner of host evasion, the HIV Tat protein binds to RON and targets it to the proteosome for degradation, a process that is dependent on the integrity of the kinase domain of RON\textsuperscript{108}. In AIDS patients, RON expression is virtually non-existent on microglia and astrocytes, suggesting that HIV is downregulating the expression of RON and may play a role in HIV induced dementia\textsuperscript{69}. 

RON expression is also reduced in the brain and spinal cord of human multiple sclerosis (MS) patients and in mice following induction of experimental autoimmune encephalitis (EAE). Specifically, immunohistochemistry revealed the absence of RON expression on glia of MS patients, while it is present in normal individuals. This may due to the observed increase in Cbl expression in MS patients, a negative feedback regulator of RON. In Ron TK-/− mice, EAE disease is exacerbated, and increased mRNA levels of the inflammatory cytokines TNFα, IL-12, IL-1β, and reduced levels of the Th2 cytokine, IL-4, are observed in the spinal cord. Conversely, in monocyte-derived macrophages from healthy individuals, MSP stimulation can reduce IL-1β and increase IL-10 expression suggesting that RON expression and activation in microglia could protect against MS.68

**Osteoclasts**: Ron expression is observed within the developing bone at sites of active remodeling. Later it was found that MSP activates murine osteoclasts and facilitates bone resorption. Furthermore, upon activation of Ron by MSP, Src kinase localizes to the surface of these cells. This finding was confirmed in human osteoclasts where the effects of MSP/RON could not be reciprocated with HGF/MET activation suggesting a unique role for RON in regulating osteoclast function.109

Peritoneal macrophages and monocyte-derived macrophages are the most readily available population available for research, and as such, much of our signaling and functional knowledge is derived from these cells. However, before we begin our conversation of Ron effects within these macrophages, it is helpful to delineate between types of macrophage activation.

**Classical vs. Alternative Macrophage Activation**: A number of models have been put forth to describe the various types of macrophage activation observed in vitro and their potential function in vivo. The model proposed by Gorden et al. will be discussed here.110, 111 In this model, macrophages serve as the focal point in immunity against pathogens, bridging the innate
and adaptive arms of the immune system. It is proposed that pattern recognition receptors (PRR) initiate the differentiation of Th1 cells, due to increased macrophage IL-12 production.

Conversely, the paradigm is shifted in macrophages stimulated by pattern associated molecular patterns (PAMPs) that promote IL-10, but not IL-12, production, and this change in cytokine production by macrophages consequently drives Th2 cell differentiation. Thus, the presentation of antigen by MHC, coupled with the production of IL-12 or IL-10 by the macrophage promotes Th1 or Th2 differentiation resulting in the production of IFNγ or IL-4, respectively. IFNγ or IL-4 then promote reciprocal classical or alternative macrophage activation in a paracrine manner.

Functionally, classically activated macrophages exhibit increased MHC II surface expression and increased inducible nitric oxide synthase (iNOS) expression, which aid in the clearance of bacteria and intracellular pathogens. Alternatively activated macrophages, on the other hand, express increased levels of IL-1 receptor antagonist (IL-1Ra) and arginase I, both of which reduce inflammation and aid in the containment or elimination of extracellular pathogens such as helminths. A common thread within classical and alternative macrophage activation is the catalysis of the shared substrate, L-arginine, by either inducible nitric oxide synthase (iNOS), or arginase I, respectively (Figure 1.2). Catalysis of L-arginine by iNOS leads to production of the cytotoxic mediator NO, and citrulline, whereas catalysis of L-arginine by arginase I promotes proline and polyamine synthesis which are important for collagen and DNA synthesis, respectively. The following discussion will highlight the known role of MSP/Ron signaling in regulating the dichotomy of macrophage activation.
Figure 1.2: Ron shifts the balance of the immune response by altering macrophage activation. Following macrophage activation by environmental stimuli such as pathogens, macrophages (MΦ) produce IL-12 or IL-10 (1) which then promote the development of Th1 and Th2 cells respectively (2). This initiates reciprocal expression of cytokines that influence the classical or alternative dichotomy of MΦ activation (3). Classically activated MΦ catalyze L-arginine via iNOS to produce NO and citrulline, while alternatively activated MΦ catalyze L-arginine to induce proline and polyamine synthesis. Ron tips the balance of MΦ activation by inhibiting iNOS and inducing arginase I expression. Tipping the balance of MΦ activation then promotes Th2 type immune responses.
Dampening Classical Activation

Both Ron/−/ − and Ron TK/−/ − mice are susceptible to endotoxic shock. While the majority of wild-type mice recover, Ron/−/ − mice succumb to death within 40 hours post LPS challenge. A predominant role for macrophages in this process has been proposed. Stimulation of primary peritoneal macrophages with LPS and IFNγ results in the upregulation of IL-12 production, and stimulation of these cells with MSP almost completely inhibits this response. Moreover, peritoneal macrophages that have been stimulated with MSP exhibit decreased iNOS mRNA expression and NO production in response to either LPS, IL-2/IFNγ, or TNFα/IFNγ stimulation. This inhibition appears to be specific to iNOS and IL-12, as little or no inhibition of the inflammatory cytokines MCP-1, IL-1, IL-6, or TNFα is observed in these cells. Interestingly, following 8 hours of in vivo LPS treatment, Ron protein expression is reduced, which is about the same time as visible shock is observed within animals (M. Ray/DR Sharda unpublished observations). In vitro analysis of this phenomenon revealed that, in the absence of MSP stimulation, LPS stimulation targets Ron for degradation via an NO dependent pathway. While the downregulation of Ron at 8 hours following LPS challenge seems to contradict the protective effect of Ron, the recent finding that Ron contains two NFκB sites in its promoter supports the potential upregulation of Ron mRNA following LPS administration as a negative feedback against excessive inflammation, and this hypothesis would fit the model of recovery in wild-type mice to LPS challenge. The importance of Ron in regulating excessive NO production in macrophages is demonstrated by the finding that Ron/−/ − mice exhibit increased inflammation and susceptibility to the intracellular pathogen Listeria monocytogenes.

The mechanisms employed by Ron in downregulating inflammatory responses in classically activated macrophages occur at several levels. LPS signals through the toll like
receptor 4 (TLR4) to induce the degradation of IκB following phosphorylation by IKK, which then leads to nuclear localization of NFκB, and transcription of NFκB dependent genes such as IL-12 and iNOS. Co-stimulation of MSP with LPS prevents IκB degradation. Moreover, MSP/Ron decreases nuclear localization of NFκB in response to LPS/IFNγ treatment, and also in HIV infected macrophages suggesting a role for the downregulation of NFκB in the regulation of IL-12 and iNOS mRNA. MSP treatment also inhibits the upregulation of ICSBP, a critical regulator of IL-12 transcription, by IFNγ and LPS. Ron also plays a role in protecting macrophages from LPS induced apoptosis. Ron signaling also limits the upregulation of MHC class II surface expression and its transcriptional regulator, CIITA, in response to IFNγ stimulation. This inhibition is associated with reduced Stat1 phosphorylation following IFNγ stimulation, and downregulation of surface IFNγ receptor expression, and is associated with the upregulation of suppressor of cytokine signaling 1 and 3 expression (SOCS1 & SOCS3) by MSP. Ron/- mice express elevated levels of IL-12 and produce increased levels of IFNγ following LPS challenge, and crossing the Ron/- mice with IFNγ receptor knockout mice diminishes the effect of Ron on survival to a wide range of LPS doses in vivo. These studies implicate the importance of regulating IFNγ responses by Ron in protection against shock in Ron/- animals.

**Promoting Alternative Activation**

In addition to downregulating inflammatory responses to LPS stimulation, in the absence of pathogenic insult, Ron promotes the alternative activation of macrophages. The first implication of MSP in the alternative activation of macrophages was the observation that MSP increased macrophage migration and increased phagocytosis of C3bi coated erythrocytes. MSP enhances binding of C3bi coated erythrocytes to the αMβ2 integrin (CD11b). Induction of this response is dependent on PI3K phosphorylation of PKCζ and subsequent actin rearrangement. As mentioned, alternatively activated macrophages often are associated with the clearance of large extracellular pathogens such as helminths. These pathogens are too large to
be phagocytosed and processed through endocytic vesicles, and therefore, alternatively activated macrophages increase pinocytosis as a way of gathering pathogenic ‘data’ from their environment. Though a role for Ron in regulating helminth infection is unknown, MSP was found to increase the presence of pinocytic vesicles as well as long cytoplasmic processes.10

Alternative macrophage activation is somewhat less well defined than classical activation and has taken on a de facto meaning of anti-inflammatory macrophage by many researchers. These macrophages are associated with clearance of parasites123 and are observed in atherosclerotic lesions124, growing tumors125, and hypoxic environments such as wounds126. Common to all of these macrophages is the upregulation of arginase I expression. Morrison et al. found that MSP stimulated macrophages increase arginase I mRNA, protein, and activity. They further demonstrated the increased expression of other alternatively activated genes such as IL-1Ra and scavenger receptor A (SR-A). The increase in SR-A expression was associated with increased uptake of acetylated LDL (AcLDL)127. As discussed previously, RON plays an important role in wound healing33, and the observation that RON is expressed on dermal macrophages post wounding58, suggests the possibility that, in addition to its regulation of keratinocyte activity, RON may also promote wound healing through its regulation of macrophage activation in the wound microenvironment.

Thus, the primary role of Ron in macrophages is to dampen the classically activated macrophage response to pathogen, and to promote the alternative activation of macrophages. This function of Ron in macrophages complements its role in limiting excessive inflammation in epithelium at sites of active pathogen encounter. This dissertation will address the following three unanswered questions:

1. What is the mechanism by which Ron upregulates arginase I expression?
2. What role does Ron play in the microenvironment of a growing tumor?
3. What is the capacity of MSP to induce alternative activation under hypoxic conditions?
Chapter 2

Regulation of the Arginase I Promoter in Primary Macrophages by the Ron Receptor Tyrosine Kinase Occurs Through a Novel AP-1 Site that is Unique From IL-4/Stat6 Driven Transcription

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Abstract

Macrophage regulation of inducible nitric oxide synthase and arginase I expression have long been studied as prototypic markers of classical versus alternative macrophage activation. The Th2 cytokine, IL-4, promotes alternative macrophage activation and is a well-characterized inducer of macrophage arginase I expression. In previous studies, we have demonstrated that the Ron receptor also tips the balance of macrophage activation away from the classically activated phenotype and promotes hallmarks of alternative macrophage activation, including arginase I expression. In the present study, we demonstrate that, while both IL-4 and the ligand for the Ron receptor, MSP, enhance arginase I expression in macrophages, MSP induction of the arginase I promoter occurs at sites independent of those induced by IL-4. MSP, but not IL-4, induces potent MAPK activation in primary macrophages and, through systematic mutagenesis, we demonstrate that induction of the arginase I promoter in response to MSP is mediated by an AP-1 binding site located 433bp upstream of the transcription start site (TSS). In contrast, IL-4 induces arginase I expression through a Stat6 site located ~2.9kb (Stat6-2888) upstream of the TSS. We also identified a novel Stat6 site (Stat6-453) adjacent to the AP-1 site that is required by both MSP and IL-4 to induce arginase I promoter activity. The role of these sites in vivo in primary macrophages is supported by results from ChIP analysis demonstrating enhanced binding of Fos to the AP-1 site following MSP, but not IL-4 stimulation, and the enhanced binding of Stat6 in response to either IL-4 or MSP to the Stat6-2888 and Stat6-453 sites. We conclude that MSP and IL-4 induce arginase I expression in macrophages by both shared and divergent mechanisms.
**Introduction**

L-arginine metabolism in macrophages is regulated by the enzymes inducible nitric oxide synthase (iNOS) and arginase I\(^{128}\). Catalysis of L-arginine by iNOS or arginase I yields the inflammatory mediator nitric oxide (NO) or prolines and polyamines, respectively. While expression of these proteins can be observed simultaneously within the same cell, this scenario is the exception rather than the rule, and has led to the dichotomous nomenclature of macrophages\(^{110,128}\). Classically-activated macrophages (also called M1 macrophages) express high levels of iNOS and low levels of arginase I, and are responsible for the clearance of pathogenic insult. Conversely, alternatively-activated macrophages (also called M2 macrophages) display the reverse expression pattern, and promote granuloma formation and resolution of inflammation and wound healing following pathogen clearance. Unregulated expression of iNOS and production of nitric oxide by classically-activated macrophages can also induce damage to host tissues. Likewise, arginase I expression by alternatively-activated macrophages can be detrimental by promoting liver fibrosis following parasitic infections\(^{129,130}\) and may play a role in the progression of diseases including tumorigenesis\(^{131-133}\), asthma\(^{134}\), while providing a protective role against atherosclerosis\(^{124,135}\).

Arginase I, commonly referred to as ‘liver-type arginase’, was originally identified in the liver as a critical enzyme involved in regulation of the urea cycle\(^{136}\). Arginase I resides in the cytoplasm, while the related enzyme, arginase II, is localized within the mitochondria\(^{137,138}\). In the liver, arginase I expression is regulated by Stat6 and C/EBP\(\beta\) within the arginase I promoter. In hepatocytes, arginase I is constitutively expressed, though its expression can be further enhanced by glucocorticoids, a process which requires *de novo* protein synthesis\(^{139}\). Arginase I levels are negligible in resting macrophages, but can be significantly upregulated by cytokines, glucocorticoids, hypoxia\(^{126}\), and LPS stimulation\(^{140}\). Th2 cytokines IL-4 and IL-13 are potent
inducers of the alternatively-activated macrophage phenotype and promote arginase I transcription. Macrophages from Stat6 knockout mice display profound defects in the ability to promote a number of responses to IL-4/13, including the upregulation of arginase I expression. An enhancer located ~3kb upstream of the transcription start site (TSS) is responsible for regulation of arginase I transcription in IL-4/13-stimulated macrophages. Binding sites within this enhancer for C/EBPβ, Stat6, and PU.1 are required for the induction of arginase I transcription by IL-4 and IL-13. Apart from this enhancer-mediated expression, a secondary mechanism requiring de novo protein synthesis has also been observed to induce arginase I expression in macrophages stimulated with IL-4.

There is an emerging understanding of the role of receptor tyrosine kinases (RTKs) in the regulation of classical vs. alternative macrophage activation. One such RTK is the Ron receptor, a member of the MET family of tyrosine kinase receptors. While Ron is not expressed on monocytes, it is expressed on mature, differentiated, tissue-resident macrophages. We and others have found that Ron-expressing macrophages express decreased levels of the inflammatory mediator iNOS upon stimulation with LPS. Furthermore, macrophages pretreated with macrophage-stimulating protein (MSP), the ligand for Ron, produce reduced levels of IL-12 in response to LPS and IFNγ stimulation. These data suggest that Ron-expressing macrophages are phenotypically less inflammatory in nature, a hypothesis which is supported by the fact that Ron-/- mice are more susceptible to endotoxic shock. Alternatively, upon MSP stimulation of Ron, macrophages exhibit upregulation of arginase I gene expression and increased arginase activity. In addition, Ron induces the expression of Scavenger receptor A (SR-A) and IL-1 receptor antagonist (IL-1Ra) in primary macrophages. Both Ron and MSP are upregulated by day 7 in excisional wounds, and by day 2-5 in burn wounds, suggesting that Ron may also aid in the resolution of inflammatory insult and in wound healing. Therefore, like IL-4, MSP stimulation promotes the alternative activation of macrophages, including the upregulation of...
arginase I. However, the mechanism used by Ron to upregulate arginase I expression has not been elucidated.

Here we show that MSP, but not IL-4, is a potent inducer of Map kinase signaling in primary macrophages. In addition, we demonstrate that, while IL-4 induces arginase I expression through the upstream enhancer described previously, MSP-induced arginase I promoter activity is independent of this element. Rather, MSP regulates the arginase I promoter via a promoter proximal region containing a novel AP-1 site that is responsive to MSP, but not to IL-4. Furthermore, we have identified a second Stat6 site within this region that promotes arginase I expression in response to both MSP and IL-4. These data indicate that IL-4 and MSP induce arginase I expression through both shared and unique regulatory mechanisms in primary macrophages.

Methods

Mice, Cells, and Reagents

We used 6-10 week old C57BL/6 mice in this study. HEK 293T cells and RAW264.7 cells were purchased from ATCC. All cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with L-glutamine, non-essential amino acids, sodium pyruvate (CellGro, Mediatech, Manassas, VA), 10% FBS (Gibco, Gaithersburg, MD), and 10 μg/mL ciprofloxacin (Serologicals Proteins, Inc., Kankakee, IL). Antibodies used for western analysis were phospho-p44/42 (phospho-ERK), phospho-Jun (S63), phospho-JNK (Thr 183/Thr 185), phospho-STAT6 (Tyr-641), phospho-Gab1 (Tyr-307), phospho-Gab2 (Tyr-452 & Tyr-159), phospho- CREB, p44/42 (ERK), Jun, Gab1, Gab2 (Cell Signaling, Beverly, MA), Actin (Sigma, St Louis, MO), STAT6 (RnD Systems, Minneapolis, MN), and Arginase (BD-Transduction
Laboratories, San Jose, CA). FC-Block, anti-CD11b APC-750 (BD Pharmingen, Inc., San Diego, CA), anti-F4/80 RPE (Serotec, Raleigh, NC), Anti-STK (RnD Systems Cat# AF431, Minneapolis, MN), and anti-goat Alexa Fluor® 647 (Molecular Probes, Eugene, OR) were used for flow cytometry. Recombinant human MSP C632A and murine IL-4 were purchased from R&D Systems (Minneapolis, MN).

The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) approved all animal experiments.

Cell Transfection

293T Transfection

We plated 6×10⁵ 293T cells in 24-well plates. Sixteen hours later, 15 ng/well arginase I, PCI-Ron or PCI-neo control, and 0.5 ng/well Renilla plasmids were mixed in 35 μL of serum-free media with 1μL Mirus Transit 293T transfection reagent for 20 minutes at room temperature. The solution was then added dropwise to each well of the 24-well plate for transient transfection. After 24 hours, cells were stimulated with MSP (100 ng/mL), and 20 hours later, media was aspirated and luciferase activity was measured using the Dual Luciferase® reporter system according to the manufacturer’s protocol (Promega, Madison, WI). For Figure 2.4A, equimolar amounts of the different arginase plasmids were added and balanced with PCI-Neo, such that each well received the same molar amount of plasmid as the arginase 3.2 construct. In the case of Src-WT and Src-DN, 80 ng/well were added. In each case, data are expressed as relative light units (normalized to Renilla).
**RAW264.7 Transfection**

We plated 1.25x10^5 RAW macrophages in 24-well plates. Sixteen hours later, an equivalent of 50 ng/well of arginase and 10 ng/well of Renilla plasmids were mixed in 25 μL serum-free media with a ration of 5μL of FuGENE-HD transfection reagent per 2 μg of plasmid DNA. During a 20 minute incubation period of the aforementioned transfection complex, media was aspirated, and 500 μL of fresh media were added to each well of RAW cells. The transfection complex was then added dropwise to each well of cells. After 24 hours, cells were stimulated with IL-4 (10 ng/mL), LPS (0.1 μg/mL), or Poly IC (20 μg/mL) for 8 hours, at which point luciferase activity was measured as above.

Table 2.1: List of primers and probes used for qRT-PCR, ChIP, and mutation constructs.

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<th>Reverse Primer (5' --&gt; 3')</th>
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<td>RAN 2 (API)**</td>
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* consensus sequence is in bold, mutated sequence is underlined in forward primer
** consensus sequence for Stat6 and AP1 are in bold and underlined, mutated sequence is in bold in forward primer
Plasmid Construction

Analysis and identification of arginase promoter binding sites were achieved using MatInspector software by Genomatix (Ann Arbor, MI). Mutagenesis of pXP2-Arginase constructs was carried out using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) per the manufacturer’s instructions. (See Table 2.1 for a list of primers used.) Construction of Ron, Src,25 and the arginase deletion constructs144 were previously published.

Macrophage Isolation

Resident

Resident peritoneal macrophages were extracted by lavage with 10 mL PBS from 6-10 week old C57Bl/6 mice. Cells were plated and allowed to adhere for 3 hours before excess cells were washed away with 2 washes of PBS. Cells were then returned in complete DMEM to 37°C for 1-2 hours prior to stimulation with either 100 ng/mL MSP or 10 ng/mL IL-4.

Thioglycollate

Thioglycollate macrophages were prepared by injecting 2 month-old C57Bl/6 mice with 2 mL of 3% aged thioglycollate media (Difco, Sparks, MD). On day 4, cells were isolated by lavage and prepared the same way as with the resident macrophage isolation stated above. For the flow cytometry analysis, cells were not plated, but were immediately used to confirm the expression of Ron.
Flow Cytometry

3×10^6 peritoneal lavage cells were incubated per tube in 100 μL FACS buffer (ice cold PBS + 2% FBS). Non-specific binding was blocked by the addition of 1 μL FC-Block for 10 minutes on ice. 3 μL of Anti-F4/80-RPE, 0.1 μL anti-CD11b-APC-750, and 5 μL anti-Ron were added to appropriate tubes for 30 minutes on ice. Cells were washed 2 times with FACS buffer and resuspended once more in 100 μL of FACS buffer. Cells were incubated with anti-goat alexa 647 as a secondary antibody to Ron for 30 minutes on ice. Cells were washed two more times, resuspended in 1mL of FACS buffer and analyzed for FLOW on a Beckman-Coulter FC500. Ron expression was measured in macrophages positive for F4/80 and CD11b.

Western Blot

1×10^6 resident peritoneal macrophages, 3×10^6 thioglycollate-elicited macrophages, or 3×10^5 293T cells were stimulated with MSP (100 ng/mL) or IL-4 (10 ng/mL) for the indicated times, washed once with PBS, and then lysed for 30 minutes at 4°C and collected in 500 μL RIPA lysis buffer (10 mM Tris-HCL pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGF, 1% Triton X-100, 0.1% SDS, 0.1% Na-Deoxycholate + protease inhibitors (same as in ChIP Dilution Buffer below)). Lysates were cleared by centrifugation at 14,000g for 15 minutes at 4°C, and supernatants containing protein were transferred to pre-chilled tubes. 75 μL of cell lysates were mixed with 4X denaturing (25 mM DTT) SDS loading buffer and heated to 100°C for 5 minutes. In each well, 40 μL were loaded for SDS-PAGE separation using 8% Bis-Acylamide, and subsequently protein was transferred onto Immobilon-P PVDF membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hour at room temperature with 5% non-fat milk/BSA per the recommendations for each antibody blotted. Primary antibody was then added for overnight
probing at 4°C. Blots were washed three times in TBST for 15 minutes/wash with rotation. Anti-rabbit and anti-mouse HRP conjugated secondary antibodies were used at 1:10,000 and 1:2,000, respectively, for 45 minutes at room temperature. Blots were again washed for 15 minutes three times in TBST at room temperature with rotation. ECL Plus (Amersham, Piscataway, NJ) was used to develop the blots. For reprobing, membranes were stripped with 62.5 mM Tis-HCL (pH 6.8), 2% SDS, and 0.7% β-mercaptoethanol at 55°C for 30 minutes.

**Quantitative RT-PCR for arginase gene expression**

1×10^6 peritoneal macrophages were stimulated with MSP or IL-4 for the indicated times, washed once with PBS, and then RNA was collected using the Qiagen RNeasy kit according to the manufacturer’s instructions. During the elution step we added 200 ng/μL yeast tRNA (Ambion, Austin, TX) to ensure stabilization of our RNA sample. Sequence-specific primers and probe were generated by the Nucleic Acids Facility at The Pennsylvania State University using Primer Express (Applied Biosystems, Foster City, CA) for quantitative Real Time RT-PCR for the gene expression characterization of arginase I (see Table 2.1 for primers and probe). Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. From this reaction, 100 ng of cDNA, using 300 nM of each primer and 20 nM of probe, were used per qPCR reaction with TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and reactions were run and analyzed using an Applied Biosystems 7300. GAPDH (Taqman®, Applied Biosystems, Foster City, CA) was used as an internal control.
ChIP Assays

ChIP assays were performed on resident and thioglycollate-elicited peritoneal macrophages by generally following the protocol outlined in Ghisletti et al. Specifically, following stimulation, cells were fixed with formaldehyde at a final concentration of 1% for 10 minutes at room temperature, and then fixation was quenched by the addition of glycine at a final concentration of 0.125 M for 5 minutes. Media was aspirated and cells were washed twice with wash buffer (ice cold PBS + protease inhibitors (10 mM sodium fluoride, 4 mM sodium othovanadate, and 1 mM phehylmethysulfonyl fluoride)). Cells were then collected with a rubber policeman in wash buffer and centrifuged at 1500g for 5 minutes at 4˚C. Cells were resuspended in 1 mL of wash buffer, counted, and 10×10⁶ cells were aliquoted to 1.7 mL Eppendorf microcentrifuge tubes. Cells were spun down at 2000g at 4˚C for 5 minutes, and resuspended in 300 μL of cell lysis buffer (50 mM Tris-HCl Tris-HCl pH 8.0, 10 mM EDTA, 1%SDS, + protease inhibitors + 10 μg/mL Aprotinin, 10 μg/mL Leupeptin, 1μg/mL Pepstatin A)). Chromatin was sonicated using the Diagenode Bioruptor (power setting high, 20 cycles of 30 seconds on, 60 seconds off) to achieve sheared DNA between 200 and 600bp in length. Samples were centrifuged at 14,000g for 15 minutes at 4˚C to remove debris. The volume equivalent of 1.5×10⁶ resident or 4×10⁶ thioglycollate-elicited macrophages was aliquoted into 10x ChIP dilution buffer (0.5% Triton-X 100, 2.2 mM EDTA, 22 mM Tris-HCl pH 8.0, 150 mL NaCl + protease inhibitors), primary antibody was added (3 μg/mL of Santa Cruz Biotechnology antibodies STAT6 (sc-1698x), C/EBPβ (sc-150x), Fos (sc-52x), Fosβ (sc-48x), Jun (sc-44x), and 4 μg/mL of phospho-Jun (2 μg/mL each of sc-7980x and sc-16311x)) and samples were incubated overnight at 4˚C with rotation. The next morning, 20 μL of protein A or G magnetic beads (New England Biolabs, Beverly, MA) were added to the samples and incubated for 2 hours at 4˚C with rotation. Beads were then collected by magnetic isolation and washed three times
with 1 mL of low salt wash buffer (0.1% SDS, 1% Triton-X 100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), and twice with TE. During the final TE wash, samples were transferred to new tubes to eliminate the possibility of non-specific binding to tube walls. Immune complexes were reverse-crosslinked by the addition of 140 μL of complete elution buffer (20 mM Tris-HCl pH 8.0, 5 mM EDTA, 50 mM NaCl, 1% SDS, 125 μg/mL proteinase K) and incubation at 65°C for 3 hours. DNA was then collected and eluted in 30 μL of water using DNA Clean and Concentrator (Zymo Research, Orange, CA). 2 μL of DNA was then used per qPCR reaction, using SensiMix SYBR (Bioline, Taunton, MA), and analyzed on an Applied Biosystems 7900HT. See Table 2.1 for a list of primers used.

Results

The Ron receptor induces arginase I promoter activity.

In a previous study, we demonstrated that stimulation of the tyrosine kinase receptor, Ron, by its ligand, macrophage stimulating protein (MSP), induces arginase I expression and activity in primary peritoneal macrophages. In order to determine whether this regulation occurs at the transcriptional level, we utilized a luciferase reporter construct containing a 3.2 kb sequence upstream of the arginase I TSS, as described previously. The arginase I reporter was transiently transfected with or without a cDNA-encoding murine Ron in 293T cells and luciferase activity was measured. Ron-expressing cells exhibited a marked increase in reporter activity (Figure 2.1), which was further induced upon stimulation with MSP. These results confirm that Ron induces arginase I expression and activity by regulating arginase I promoter activity.
Figure 2.1: Ron induces arginase reporter activity in a ligand-dependent and -independent manner. 293T cells were co-transfected with Ron/Neo and the arginase reporter construct. Following transfection, cells were either left unstimulated, or stimulated with MSP for 20 hours, and luciferase reporter activity was measured and normalized to Renilla.
Previous studies have demonstrated that Ron kinase domain tyrosines 1175, 1265, and 1294 are required for ligand-independent phosphorylation of Erk, upregulation of Fos expression, and activation of an AP-1 luciferase reporter by the Ron receptor in a Src kinase-dependent manner. To test the hypothesis that the three tyrosines in the kinase domain of Ron similarly mediate ligand-independent induction of the arginase I promoter, we co-transfected a kinase-dead mutant of Ron (K1091M) or a Ron mutant harboring tyrosine-to-phenylalanine mutations at positions 1175, 1265 and 1294 (Y3F) with the arginase I promoter construct and compared reporter activity with that induced by the WT receptor. The kinase-dead mutant was unable to induce reporter activity in the presence or absence of ligand (Figure 2.2A). The Y3F mutant was also unable to induce reporter activity in a ligand-independent manner. However, upon MSP stimulation, Y3F Ron retained its ability to induce arginase I reporter activity. To determine whether this induction is mediated by Src, we co-transfected Ron with either a WT or a dominant-negative Src (Src-DN) expression plasmid, and then observed arginase I reporter activity. Co-transfection with Src-DN inhibited Ron-mediated arginase I reporter activity to control levels, but MSP-induced activity was retained in a manner similar to that observed with the Ron-Y3F mutant (Figure 2.2B). From this, we concluded that the three tyrosines in the kinase domain of Ron and Src kinase, which were previously demonstrated to be required for ligand-independent Erk activation and induction of AP-1 activity, also mediate the ligand-independent upregulation of the arginase I promoter by Ron.

Two docking site tyrosines (Y1330 and Y1337) in the C-terminal tail of Ron are responsible for mediating cell signaling and phenotypic events in response to MSP. In a previous study, we found that mutation of these two tyrosines abrogates the ability of Ron to induce phosphorylation of Erk, upregulate Fos and activate an AP-1 luciferase reporter in response to MSP. Therefore, we co-transfected Ron Y2F (Y1330/37F), Ron Y1330F, or
Figure 2.2: Kinase domain tyrosines 1175/1265/1294 play an instrumental role in ligand-independent Ron-mediated arginase expression via Src kinase. (A) Upper panel: Kinase domain tyrosines 1175/1265/1294 were mutated to phenylalanine (Y3F), co-transfected into 293T cells, and luciferase activity was measured. Kinase-dead (K1091M—KD) was also used to examine the importance of the kinase domain of Ron in regulating arginase reporter activity. Lower panel: Lysates used in the luciferase assay were kept for analysis of protein expression by western blot. Data from the western blot also display results from lysates used in Figure 2.3. (B) Src-WT and Src-DN plasmids were co-transfected with Ron and the arginase reporter construct in 293T cells and luciferase activity was measured. Insert: Lysates from the luciferase assay were analyzed by western blot to insure equal expression of mutant plasmids.
Ron Y1337F with the arginase I reporter plasmid in 293T cells to determine whether these docking site tyrosines are required for induction of arginase I promoter activity in response to MSP. Ron Y2F induced arginase I reporter activity to a similar level as WT Ron when co-transfected in 293T cells (Figure 2.3).

However, addition of MSP did not further upregulate activity, indicating that the two docking site tyrosines are required for induction of arginase I promoter activity in response to MSP. Upon transfection of either the Y1330F or the Y1337F single docking site mutants, MSP retained its ability to induce reporter activity, suggesting that these two tyrosines induce overlapping signaling pathways.

**Ron regulates arginase I expression through an AP-1 site in the promoter proximal region.**

To identify which region of the arginase I promoter is stimulated in response to Ron/MSP, we utilized a series of N-terminally deleted promoter constructs, as described previously. Stat6 and C/EBPβ elements in the region between -3.29 kb and -2.78 kb are known to play a central role in the induction of arginase I promoter activity by IL-4. However, deletion of this region did not result in a significant loss in promoter activity induced by Ron in the presence or absence of MSP (Figure 2.4A).

Moreover, mutation of Stat6 and C/EBPβ sites that mediate IL-4-induced arginase I expression did not affect the upregulation of arginase I promoter activity induced by Ron (Figure 2.4B). On the other hand, the region between -0.3 and -0.8 kb of the promoter was critical for induction of arginase I promoter activity by Ron, as the -0.3 kb plasmid exhibited almost no reporter activity while the -0.8 kb plasmid retained robust activity (Figure 2.4A). This result suggests that induction of the arginase I promoter by Ron in the presence or absence of ligand occurs via a mechanism distinct from that observed for IL-4.
Figure 2.3: MSP induces arginase reporter activity through the multifunctional docking site of the Ron receptor. 293T cells were co-transfected with the arginase reporter, Renilla, and one of several forms of Ron (either wild-type Ron, Ron for which both docking site tyrosines were mutated (Y2F), or Ron for which either tyrosine was individually mutated (Y1330F or Y1337F)). See Figure 2.2A, lower panel, for expression confirmation of the various Ron plasmids.
Figure 2.4: The Ron receptor induces arginase reporter expression via a site positioned in a unique manner in relation to the sites identified for IL-4 induced expression. (A) Equi-molar amounts of successive deletions of the arginase promoter were co-transfected with the Ron receptor in 293T cells and analyzed for reporter expression. Plasmids are labeled in kilobases for the length of promoter they contain upstream of the transcription start site. (B) Stat6-2888 and C/EBPβ mutated arginase plasmids were co-transfected with Ron in 293T cells and luciferase activity was measured.
We employed MatInspector software to search the region between -0.8 kb and -0.3 kb of the arginase I promoter for potential transcription factor binding sites. Of the 80 sites identified, two sites that were of interest to us were binding sites for Stat6 and AP-1, located at -453 bp and -433 bp upstream of the TSS, respectively (Figure 2.5A). To test whether these binding sites contributed to induction of arginase I promoter activity by Ron, we made systematic mutations in the promoter binding sites of both Stat6 (Stat6-453) and AP-1 (AP-1-433) in the context of the -3.2 kb and -0.8 kb arginase I reporter constructs, co-transfected these reporter constructs with Ron in 293T cells, and measured luciferase activity. In the context of both the -3.2 kb (Figure 2.5B) and the -0.8 kb promoter fragment (data not shown), the AP-1-433 mutation resulted in the ablation of Ron-induced reporter activity.

The Stat6-453 mutant also strongly inhibited Ron-induced promoter activity, whereas mutation of the upstream Stat6 element (Stat6-2888), previously identified as an IL-4-responsive site, did not. To confirm specificity of the Stat6 and AP-1 mutagenesis, we also made two random mutations of 3bp each in the region directly adjacent to the AP-1-433 and Stat6-453 binding sites. These mutants retained full reporter activity in the presence of Ron (Figure 2.5B).

To determine whether these sites also play a role in IL-4-induced arginase I transcription, we utilized the macrophage cell line RAW264.7, which responds to IL-4 but fails to express Ron. Consistent with our previous observations, using the -3.2 kb reporter, arginase I promoter activity was induced in transiently-transfected RAW264.7 cells in response to IL-4. Mutation of AP-1-433 in the context of this promoter did not result in a significant reduction of reporter activity compared with the WT reporter (Figure 2.5C). As demonstrated previously, mutation of Stat6-2888 completely eliminated IL-4-induced reporter activity. Moreover, mutation of the Stat6-453 site resulted in a significant reduction in reporter activity. Together, these data suggest that, in addition to the previously identified Stat6-2888 site, the newly identified Stat6-453 site is also important in inducing arginase I transcriptional activity in response to IL-4. However, induction
Figure 2.5: The Ron receptor induces arginase reporter expression via an AP-1 site located 433 bp upstream of the TSS while IL-4 induces via a Stat6 site. (A) Schematic of the arginase I promoter. (B) The AP-1-433 and Stat6-453 sites identified \textit{in silico} were mutated and co-transfected with Ron into 293T cells. In addition, random mutations (Ran1 and Ran2) were made in sites flanking the AP-1-433 and Stat6-453 sites, respectively. Reporter activity was measured. (C) RAW264.7 cells were co-transfected with arginase plasmids and Renilla, and stimulated with IL-4 for 8 hours. Reporter activity was measured and, due to transfection variability, was self-normalized to show fold induction by IL-4 vs unstimulated cells of the same transfected plasmid.
of arginase I promoter activity by Ron is dependent on both Stat6-453 and AP-1-433 binding sites.

**MSP and IL-4 induce disparate signaling events.**

Because MSP and IL-4 regulate the arginase I promoter in different ways, we examined signaling events mediated by stimulation with MSP or IL-4 in primary macrophages. To our surprise, we routinely detected stronger phosphorylation of Stat6 by MSP than IL-4, even though IL-4 more potently induces arginase I expression (Figure 2.6A). The Mapk pathway induces AP-1 complex formation and activity, and we observed strong and sustained phosphorylation of the Mapk, Erk, in macrophages stimulated with MSP, while a reduced and transient induction was observed in IL-4 stimulated cells (Figure 2.6B). We also found that Jun, was strongly phosphorylated in macrophages stimulated by MSP but not IL-4 (Figure 2.6B). Ron and the closely-related family member MET induce MAPK signaling through recruitment and phosphorylation of adaptor molecules Gab1 and Gab2. We observed strong phosphorylation of both Gab1 and Gab2 by MSP, but not by IL-4 (Figure 2.6C). Thus, MSP, but not IL-4, seems to be a strong inducer of the Mapk pathway in primary macrophages, which supports the hypothesis that AP-1 mediates the upregulation of arginase I transcription by Ron, but not IL-4.

In order to determine the timeframe in which arginase I transcription is induced by MSP or IL-4, arginase I mRNA levels were quantified by qRT-PCR. While both MSP and IL-4 induce arginase I expression as early as 1 hour post-stimulation, the pattern of induction for MSP stimulation displayed a biphasic level of mRNA expression, whereas IL-4 induced sustained mRNA expression (Figure 2.7). The timing of the early phase (1-4 hours post stimulation) of arginase I induction by MSP mirrored that observed for the phosphorylation of Erk, Jun, and Gab1/2. By 24 hours post-stimulation there was renewed induction of arginase I mRNA by MSP,
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- Phospho Erk
- Total Erk
- Phospho Jun
- Total Jun
- Phospho CREB

Fold Induction (Δ1hr MSP)

Fold Induction (Δ2hr MSP)

Phospho-ERK (Time)

Phospho-Jun (Time)
Figure 2.6: MSP and IL-4 induce divergent signaling patterns in primary peritoneal macrophages. (A-C) Primary peritoneal macrophages were stimulated with MSP or IL-4 for the indicated times, lysed, and then immunoblotted for the proteins indicated. Arrow in 5C denotes expression of phospho-Gab1. Densitometric analysis is displayed for phospho-Stat6, phospho-ERK, phospho-Jun, and phospho-Gab2-Y452.
Figure 2.7: Time course for arginase I mRNA expression in primary peritoneal macrophages stimulated with IL-4 or MSP. Primary peritoneal macrophages were stimulated with IL-4 or MSP for the indicated times, and RNA was collected for quantitative RT-PCR analysis. Data are presented in a manner which emphasizes the temporal regulation by both IL-4 and MSP. Note the separate Y-axes for each stimulant.
suggesting that, like IL-4, MSP may require de novo protein synthesis to induce a secondary response that mediates robust arginase I expression.

**MSP and IL-4 induce differential binding of AP-1 and Stat6 to the arginase I promoter in vivo.**

Having established a time course for MSP- and IL-4-induced arginase I expression, we sought to determine whether this induction is associated with binding of AP-1 and/or Stat6 to the AP-1-433 and Stat6-453 or Stat6-2888 sites in primary macrophages by chromatin immunoprecipitation (ChIP). Primary peritoneal macrophages were stimulated with MSP or IL-4, and qPCR for the AP-1-433 site was performed on chromatin immunoprecipitates for AP-1 components phospho-Jun, Jun, Fos, and Fosβ. We observed an enrichment of arginase I DNA by Fos ChIP in response to MSP, whereas IL-4 failed to induce significant Fos binding (Figure 2.8A). We did not detect DNA enrichment by phospho-Jun, Jun, or Fosβ (data not shown), though this may be due to limited cell numbers available from resident peritoneal macrophages or limitations of the antibodies available.

Next, we performed ChIP for Stat6 and examined Stat6 binding to both the Stat6-453 and Stat6-2888 binding sites. While MSP induced strong phosphorylation of Stat6, only moderate enrichment of arginase I DNA was observed at either Stat6 site (Figure 2.8B). However, IL-4 induces as much as a 35-fold enrichment of DNA at the Stat6-2888 site (S2.1), a phenomenon previously observed in macrophage cell lines. We are the first to report that IL-4 induces binding of Stat6 to the proximal Stat6-453 site, while MSP induces moderate enrichment at this site (Figure 2.8B).
Figure 2.8: Macrophages stimulated with MSP or IL-4 display differential enrichment of DNA-binding elements for AP-1 and Stat6. Primary peritoneal macrophages were stimulated with MSP (100 ng/mL) for two hours or IL-4 (10 ng/mL) for one hour and chromatin immunoprecipitation (ChIP) and subsequent qPCR were performed for AP-1 member Fos (A), Stat6 (B), and C/EBPβ (C). Data are normalized to either unstimulated (A and B) or IgG (C). D. Schematic of the arginase I promoter. Sites are labeled at positions relative to the transcription start site.
Previous studies have found that C/EBPβ is required for IL-4-induced arginase I expression in RAW macrophages. Our ChIP assays on primary macrophages revealed that C/EBPβ binds the arginase I promoter very strongly in the absence of stimulus, as has been previously demonstrated in RAW macrophages. Moreover, moderate induction of C/EBPβ binding was observed upon stimulation with either IL-4 or MSP (Figure 2.8C). These results suggest that MSP primarily regulates arginase I transcription through induction of AP-1 binding, whereas IL-4 primarily induces Stat6 binding. In addition, C/EBPβ seems to be an intrinsic element that is likely shared by both MSP and IL-4 in the induction of arginase I. All of the ChIP studies were repeated in thioglycollate-elicited macrophages. Ron expression was confirmed in the elicited macrophages, as well as a response to MSP-induced ERK phosphorylation and the upregulation of arginase I expression in these cells (Figures S.2.1, S.2.2).

**TLR-induced arginase I expression is independent of both AP-1 and Stat6, and requires C/EBPβ.**

Toll-Like Receptor (TLR) ligands, including LPS, potently enhance arginase I expression, a mechanism that some pathogens utilize to evade host immune responses. Moreover, arginase I protein expression is observed as early as 1 hour post-TLR stimulation, which is much earlier than that observed in either IL-4- or MSP-stimulated macrophages. This expression is independent of Stat6 but at least partially dependent on C/EBPβ. Since TLR ligands LPS and Poly IC are also potent inducers of Mapk signaling and AP-1 activity, we tested the hypothesis that these ligands also utilize the AP-1 binding site in the arginase I promoter in addition to the C/EBPβ site. We transfected RAW cells with the full-length arginase I promoter, stimulated cells with LPS and Poly IC, and examined reporter expression. In contrast to results obtained with stimulation of IL-4 or MSP, we observed strong induction of arginase I reporter
activity by LPS and PolyIC in the presence of constructs in which either of the Stat6 binding sites was deleted (Figure 2.9). As previously reported, C/EBPβ mutation markedly reduced reporter activity in response to LPS and Poly IC treatment, although luciferase activity was not entirely eliminated under these conditions. Despite the robust induction of Mapk signaling and AP-1 activity by LPS and Poly IC\textsuperscript{151}, induction of the arginase I promoter by these stimuli was independent of the AP-1 binding site, suggesting that other unidentified elements may promote arginase I induction by TLR ligands.

Discussion

The mechanisms that govern arginase I expression in macrophages have been the focus of research over the years due to its potential importance in disease. While much of the body of work has focused on IL-4-mediated production of arginase I, here we describe mechanisms regulating arginase I expression by MSP. Specifically, we found that MSP primarily regulates arginase I expression via the Mapk pathway and an AP-1 site located in the promoter proximal region of the arginase I promoter, while IL-4 regulates expression of arginase I primarily through activation of the Jak/Stat pathway and two Stat6 binding sites in the arginase I promoter. While the regulation of arginase I by IL-4 is mediated by an enhancer located ~3 kb upstream of the transcription start site (TSS), we demonstrate that MSP stimulation of the Ron receptor induces arginase I expression through a region located ~430 bp upstream of the TSS.

Our previous studies demonstrated that Ron induces Mapk signaling and AP1 transcriptional activity in both a ligand-independent and ligand-dependent manner\textsuperscript{25}. 
Figure 2.9: LPS and Poly IC require the C/EBPβ site located in the enhancer but not the AP-1 or Stat6 sites to induce arginase reporter activity. RAW264.7 cells were co-transfected with Arginase I and Renilla plasmids, and stimulated with LPS (0.1 μg/mL) or Poly IC (20 μg/mL) for 8 hours. Reporter activity was measured and analyzed as in Figure 2.5B.
We further demonstrated that, while two docking site tyrosines in the c-terminal tail of Ron are critical for activation of this pathway in response to MSP, three tyrosines in the kinase domain mediate ligand-independent Mapk signaling. The present study indicates that the ligand-dependent and ligand-independent induction of arginase I promoter activity by Ron is dependent on these same sets of tyrosines, supporting the role of Mapk signaling and AP1 activation in this response. Mutation of both docking site tyrosines in Ron ablated MSP induced upregulation of arginase I promoter activity. However, Ron constructs harboring individual mutations of either docking site tyrosine maintained full activation of the reporter construct. These docking site tyrosines have been shown to recruit multiple SH2 domain-containing signaling partners, including the IRS-related adaptor proteins, Gab1 and Gab2. Docking site Y1330 has been shown to bind directly to Gab1 via a MET binding domain\(^{32}\), whereas Grb2 can bind to the second docking-Y1337 site tyrosine\(^ {28}\) and thereby indirectly recruit either Gab1\(^ {152}\) or Gab2\(^ {30,153}\). Propagation of signaling by either Gab1 or Gab2 can lead to the activation of Mapk signaling through the subsequent recruitment of Shp2. Thus, the requirement for only one docking site tyrosine in Ron in the induction of arginase I promoter activity in response to MSP is consistent with the ability of either docking site alone to promote Mapk signaling.

We observed strong phosphorylation of Gab1, Gab2, Erk, and Jun in response to MSP in primary macrophages, and the time frame of this activation corresponded to the expression of the first wave of arginase I mRNA. The timing of Erk phosphorylation following stimulation with MSP also correlates with binding of the AP-1 component, Fos, to the arginase I promoter. Despite the fact that MSP induces stronger phosphorylation of Stat6 than IL-4 in primary macrophages, a 7-35 fold enrichment of DNA is observed in response to IL-4 at the Stat6-453 and Stat6-2888 sites, respectively, while only moderate enrichment is observed by MSP. Thus, other signals induced by IL-4 likely play a critical role in the nuclear localization and/or binding of Stat6 to the arginase I promoter (Figure 2.10). This hypothesis is supported by previous
Figure 2.10: Proposed model of transcriptional activation by MSP and IL-4. Macrophage stimulation by IL-4 induces phosphorylation and dimerization of Stat6. IL-4 stimulation also induces the removal of an unknown cytoplasmic retention factor allowing for nuclear localization of Stat6 and activation of the arginase I promoter. MSP stimulation of Ron induces MAPK signaling leading to activation of AP-1 members Jun and Fos, which then translocate to the nucleus and induce arginase I transcription. Stimulation of the Ron receptor also induces phosphorylation of Stat6, but does not induce the removal of the cytoplasmic retention factor thus limiting nuclear localization and binding to the arginase I promoter. C/EBPβ is positioned at the promoter in a constitutively bound manner, and is likely a common regulator of transcription that is shared with IL-4, MSP, and TLR agonists.
findings whereby phosphorylated Stat6 isolated from the cytoplasm is unable to bind DNA, and that upon the removal of an unidentified molecule by detergent, DNA binding activity is restored, confirming that another factor(s) is involved with mediating the DNA binding activity and nuclear localization of Stat6\textsuperscript{154}.

Others have shown that while Stat6 is important for early regulation of arginase I expression in liver cells in response to glucocorticoids, \textit{de novo} protein synthesis, via a Stat6 dependent mechanism, is required for a secondary response\textsuperscript{139, 155, 156}. In macrophages, cycloheximide, an inhibitor used to block \textit{de novo} protein synthesis, diminishes arginase I mRNA expression in response to IL-4\textsuperscript{145}. In the present study, the time course for arginase I mRNA expression suggests that MSP induces a biphasic arginase I transcriptional response, and this second phase occurs after six hours of stimulation. These findings present the possibility that, while IL-4 and MSP induce divergent pathways that initiate arginase I transcription at early time points, they may share a common mediator of transcription at later times that requires \textit{de novo} protein synthesis. Pu.1 interacts with the arginase I promoter in response to IL-4 in RAW cells\textsuperscript{143}. We have found that Pu.1 is upregulated in response to the activation of a truncated Ron receptor by the Friend erythroleukemia virus, and that this upregulation of Pu.1 is important in promoting erythroleukemia, a hallmark of Friend virus pathogenesis\textsuperscript{157}. However, we failed to observe an increase in Pu.1 expression in either MSP- or IL-4-stimulated peritoneal macrophages (data not shown), rejecting the hypothesis that an upregulation of Pu.1 accounts for increased arginase I transcription at the 24 hour time point in response to either ligand. This is consistent with the finding in RAW cells that Pu.1 binds to the arginase I promoter even in the absence of IL-4 stimulation\textsuperscript{143}.

Even though we observed an enrichment of DNA using a C/EBPβ antibody by ChIP in response to both MSP and IL-4, C/EBPβ is also highly bound to the promoter in the absence of ligand stimulation. Consistent with a universal role for C/EBPβ in the regulation of arginase I
expression, deletion of C/EBPβ impairs induction of arginase I in response to a wide variety of stimuli including IL-4\textsuperscript{140, 143, 144}, LPS, hypoxia\textsuperscript{126, 128, 140}, and TLR ligands\textsuperscript{150}. It is therefore likely that C/EBPβ, like Pu.1, is constitutively bound to the arginase I promoter, and cooperates with Stat6 and/or AP-1 which are inducibly recruited to the arginase I promoter by IL-4 or MSP, respectively (Figure 2.10).

Sequence alignment using the UCSC genome browser did not reveal homology of the Stat6-2888 site between the mouse and human promoters (Figure 2.11A). However, as previously alluded to\textsuperscript{144}, there is a putative STAT6 site in the human promoter that is ~2.4 kb upstream of the TSS of the human promoter, the empirical analysis of which has yet to be performed. In contrast to observations in murine macrophages, IL-4 and IL-13 alone are weak inducers of arginase activity in human macrophages\textsuperscript{158-160}, despite high levels of arginase I expression in macrophages in the context of tumors\textsuperscript{133}, asthma\textsuperscript{161}, and infection, and decreased expression in foam cells of atherosclerotic lesions\textsuperscript{162}, a phenomenon shared with murine models. This difference is unique to arginase I expression as upregulation of other hallmarks of alternatively activated macrophages by IL-4 such as IL-1Ra\textsuperscript{159} and MGL\textsuperscript{163} is preserved in human macrophages. However, with the addition of cAMP activators, IL-4 can potently induce arginase I expression in human alveolar macrophages\textsuperscript{134}. These observations indicate that while STAT6 may contribute to the induction of arginase I in human macrophages, activation of STAT6 alone is not sufficient to induce arginase I expression in these cells. cAMP is a second messenger that induces the phosphorylation of CREB, a transcription factor that binds DNA via cAMP-response elements (CRE). CREB, like AP-1, is a basic-region leucine zipper transcription factor, but is distinct in binding preference from AP-1 via a 2 bp insertion between the shared TGA/TCA half sites (e.g., TGACGTCA for CREB vs TGACTCA for AP-1)\textsuperscript{164}. Nerve growth factor has been shown to phosphorylate CREB in an Mapk/Erk-dependent manner\textsuperscript{165, 166}, and the present study demonstrates that MSP also stimulates the phosphorylation of CREB (Figure 2.6B). The
**Figure 2.11:** Conservation of the arginase I promoter between species. The arginase I promoter surrounding the distal (A) and proximal (B) sites identified in this study were subjected to sequence alignment using the UCSC genome browser. (A) Blue: C/EBPβ site; red: Stat6-2888 site in the murine promoter. (B) Yellow: AP-1-433 site; red: Stat6-453 site in the murine promoter. In A and B, bases in bold represent conserved nucleotides, and underlined segments represent half sites for their respective DNA-binding elements. Consensus sequences for Stat6, C/EBPβ, and AP-1 are TTCNNNNGAA, (A/G)TTGCG(C/T)AA(C/T), and TGA(C/G)TCA, respectively.
physiological role that CREB phosphorylation plays in the induction of arginase I remains to be clarified, but there may be a role for MSP and IL-4 working in tandem to induce CREB and STAT6, respectively, in human macrophages.

Regulation by MSP and IL-4 at the Stat6-453 site may well be a phenomenon specific to rodent macrophages as it is poorly conserved in other species (Figure 2.11B). Alternatively, while the AP-1 site as a unique putative DNA binding element is not highly conserved, as a half site, this element is highly conserved, which presents the possibility that, in the human promoter, AP-1 binds with another factor at or near this site. AP-1 components have been shown to cooperate through such a half site with a number of other regulatory factors including NFAT\textsuperscript{167}, nuclear factor-\textgamma\textsuperscript{168}, and C/EBP\textalpha\textsuperscript{169}.

We further scanned the human arginase I promoter region using MatInspector and found that there are predicted STAT6 and AP-1 sites located \~1.5kb upstream of the transcription start site separated by 33 bp. It is unclear whether or not these sites act in tandem or individually to regulate arginase I expression in human macrophages.

Hepatocytes constitutively express high levels of arginase I, and this expression is necessary for the regulation of the urea cycle. On the other hand, macrophages express relatively little arginase I, and upon stimulation, can significantly increase arginase I expression. Thus, cell context is clearly an important factor to consider in the regulation of arginase I. Since macrophage arginase I expression has been implicated in a wide variety of diseases, finding a means of intervention that specifically targets the expression of arginase I in macrophages without impairing its expression in hepatocytes is of pharmacological interest. Future studies will determine whether MSP induces expression of arginase in human macrophages and/or enhances arginase expression in these cells in cooperation with IL-4.
Figure S.2.1: Day 4 thioglycollate-elicited macrophages express Ron and respond to MSP stimulation by inducing ERK phosphorylation and increasing arginase I transcription. (A) Day 4 thioglycollate-elicited macrophages and resident peritoneal macrophages were harvested and analyzed by FLOW cytometry for Ron expression. (B) Thioglycollate-elicited macrophages were stimulated with MSP (100 ng/mL) for the indicated times and lysates were immunoblotted for phospho-Erk. (C) Thioglycollate-elicited macrophages were stimulated with MSP for two hours and RNA was analyzed by qRT-PCR for arginase expression.
Figure S.2.2: Thioglycollate-elicited macrophages stimulated with MSP or IL-4 display differential enrichment of DNA binding elements for AP-1 and Stat6. Day 4 thioglycollate-elicited macrophages were stimulated with MSP (100 ng/mL) for two hours or IL-4 (10 ng/mL) for one hour and chromatin immunoprecipitation (ChIP) and subsequent qPCR were performed for Fos (A), Stat6 (B), and C/EBPβ (C). Data are normalized to either unstimulated (A and B) or IgG (C). (D) Schematic of the arginase I promoter. Sites are labeled at positions relative to the transcription start site.
Chapter 3

Reduced syngeneic tumor growth in mice with a targeted deletion in the Ron receptor tyrosine kinase

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Abstract

The Ron receptor tyrosine kinase is an oncogene that is overexpressed in a number of human carcinomas. However, Ron is also expressed on macrophages of the innate immune system and can shift the balance of immune activation from Th1-type to Th2-type responses. These responses have been well characterized in the murine model of septic shock where Ron−/− mice produce elevated levels of IFNγ and succumb to death while wild-type animals survive. Thus a role for Ron in mediating immune responses exists, yet the potential role of Ron in the regulation of tumor growth by way of altering tumor immunity is currently unknown. In the present study, we find that the growth of the syngeneic tumors 3LL, B16-F10, and EG.7 is reduced in Ron−/− mice. Concurrently, Ron−/− mice exhibit reduced induction of myeloid derived suppressor cells (MDSCs) as well as cytokines produced upon tumor onset that promote the recruitment of MDSCs to the tumor microenvironment where they differentiate into tumor associated macrophages (TAMs). While the overall percentage of TAMs associated with the tumors are not affected by the absence of Ron, expression of arginase I by these cells is severely reduced in Ron−/− mice. Expression of arginase I by TAMs has been implicated in the inhibition of T cell activity. Consistent with these observations, we demonstrate that Ron−/− tumor bearing mice (TBM) harbor increased numbers of CD4 and CD8 T cells. In addition, splenocytes from Ron−/− TBM produce increased levels of IFNγ and decreased levels of IL-10 in response to mitogenic challenge, and exhibit increased proliferation to allogeneic challenge in a mixed lymphocyte reaction. Together, the present findings suggest that, in addition to the well characterized role of Ron as an oncogene, Ron also modulates the immune response to tumor challenge such that the absence of Ron in the tumor microenvironment enhances tumor immunity and reduces tumor growth. These studies have important implications for the therapeutic intervention of a broad range of solid tumors through the targeting of Ron.
Introduction

Tumor growth and progression is dependent on evasion of the host immune response, and suppression of both the innate and adaptive branches of the immune response by tumors has been documented. Accessory cells of the innate immune system are positioned as primary players in suppressing the immune response to tumor growth.

CD11b\(^+\)/Gr-1\(^+\) myeloid derived suppressor cells (MDSCs) increase in numbers within secondary lymphoid organs upon tumor onset where they suppress T cell activation in a manner that is dependent on both inducible nitric oxide synthase (iNOS) and arginase I enzymes\textsuperscript{112}. These MDSCs can potently inhibit T cell proliferation in response to both mitogen stimulation and alloreactivity\textsuperscript{170}. In addition to their role in suppressing the immune response in secondary lymphoid organs, MDSCs home to the tumor where they can become CD31\(^+\) endothelial cells\textsuperscript{171} or differentiate into macrophages displaying CD11b and F4/80, markers characteristic of mature, tissue-resident macrophages\textsuperscript{172}.

Tumor associated macrophages (TAMs) constitute a substantial part of the tumor mass\textsuperscript{173}, and are phenotypically anti-inflammatory, or M2, in nature\textsuperscript{174-176}. Indeed, in some carcinomas, including breast\textsuperscript{177, 178}, glioma\textsuperscript{179}, prostate\textsuperscript{131}, and lung\textsuperscript{180} to name a few, macrophage infiltration has been indicative of tumor progression and poor prognosis. In particular, tumor macrophages can prevent T cell proliferation and induce apoptosis in response to antigen stimulation. Like MDSCs, TAMs also exhibit dependence on the enzyme arginase I to inhibit T cell proliferation\textsuperscript{170, 172}. In fact, IL-4 and IL-13, two cytokines associated with M2-macrophage activation, have been shown to enhance arginase I mediated immunosuppressive effects\textsuperscript{170, 181}. Furthermore, inhibition of arginase enzyme activity in tumor bearing mice (TBM) reduces tumor growth, a phenomenon that is lost in \textit{scid} mice, indicating that arginase expressing MDSCs and TAMs mediate their effects on tumor growth through the regulation of T cell responses\textsuperscript{132}. 
While the role of receptor tyrosine kinase (RTK) activation in the transformation of tumor cells has been widely studied, the potential function of RTKs in accessory cells within the tumor microenvironment in regulating tumor progression has not previously been addressed. The Ron RTK is expressed at elevated levels, or as a constitutively active mutant, in various carcinomas including breast, colon, pancreatic, and lung. Furthermore, autocrine stimulation of the Ron receptor in breast cancer cells by its ligand, macrophage stimulating protein (MSP), promotes metastasis to the bone, and elevated expression of MSP in breast cancer patients is associated with poor prognosis. Recently, in a murine breast cancer model, transgenic mice expressing the polyoma virus middle T antigen (pMT) under the control of the mouse mammary tumor virus promoter develop both delayed onset and reduced growth of spontaneous tumors in the absence of Ron. However, it is not clear whether the decreased tumor progression observed in these models is due to the absence of Ron in the tumor cells themselves or in the tumor microenvironment.

We have previously demonstrated that Ron expressing macrophages display an M2 activation phenotype including the upregulation of arginase I expression, and are less inflammatory than Ron-/− macrophages. Consistent with these observations, Ron-/− mice produce enhanced levels of IFNγ following LPS challenge, and are more susceptible to septic shock compared with wild-type control mice. Therefore, the possibility exists that Ron not only promotes tumor growth in a cell autonomous manner, but could also play a role in modulating tumor immunity. Here we present evidence that syngeneic tumor growth in Ron-/− mice is reduced, and that this phenomena is associated with decreases in both arginase I expression in TAMs and T cell responses to tumor challenge. These results demonstrate, for the first time, that Ron expression in the tumor microenvironment regulates tumor growth and suggest the possibility that the therapeutic targeting of Ron on immune cells, as well as tumor cells themselves, could improve tumor outcome.
Methods

Mice, cells, and reagents

6-10 week old C57BL/6 and BALB/c mice were used in this study. 3LL and B16-F10 cells were purchased from ATCC (Manassus, VA). EG.7 cells were a kind gift from Dr. Avery August. All cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with L-glutamine, Non-Essential Amino Acids, Sodium Pyruvate (CellGro, Mediatech, Manassas, VA), 10% FBS (Gibco, Gaithersburg, MD), and 10ug/mL ciprofloxacin (Serologicals Proteins, Inc., Kankakee, Ill). Antibodies used for western blot are Arginase I (BD-Transduction Labs, San Jose, CA) and actin (Sigma, St Louis, MO). Antibodies used for FLOW/FACS: FC-Block, CD11b-APC-750, Gr-1-FITC, CD4-Cy5, CD8-FITC, and CD3-PE (BD-Pharmingen, San Diego, CA), F4/80-RPE (Serotec, Raleigh, NC).

The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) approved all animal experiments.

Tumor growth

Tumor cells were grown to confluence, trypsonized, and resuspended in PBS. 100uL containing 5×10^5 cells were injected subcutaneously into the shaved right flank of 6-8 week old wild-type or Ron/- knockout C57BL/6 syngeneic mice. Upon onset of palpable tumor growth, mice were examined every other day by caliper for tumor volume as determined by the formula \( ab^2 \times 0.4 \), where \( a \) is the length and \( b \) is the width.
Multiplex cytokine assay

Serum was collected from euthanized mice on day 7, 11, and 15 from EG.7 wild-type and Ron-/− TBM. Serum was analyzed for cytokines/chemokines using the LincoPlex assay kit according to the manufacturer’s instructions (Millipore). Cytokines analyzed were: MCP-1, IL-1α, G-CSF, IP-10, IL-6, IL-17, IL-4, IL-13, MIP-1α, GM-CSF, KC, RANTES, IFNγ, IL-1β, IL-2, IL-5, IL-10, IL-12p70, TNFα, IL-9, and IL-15. Italicized cytokines were the only cytokines detected above baseline.

Flow Cytometry

Splenocytes

Spleens from tumor bearing mice were harvested on the indicated days post tumor implantation, and single cell suspensions were achieved using a Dounce homogenizer. RBCs were lysed for 15 minutes on ice using ACK cell lysis buffer (0.15M NH₄Cl, 1mM KHCO₃, 0.1mM Na₂EDTA pH7.4). Splenocytes were washed one time with FACS buffer (ice cold PBS + 2% FBS). Cells were counted using the Advia 120 (Bayer), and 3×10⁶ cells per sample were then stained in 100uL FACS buffer with 0.1uL CD11b and 1uL Gr-1 for MDSCs, or 1uL each of CD4, CD8, and CD3 for T cells. MDSCs were determined by gating on CD11b⁺/Gr-1 double⁺ positive population, and CD4 and CD8 T cells were determined by gating on CD4⁺/CD3⁺ and CD8⁺/CD3⁺ double positive populations.
Tumors

Tumors were dissected from the mice at the indicated time points and diced to sizes smaller than 1mm using a razor blade. Tumors were then treated with 0.05% collagenase type IV (sigma) for 30 minutes at 37°C. Digested tumors were washed twice with PBS, and filtered through a 70μm nylon mesh (BD Falcon) to achieve single cell suspensions. Cells were stained with 0.1uL CD11b, 6uL F4/80, and 1uL of Gr-1. Tumor macrophage percentages were determined based on cells staining positive for both CD11b and F4/80 with the aid of FLOW analysis on a Beckman FC500 FLOW cytometer.

Re-challenge of splenocytes and ELISA

Splenocytes were collected from day 7, 11, and 15 TBM, and lysed with ACK lysis buffer as described above. 5×10^6 splenocytes were added per well of a 24 well plate in 600uL complete DMEM. 1mL containing 4×10^5 γ-irradiated EG.7 cells (5000RADS) were added to each well of the co-culture. Cells were incubated at 37°C for 3 days at which time 400uL containing 6×10^5 γ-irradiated EG.7 cells were added per well of the original culture bringing the total volume to 2mL. On day 4, plates were spun down at 250g for 5 minutes, and 1mL of supernatant was collected for ELISA analysis.

ELISA analysis for IFNγ, IL-10, IL-4, IL-2, and IL-12 was carried out using the BD OptEIA kits according to the manufacture’s protocol (BD-Pharmlingen, San Diego, CA).

Western blot

Tumors and spleens were dissected and stained for TAMs and MDSCs as above. Cells were sorted using a Cytopeia Influx cell sorter. Sorted cells were lysed in 300uL RIPA lysis
buffer (10mM Tris-HCL pH 7.5, 140mM NaCl, 1mM EDTA, 0.5 mMEGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-Deoxycholate + protease inhibitors: 10mM sodium fluoride, 4mM sodium othovanadate, 1mM pheylmethylsulfonyl fluoride, 10ug/mL Aprotinin, 10ug/mL Leupeptin, and 1ug/mL Pepstatin A). Lysates were cleared by centrifugation at 14,000g for 15 min at for 4°C, and supernatants containing protein were transferred to pre-chilled tubes. 75uL of cell lysates were mixed with 4X denaturing (25mM DTT) SDS loading buffer and heated to 100°C for 5 minutes. 40uL was loaded per well for SDS-PAGE separation using 8% Bis-Acylamide, and subsequently protein was transferred onto Immobilon-P PVDF membranes (Millipore, Bedford MA). Membranes were blocked for one hour at room temperature with 5% non-fat milk/BSA per the recommendation spec sheet of each antibody blotted. Primary antibody (arginase I) was then added for overnight probing at 4°C. Blots were washed three times in TBST for 15 minutes for each wash with rotation. Anti-rabbit and anti-mouse HRP conjugated secondary antibodies were used at 1:10,000 and 1:2,000 respectively for 45 minutes at room temperature. Blots were again washed for 15 minutes three times in TBST at room temperature with rotation. ECL Plus (Amersham, Piscataway, NJ) was used to develop the blots. For reprobing with actin, membranes were stripped with 62.5mM Tis-HCL (pH 6.8), 2% SDS, and 0.7% β-mercaptoethanol at 55 degrees for 30 minutes.

**Real time qRT-PCR**

Real time qRT-PCR for Ron expression was performed as previously described. TAMs were sorted and collected as above. Resident peritoneal macrophages were extracted by lavage with 10mL PBS from 6-10 week old C57Bl/6 mice. Cells were plated and allowed to adhere for 3 hours before washing non-macrophages away with 2 washes of PBS. Bone marrow
was harvested by flushing femurs of mice, and then plated with 30% L929 conditioned media for 5 days at which point bone-marrow derived macrophages were harvested.

**Mixed lymphocyte reaction**

Splenocytes were collected as above. Splenocytes from non-TBM BALB/c mice were γ-irradiated with 2000 RADS and plated at a concentration of 2–4×10^5 per well as stimulators. 2–8×10^5 C57BL/6 splenocytes per well were plated as responders. Cells were cultured for 5 days at 37°C, and for the final 24 hours cells were pulsed with 0.5μCi per well of [^3]H-Thymidine. Cells were collected by a semi-automated cell harvester and analyzed on a β-counter for[^3]H-Thymidine content as a measure of proliferation.

**Statistical analysis**

Minitab software was used for statistical analysis. Students T test was used for statistical analysis unless noted otherwise. Pearson Correlation, ANOVA, and MANOVA were used as indicated.

**Results**

**Ron-/- mice display reduced syngeneic tumor growth.**

In order to distinguish between the role of Ron as an oncogene and its potential function in the modulation of tumor immune responses, we elected to use syngeneic carcinomas that do not express Ron so that any differences observed in tumor growth between wild-type and Ron-/-
mice would be strictly due to the effects of Ron in the tumor microenvironment. To that end, we injected syngeneic non-small-cell lewis lung carcinoma (3LL), melanoma (B16-F10), and lymphoma (EG.7) cell lines subcutaneously into the flanks of wild-type and Ron/-/ mice, and measured tumor growth over time. As shown in Figure 3.1A-C, wild-type mice exhibit significantly larger tumors than their Ron/-/ counterparts, and in all cases, differences in tumor growth are observable as early as can reasonably be assessed by caliper measurement and persist throughout the duration of the experiment. From these results we conclude that Ron plays a role in modulating the host response to tumorigenesis such that the absence of Ron results in decreased tumor growth.

**Ron/-/ mice harbor fewer myeloid derived suppressor cells.**

The number of myeloid derived suppressor cells (MDSC) increases upon tumor onset within the tumor itself as well as in lymphoid organs, and these cells play a critical role in suppressing host immune responses against the growing tumor. In order to determine whether Ron regulates the development of these cells upon tumor onset, we isolated spleens from wild-type and Ron/-/ tumor bearing mice (TBM) and analyzed the fraction of CD11b^+/Gr-1^+ MDSCs. We observed a significant increase of MDSCs in wild-type TBM compared with control mice, a response that is attenuated in Ron/-/ animals (Fig 3.2A). Moreover, as previously demonstrated, this phenomenon displays correlation with tumor size itself (Figure 3.2B). MDSCs originate from the bone marrow of TBM, migrate to the spleen, and then the tumor where they further differentiate into tumor associated macrophages (TAMs). Recruitment and mobilization of MDSCs is mediated by pro-inflammatory cytokines such as MCP-1, G-CSF, IL-6, and IL-1, the levels of which increase upon tumor onset. Therefore, we collected serum from TBM at various time points and assayed for chemokine expression using a multiplex cytokine/chemokine
Figure 3.1: Reduced syngeneic tumor growth in Ron−/−. Wild-type and Ron−/− mice were injected subcutaneously with 1×10⁶ 3LL (A), B16-F10 (B), or EG7 (C). Upon tumor onset, tumors were measured every other day by caliper and growth was determined by the formula \(ab^2 \times 0.4\), where \(a\) is the length and \(b\) is the width. 3LL: WT (n=22), Ron−/− (n=23). B16-F10: WT (n=12), Ron−/− (n=13). EG7: WT(n=9), Ron−/− (n=10). *p<0.05.
Figure 3.2: Ron-/- TBM display attenuated expansion of MDSCs. (A) Splenocytes from day 17 mice harboring B16-F10 tumors (p<0.025 for growth) were collected and analyzed by FACS for CD11b and Gr-1 expression. Total splenocyte count was multiplied by percentage of CD11b/Gr-1 double positive cells. Data are displayed as mean +/- standard error. *p<0.05. (B) MDSCs are plotted against tumor growth (p<0.05 Pearson correlation). WT (n=12), Ron-/- (n=13).
As shown in figure 3.3A-D, we observed a significant increase in the expression of MCP-1, G-CSF, IL-6, and IL-1α over time in wild-type mice, while this increase is diminished in Ron-/- animals. The levels of chemokines IL-1α, IL-6, and G-CSF in these mice also correlated with tumor size as shown for G-CSF (p<0.005, Pearson correlation, Figure 3.4A, B). Moreover, Th17 cells, the differentiation of which is driven by IL-6 and IL-1, can impair CD8 cytotoxic ability. Subsequently, we observed significantly reduced levels of IL-17 in the serum of Ron-/- TBM, suggesting that these mice exhibit reduced Th17 cell development compared with wild-type mice (Figure 3.5).

Tumor associated macrophages from Ron-/- mice express reduced levels of arginase I

Tumor associated macrophages have been widely characterized as M2-type macrophages that express arginase I, and this expression has been implicated in the inhibition of T cell responses and enhanced tumor growth. Moreover, MDSCs from TBM inhibit T cell responses to antigen stimulation, and this inhibition requires both arginase I and iNOS expression. To determine whether tumor associated macrophages or MDSCs from Ron expressing mice exhibit higher levels of arginase I, we isolated CD11b<sup>+</sup>/F4/80<sup>+</sup>TAMs and CD11b<sup>+</sup>/Gr-1<sup>+</sup> MDSCs from tumors and spleens of wild-type and Ron-/- mice by FACS, harvested cell lysates, and immunoblotted for arginase I expression. As shown in Figure 3.6A, Ron-/- TAMs express relatively low levels of arginase I when compared with wild-type TAMs, and this phenotype appears to be restricted to the macrophage population, as no arginase I expression was detected by western blot analysis in MDSCs from either wild-type or Ron-/- mice. Despite the decrease in arginase I expression in TAMs from Ron-/- TBM, we failed to observe differences in the percentages of TAMs (F4/80<sup>+</sup>/CD11b<sup>+</sup>) between wild-type and Ron-/- (Figure 3.6B), suggesting that it is the function of these cells, rather than their recruitment to the tumor microenvironment that is altered in Ron-/- mice.
Figure 3.3: Ron-/- TBM produce fewer inflammatory cytokines than do their wild-type counterparts. Wild-type and Ron-/- EG.7 TBM were euthanized on days 7, 11, and 15 and serum was collected by cardiac puncture. Serum was analyzed using a lincoplex assay according to the manufacturers’ instructions for MCP-1 (A), G-CSF (B), IL-6 (C), and IL-1α (D). For WT and Ron-/-, n=7 and 8 for Day 7, n=8 and 8 for Day 11, and n=9 and 10 for Day 15, respectively. Data are displayed as mean +/- standard error. *p<0.025, ‡p<0.07, †p<0.09, n.d. = none detected.
Figure 3.4: G-CSF, IL-1, IL-6, and MCP-1 expression correlate with tumor size. (A) Distribution of G-CSF expression as shown in Figure 3.3 on days 7, 11, and 15 TBM. (B) Cytokine expression correlates with tumor size, as shown here for day 15 G-CSF expression. Pearson correlation with size: p<0.005 for G-CSF, IL-1, IL-6. p=0.07 for MCP-1. *p<0.025, †p<0.09
Figure 3.5: Ron/- TBM produce significantly less IL-17. IL-17 cytokine production was analyzed as in Figure 3.3. *p=0.018.
Figure 3.6: TAMs from Ron−/− mice produce less arginase I. (A) Ron−/− TAMs produce less arginase I, while expression is not observed in MDSCs. Tumors and spleens were isolated from Day 15 3LL TBM. TAMs and MDSCs were isolated by FACS as CD11b+/F4/80+ double positive cells and CD11b+/Gr-1+ double positive cells, respectively. Cells were lysed and immunoblotted for arginase I expression. Actin was used as a control. N=2-4. Data are representative of three experiments. (B) Percentage of TAMs is similar between wild-type and Ron−/−, as assessed by Flow cytometry. Tumors from day 15 EG7 tumor-bearing Ron−/− and WT mice (p=0.05 for growth) were collected and analyzed for F4/80+ TAMs. n=9 and 10 for wild-type and Ron−/−, respectively. (C) Neither TAM nor MDSC express Ron. Ron TAM were isolated as in (A), lysed, and then real time qRT-PCR was used to analyze Ron expression.
We have previously shown that MSP stimulation of the Ron receptor in primary peritoneal macrophages induces arginase I expression and promotes an alternatively activated macrophage phenotype. Therefore we predicted that expression of Ron on TAMs would promote arginase I expression by these cells. However, we failed to detect Ron expression by qRT-PCR (Figure 3.6C), FACS, or western blot analysis (data not shown) in TAM or MDSC populations from tumors or spleens of TBM, suggesting that the differences in arginase I expression observed between wild-type and Ron-/ mice is an indirect effect of Ron, rather than a direct effect of Ron expression on these cells.

**Ron-/ TBM exhibit more robust immunologic responses.**

Based on the observation that Ron-/ TBM exhibit reduced tumor growth, fewer MDSCs, and reduced arginase I expression in TAMs, we tested the hypothesis that Ron-/ TBM exhibit enhanced tumor immunity. Toward that end, we isolated spleens from day 15 TBM, and examined the number of CD4 and CD8 T cell populations in the spleen. As shown in Figure 3.7A, Ron-/ and wild-type control mice have comparable percentages of CD4 and CD8 splenic T cells. However, Ron-/- tumor bearing mice harbor significantly more CD4 and CD8 T cells than wild-type TBM suggesting that these mice are better able to mount an immune response to tumor challenge (Figure 3.7B).

In order to determine whether splenocytes from wild-type TBM are functionally impaired in response to mitogenic challenge, we isolated splenocytes from EG.7 TBM, re-challenged these splenocytes by co-culture with γ-irradiated EG.7 cells, and then examined secreted cytokine production *in vitro* by ELISA. While we did not observe any differences in IL-12p70, IL-4, or IL-2 production by these cells, we did observe an increase in production of the pro-inflammatory
Figure 3.7: Ron-/- TBM exhibit elevated numbers of CD4 and CD8 T cells. (A) CD8 and CD4 T cells are comparable in non-tumor bearing WT and Ron-/- mice, whereas CD4 and CD8 numbers are elevated in Ron-/- TBM (B) Splenocytes were collected on day 17 from B16-F10 wild-type and Ron-/- TBM (p=0.024 for tumor growth) and analyzed for CD4 and CD8 expressing T cells by Flow cytometry. **p<0.01.
Figure 3.8: Ron-/- mice display an enhanced immune response to tumor re-challenge by producing more IFNγ and less IL-10. Splenocytes from EG.7 TBM were collected on day 15 and re-challenged with γ-irradiated EG.7 cells. Supernatants were analyzed by ELISA for IFNγ (A) and IL-10 (B). (C) IFNγ-inducible IP-10 is elevated in Ron-/- TBM, as assessed by multiplex assay as in Figure 3.3. p*<0.05, ‡p<0.05 with exclusion of outlier. N.B. The data for IFNγ, IP-10, and IL-10 each include one outlier that is more than 2 standard deviations away from the mean (one outlier in wild-type IFNγ, one outlier in wild-type IP-10, and one outlier in Ron-/- IL-10). Removal of those outliers yields a statistically significant difference. Hash marks represent means.
cytokine IFNγ, and a decrease in the production of the anti-inflammatory cytokine IL-10 by splenocytes isolated on day 15 from Ron−/− TBM (Figure 3.8A, B). Moreover, consistent with the increase in IFNγ production by re-stimulated splenocytes, levels of the IFNγ inducible chemokine, IP-10, were elevated in the serum of Ron−/− TBM (Figure 3.8C).

Finally, we tested the ability of splenocytes from TBM to proliferate in response to allogenic splenocytes in a mixed lymphocyte reaction (MLR). When C57BL/6 TBM were used as responders to γ-irradiated BALB/c splenocytes, a stronger proliferative response was observed by splenocytes from Ron−/− mice compared with wild-type mice, suggesting that T cells from Ron−/− TBM mount a more robust allogeneic immune response (Figure 3.9). Together these data suggest that TBM lacking Ron expression exhibit a stronger immune response than their wild-type counterparts.

**Discussion**

A clear role for the Ron receptor as an oncogene has been previously demonstrated. In the present study, we present findings that demonstrate a novel role for Ron expression in the tumor microenvironment in promoting syngeneic tumor progression through modulation of the immune response to the tumor. The underlying mechanisms that govern these observations remain unclear. However, published findings may shed some light on the context in which Ron mediates its effects and leads to changes in tumor growth.

There is considerable consensus regarding the importance of MDSCs in promoting tumor growth through arginase-mediated suppression of the immune response. MDSCs produce elevated levels of reactive oxygen species (ROS) and this can inhibit the production of IFNγ by CD8 T cells. Use of the arginase inhibitor nor-NOHA prevented ROS production and restored
Figure 3.9: Splenocytes from Ron-/- TBM have a stronger proliferative response to allogeneic challenge. Splenocytes were isolated from day 17 B16-F10 wild-type or Ron-/- TBM and co-cultured with γ-irradiated Balb/C allogeneic splenocytes as stimulators. Cells were co-cultured for 5 days, and during the final 24 hours, [³H]-Thymidine was added. Following this, cells were harvested and counted with a β-counter for proliferation response. In each case, spleens from 12 wild-type and 12 Ron-/- TBM were isolated and pooled in groups of 4 for analysis. *p<0.05, **p<0.01, ***p<0.001. Data are presented as mean +/- standard error.
CD8 T cell production of IFNγ. Inhibition of T cells by MDSCs is also reported to be co-dependent on iNOS, leading to the hypothesis that arginase and iNOS co-expression results in depletion of the common substrate, L-arginine, which subsequently results in enhanced iNOS reductase domain activity and increased O₂⁻ production.

Antigen uptake and presentation via class I MHC is also required for the ability of MDSCs to promote inhibition and tolerance of T cells. IL-6 is associated with poor prognosis in cancer patients. Not only does IL-6 lead to the recruitment of MDSCs, but it also inhibits macrophage and dendritic cell maturation by inhibiting the optimal expression of molecules associated with antigen presentation. Many of these observations have been made in the spleen, lymph nodes, or tumor, and thus, this effect on antigen presentation seems to be both wide spread and diverse in scope in TBM. Moreover, MDSC expansion in the livers leads to the upregulation of the negative costimulatory molecule PDL-1 on kupffer cells, which then inhibit T cell responses to the tumor.

In addition to their role in lymphoid organs in suppressing the immune response, expansion of MDSCs is a prerequisite for the development of tumor associated macrophages (and D.R. Sharda unpublished observations). Once differentiated, TAMs retain some of the same properties they had as MDSCs, but also appear to gain new functions that promote tumor development. With regard to the immune system, TAMs induce apoptosis of T cells in a TNF/IFNγ dependent manner. TAMs also represent a substantial fraction of the growing tumor mass and produce copious amounts of arginase I, which depletes L-arginine from the extracellular environment. The depletion of L-arginine in the extracellular environment by arginase I-expressing macrophages in vitro, or by TAMs in vivo, decreases the expression levels of the CD3ζ chain in co-cultured T cells. This decrease in CD3ζ chain expression results in decreased activation of T cells as the ζ-chain is critical for T cell receptor proximal signaling.
While we observed a decrease in arginase I expression in TAMs from Ron-/- TBM compared with those from wild-type animals, this was not due to expression of Ron on the TAMs themselves. IL-4 and IL-13 are potent Th2 cytokine inducers of arginase I expression in TAMs\(^{110}\). However, we failed to detect either of these cytokines in the serum of TBM, and rechallenged splenocytes from EG.7 TBM also failed to produce IL-4. Therefore, the mechanism underlying the observed difference in arginase I expression between wild-type and Ron-/- TAMs remains unclear. Interestingly, IL-6\(^{200}\) or IL-1\(^{201}\) can induce the production of prostaglandin E2 (PGE\(_2\)) in myeloid cells, which in turn can induce arginase I expression in TAMs\(^{202}\). Moreover, G-CSF can skew CD4 cytokine production toward a Th2 phenotype, and G-CSF supports tumor growth in vivo but not in vitro, suggesting that the presence of accessory cells are important for this response\(^{203}\). Our observation that Ron-/- TBM exhibit decreased levels of IL-6, IL-1\(_\alpha\), and G-CSF in the serum, suggest that these cytokines are potential candidates for mediating the enhanced expression of arginase I in wild-type TAMs vs. Ron-/- TAMs.

The complexity of tumor immunity is exemplified by the role of Th17 cells in this response. Th17 cells, paradoxically, exhibit both pro-tumor and anti-tumor responses\(^{204}\). While the mechanism by which Th17 cells mediate these diverse functions remains unclear, studies suggest that the function of IL-17 is dependent on the co-expression of other factors. For instance, while expression of IL-23 is important for maintaining the Th17 phenotype, high levels of IL-23 can promote CD8 T cell cytotoxicity against tumor cells\(^{205}\). Conversely, Th17 cells can enhance tumor growth by both promoting angiogenesis and inhibiting the function of CD8 T cells in combination with IL-6 and TGF\(\beta\)\(^{188}\).

Th17 cell development can be propagated by IL-6, either in combination with TGF\(\beta\) or IL-1 stimulation\(^{206}\). Th17 cells express the IL-1 receptor,\(^{207}\) and myeloid cells produce IL-1\(_\alpha\) and IL-1\(_\beta\), both of which bind with similar kinetics and activation potential to the IL-1 receptor. While IL-1\(_\alpha\), is typically a membrane bound protein, it can be secreted by activated monocytes
and macrophages. Th17 cells fail to develop in mice lacking the IL-1 receptor, yet maintain normal CD4 and CD8 T cell development suggesting a critical role for IL-1 in Th17 cell development. In the present study, we observed a significant decrease in IL-17 production in Ron/-/ TBM. Expression of IL-17 in wild-type vs. Ron/-/ TBM correlates with production of the Th17 inducing cytokines IL-6 and IL-1α. Moreover, the receptor for IL-17 is ubiquitously expressed and stimulation of stromal cells with IL-17 induces the expression of MCP-1 and G-CSF, cytokines we report here to be similarly decreased in Ron/-/ TBM. Together, these findings support a role for Th17 cells in suppressing the immune response to tumor challenge in wild-type mice, while Ron/-/ TBM avoid the suppressive effect of these cells.

Thus, the data presented here suggest that the intervention at any one of a number of levels could provide a means by which Ron modulates tumor growth (Figure 3.10). Enhanced production of IL-17 in Ron expressing mice could induce the expression of IL-6 and IL-1α in stromal cells in what is likely to be a positive feedback manner at the tumor site. Alternatively, enhanced production of IL-6 and IL-1α in Ron expressing mice could combine to drive Th17 development, or together with enhanced G-CSF and MCP1, promote the recruitment of MDSCs. Furthermore, enhanced production of IL-6, IL-1α, and IL-17 by Ron expressing TBM could induce the expression of PGE2, and in turn, induce the expression of arginase I in TAMs, thereby enhancing their inhibitory potential. Together, the increase in Th17 cells and arginase I expressing TAMs in Ron expressing mice could result in the inhibition of CD8 T cell mediated tumor immunity. The targeted inhibition of Ron could, therefore, affect any or all of these levels of regulation in a manner sufficient to reduce tumor growth.
A. Ron WT

B. Ron -/-
Figure 3.10: Ron reduces tumor immunity and promotes tumor growth. A. In the presence of Ron expression, GCSF, IL-6, MCP-1, and IL-1 are enhanced; presumably by either the tumor microenvironment such as stromal cells, or by the tumor itself. Production of these cytokines leads to the recruitment of MDSCs in secondary lymphoid organs which subsequently home to the tumor where they become TAMs. IL-1 and IL-6 production from various sources including MDSC or TAM can increase PGE$_2$ expression which then can lead to an increase in Arginase I expression in TAM. IL-1 and IL-6 can also cooperate to drive Th17 cell differentiation. Increased IL-17 Ron expressing animals can increase the expression of GCSF, IL-6, MCP-1 as well as PGE$_2$. These cytokines feed forward to increase the recruitment of MDSCs as well as increase the expression of arginase I in TAMs and further drive Th17 cell differentiation. IL-17 and TAM arginase I inhibit T cell activation, including the reduction of IFN-$\gamma$ production and enhancement of IL-10 expression. Together these reduce tumor immunity and allow for increased tumor growth. B. Absence of Ron reduces the expression of cytokines and flips a switch in tumor immunity by reducing TAM arginase I expression and also by restoring production of IFN-$\gamma$ and reducing the production of inhibitory IL-10 expression by T cells, thereby reducing tumor growth.
Chapter 4
Conclusions and Discussion

Maintaining homeostasis is a central tenet in normal biological function. The manner by which such maintenance occurs is both wide ranging and tightly regulated, and the Ron receptor tyrosine kinase is centrally positioned as a negative regulator of inflammatory responses. Ron is expressed in epithelium lining sites of pathogen encounter, and also in tissue resident macrophages where it modulates the inflammatory milieu. The purpose of the work herein was to investigate the mechanisms by which Ron regulates these responses and to further this understanding.

We had previously shown that MSP stimulation of the Ron receptor induces arginase I expression. The first objective was to determine the mechanism by which Ron regulates arginase I transcription. Using an arginase I luciferase reporter construct, we mapped the site of arginase I promoter activation by Ron to a region between 0.3 and 0.8kb upstream of the transcription start site. Transcription database analysis revealed the presence of a novel AP-1 site that, when mutated, substantially reduced the induction of promoter activity by Ron. This was in contrast to the ~2.9kb Stat6 site upstream that is responsible for induction of arginase I by IL-4. Peculiarly, western analysis revealed more robust phosphorylation of Stat6 by MSP than IL-4 in primary macrophages. The reverse trend was observed by ChIP analysis of the Stat6 binding sites within the arginase 1 promoter suggesting that an unknown mechanism of Stat6 nuclear localization by IL-4, or cytoplasmic trapping by MSP could mediate this difference. Further characterization of signaling events revealed strong MAPK activation by MSP stimulation of Ron, compared with relatively weak activation by IL-4, which supported the observation that AP-1 activation of the
arginase I promoter was induced by MSP/Ron. We then confirmed the binding of AP-1 family member Fos to the arginase I promoter by ChIP in primary macrophages stimulated with MSP.

Having established a role for Ron in promoting alternative macrophage activation, we wanted to determine if this modulation had in vivo relevance in disease. Therefore, the second objective was to test the hypothesis that Ron expression in the microenvironment promotes tumor growth. We demonstrated that Ron knockout mice exhibited significantly reduced syngeneic tumor growth from onset of palpable tumor detection that persisted throughout experimental analysis. While TAMs from wild-type mice produced significantly more arginase I than those from Ron-/- animals, it was not due to Ron expression on the TAMs themselves. We also detected reduced levels of MDSCs and cytokines associated with their accumulation in Ron-/- animals. Arginase I production by TAMs and MDSCs can inhibit T cell activation. Consistent with these findings, Ron-/- TBM, but not tumor free mice, harbored increased numbers of CD4 and CD8 T cells compared with wild-type counterparts. Moreover, splenocytes from Ron-/- TBM produced significantly elevated levels of IFNγ and decreased levels of IL-10 in response to secondary tumor challenge, and these cells exhibited enhanced proliferation in an allogeneic assay. Together, these results suggest that upon tumor onset, the immune response in Ron-/- mice is tipped away from a Th2 type response favoring alternative macrophage activation, and toward a Th1 response, which favors stronger tumor immunity than that observed in Ron expressing mice. Central to this shift in immune balance may be the role of Th17 cells, as they can inhibit CD8 T cell activation. Here we observed low levels of the Th17 effector cytokines, IL-17, and reduced IL-6, IL-1α, MCP-1, and G-CSF in Ron-/- TBM; cytokines associated with Th17 cell differentiation and/or effector responses.

In light of these findings and in thinking of future in vivo investigations of Ron function, three outstanding questions remain:
1. What is the relative contribution of Ron expressing epithelium and Ron expressing macrophages in promoting tumor growth/inflammation?

Despite increased expression of arginase I on TAMs in wild-type mice, we failed to detect Ron expression in these cells. Though a direct role for Ron in TAMs seems less likely, its contribution in accessory cells of the tumor microenvironment cannot be excluded. Ron is expressed on tissue resident macrophages. The origin of tissue resident macrophages is thought to be Ly6C^- monocytes, which roll along the endothelium at 12um per minute in the vasculature, and constitute ~5% of monocytes (less than 0.5% of total mononuclear cells in the blood). Though it has not been empirically determined, it is tempting to speculate that these cells may be the source of Ron expressing tissue-resident macrophages. The origin of tissue-resident macrophages is distinct from the origin of TAMs and MDSCs, which are recruited from the bone marrow and express Gr-1 (Ly6C/G). Because of the substantial increase in Gr-1 expressing MDSCs that differentiate into TAMs, the relative contribution of Ly6C^- monocyte-derived macrophages may be small and undetectable in the experiments detailed within this study. Yet, their presence may be sufficient to tip the balance of immune inactivation in tumor development.

The contribution of the tumor stroma, including epithelium, was not investigated here. However, the expression of Ron on stromal cells in and around the tumor cannot be excluded. As discussed, IL-6 can participate in driving Th17 cell differentiation, aid in recruitment of MDSCs, promote arginase I expression in TAMs by upregulating PGE₂, and prevent maturation of antigen presentation in macrophages and dendritic cells; all of which could participate in the inhibition of T cell activation and thereby promote tumor growth. One source of IL-6 production could be the stroma of the tumor microenvironment. Here, we found that Ron regulates IL-6 expression in TBM, but the source of IL-6 was not identified. Since Ron/- TBM produced significantly lower
levels of IL-6, it is conceivable that Ron acts within tissue resident macrophages and/or tumor stromal cells to promote IL-6 production.

Understanding the relative contribution of Ron on macrophages vs epithelium is not unique to tumor growth, and is a common and unanswered question for other Ron mediated inflammatory diseases. Further clarification will likely arise from transgenic mice that either express Ron exclusively in the macrophage lineage, or in tissue-specific knockouts of Ron. Alternatively, hematopoietic stem cell transplantation would address whether the source of Ron mediated disease arises from the bone marrow or the epithelium. However, the latter approach is complicated by the fact that tissue-resident macrophages are much slower to turn over following irradiation and bone marrow transplantation than other cells of the immune system. An alternative approach to explore the importance of macrophages in promoting tumor growth would be to carry out carrageenan\textsuperscript{212}, or clodronate liposome\textsuperscript{131, 213}, macrophage depletion prior to tumor inoculation. These approaches induce the apoptosis of macrophages, and while they have been used to study the role of TAMs, the inflammatory state that may ensue from such depletion is not clear.

2. What came first, the chicken or the egg?

A constant issue in studying the immune response to \textit{in vivo} tumor growth has been to place the results into a linear cause and effect model. IL-6 production, like many of the observations made in this study, was correlated with an increase in tumor growth. Was IL-6 production decreased in Ron\textendash/- mice because tumor growth was decreased, or was tumor growth decreased in Ron\textendash/- mice because IL-6 production was decreased? This question is not unique to Ron mediated tumor growth as others studying the immune/host response to tumor growth are faced with similar questions. One approach may be to cross the Ron\textendash/- mice with IL-6\textendash/- mice, or to perform antibody mediated IL-6 inhibition, to determine if Ron\textendash/- mice still exhibit reduced
tumor growth. However, these experiments may not provide concrete answers regarding the primary role of Ron in this process as lack of IL-6 itself would likely act as a bottle neck at multiple check points to prevent deleterious immune inhibition and reduce tumor growth. This is likely to be true for other players in immune-modulated tumor bearing hosts.

To better address the role of Ron in the chicken or the egg conundrum, application of small molecule inhibitors against Ron or Ron blocking antibodies will be of foremost importance. Inhibition of Ron could be applied prior to administration of tumor cells or at different times following tumor onset. This would help clarify both the question of whether Ron mediated effects were developmental in nature, and also whether Ron was a causative agent of tumor growth prior to tumor growth, or upon tumor onset. From a therapeutic stand point, this will be important information to glean.

3. In what other diseases might Ron participate?

Because of Ron’s unique expression pattern on the apical surface of epithelial cells and on tissue resident macrophages, it seems likely that inflammatory diseases that initiate at sites of epithelial and macrophage intervention could be regulated by Ron. Atherosclerosis seems to be a prime candidate for this phenomenon as it is a disease that is typified by inflamed epithelium with a large influx of classically activated macrophages (foam cells). The importance of macrophages in the development of atherosclerosis is supported by the observation that mice deficient in genes that are either involved in monocyte recruitment (MCP-1, CCR) or macrophage differentiation (MCSF) exhibit reduced atherosclerosis. These inflammatory, classically activated, macrophages are involved in multiple stages of disease progression, beginning with the uptake of oxLDL, inhibition of LDL efflux by ABC-A1, and subsequent foam cell development. Moreover, it has been demonstrated that foam cells exhibit an 11-fold decrease in arginase I expression and a corollary 3-fold increase in NO production. Furthermore, rabbits that exhibit
reduced susceptibility to atherosclerosis have enhanced macrophage arginase I expression\textsuperscript{124}. Though it is not known whether Ron is expressed on foam cells, Ron expression has been detected on the vascular endothelium within wounds\textsuperscript{58}. Should Ron be expressed on foam cells or epithelium of the vasculature in atherosclerotic lesions, it would quite frankly be shocking if Ron\/- mice did not have an exacerbated phenotype.

The body of work presented within provides evidence for a direct role for Ron in the alternative activation of macrophage arginase I expression \textit{in vitro}, and a novel role \textit{in vivo} for Ron in mediating the immune response to tumor challenge. Therefore, these studies add to our current understanding regarding the role of the Ron receptor in tipping the balance of the immune system. While tipping this balance is important for downregulating inflammatory responses to pathogenic insult in order to achieve homeostasis, it is also hijacked by tumor cells to promote immune evasion. These results further support the hypothesis that activation or inhibition of Ron could have therapeutic effects in the treatment of chronic inflammation or tumorigenesis, respectively.
Appendix

**Hypoxia Induces Expression of the Ron Receptor in Macrophages and Stimulation of Ron by MSP Augments the Expression of Hypoxia Inducible Genes**

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Introduction

Sites of wounding and infectious environments pose the stress of hypoxia on neighboring cells. Macrophages within these sites must potentiate responses under low oxygen tension (hypoxia) and are important cells not only for combating infectious agents in this limiting environment, but also for restoring the natural function of the local environment through the stimulation of proliferation and the recruitment of new cells. Arginase I expression is important in promoting proliferation and extracellular matrix synthesis, two processes associated with wound healing. Increased arginase I expression has been reported in wound macrophages, tumor associated macrophages, and macrophages grown under hypoxic conditions. In the case of the growing tumor, arginase I expression suppresses the immune system, thus potentiating growth of the tumor. Macrophages in hypoxic environments also play a role in recruiting endothelial cells through the production of vascular endothelial growth factor (VEGF) and thereby aiding in restoration of blood flow.

The tyrosine kinase receptor Ron is upregulated on macrophages in wound environments, and its ligand, macrophage stimulating protein (MSP), undergoes proteolytic cleavage to the active form at these sites. Moreover, we have found that activation of Ron by MSP favors upregulation of genes associated with the alternative activation of macrophages such as arginase I. Recently a HIF-1α site was identified in the human RON promoter, and under hypoxic conditions, the expression of Ron is upregulated in breast cancer cells. However, the expression of Ron in macrophages under hypoxic conditions and the effects of Ron on hypoxia-induced responses in these cells have not been investigated.

Here, using real time PCR, we detected a 3-4 fold increase in Ron expression in primary-peritoneal macrophages cultured under hypoxic conditions. Furthermore, we demonstrate that MSP and hypoxia cooperatively induce the expression of VEGF, arginase I and enzymes that
promote anaerobic glycolysis including glucose transporter-1 (GLUT-1) and phosphoglycerate kinase (PGK). Together our results indicate that the Ron receptor plays a role in augmenting the macrophage response in hypoxic conditions. These effects could be important in restoring levels of normoxia and in promoting healing.

Methods

Stimulation of primary peritoneal macrophages

Primary-peritoneal macrophages were isolated from CD1, Balb/C, and C57BL/6 mice and cultured as described in chapter 2. In each reported figure, results were confirmed at least one time in C57BL/6 mice. Each experiment was repeated a minimum of three times.

Macrophages were stimulated with MSP (100 ng/mL), IL-4 (10 ng/mL), dexamethasone (0.1 μM), LPS (0.1 μg/mL) in the presence of normoxia (20% O₂) or hypoxia (1% O₂) for indicated times. To generate hypoxia, cells were placed in a chamber and flushed with controlled gas containing 1% O₂ for 15 minutes. The chamber was sealed, and placed in a 37°C incubator for the remainder of the experiment. The ERK inhibitor PD 98059 was used at 10 μM by pre-treating the cells for 30 minutes before stimulation with MSP.

RT-PCR analysis

Semi-Quantitative RT-PCR

RNA was collected from 1×10⁶ macrophages exactly as in chapter 2. Reverse transcription was conducted for 40 minutes at 42°C using oligo(dT) primers and MuLV (Applied
Biosystems). The primer sequences for arginase I\textsuperscript{127}, β-actin\textsuperscript{7}, and VEGF\textsuperscript{220} have been previously described. The conditions for PCR were empirically determined for each gene so that the linear doubling phase would be analyzed. Touchdown PCR was carried out as follows: 94C for 3 minutes, followed by 12 cycles of 94C for 25 seconds, 68C for 30 seconds (-0.5C per cycle), 72 degrees for 50 seconds, and then x cycles of 94C for 25 seconds, 60.5C for 30 seconds, 72 degrees for 50 seconds (x=14 for arginase I, x= 22 for VEGF, x=16 for β-actin). Resolution of PCR products was performed on a 2% agarose gel, followed by densitometric analysis with Image J software (NIH v. 1.28). All samples were self normalized to β-actin expression.

**Real Time qRT-PCR**

RNA was collected from macrophages as mentioned above. Real Time qRT-PCR and analysis was carried out exactly as in Chapter 2. Sequence specific primers and probe were generated by the Nucleic Acid Facility at The Pennsylvania State University using Primer Express (Applied biosystems). Primers and probes for arginase I can be found in Chapter 2, mGlut-1 and mPGK here\textsuperscript{221}, and those of mRon here\textsuperscript{148}.

**ELISA**

3×10\textsuperscript{6} peritoneal macrophages were plated in each well of a 24 well plate. Cells were washed one time with PBS after two hours of adherence, and stimulated with MSP in normoxia or hypoxia for 24 hours. Supernatants were collected, and 100uL were used in triplicate from each set of cells in the VEGF ELISA kit per the manufactures instructions (RnD Systems).
**Western Blot**

Macrophages were collected and stimulated with MSP in normoxia or hypoxia. Cells were collected and lysed in 300uL RIPA lysis buffer (10mM Tris-HCL pH 7.5, 140mM NaCl, 1mM EDTA, 0.5 mMEGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-Deoxycholate + protease inhibitors: 10mM sodium fluoride, 4mM sodium othovanadate, 1mM phehylmethylsulfonyl fluoride, 10ug/mL Aprotinin, 10ug/mL Leupeptin, and 1ug/mL Pepstatin A). Lysates were cleared by centrifugation at 14,000g for 15 min at for 4˚C, and supernatants containing protein were transferred to pre-chilled tubes. 75uL of cell lysates were mixed with 4X denaturing (25mM DTT) SDS loading buffer and heated to 100˚C for 5 minutes. 40uL was loaded per well for SDS-PAGE separation using 8% Bis-Acrylamide, and subsequently protein was transferred onto Immobilon-P PVDF membranes (Millipore, Bedford MA). Membranes were blocked for one hour at room temperature with 5% non-fat milk. Primary antibody (phospho-Erk) was then added for overnight probing at 4˚C. Blots were washed three times in TBST for 15 minutes for each wash with rotation. Anti-rabbit HRP conjugated secondary antibodies was used at 1:10,000 for 45 minutes at room temperature. Blots were again washed for 15 minutes three times in TBST at room temperature with rotation. ECL Plus (Amersham, Piscataway, NJ) was used to develop the blots. For reprobing with total Erk, membranes were stripped with 62.5mM Tris-HCL (pH 6.8), 2% SDS, and 0.7% ß-mercaptoethanol at 55 degrees for 30 minutes.

**Macrophage Migration**

1.25×10^5 peritoneal macrophages from C57BL/6 mice were plated into 5uM transwells (Costar) and allowed to adhere in normoxic conditions for 2 hours in a modified Boyden chemotaxis assay. Media was left in the wells and MSP (100ng/mL) or MCP-1 (10ng/mL) was
added to bottom half of the chamber, or in for sample “Both”, MSP was added to both the top and bottom chambers. Migration was allowed to proceed for 2 hours in normoxia (20% O\textsubscript{2}) or hypoxia (1% O\textsubscript{2}). Cells were was one time with DMEM to remove chemoattractant and fresh DMEM with 0.5uM of Cell Tracker Green was added for 50 min. Cells were then washed with PBS one time and incubated with 3.7% formaldehyde for 10 minutes to fix cells. Cotton swabs were used to remove cells from the top chamber, and remaining cells were counted in three random high power fields using fluorescent microscopy.

**Results**

**Hypoxia upregulates the expression of Ron in macrophages.**

The expression of Ron is upregulated on at sites of wounding, and it’s ligand, MSP, is active at these sites\textsuperscript{58,72}. Therefore, we sought to determine whether Ron expression in macrophages is enhanced under conditions of hypoxia using real time qRT-PCR. As shown in Figure A.1, Ron expression is enhanced 3-4 fold in primary peritoneal macrophages following 24 hours of culture in hypoxic conditions (1% O\textsubscript{2}). Moreover, IL-4 and dexamethasone, two factors associated with the alternative activation of macrophages, also enhance Ron expression, while IL-10 and IL-6 do not significantly affect Ron expression.
Figure A.1: Ron expression is increased by hypoxia, dexamethasone, and IL-4. Macrophages were isolated by peritoneal lavage and plated in complete DMEM with 10% FBS. Primary peritoneal macrophages were stimulated by dexamethasone (0.1 μM), hypoxia (1% O₂), and IL-4 (10 ng/mL) for 24 hours and mRNA was collected. Ron expression in murine peritoneal macrophages was examined using quantitative Real Time PCR. * p<0.05 compared with control.
MSP and hypoxia cooperate to induce arginase I and VEGF expression.

Increased arginase I expression has been reported in wound macrophages, tumor macrophages, and hypoxic treated macrophages. Furthermore, we have shown that stimulation of Ron by MSP induces arginase I expression and activity in primary peritoneal macrophages under conditions of normoxia. Therefore, we sought to determine whether arginase I expression is further enhanced under hypoxic conditions in macrophages following stimulation of Ron with MSP. Utilizing semi-quantitative RT-PCR, we found that arginase I mRNA expression is enhanced by both MSP stimulated macrophages and hypoxic treated macrophages (Figure A.2A). Moreover, when macrophages are stimulated with MSP under conditions of hypoxia, the expression of arginase I mRNA is further augmented. Hypoxic macrophages also upregulate the expression of vascular endothelial growth factor (VEGF), a critical stimulator of angiogenesis. Here we demonstrate by semi-quantitative RT-PCR and ELISA, that MSP stimulated macrophages enhance their expression of VEGF mRNA and protein respectively. Moreover, this expression is amplified when macrophages are cultured in hypoxic conditions (Figure A.2B, C).
Figure A.2: Ron induces arginase I and vascular endothelial growth factor (VEGF) expression and production in hypoxia. (A) Arginase I expression was measured using semi-quantitative RT-PCR. Peritoneal macrophages were incubated with MSP (100 ng/mL), with or without hypoxia (1% O$_2$). Both MSP and hypoxia were able to induce arginase I expression, and an augmented response was observed for both treatments combined. (B, C) MSP alone induces a moderate increase in both VEGF mRNA (B) and Protein (C), but MSP and hypoxia together exhibit a further increase. Peritoneal macrophages were incubated in the presence or absence of hypoxia (1% O$_2$), and MSP (100 ng/mL) for 24 hours and analyzed by semi-quantitative RT-PCR (mRNA) and ELISA (protein). *p<0.05, ***p<0.001 compared with control.
MSP induced ERK phosphorylation is required for induction of VEGF in normoxia and hypoxia.

MSP is a potent inducer of ERK phosphorylation in primary macrophages (Chapter 2). Moreover, phosphorylated ERK stimulates the transcriptional activity of HIF-1α. Therefore, we examined ERK phosphorylation in primary peritoneal macrophages in response to MSP by Western blot analysis under normoxic and hypoxic conditions. While hypoxia alone does not induce ERK activation, we found that MSP induces comparable levels of ERK phosphorylation under both normoxic and hypoxic conditions (Figure A.3), suggesting a potential mechanism by which MSP could enhance the expression of HIF-1α dependent genes. In order to determine whether ERK mediates the regulation of VEGF by MSP, we utilized the ERK inhibitor, PD 98059. Inhibition of ERK signaling completely abolished the ability of MSP to enhance the expression of VEGF protein under hypoxic conditions (Figure A.3). Moreover, VEGF mRNA expression was also inhibited under these conditions, suggesting that the ability of MSP to enhance expression of VEGF in hypoxia occurs at the transcriptional level in an ERK dependent manner (Figure A.3).
Figure A.3: Extracellular signal-regulated kinase (ERK) is necessary for the induction of VEGF by MSP. (A) ERK is phosphorylated after 15 minutes of stimulation by MSP in primary macrophages, and phosphorylation is maintained at 60 minutes in both normoxia and hypoxia as analyzed by western blotting. (B, C) An ERK inhibitor (PD 98059-10 μM) was used on peritoneal macrophages stimulated with hypoxia, normoxia, and MSP. Cells were harvested for mRNA (B), and cell supernatants were collected and analyzed by ELISA for VEGF protein (C). *p<0.05, ***p<0.001, ‡p<0.01 compared with MSP treatment in normoxia, †p<0.01 compared with MSP treatment in hypoxia.
MSP induces anaerobic glycolysis in normoxia and this response is augmented in hypoxia.

In anaerobic conditions, cells cannot generate ATP from the citric acid cycle, and must generate ATP by producing the glycolytic end product, lactate. Since the majority of the steps in glycolysis are reversible, in order to increase energy generating capabilities in hypoxic environments, cells must tip the balance toward the forward reaction. Macrophages have been reported to upregulate the glycolytic enzyme phosphoglycerate kinase (PGK) and glucose transporter-1 (GLUT-1) in hypoxic conditions. In order to determine whether MSP stimulation of Ron enhances this response, we stimulated peritoneal macrophages with MSP under conditions of normoxia and hypoxia and examined expression of PGK and GLUT-1 by real time qRT-PCR. We observed an increase in the expression of both PGK and GLUT-1 in response to MSP under normoxic conditions, and MSP further augmented the induction of both PGK and GLUT-1 under conditions of hypoxia (Fig A.4A, B). Similarly, we observed an increase in the end product lactate in response to MSP in both normoxic and hypoxic conditions (Figure A.4C). These results suggest that stimulation of the Ron receptor enhances the ability of macrophages to generate ATP via the glycolytic pathway in hypoxic environments.
Figure A.4: PGK and GLUT-1 are strongly induced by MSP in hypoxia. Peritoneal macrophages were cultured in either hypoxia or normoxia for 24 hours with or without MSP. Cells were lysed and RNA was collected for Real Time qRT-PCR analysis. Macrophages stimulated with MSP in normoxia showed mild induction of PGK (A) and GLUT-1 (B), and a greater induction in hypoxia alone. Together, MSP and hypoxia elicit a synergistic response. (C) Furthermore, this resulted in increased levels of lactate production in macrophages indicating that anaerobic metabolism was increased, and that STK was enhancing energy production in the limiting environment of hypoxia. **p<0.01, ***p<0.001 compared with control.
Gene regulation of arginase I, GLUT1, and PGK are uniquely regulated.

In order to partially determine if MSP regulated expression of arginase I, GLUT-1, and PGK occur in the same manner, we analyzed the expression of these genes by real time qRT-PCR over time. As reported in chapter 2, arginase I exhibits a biphasic induction response (Figure A.5A). However, GLUT-1 and PGK did not share this response pattern. GLUT-1 is most potently upregulated at 1 hour post MSP stimulation and tappers off before returning to resting levels at 24 hours (Figure A.5B). In contrast, PGK expression is relatively weak at 1 hour, but progressively increases through 10 hours post MSP stimulation (Figure A.5C). As shown in Figure A.5D, the differential time regulation of these genes by MSP suggest that distinct mechanisms are governing their activation.
Figure A.5: Differential expression of arginase I, GLUT-1, and PGK in normoxia. Peritoneal macrophages were stimulated with MSP for the indicated times and analyzed for arginase I (A), GLUT-1 (B), and PGK (C) expression by Real Time qRT-PCR. (D) Overlay of arginase, PGK, and GLUT-1 expression in primary macrophages. **p<0.01, ***p<0.001 compared with control.
Macrophage migration is enhanced by MSP in normoxia and hypoxia alike.

MSP induces the migration of macrophages\(^1\), keratinocytes\(^99\), and has been implicated in the invasive potential of tumors\(^77\). However, hypoxia has been shown to inhibit the migratory potential of macrophages\(^224, 225\). In order to determine whether MSP retains the ability to promote migration of primary peritoneal macrophages, we utilized a transwell chemotaxis assay. Results from these studies indicate that peritoneal macrophage migration in response to MSP is induced in conditions of both normoxia and hypoxia. While this may in part be due to the induction of cell spreading, chemokinesis alone (MSP added to both the top and bottom chamber of the transwell) does not induce the same level of migration, indicating that MSP is an inducer of chemotaxis (Figure A.6).

![Graph showing macrophage migration](image)

**Figure A.6:** Macrophage migration by MSP stimulation is robust in both normoxia and hypoxia. Corning transwell inserts were used to analyze migration of macrophages to MSP (100 ng/mL) and MCP-1 (10 ng/mL) in normoxia (20% O\(_2\)) or hypoxia (<1% O\(_2\)). After 2 hours, cells were fixed and counted in 3 random high power fields by fluorescent microscopy. ***p<0.001 compared with control.
References


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Education

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Publications


Presentations

2003 The expression of the STK receptor tyrosine kinase in primary peritoneal macrophages is induced by hypoxia and the STK/MSP signaling pathway cooperates with hypoxia to regulate the expression of VEGF. Poster presentation at the American Association of Immunologists FASEB conference. Denver, CO, USA.

2004 The stem cell-derived tyrosine kinase receptor (STK) regulates the response of macrophages to hypoxic conditions. Poster and oral presentation at the American Association of Immunologists FASEB conference. Washington, DC, USA.

2006 Tumor macrophages expressing the stem cell-derived tyrosine kinase receptor regulate tumor growth. Poster presentation at the Society of Leukocyte Biology, Innate Immunity conference. San Antonio, TX, USA.