REGULATION OF MACROPHAGE HETEROGENEITY AND DIET-INDUCED METABOLIC DISEASES BY THE RECEPTOR TYROSINE KINASE RON

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by
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ABSTRACT

Tissue resident macrophages present in virtually all tissues are important immune cells that regulate the progression of many diseases. Classically activated macrophages (M1) elicit immune responses and eliminate pathogens, while excessive products generated in this inflammatory process cause host tissue damage and potentiate the progression of chronic inflammatory diseases. Alternatively activated macrophages (M2) attenuate inflammation and produce mediators that are involved in tissue regeneration. Our previous results showed that Ron receptor tyrosine kinase tilts the balance of macrophage activation towards an M2 phenotype and away from an M1 phenotype. In acute inflammatory models, absence of Ron results in impaired inflammatory regulation due to M1 and M2 imbalance and lead to an accelerated disease progression. However, the mechanism through which Ron inhibiting pro-inflammatory M1 activation is not well understood. Here, we demonstrated that Ron and its ligand, macrophage stimulating protein (MSP), regulated LPS-induced inflammatory signals through inhibiting activation of IKK, retarding IkBα degradation and reducing the DNA binding of NF-κB in response to LPS in primary macrophages. In addition, MSP inhibited the NF-κB-dependent upregulation of IkBζ, which inhibits transactivation of p65 and its DNA binding. However, MSP did not inhibit the MyD88-dependent TAK1 and MAPK activation or the MyD88-independent activation of IFNβ production in response to LPS. Altogether, we revealed the mechanism of Ron inhibiting M1 macrophage activation in primary macrophages.

We also investigated the ability of Ron to regulate macrophage activation in a diet-induced chronic systemic inflammation model, the disease phenotypes of which include atherosclerosis, fatty liver disease and obesity. We showed that the expression of Ron in adipose tissue macrophages, aortic macrophages, and fatty liver Kupffer cells was tightly associated with an M2 phenotype. Ron expression in these tissue resident macrophages was reduced with the
progression of these chronic diseases. Additionally, Ron deficient mice exhibited enhanced severity of atherosclerosis and liver injury with more lipid accumulation when challenged with a high cholesterol diet. This phenotype was associated with elevated expression of inflammatory markers in atherosclerotic aortas and fatty livers. Our results demonstrated that Ron is important in regulating macrophage phenotype in diet-induced inflammation, and thus attenuates the progression of diet-induced atherosclerosis, fatty liver disease and obesity.

Taken together, the results presented in this thesis indicated that Ron balances macrophage activation by inhibiting M1 macrophage activation while promoting an M2 macrophage phenotype, and plays a protective role in the progression of diet-induced metabolic inflammation. This work provides evidence that Ron might be used as an M2 macrophage marker in certain diet-induced chronic inflammatory diseases. Ron and its signaling components are potential therapeutic targets for treating these diet-induced metabolic diseases.
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<tbody>
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<td>AIDS</td>
<td>Acquired immunodeficiency syndrome</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
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<tr>
<td>AP</td>
<td>Activator protein</td>
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<tr>
<td>ApoE</td>
<td>Apolipoprotein E</td>
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<tr>
<td>ARG1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>BCL</td>
<td>B-cell lymphoma</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>DKO</td>
<td>Double knockout</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalitis</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>Erk</td>
<td>Extracellular signal related kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>Gab</td>
<td>Grb2 associated binding protein</td>
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<td>Grb</td>
<td>Growth factor receptor bound protein</td>
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<td>HCD</td>
<td>High cholesterol diet</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HFD</td>
<td>High fat diet</td>
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<td>Hepatocyte growth factor</td>
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<td>HGFL</td>
<td>Hepatocyte growth factor ligand</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional animal care and use committee</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of kappa B</td>
</tr>
<tr>
<td>IκB₅</td>
<td>Inhibitor of kappa B, zeta</td>
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<tr>
<td>IKK</td>
<td>Inhibitor of kappa B kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MAL</td>
<td>MyD88 adaptor like</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
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<tr>
<td>MCM</td>
<td>Macrophage-conditioned media</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>MSP</td>
<td>Macrophage stimulating protein</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MyD</td>
<td>Myeloid differentiation</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized low-density lipoprotein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PMAC</td>
<td>Peritoneal macrophage</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPRE</td>
<td>PPAR response element</td>
</tr>
<tr>
<td>RD</td>
<td>Regular chow diet</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Ron</td>
<td>Receptor d’origine nantais</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
</tr>
<tr>
<td>SHP</td>
<td>Small heterodimer partner</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SR</td>
<td>Scavenger receptor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SVC</td>
<td>Stromal vascular cells</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal vascular fraction</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β activated kinase</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor associated macrophage</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>T helper</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF related adaptor molecule</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor associated factor</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR domain containing adaptor protein inducing interferon β</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

Looking back at the time that I have spent in Dr. Pamela Hankey’s lab, I cannot believe how fast time flies. When I first started, I knew little about immunology and macrophages. I am very thankful to Dr. Hankey for the opportunity to learn and grow in her lab, and for the continuous support and encouragement all these years. I have felt that I might be the luckiest graduate student and I could not have imagined having a better advisor for my PhD study.

When I finished writing this thesis, I could hardly believe how much I have learned through these years at Penn State. I have received so much help and support from people that I met here, to whom I owe a tremendous amount of gratitude. I would like to thank my committee members for providing constructive advice on my project. I would like to thank everyone in my lab, as well as fellow students, faculty and staff members in the Veterinary and Biomedical Sciences department for their help in a daily basis. I would like to thank people in the physiology program and the Huck Institute office for support and advice on my academic and personal issues. I would like to thank people in Penn State’s core facilities for all their assistance with my project. I would like to thank all my friends who were there with me when I needed them. I am not listing particular names, because if I do, it will take up half of this thesis. However, you all know who you are, and you are all appreciated for everything that you’ve done to support my growth throughout these years while I was in the United States. Lastly and most importantly, I would like to thank my family in China, especially my mom and my grandmother for all their love and support.
Chapter 1

Literature Review: Ron regulates homeostasis of immune responses in macrophages

Macrophages and disease

Infectious disease is associated with activation of the immune system, which protects against foreign pathogens [1]. Epithelial and mucosal barriers constitute the outer layer of defense, while both the innate and adaptive immune responses comprise the inner defense against pathogens. Acting as a link between the innate and adaptive immune systems, macrophages secrete various cytokines and inflammatory mediators in response to foreign pathogen insult. Macrophages, found in virtually all tissues are differentiated from either hematopoietic stem cells or primitive hematopoietic progenitors in the yolk sac [2] [3]. In the hematopoietic lineage, Ly6C- monocytes which develop from common myeloid progenitor cells are released from the bone marrow into the bloodstream under homeostatic conditions, and further recruited by the chemokine CX3XL1 into tissues including the liver (Kupffer cells), spleen, gastrointestinal tract, bone (osteoclasts), alveoli, adipose tissue, central nervous system (microglial cells) and peritoneum [4]. Alternatively, Ly6C+ monocytes are recruited by chemokine CCL2 into inflamed tissues where they actively clear pathogens and regulate the immune response. More recent studies suggest that tissue-resident macrophages also arise in the yolk sac and migrate into various organs during the early stages of embryonic development. These yolk sac-derived tissue-
resident macrophages are long-lived and regenerative compared to hematopoietic stem cell-derived macrophages[3] [5] [6].

In order to detect invading pathogens, vertebrate genomes encode a variety of pathogen-associated molecular pattern (PAMP) receptors, such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), that recognize a broad spectrum of pathogens through pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs)[7].

Macrophages are not only capable of mounting a localized and intense response to target small highly replicative bacterial pathogens using toxic reactive oxygen and nitrogen species, but can also defend against larger pathogens in an eosinophil-coordinated fashion at mucus barriers in gastrointestinal and respiratory tracts. Although the innate immune response is effective in clearing some offending organisms, activation of the adaptive immune response promotes effector lymphocyte responses and the production of antibodies against specific-molecular epitopes on pathogens [8]. Mediators produced by macrophages, including cytokines, chemokines, and small molecular metabolites play an important role in linking innate immunity with adaptive immunity.

While macrophages play an essential role in fighting infections, excessive inflammatory responses cause energy metabolism dysfunction and host tissue damage. To prevent this scenario, the immune system has evolved to eliminate excessive inflammation by transiting into a resolution phase. This stage of inflammation is an active and intricate process, which keeps the immune response balanced. Macrophages that are involved in cell clearance may help to prepare tissues for the resolution of inflammation and tissue repair. Mediators that are produced locally by macrophages during the resolution phase include lipoxins, resolvins, prostaglandins of the J series, anti-inflammatory cytokines, microRNAs and inhibitors of pattern-recognition receptors. The deletion of genes encoding inhibitors of TLRs and NLRs leads to a state of hyper-
inflammation, supporting the notion that active control of inflammation is important to prevent collateral tissue damage and chronic inflammation[9].

Macrophages in a resting state are activated by a variety of stimuli. PAMPs, such as LPS, peptidoglycans and flagellins or Th1 cytokines, such as IFNγ, TNFα, IL-1β, IL-12, induce macrophage activation through the classical or M1 pathway. Upon classical activation, macrophages exhibit more potent phagocytic activity and increased microbial activity by expressing high levels of inducible nitric oxide synthase (iNOS), an enzyme that oxidizes L-arginine to yield L-citrulline and nitric oxide (NO). They also secrete inflammatory cytokine mediators, such as TNFα, IL-1β, IL-6 and IL-12. Increased expression of MHC class II molecules on these macrophages promotes the presentation of antigen to T helper (Th) cells to enhance the adaptive immune response. The critical role of classically activated macrophages in host defense to intracellular pathogens has become increasingly clear [10] [4] [11]. Mice and humans deficient in IFNγ are more susceptible to various bacterial, protozoal, viral, or parasitic infections. While classical macrophages play a critical role in the killing of intracellular microorganisms, their activation must be tightly controlled because the cytokines and mediators that they produce can lead to host-tissue damage. Indeed, classical macrophages are key mediators of some autoimmune diseases, including multiple sclerosis, rheumatoid arthritis [12] and inflammatory bowel disease [13].

Resting macrophages can also be activated through an alternative or M2 pathway by the Th2 cytokines IL4 or IL13, IL-10 or TGFβ, immune complexes and IL-1β or LPS, or immune suppressing glucocorticoids[14]. These M2 macrophages are less pro-inflammatory and have distinct secretory and functional capacities. These macrophages in tissues or Th2-type inflammatory site can be identified by their distinctive functions, including their enzymatic (Arg1), secretory (Chi3l3, Chi3l4 and Retnla) and phagocytic (Mrc1, Clec7a and Clec10a)
activities. The expression of Arg1 by these macrophages allows them to convert L-arginine into urea and ornithine, which are the precursors for synthesis of polyamines and proline produced by M2 macrophages promote cell proliferation, angiogenesis, tissue repair and regeneration. [16] [17]. M2 macrophages can also cause host damage when their function is dysregulated. Immunopathology and tissue fibrosis occur during chronic schistosomiasis due to the uncontrolled activation of M2 macrophages [18]. M2 macrophages are also present in the

Figure 1-1: Macrophage activation pathways contribute to different aspects of disease progression. Resting macrophages can be activated through a classical activation pathway by various stimuli including LPS, peptidoglycan, flagellin, IFNγ, IL-1β, TNF, IL12, oxLDL or saturated fatty acids to become M1, or through an alternative activation pathway by IL4/IL13, TGFβ/IL10, LPS with immune complexes, or Glucocorticoids to become M2. M1 and M2 macrophages both utilize L-arginine as a substrate to produce NO and amino compounds, respectively. Activated macrophages produce various inflammatory mediators to augment their phenotypes and contribute to disease progression. This figure is adapted from Designer macrophages: Oxidative metabolism fuels inflammation repair. 2006.
lungs of mice with experimental asthma and contribute to disease progression in this model. Additionally, M2 macrophages promote tumor growth by promoting angiogenesis while decreasing tumor immunity (Figure 1-1).

Given that M1 and M2 macrophages preferentially express iNOS and Arg1 respectively and the distinct role of iNOS and Arg1 in the regulation of L-arginine metabolism, the expression of iNOS and Arg1 must be tightly controlled in macrophages during both the initiation and resolution of inflammation. Macrophages activated by different pathways may either prevent disease progression or assist the resolution of inflammation. Enhanced and persistent activation of macrophages, together with other aspects of dysregulated immune functions leads to the development of various chronic inflammatory diseases. Therefore, identifying therapies that promote balanced macrophage activation is an attractive option for the treatment of inflammatory diseases. While M1 and M2 macrophages are thus classified for convenience, these two phenotypes represent two ends of a broad continuum of activated macrophage phenotypes. However, I will use the M1 and M2 nomenclature herein for simplicity.

Pathogenesis of diet-induced metabolic disorders

The obese population has increased dramatically in the past decades around the world. Consequently, obesity and its associated metabolic disorders, including diabetes, atherosclerosis, fatty liver disease, degenerative disorders, airway disease and some cancers [19] are now serious threats to global public health. In the classic literature, the immune response was recognized as a process that is elicited during injury, the features of which include swelling, redness, pain and fever [20]. However, it has been increasingly clear that immune system is also a key regulator in the progression of obesity and its associated metabolic disorders. While injuries are considered acute inflammation and nutrient-induced inflammation is usually referred to as ‘low grade’ or
‘chronic’ inflammation, these two types of inflammation engage a similar set of pathological mediators and signaling pathways. Metabolic signaling is important in efficiently converting nutrients into energy or other sources for storage. However, excessive nutrients not only activate the metabolic pathway, but also cause aberrant immune responses, leading to severe inflammatory diseases. Therefore, a delicate balance between metabolic signaling and inflammatory signaling is required to maintain homeostasis. Given the trend of increased metabolic overload and reduced physical activity observed in most developed countries, the link between excessive intake of nutrients and inflammatory responses needs to be further investigated, and prevention and treatment of diet-induced metabolic disorders could be provided by regulating obesity-induced inflammation[21]. In the following section, I will review the most common inflammatory diseases associated with energy imbalance caused by metabolic overload.

**Obesity and inflammation**

The World Health Organization estimates that more than 1 billion adults worldwide are overweight, 300 million of whom are clinically obese – defined as having a body mass index equal to or greater than 30 kg m$^{-2}$. Obesity associated inflammation has been widely studied in the past decade. The innate immune system plays an important role, not only in defending against pathogen invasion but also in regulating the progression of obesity induced by dysregulated metabolism of nutrients. Adipose tissue used to be thought of as an energy storage compartment lacking immune function. However, the observation that TNF$\alpha$, a pro-inflammatory cytokine that promotes insulin resistance was detected in adipose tissue of obese mice, provided the first clear link between obesity, diabetes and chronic inflammation[22]. In addition to TNF$\alpha$, adipocytes can also induce expression and secretion of several acute phase reactants and mediators of inflammation, including IL-1, IL-6, IL-8, IL-10, IL-15, leukemia inhibitory factor, hepatocyte
growth factor, SAA3, macrophage migration inhibitory factor, prostaglandin E2, and potential inflammatory modulators such as leptin, adiponectin and resistin [23]. These pro-inflammatory mediators provide cues for infiltration and activation of immune cells, which contribute to inflammation in adipose tissue and promote insulin resistance [24].

Among the infiltrating cells found in enlarging adipose tissue, macrophages are dominant. In lean mice, only 10% to 15% of cells are F4/80 expressing macrophages, while 45% to 60% of F4/80+ macrophages are found in adipose tissue of obese mice [25]. In addition to a difference in their numbers, macrophages in lean and obese animals display distinct functional characteristics in regulating inflammation. Adipose tissue macrophages in lean animals exhibit an M2 phenotype (Arg1+ CD206+ CD301+ CD11c-), and are dispersed throughout the adipose tissue, whereas adipose tissue macrophages in obese animals predominantly exhibit an M1 phenotype (NOS2+ TNF+ CD11c+)[26] [27], which phagocytose dead adipocytes and become multinucleated giant cells, forming a ‘crown-like’ structure. M2-like adipose tissue macrophages release anti-inflammatory cytokines, such as IL-10, and are important in maintaining adipose tissue insulin sensitivity, whereas M1 adipose tissue macrophages secrete pro-inflammatory cytokines that attenuates insulin action in adipocytes, resulting in increased lipolysis and free fatty acid release [28] [29], which exert an enhanced effect on inflammation.

There is currently an avalanche of evidence suggesting that M1 macrophages contribute to the pathogenesis of obesity-induced inflammation, whereas M2 macrophages attenuate the inflammatory milieu. Depletion of pro-inflammatory CD11c+ M1 macrophages in adipose tissue in CD11c-DTR mice reduces adipose tissue inflammation and improves insulin sensitivity without any significant impact on diet-induced obesity [30]. Furthermore, genetic manipulation or chemical inhibition of NF-κB dependent pro-inflammatory signals is effective in protecting mice from diet induced obesity and insulin resistance. For instance, myeloid cell specific genetic
deletion of the pro-inflammatory signaling protein IKKβ[31] or reconstitution of mice with JNK-deficient bone marrow cells reduces myeloid cell-mediated inflammation in adipose tissue[32]. Chemical inhibition or genetic ablation of IKKe, another pro-inflammatory protein elevated during NF-κB activation was protective against diet-induced obesity and insulin resistance[33][34]. Additionally, mice deficient of eosinophils, primary sources of IL-4, which mediates M2 macrophage activation, gained more weight on a high fat diet and exhibited a worsened metabolic profile when compared with control mice[35]. Similarly, myeloid cell specific disruption of PPARγ impaired M2 activation both in vitro and in vivo, and reduced the number of Arg1 expressing macrophages in adipose tissue of lean mice. Subsequently, these PPARγ deficient mice developed more severe obesity and inflammation when challenged with a high fat diet[36]. These studies provide evidence that M1 macrophages infiltrate adipose tissue and promote diet-induced obesity and inflammation by secreting pro-inflammatory cytokines, while M2 macrophages limit inflammation and enhance the anabolic action of insulin.

**Atherosclerosis and inflammation**

Atherosclerosis, the primary cause of coronary artery disease, stroke and peripheral vascular disease, is one of the complications associated with diet-induced obesity. According to the National Institutes of Health, cardiovascular diseases, including atherosclerosis, are responsible for 30% of deaths worldwide, exceeding all others including cancer. For many years, atherosclerosis was thought to be a passive process of accumulation of lipids in the arterial wall. Currently, atherosclerosis is understood to result from chronic inflammation driven by lipids, particularly oxLDL, and immune cells, including T cells, monocytes and macrophages. In response to intimal lipid accumulation, disturbed blood flow, low shear stress, and other stimuli,
monocytes passage across the endothelium under the guidance of chemotactic cues. These infiltrated monocyte-derived macrophages recognize and ingest lipids that have accumulated in the intima. In the early stages of disease, these lipid-laden macrophages, also known as foam cells, are effectively removed by efferocytosis, a process that is helpful in eliminating inflammation. However, accelerated disease progression leads to defective efferocytosis, and subsequent accumulation of macrophages and other apoptotic cells in the lesion. This failure in efferocytosis contributes to secondary necrosis, which enhances the inflammatory response and leads to the accumulation of cell debris and lipids in the necrotic core, eventually developing into a vulnerable plaque [37].

As important immune regulators, macrophages rely on various strategies to sense, internalize, and process the diverse lipid components they encounter. Pattern recognition receptors (PRRs) expressed on the plasma membrane recognize various native and oxidized lipoproteins and facilitate their uptake for lysosomal degradation. For example, SR-A and CD36, responsible for recognizing and ingesting lipids, are indispensable for lipid-laden macrophage foam-cell formation[38]. Cytoplasmic sensors such as the NLRP3 inflammasomes respond to cholesterol crystals and fuel inflammation by promoting the release of the pro-inflammatory cytokine IL-1β[39]. TLRs are well-characterized PRRs that respond to specific pathogen-associated molecules or endogenous ligands, initiating signaling cascades that control the activation of transcription factors and initiation of pro-inflammatory cytokine transcription. TLR4 is the most conserved TLR across species. It regulates immune responses by activating NF-κB, the central master transcriptional regulator of inflammatory genes.

Upon ligand binding, TLR4 recruits adaptor proteins to the membrane and initiates activation of NF-κB via a MyD88 dependent pathway. In the resting state, NF-κB is sequestered in the cytoplasm by binding to IκB. Upon activation of TLR4, IKappaB Kinase (IKK)
phosphorylates IκB, leading to its ubiquitination and subsequent degradation. Free NF-κB moves into the nucleus and activates the transcription of genes encoding inflammatory molecules. Recently, a linkage between TLRs and the endogenous ligand oxLDL, which induces inflammation and causes atherosclerosis in the aorta was revealed. TLR4 and TLR6 heterodimerization promotes a six-fold increase in NF-κB activation following exposure to oxLDL compared with cells expressing these receptors individually. [40] Defective TLR signaling reduces the development of atherosclerosis in individuals who suffer from hypercholesterolaemia[41, 42]. TLR4/ApoE double knockout mice develop less severe atherosclerosis compared to ApoE knockout mice, and plaques from both TLR4/ApoE and MyD88/ApoE double knockout mice have reduced lipid content. NF-κB inhibition also results in decreased atherosclerosis, which was observed in mouse models employing either NEMO ablation to interfere with IKK activation or transgenic expression of degradation-resistant DNIκB to block nuclear translocation of NF-κB. Endothelial cell-specific NF-κB inhibition results in strongly reduced atherosclerotic plaque formation in ApoE-/- mice maintained on a high cholesterol diet. These observations provide evidence suggesting atherosclerosis is an inflammatory process that is tightly regulated by the TLR4 - NF-κB axis, which is a potential therapeutic target.

Both classically and alternatively activated macrophages have been identified in the atherosclerotic lesions. M2 macrophages predominate in non-inflamed aortas or early aortic lesions, while M1 macrophages are associated with the later stages of disease. In the advanced stage, M1 macrophages are found in the necrotic core within a plaque, whereas M2 macrophages are present in the shoulders of the plaque and produce components of the extracellular matrix, which promotes plaque stability. The plasticity of macrophages within the lesion is highly correlated with atherosclerosis progression. Apoptotic macrophages abundant in the atheroma can
produce proteolytic enzymes, such as MMPs, capable of degrading the collagen that lends strength to the plaques’s protective fibrous cap, resulting in cap thinning, weakening, and susceptibility to rupture. Inhibition of MMP activity by adenovirus-mediated delivery of the gene encoding human tissue inhibitor of metalloproteinases (TIMP)-1 reduced lesion size in the aortic root of ApoE knockout mice. However, MMP family members exhibit diverse roles in atherosclerotic plaque stability. For example, both MMP3 and MMP9 limit plaque size and protect the stability of plaques, while MMP12 reduces smooth muscle cells and increases macrophage content in the plaque, resulting in a less stable plaque[43]. Inflammatory mediators regulate tissue remodeling by macrophages, demonstrating an essential link between inflammation and progression of atherosclerosis and thrombosis [44].

Overall, aortic macrophages with various phenotypes are important in regulating the progression of atherosclerosis. Given the high morbidity and mortality rate caused by cardiovascular diseases worldwide, therapies need to be developed by addressing the following aspects of these diseases: attenuating inflammation, stabilizing plaques and eventually improving resolution of inflammation.

Non-alcoholic fatty liver disease (NAFLD) and inflammation

NAFLD has been one of the most frequent causes of chronic liver disease worldwide in the past two decades[45]. An estimated 20-30% of adults and 3-10% of children in the Western countries are thought to be affected by NAFLD[46, 47]. NAFLD is caused by the accumulation of fat in liver cells and is associated with varying degrees of inflammation. It includes a wide spectrum of liver diseases ranging from fatty liver (steatosis), to non-alcoholic steatohepatitis, to cirrhosis, which is a stage characterized by irreversible and advanced scarring of the liver. In the early stages of NAFLD, the accumulation of fat in the liver is a benign condition, which does not
cause significant liver damage. The next stage, steatohepatitis, involves the accumulation of fat as well as inflammation in the liver, where tissue resident macrophages, Kupffer cells, produce a variety of cytokines, which activate collagen-producing cells, called stellate cells, and damage hepatocytes. These injured hepatocytes release aspartate aminotransferase (AST) and alanine aminotransferase (ALT) into the circulation, which are commonly used as markers for liver injury. Deposition of collagen and extracellular matrix (ECM) proteins causes liver fibrosis in advanced HAFLD stages. The accumulation of ECM proteins disrupts the hepatic architecture by forming a fibrous scar, and the subsequent development of regenerating hepatocytes defines cirrhosis. Cirrhosis is characterized by increased intrahepatic blood flow resistance and hepatocellular dysfunction[48], resulting in severe liver damage.

Kupffer cells, liver resident macrophages, can also be designated as either classically or alternatively activated macrophages. Unlike adipose tissue and aortic macrophages, Kupffer cells are not recruited by circulating macrophage infiltration upon inflammation; instead, they are present in the liver throughout their development. In lean individuals, these Kupffer cells display a mostly M2 phenotype, which protects the liver from inflammatory damage. However, adipokines and cytokines produced by adipose tissue responding to the dysregulated lipid metabolism during obesity induces the switch of these Kupffer cells from an M2 phenotype to an M1 pro-inflammatory phenotype. This phenotypic change in Kupffer cells results in hepatic de novo lipogenesis which causes steatosis.

There is some evidence that the presence of pro-inflammatory M1-like Kupffer cells potentiates liver disease progression, while attenuating this phenotype or a switch to an M2 phenotype alleviates disease progression. As a central master regulator of M1 macrophage activation, NF-κB promotes the expression of pro-inflammatory mediators, including IL-6, TNFα and IL-1β, the levels of which were found to directly correlate with increased severity of NAFLD
in human patients. Genetic ablation of IKKβ in the myeloid cells reduced Kupffer cell-mediated inflammation, and improved systemic and hepatic insulin resistance [31]. Conversely, LPS-induced pro-inflammatory Kupffer cell activation leads to hepatotoxicity in obese mice[49]. Whereas M1 Kupffer cell activation promotes hepatic inflammation, complete genetic ablation of PPARδ, a transcription factor that directs M2 activation, or reconstitution of mice with PPARδ null bone marrow cells, resulted in hepatic dysfunction and insulin resistance in obese mice[50].

Unlike the increasingly clear role of alternatively activated adipose tissue macrophages and aortic macrophages, the metabolic function of M2 Kupffer cells still remains largely unknown. In order to further assess the therapeutic potential of these Kupffer cells, the molecular mechanism of Kupffer cell activation needs to be further delineated.

**Ron and its ligand MSP mediated macrophage immune responses**

**Ron and its ligand MSP**

Ron, a member of the hepatocyte growth factor (HGF)/MET family of receptor tyrosine kinases (RTKs), plays a vital role in transducing signals from the extracellular environment into the intracellular compartment, and triggers biological responses, such as proliferation, migration, invasion and angiogenesis. Ron, also referred to as macrophage stimulating 1-receptor (MST1R), is synthesized as a 185 kDa precursor glycosylated protein and is further processed by furin-like proteases before being delivered to the cell surface as a mature protein. On the cell surface, it is composed of a disulfide-linked extracellular α chain (35 kDa) and a transmembrane β chain (150 kDa), which contains the kinase domain and critical signaling residues[51]. According to the latest NCBI gene analysis, homologs of Ron have been identified by sequence analysis in 29 mammalian and nonmammalian species. Ron was first identified by screening a library prepared
from a mixture of human tumors. The full-length Ron cDNA was subsequently identified in a human foreskin keratinocyte library[52]. The mouse Ron, first cloned from hematopoietic stem cells, is also referred to as stem cell derived tyrosine kinase (STK) [53]. Human and mouse Ron cDNAs share about 74% identity overall, with about 88% identity in the intracellular domains[53]. The human Ron mRNA is composed of 20 exons, while mouse Ron consists of 19 exons. The altered splicing of the mouse Ron gene generates a deletion in the juxtamembrane region compared to human Ron [54].

Ron signaling is activated upon binding to its ligand, macrophage stimulating protein (MSP) [55] [51] [56]. MSP was first cloned from a human genomic library by screening for the characteristic kringle domains present in proteins of the blood coagulation system [57]. MSP, also known as hepatocyte growth factor-like (HGFL) protein, is primarily produced in the liver as pro-MSP, with low levels detected in lung, adrenal gland, and placenta. Pro-MSP is secreted as an inactive form of 80 kDa at a concentration around 400 ng/ml, and is transported in an endocrine manner to distant sites of inflammation, where it is activated upon cleavage by the serine protease family including coagulation enzymes (kallikrein, factor XIIa, and factor Xla) and by nerve growth factor-γ (NGFγ) and epidermal growth factor-binding protein (EGF-BP). Membrane bound proteases produced by macrophages have pro-MSP proteolytic activity, such that both activation and degradation of pro-MSP occurs at the cell surface [58]. The inhibitor of the MSP degrading enzyme α 1-antichymotrypsin, associated with poor prognosis of patients of breast adenocarcinoma, is overexpressed in human breast tumors, which might allow for the increased activation of MSP[59]. Upon cleavage, MSP is composed of a disulfide linked heterodimer including an α-chain (50 kDa) and a β-chain (35 kDa). The α-chain of MSP contains four kringle domains, while the β-chain contains a serine protease-like domain[57, 60]. The two chains have
distinct functions: the α-chain is important for regulating the functional activities of Ron, while the β-chain regulates the its binding to the receptor[61][62].

The stimulation of Ron by MSP induces the phosphorylation of two tyrosines in the activation loop, leading to the upregulation of the kinase activity and the recruitment of downstream signaling molecules to the two tyrosine docking sites in the c-terminal tail [63][64].

**Ron/MSP mediated macrophage activation**

Macrophages exert diverse effects on surrounding cells and tissues, depending on the signals present in the microenvironment. As discussed earlier, macrophages activated through either a classical or an alternative pathway display distinct functions in various diseases. Ron, expressed on tissue resident macrophages, including resident peritoneal macrophages, alveolar macrophages, osteoclasts, Kupffer cells, mesangial cells and microglial cells, regulates the balance of macrophage activation[65]. Our previous studies demonstrated that Ron negatively regulates inflammatory responses by inhibiting cytokine expression. IL-12 produced by M1 macrophages induces IFNγ production by NK and γδT cells, and promotes Th1 differentiation. The IFNγ produced by these cells then feeds back to enhance M1 macrophage activation[66]. Ron inhibits not only the production of IFNγ by inhibiting IL-12 expression in macrophages, but also inhibits the subsequent response of macrophages to IFNγ by suppressing IFNγ-induced STAT1 phosphorylation [67]. NF-κB, an important mediator for M1 macrophage activation, regulates inflammatory responses through activating genes that encode pro-inflammatory cytokines, chemokines and inducible enzymes, such as COX2 and iNOS. Ron inhibits NF-κB activation and production of pro-inflammatory cytokines in response to LPS, by reducing the activity of the upstream kinase, IkB kinase (IKK) [68]. Conversely, Ron upregulates the expression of arginase
I, scavenger receptor (SR-A) and IL-1ra in primary macrophages, and promotes the M2 phenotype [69]. Ron/MSP signaling induces AP-1 binding to a novel site in the arginase promoter, and enhances STAT6 phosphorylation and binding to its promoter [70]. We will present evidence in next chapter that suggests Ron tips the balance of macrophage activation away from the M1 phenotype and promotes the M2 phenotype.

**Mouse models used for studying Ron function**

In order to study the role of Ron in regulating biological and physiological responses in vivo, several mouse models with defects in Ron have been described. The first mouse model of Ron deficiency was produced by replacing a 850bp region within the first translated exon of the Ron gene with a LacZ gene containing its own ATG under the transcriptional control of the Ron promoter. This insertion of LacZ gene permits visualization of Ron expression, while the translation and function of Ron is abrogated, such that its ligand MSP is incapable of binding to the Ron receptor. These homozygous mutant mice are viable and phenotypically normal, while exhibiting enhanced response to inflammation when challenged with endotoxin by producing excessive nitric oxide [71], which demonstrates that Ron plays a role in regulating immune responses. All of the studies on loss of function of Ron performed in this dissertation used this mouse model.

Another mouse model of a total loss of Ron protein function was produced by a global deletion of exon 1-14 of the mouse Ron gene. This knock-out strategy completely deleted a large genomic region of Ron containing Ron 5’-flanking sequences, the extracellular domain, the transmembrane domain, and some intracellular domain. This deletion of Ron in mice was embryonic lethal [72]. However, mice that were hemizygous for this deletion of Ron were viable
and fertile, and also displayed a phenotype of increased susceptibility to endotoxic shock and enhanced responses to inflammation.

A mouse model was produced by the preserving extracellular and transmembrane domains of Ron, along with eight amino acids of the intracellular domain, while deleting the remainder of the cytoplasmic domain of Ron. In this mouse model, the intracellular signaling of Ron is completely abrogated. Homozygous mice with this germline deletion are viable, fertile and display normal biological phenotype. Nevertheless, this deletion of Ron in mice also shows an enhanced responses to both acute and cell-mediated inflammatory stimuli [73]. Overall, studies in all three mutant mouse models suggest that Ron is an important negative regulator in inflammatory responses.

**Ron/MSP activation mediated immunity in diseases**

Immune and inflammatory responses are tightly regulated to maintain a homeostatic balance between an effective immune response and tissue damage to the host. It has been increasingly clear that Ron signaling plays an important role in tipping the balance of macrophage inflammatory response in various disease models of septic shock, delayed type hyper-sensitivity, experimental autoimmune encephalitis (EAE), acute liver injury, acute lung injury, central nervous system (CNS) inflammation and tumorigenesis (Figure 1-2).

**Acute immune responses**

The observation that increased mortality rate of Ron deficient mice when challenged with endotoxin drew attention to the immune regulatory function of this receptor tyrosine kinase[71]. The regulation of iNOS is critical in the pathogenesis of septic shock, which is a systemic
inflammatory response to bacterial-derived endotoxin. This decreased survival rate of Ron deficient mice was due to failed suppression of endotoxin-induced iNOS expression and subsequent NO production, resulting in immune response-induced tissue damage[66, 73].

Subsequently, the role of Ron expressed by liver and lung tissue resident macrophages in regulating acute inflammation was revealed. A study using LPS to induce acute liver inflammation showed that Ron plays a role in both Kupffer cells and endothelial cells in the liver.
to suppress pro-inflammatory protein expression [74]. Another study used acetaminophen to induce liver injury [75], while the third group used a combination of hepatocyte-specific transcriptional inhibitor galactosamine (GalN) and LPS [76]. Both studies corroborated the first finding that loss of Ron on Kupffer cells exacerbates toxin-induced liver inflammation by increasing expression of cytokines and chemokines, such as TNFα, IL-1β, IL-12, MCP-1, CCR2, while reducing expression of IL-10. In addition, toxin-challenged Kupffer cells deficient in Ron are more detrimental to hepatocytes by inducing hepatocyte cell death. Likewise, Ron deficient mice displayed enhanced immune responses to nickel-induced acute lung injury, by expressing increased levels of IL-6, MCP-1 and MIP-2 [77]. Overall, these experiments provide evidence that Ron plays an essential role in regulating the progression of acute inflammation by alleviating expression of pro-inflammatory mediators.

**Tumorigenesis:**

RTKs, including Ron, are involved in the initiation and progression of variety of human malignancies. In addition to its ability to promote cell migration, Ron is highly expressed in metastatic tumors and plays a critical role in metastatic tumor development. Expression of Ron is markedly elevated in a large number of epithelial cancers derived from breast (56%), colon (51%), lung (48%), thyroid (42%), skin (37%), bladder (36%) [78], pancreas (33%) [79], gastroesophagus (74%) [80], and Burkitt’s lymphoma [81]. In addition, constitutively active splice variants of Ron have been isolated from human colorectal carcinoma cells [82]. A dramatic increase in Ron expression and constitutive phosphorylation of Ron is associated with tumor malignancy, while normal epithelial cells and benign lesions, including adenomas and papillomas, express comparatively low levels of Ron [83]. Overexpression of the Ron ligand, MSP, in breast cancer cells promotes aggressive disease with poor prognosis [84]. Current
therapeutic strategies to target Ron include Ron blocking proteins, small interfering RNAs, monoclonal antibodies [85], and small molecule inhibitors[86].

A malignant tumor is comprised of cancer cells and the components of tumor microenvironment. Tumor-associated macrophages (TAMs), which are abundantly present in malignant mouse and human tumors, generally exhibit hallmarks of M2-like phenotype and enhance tumor progression by decreasing tumor immunity while promoting tumor invasion, migration and angiogenesis[87, 88]. In turn, tumor cells produce chemokines, and polarizing cytokines to escape macrophage phagocytosis and promote an M2-like polarization. These M2-like TAMs express higher levels of Arg1 and Tgfb1, but lower levels of M1 genes, such as Inos and Il6. Selective depletion of these M2-like TAMs results in inhibition of both angiogenesis and tumor growth[89]. Thus, targeting the tumor-promoting activity of TAMs, including the expression of Arg1, has important therapeutic implications.

While the role of RTKs, including Ron, in the promotion of tumor cell growth and metastasis is well characterized, the potential role of Ron expressed by macrophages in the tumor stromal microenvironment has been recently revealed. Our result shows that Ron induces the M2 macrophage marker Arg1 expression both in vitro in primary macrophages and in vivo in TAMs, and that Ron promotes tumor growth and suppresses T cell-mediated tumor immunity [70]. The significance of myeloid-specific expression of Ron in tumorigenesis was recently confirmed by studies, in which loss of Ron in myeloid cells is sufficient to inhibit prostate cancer cell growth, at least in part, by suppressing the activity of CD8+ T cells in the tumor microenvironment[90]. Taken together, these results provide evidence that Ron is a promising target for therapeutic intervention against malignant tumors.
**Chronic HIV and CNS inflammatory diseases:**

A number of studies show that Ron, expressed in microglial cells in both human and mouse, regulates neuroinflammation of central nervous system (CNS). AIDS patients with CNS diseases and multiple sclerosis (MS) patients exhibit lower levels of Ron protein expression in the CNS compared to control patients. In an MS animal model or experimental autoimmune encephalomyelitis (EAE), Ron deficient mice displayed rapid onset, increased severity, and enhanced inflammation after EAE induction associated with increased expression of TNFα and MMP12 in the CNS [91]. These findings suggest that Ron might play a role in eliminating inflammation and progression of these chronic CNS diseases.

In the early stage of HIV infection, tissue resident macrophages are productively infected by HIV-1 and become long-term virus reservoirs because of their resistance to the cytopathic effects of HIV. HIV decreased Ron expression levels in the CNS through the HIV-1 Tat trans activation domain, in a manner which is dependent on the integrity of the Ron receptor. A decrease in Ron expression is advantageous for viral replication and leads to dysregulation of the cytokine network and compromised innate immunity, increasing the susceptibility to opportunistic infection and facilitating disease progression [92]. Conversely, overexpressing Ron in monocytes and macrophages reduces the binding activity of NF-κB to the HIV-1 long terminal repeat, which is important in HIV-1 proviral transcription and replication[93]. In addition, Ron limits HIV-induced activation of macrophages and microglia cells and production of inflammatory mediators by reducing RNA polymerase II processivity[94], which is essential for HIV replication. These findings suggest Ron not only inhibits the HIV replication, but also attenuates HIV-induced chronic CNS inflammatory diseases.

Despite the correlation between Ron expression and elevated inflammation in CNS of both MS human patients and EAE mouse model, the role of Ron in regulating polarization of
microglial cells has not been investigated. The mechanism of macrophage Ron expression in regulating CNS inflammatory diseases needs to be further delineated and established.

**Diet-induced metabolic diseases:**

While an anti-inflammatory function of Ron has been firmly established, nothing is known about the role of Ron-mediated macrophage activation in the regulation of inflammation associated with diet-induced metabolic diseases. The evolution of these diet-induced metabolic diseases is associated with cell apoptosis and necrosis due to ER stress and inflammasome activation. Tissue resident macrophage apoptosis and defective clearance of these apoptotic cells leads to necrosis-induced inflammation[37] [24] [26]. Ron inhibits cell apoptosis in cancer cells and in LPS-stimulated macrophages. [95] [96] [97]. Thus, in additional to its role in direct regulation in inflammation, Ron could also retard inflammatory stimuli-induced cell apoptosis in atherosclerotic lesions. Furthermore, Ron promotes macrophage phagocytosis by enhancing expression of Scavenger receptor –A (SR-A) and has been shown to increase phagocytosis of C3bi-coated erythrocytes [69] [98]. Therefore, Ron could also participate in phagocytosis of apoptotic cells in the atherosclerotic lesions, preventing secondary necrosis. Macrophages defective in MER, a receptor tyrosine kinase closely related to Ron, exhibit impaired clearance of apoptotic thymocytes [99]. Atherosclerosis studies using the ApoE KO mouse model demonstrated that MER facilitates the clearance of apoptotic cells in the atherosclerotic lesions, resulting in decreased severity of atherosclerosis [100].

In this work, we test the hypothesis that Ron attenuates **diet-induced metabolic diseases by reducing pro-inflammatory immune responses and promoting anti-inflammatory signals and tissue regeneration** (Figure 1-3). Results from this study will
determine the role of Ron in regulating the progression of these metabolic diseases and whether Ron is a potential therapeutic target for the treatment of these diseases.

Figure 1-3: Hypothesis: Ron attenuates metabolic disorders by eliminating M1 macrophages while promoting an M2 phenotype. Predominant existence of M1 macrophages in tissues contributes to the progression of metabolic disorders. Expression of the Ron receptor tyrosine kinase on these tissue resident macrophages tilts the balance of macrophage activation towards an M2 phenotype, which alleviates the disease progression.
Chapter 2

Inhibition of TLR4-mediated inflammation by the Ron receptor tyrosine kinase and its ligand Macrophage-Stimulating Protein

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Abstract

The Ron receptor tyrosine kinase regulates the balance between classical (M1) and alternative (M2) macrophage activation. MSP, the ligand for the Ron receptor, inhibits the expression of the pro-inflammatory genes Il12b, Inos, and Tnf, while promoting the expression of arginase I, a hallmark of alternatively activated macrophages. Ron deficient mice express increased levels of IL-12 following LPS challenge, resulting in increased production of IFNγ and rendering them more susceptible to septic shock. In this study, we examine the mechanism by which Ron inhibits LPS-induced inflammatory signals in primary macrophages. We show that MSP significantly reduces LPS-dependent IL-12p40 production through activation of the Ron receptor. We further demonstrated that MSP reduces activation of IKK, delays IκBα degradation and inhibits the DNA binding of NF-κB in response to LPS in primary macrophages. In addition, MSP inhibits the NF-κB-dependent upregulation of IκBζ, a positive regulator of IL-12p40. However, MSP does not inhibit the IFNβ in response to LPS, nor does it inhibit TAK1 or MAPK activation. These results indicate that Ron does not inhibit receptor proximal TLR4 signals, but rather targets the activation of IKK and NF-κB. Therefore, Ron could be targeted for the treatment of a number of chronic inflammatory diseases.

Introduction

Macrophages are a heterogeneous population of cells that exhibit a wide range of functions depending on their physical location, as well as external cues received from the tissue microenvironment. M1 macrophages produce a variety of inflammatory mediators, including cytokines and iNOS to convert L-arginine to NO, which plays an important role in pathogen killing and host defense. M2 macrophages utilize arginase I to convert the same substrate L-
arginine to urea and ornithine, which promotes tissue regeneration and wound healing. An imbalance in M1 and M2 macrophage populations could potentiate host tissue damage and potentiate the progression of a number of chronic inflammatory diseases. Therefore, macrophage activation in immune responses must be tightly regulated.

It has become increasingly clear that receptor tyrosine kinases play a critical role in the regulation of macrophage activation [101][102]. The Ron receptor tyrosine kinase is expressed by tissue-resident macrophages and inhibits classical macrophage activation while promoting hallmarks of alternative macrophage activation. Macrophage stimulating protein (MSP), the ligand for the Ron receptor, regulates L-arginine metabolism in macrophages by inhibiting the expression of iNOS while promoting the expression of Arg1 [66, 69, 103]. Furthermore, mice with a targeted deletion in the gene encoding Ron display enhanced inflammation and tissue damage in models of Th1 mediated diseases, such as septic shock, EAE, acute liver injury and acute lung injury[71, 73, 74, 91, 104]. Conversely, mice with both a global deletion and macrophage-specific deletion of Ron exhibit reduced tumor growth [70][90]. Thus, understanding the mechanism by which the Ron receptor promotes these responses is of potential clinical significance.

Previous results from our laboratory have demonstrated that Ron promotes the M2 phenotype in primary macrophages, as exemplified by the upregulation of Arg1, scavenger receptor, and IL-1 antagonist expression[69]. Ron induces Arg1 expression through an AP-1 binding site in the Arg1 promoter, and increases Arg1 activity in the primary macrophages. In vivo, Ron induces Arg1 expression in tumor associated macrophages and promotes tumor growth[70]. Ron also augments the phagocytic capacity of macrophages by increasing scavenger receptor expression and inducing phagocytosis of opsonized erythrocytes [98].
In addition to its role in promoting the M2 macrophage phenotype, we have shown previously that Ron is a critical regulator of IL-12 production by macrophages both in vitro and in vivo[67, 105]. IL-12, a product of classically activated macrophages, plays a central role in promoting both the innate and acquired immune response to infection[106]. In the early stages of infection, IL-12 produced by macrophages induces the expression of IFNγ by NK cells, γδ T cells, and innate CD8+ T cells. At later stages, IL-12 promotes the differentiation of Th1 cells, which produce increased levels of IFNγ and drive the acquired immune response to infection. IFNγ produced by both innate and acquired immune cells feeds back to prime macrophages for further activation of the M1 phenotype. Enhanced production of IL-12 by Ron−/− splenocytes after injection with LPS, a ligand for TLR4, results in increased IFNγ production by NK cells and enhanced susceptibility of Ron−/− mice to endotoxic shock. Furthermore, MSP stimulation of the Ron receptor on primary macrophages inhibits the response of these cells to IFNγ through inhibition of Stat1 tyrosine phosphorylation induced by IFNγ. MSP stimulation of primary macrophages induces the expression of suppressor of cytokine signaling (Socs) 1 and Socs3 delineating a mechanism by which the inhibition of IFNγ-dependent responses in these cells could occur[67].

It has been demonstrated that the RON receptor inhibits LPS and cytokine-induced NF-κB activation in various macrophage populations[66, 93, 107]; however, the molecular targets of Ron downstream of TLR activation in primary macrophage have not been systematically addressed. Herein, we show that stimulation of primary peritoneal macrophages with MSP, the ligand for Ron, inhibits expression of a variety of inflammatory mediators in response to LPS. This inhibition is not due to the inhibition of receptor proximal signaling events initiated by LPS stimulation of TLR4, but rather by specific inhibition of the activation of IκB kinase (IKK) and
its downstream targets by LPS. These studies identify potential regulatory mechanisms that could be targeted in the treatment of a wide range of inflammatory diseases.

**Material and Methods**

*Animals.* The generation of mice with a targeted mutation in Ron was described previously[108]. Primary peritoneal macrophages or bone marrow-derived macrophages were isolated from wild-type and Ron−/− mice on the BALB/c or C57BL/6 backgrounds. The Pennsylvania State University Institutional Animal Care and Use Committee (IACUC) approved all animal experiments.

*Reagents.* The following reagents were obtained from the indicated sources: FBS and PBS (Invitrogen, Grand Island, NY); recombinant human MSP (R&D Systems, Mineapolis, MN); lipopolysaccharide (LPS) (055:B5) (Sigma, St. Louis, MO); DMEM, L-glutamine, Non-Essential Amino Acids and Sodium Pyruvate (CELLGRO, mediatech, Manassas, VA). Antibodies for Western blot against IκBα, phosphoThr<sup>184/187</sup>Tak1, total Tak1, IκBζ, phosphoT<sup>202</sup>Y<sup>204</sup>p44/42, total p44/42, phosphoT<sup>183</sup>Y<sup>185</sup> stress-activated protein kinase/JNK, total stress-activated protein kinase/JNK, phosphoT<sup>180</sup>Y<sup>182</sup>p38, total p38, phosphoY<sup>307</sup>Gab1, total Gab1, phosphoY<sup>452</sup>Gab2, total Gab2, phosphoS<sup>536</sup>p65, total p65 were purchased from Cell Signaling (Beverly, MA). Antibodies for flow cytometry, F4/80-RPE, CD11b-APC-750, and Ron were purchased from AbD Serotec (Raleigh, NC), eBioscience (San Diego, CA), and R&D Systems (Mineapolis, MN), respectively.

*Cells.* Murine resident peritoneal macrophages were harvested and cultured as described previously[109]. Briefly, 8 ml ice-cold DPBS was injected into the peritoneum of a euthanized mouse. Following a gentle shake of the abdomen, the peritoneal cells were aspirated with a syringe. The cells were collected in a 50 ml conical tube and centrifuged at 1500 rpm for 5 min at
4°C. The cell pellet was resuspended in a complete cell culture medium (DMEM with L-glutamine, Non-essential amino acids, Sodium Pyruvate, ciprofloxacin, and 10% FBS), and plated in cell culture dishes. Thioglycollate elicited macrophages were prepared by injecting 2 month old BALB/c mice with 2 mL of 3% aged thioglycollate media (Difco, Sparks, MD). On day 4, cells were harvested by lavage and macrophages were isolated and cultured as described for resident macrophages. 0.2 X 10^6 cells were seeded in each well of a 24-well plate for the cytokine analysis. 3.2 X 10^6 cells were seeded in each 60 mm plate for gene expression and western blot analysis. 8.8 X 10^6 cells were seeded in each 100 mm plate for in vitro kinase assay and NF-κB binding assay.

**Cytokine analysis.** Cells were cultured with or without 100 ng/ml MSP overnight followed by stimulation for 24 hours with 100 ng/ml LPS. Cell supernatants were collected and assayed for IL-12p40 by ELISA using the manufacturer’s protocol (BD Biosciences, San Jose, CA).

**Western blot analysis.** Cell lysates were collected using the following lysis buffer (150 mM NaCL, 10 mM Tri-HCl (pH 7.5), 0.5% Triton X-100), supplemented with protease and phosphatase inhibitors (10 µg/ml leupeptin, 2 µg/ml pepstatin A, 10 µg/ml aprotinin, 1 mM sodium orthovanadate, 1 M sodium fluoride and 1mM PMSF). SDS-PAGE was performed using 10% Bis-Acrylamide gels. The protein was transferred onto Immobilon P PVDF membranes (Millipore, Bedford, MA) and the blots were blocked with 5% non-fat dry milk or 5% BSA (Rockland, Gilbertsville, PA) in Tris-buffered saline containing 0.1% Tween 20 for 1 hr at room temperature. The blots were then incubated overnight with primary antibody at 4 °C. Anti-rabbit or anti-mouse secondary antibodies conjugated with HRP were used at concentrations according to the manufacturer’s instructions for one hour at room temperature. ECL Plus (Amersham, Piscataway, NJ) was used to develop the blots.
**In Vitro Kinase Assay.** Thioglycollate elicited macrophage lysates was prepared using the M-PER buffer (Pierce) with protease and phosphatase inhibitors as described above. The cell lysates (100 µg of protein) were incubated with GST-IκBα, ATP and [γ-32P]ATP (1 µCi, Amersham Biosciences) for 1.5 h in the kinase wash buffer (50 mM Tris-Cl, pH 8.0, containing 100 µM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate) on a shaker at room temperature. Glutathione sepharose beads were added to the mixture and incubated at room temperature for 1 hour with gentle shaking followed by centrifugation. The pellet was washed two times with sterile PBS and phospho-labeled GST-IκB was separated by SDS-PAGE, followed by autoradiography.

**NF-κB binding assay.** Thioglycollate elicited peritoneal macrophages were cultured with or without 100 ng/ml MSP overnight followed by stimulation with 100 ng/ml LPS for the indicated times. Nuclear proteins were collected and assayed for NFκB binding activity by ELISA according to the manufacturer’s protocol (Active Motif, Carlsbad, CA).

**Flow cytometry.** Peritoneal lavage cells were plated overnight with or without MSP stimulation. 12 hours following adherence to the plate, cells were collected. 1 X 10⁶ 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 (0.5mg/ml) for 10 minutes on ice. F4/80-RPE, CD11b-APC-750 and Ron antibodies were added to the tubes for 30 minutes on ice. Cells were washed 2 times with FACS buffer and resuspended in 100 µl FACS buffer. Cells were incubated with anti-goat Alexa647 as a secondary antibody to Ron for 30 minutes on ice. Cells were washed two more times, resuspended in 500 µl of FACS buffer and analyzed on a Beckman-Coulter FC500. Ron expression was measured in macrophages positive for F4/80 and CD11b.

**RNA extraction and real-time PCR.** RNA was extracted from cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. Real-time PCR
was carried out on an Applied Biosystems 7300 or 7900. Reverse transcription for real-time PCR experiments was carried out using the Applied Biosystems High Capacity RT Kit according to the manufacturer’s recommendations. All gene specific Taqman probes were purchased from Applied Biosystems (San Francisco, CA).

Statistical Analysis. Statistical analyses were performed using the Students T Test or One-way ANOVA with a Tukey’s post-test with * P<0.05, ** P<0.01, *** P<0.001.

Results

Ron inhibits expression of inflammatory mediators in primary macrophages

M1 macrophages polarized by IFNγ and TLR agonists function to sense microbial components and promote immunity to infection, whereas M2 macrophages polarized by IL-4 and IL-13 promote the resolution of inflammation and tissue repair. To analyze the role of Ron in regulating macrophage polarization, we stimulated primary peritoneal macrophages with MSP and assessed the expression of a panel of M1- and M2- associated genes by real-time PCR. MSP alone induced expression of Il1b and Il6, markers of M1 activation, and Arg1 and Clec7a (encoding Dectin-1 protein), markers of M2 activation (Figure 2-1 A). Primary macrophages were also stimulated with LPS or IL-4 to polarize these cells towards either M1 or M2 macrophage phenotype, respectively, following an overnight stimulation with MSP. We find that MSP inhibits LPS-induced expression of the M1 markers Inos, Il12b (encoding IL-12p40), Tnf and Il1b, but not Il6 (Figure 2-1 B). MSP also significantly reduces the expression of the M2 markers Mrcl, Chi3l3 (encoding Ym-1 protein), and Retnla (encoding Fizz-1 protein) induced by
IL-4, while enhancing Arg1 expression in the presence of IL-4 (Figure 2-1 C). This observation suggests that Ron might play an important role in regulating both Th1- and Th2-mediated immune responses.

**Figure 2-1: Ron regulates M1 and M2 macrophage activation.** A) Primary resident peritoneal macrophages were stimulated for 4 h with 100 ng/ml MSP, and expression of genes associated with M1 and M2 macrophage activation were assessed by real-time PCR. B) Primary peritoneal macrophages were cultured overnight with or without MSP followed by stimulation with 100 ng/ml LPS for 4 h, and the expression of genes associated with M1 macrophage activation was assessed by real-time PCR. C) Primary peritoneal macrophages were cultured overnight with or without MSP followed by stimulation with 5 ng/ml IL-4 for 4 h, and expression of genes associated with M2 macrophage activation was assessed by real-time PCR. Values are expressed as mean ± SEM, and results are the average of two independent experiments with duplicates in each real-time PCR experiment. * P<0.05, ** P<0.01, *** P<0.001.
MSP inhibits IL-12p40 production by primary macrophage in response to LPS in a Ron receptor dependent manner

In order to confirm the role of MSP in the regulation of IL-12p40 protein expression, we treated primary macrophages with MSP either overnight followed by LPS stimulation or simultaneously with LPS, and measured IL-12p40 production by ELISA. We observed that the inhibition of IL-12p40 by MSP was enhanced when the cells were pre-treated with MSP overnight (Figure 2-2 A). The sustained expression of Ron on primary macrophages after overnight culture was confirmed by flow cytometry (Figure 2-2 B). To determine whether this inhibition is mediated by the Ron receptor, we treated primary macrophages of WT and Ron-/- with MSP prior to stimulation with LPS. Results from these studies indicate that MSP inhibits *Il12b, Inos, Il1b, Il6* and *Tnf* expression in a Ron-dependent manner (Figure 2-2 C). To confirm that MSP induces Ron-dependent signaling in primary macrophages, we examined the phosphorylation of Gab1 and Gab2, two adaptors that are recruited to Ron and mediate downstream signaling events induced upon Ron activation. We observed MSP induces robust phosphorylation of both Gab1 and Gab2 in primary macrophages, and this phosphorylation is dependent on the Ron receptor (Figure 2-2 D), suggesting that MSP inhibition of LPS-induced pro-inflammatory cytokine expression is dependent on the Ron receptor.
Figure 2-2: MSP stimulation of the RON receptor inhibits IL-12p40 and other pro-inflammatory markers expression in primary macrophages. A) Primary peritoneal macrophages were cultured overnight in the presence or absence of 100 ng/ml MSP followed by stimulation with LPS with or without MSP. IL-12p40 production was assessed by ELISA. B) Primary resident peritoneal macrophages were collected from wild-type and Ron/- BALB/c mice by lavage. Macrophages were isolated by adherence to plastic. Macrophages were cultured overnight before they were collected by scraping and analyzed by flow cytometry. C) Peritoneal macrophages were isolated from WT and Ron/- mice and were stimulated with 100 ng/ml MSP overnight followed by activation with 100 ng/ml LPS for 4h. Il12b, Inos, Il1b, Il6 and Tnf mRNA expression was assessed by real-time PCR. D) Primary peritoneal macrophages were isolated from wild-type and Ron/- mice by lavage, and macrophages were isolated by adherence to plastic. After overnight culture, the cells were stimulated with MSP for the indicated times, and phosphorylation of Gab1 and Gab2 was assessed by Western blot analysis. Values are expressed as mean ± SEM, and the results are representative of three independent experiments. * P<0.05.
MSP does not inhibit LPS/TLR4 proximal signaling. MyD88 independent signaling by TLR4 mediated through adaptor proteins TRIF and TRAM, and results in the upregulation of IFNβ promoting anti-viral immunity. In order to determine whether Ron regulates activation of IFNβ, we stimulated primary peritoneal macrophages with MSP followed by LPS and assessed IFNβ levels. Our results indicate that MSP does not inhibit the upregulation of IFNβ as assessed by RT-PCR (Figure 2-3 A) and ELISA (Figure 2-3 B) in response to the LPS-induced activation of TLR4.
MyD88-dependent signaling by TLR4 mediated through the adaptors MyD88 and Mal induces Tak1 kinase activation, which leads to MAPK activation [110]. In order to determine whether Ron regulates the upstream signals lead to MAPK activity, we examined the effect of MSP on the phosphorylation of Tak1 in peritoneal macrophages. Our results indicate that MSP does not inhibit Tak1 phosphorylation in response to LPS (Figure 2-4 A). We further assessed the effect of MSP on the induction of MAPK signaling in response to LPS. We stimulated primary peritoneal macrophages with MSP followed by LPS stimulation, and examined phosphorylation

Figure 2-4: MSP enhances MAPK activities but does not affect Tak1 activity in LPS-stimulated macrophages. A) Primary peritoneal macrophages were stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. Cell lysates were extracted at the indicated times, and phosphorylated Tak1 was assessed. Blot was stripped and re-probed with anti-Tak1. B) Primary peritoneal macrophages were stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. Cell lysates were extracted at the indicated times, and phosphorylation of ERK, p38, and JNK were assessed by Western blot analysis with phosphor-specific antibodies. Blots were stripped and re-probed with anti-ERK, anti-p38, and anti-JNK. The results are representative of three independent experiments.
of ERK, p38, and JNK at various times after LPS stimulation. The results showed that LPS induces the phosphorylation of ERK, p38, and JNK in primary macrophages, and this induction is further enhanced by MSP (Figure 2-4 B).

**MSP inhibits LPS-induced activation of IKK and its downstream targets**

Downstream of Tak1, MyD88-dependent signaling by TLR4 promotes TRAF6-dependent activation of the IKK complex. Activated IKK phosphorylates IκB, which constitutively binds to NF-κB in a resting state. The phosphorylation of IκB leads to its degradation, and subsequent release of NF-κB, which allows NF-κB to translocate into the nucleus from cytosol. IKK and its downstream effector molecules including IκB and NF-κB are important regulators of a wide range of pro-inflammatory mediators, including IL-12p40. To determine whether Ron regulates IKK activity induced by LPS in primary macrophages, we used thioglycolate-elicited macrophages. We previously confirmed that these cells express Ron, and that MSP inhibits LPS-induced IL-12p40 expression in these cells. Elicited macrophages were stimulated with LPS in the presence or absence of MSP, and IKK activity was assessed by in vitro kinase assay using IκB as a substrate. We observed a significant upregulation of IKK activity within 15 min of LPS stimulation in these cells, and this induction was significantly reduced in the presence of MSP. IKK activity returned to basal levels by 60 min in both the presence and absence of MSP (Figure 2-5 A). IKK phosphorylation of cytoplasmic IκB family members results in the proteasome-mediated degradation of IκB. We next examined IκBα protein levels in primary resident macrophages stimulated with LPS in the presence or absence of MSP. We observed reduced IκB degradation in the presence of MSP; however, by 15 min, IκB protein was undetectable in LPS-stimulated cells in either the presence or absence of MSP (Figure 2-5 B).
To identify the effect of MSP on LPS-induced NF-κB activation, we stimulated primary peritoneal macrophages with LPS in the presence or absence of MSP. The serine phosphorylation of p65 was assessed by western blot analysis. We observed that MSP inhibited p65 serine phosphorylation within 15 min of LPS stimulation (Figure 2-6 A). We also assessed the effect of MSP on LPS-induced NF-κB DNA binding by extracting nuclear lysates from elicited macrophages stimulated with LPS in the presence or absence of MSP. DNA binding of p65 from nuclear lysates was assessed by ELISA. The results showed a significant reduction in the DNA binding of NF-κB 15 min following LPS stimulation in the presence of MSP (Figure 2-6 B). These results suggest that MSP delays the activation of NF-κB signaling through the canonical pathway after stimulation of primary macrophages with LPS.

Figure 2-5: MSP inhibits IKK activity and retards IκB degradation in primary macrophages. A) Thioglycolate-elicited macrophages were isolated by adherence and stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. At the indicated times, cell lysates were extracted and IKK activity was measured by in vitro kinase assay using IκB as a substrate. B) Primary resident peritoneal macrophages were stimulated overnight with 100 ng/ml MSP followed by 100 ng/ml LPS. Cell lysates were extracted at the indicated times, and IκBα levels were assessed by Western blot analysis. The blots were stripped and re-probed with anti-Actin. The results are representative of two independent experiments.
Unlike conventional IκB family members, nuclear IκBs contain a nuclear localization signal and reside primarily in the nucleus, rather than the cytoplasm. These IκB family members exhibit both positive and negative effects on NF-κB-dependent transcriptional activation. IκBζ is

**Figure 2-6:** MSP inhibits NF-κB transcriptional activity and delays serine phosphorylation of p65 in primary macrophages. A) Primary resident peritoneal macrophages were harvested by lavage and isolated by adherence to plastic. The cells were stimulated with 100 ng/ml MPS overnight followed by 100 ng/ml LPS. Cell lysates were harvested at the indicated times, Serine phosphorylation of p65 was assessed by Western blot analysis. B) Thioglycolate-elicited macrophages were isolated by adherence to plastic and stimulated with 100 ng/ml MSP overnight followed by 100 ng/ml LPS. Nuclear extracts were isolated at the indicated times, and binding to DNA was assessed by ELISA. C) Primary resident peritoneal macrophages were stimulated with LPS for the indicated times in the presence or absence of MSP. IκBζ expression was assessed by Western blot analysis. The blot was stripped and re-probed with anti-actin. The results of A) and C) are representative of two independent experiments. Values of B) was expressed as mean ± SEM, and results are the average of two independent experiments with duplicates in each real-time PCR experiment. * P<0.05.
a nuclear IκB family member that is upregulated at the transcriptional level by LPS in an NF-κB-dependent manner. IκBζ-deficient macrophages display impaired expression of a subset of LPS-inducible genes including IL-12p40. To examine the effect of MSP on the induction of early NF-κB response genes, we assessed the expression of IκBζ in response to LPS in the presence or absence of MSP in primary peritoneal macrophages. The results showed that MSP significantly reduced LPS-induced IκBζ expression (Figure 2-6 C).

**Discussion**

Previous studies from our laboratory indicate that the MSP/Ron signaling pathway significantly reduces the expression of IL-12p40 both in vitro and in vivo, resulting in increased susceptibility of Ron deficient mice to septic shock [67, 105]. Here, we identified molecular targets downstream of TLR4 regulated by MSP. We demonstrated that MSP inhibits TLR4-induced NF-κB activity by reducing IKK activity, retarding IκB phosphorylation and degradation and limiting DNA binding of p65. Additionally, MSP inhibits the upregulation of the unconventional IκB family member, IκBζ, in response to LPS (Figure 2-7).

The data presented here indicates that the ability of MSP to inhibit LPS signaling in primary macrophages is enhanced significantly when the cells were cultured overnight in MSP before LPS stimulation. It is speculated that Ron activation might promote the synthesis of a wave of new proteins, which could inhibit LPS signaling. We previously demonstrated that Stat3 phosphorylation is promoted by MSP in resident macrophages. Our studies suggest that the activation of Stat3 by Ron occurs via the recruitment of a Grb2/Gab2 signaling complex to Ron, and the subsequent recruitment of Stat3 to the complex through a YxxQ motif present in Gab2. STAT3 is also known to be activated through an IL-6-dependent manner [111]. MSP alone
induces expression of IL-6, which could further lead to STAT3 activation. STAT3 promotes a gene expression program, the products of which inhibit pro-inflammatory mediator induction and inhibit NF-κB-dependent gene expression. Thus, the ability of MSP to inhibit LPS-induced pro-inflammatory gene expression could be the result of MSP-induced STAT3 signaling.

MAPK signaling is directly involved in cellular responses to various inflammatory stimuli. The TLR4-dependent activation of MAPK signaling promotes the expression of many LPS-dependent genes. However, recent studies suggest that suppression of ERK signaling results
in substantial production of IL-12p40, and hyper-activation of ERK activity, which induces activation of repressor element GA-12, reduces LPS-induced IL-12p40 production in primary macrophages [112]. We observed that MSP enhances phosphorylation of MAPKs in LPS-stimulated macrophages. This result suggests another possible mechanism by which MSP could inhibit LPS-induced IL-12p40 production.

Recently, a role for IκBζ, an atypical IκB family member, has been described in promoting LPS-induced gene expression of IL-6 and IL-12p40. IκBζ positively regulates of LPS-induced transcriptional activation by binding to p50 homodimers on the promoters of genes of interest [113] [114]. P50 homodimers binds to the promoters and blocks access of p50p65 heterodimers, thus blocks transactivation of classical NF-κB and transcription of pro-inflammatory genes that are driven by NF-κB [115, 116]. We demonstrated here that MSP inhibits the NF-κB-dependent upregulation of IκBζ in primary macrophages, suggesting that MSP could inhibit IL-12p40 expression both through regulation of IKK activity and through the regulation of IκBζ. However, the fact that MSP inhibits IL-12p40, but not IL-6, production by LPS-activated macrophages, both targets of IκBζ, indicates that the effects of MSP on LPS-dependent gene expression mediated by its regulation of IκBζ are likely to be context dependent.

The prostaglandin 15-deoxy-delta 12, 14-PGJ2 (15d-PGJ2), an arachidonic acid derived endogenous electrophile, is a potent inhibitor of NF-κB signaling. 15d-PGJ2 directly inhibits IKKβ activity by forming an adduct with Cys-179 in IKKβ [117]. At higher concentrations, PGJ2 serves as an endogenous ligand for peroxisomal proliferator-activated receptor gamma (PPARγ). PPARγ plays a central role in the regulation of NF-κB signaling through the ligand-dependent transrepression of NF-κB [118]. We observed that treatment of macrophages with MSP induces time-dependent production of PGJ2 (S. Figure 2A). MSP also enhances PGJ2 production in the presence of LPS, suggesting the ability of MSP to limit LPS-induced inflammation could result
from PGJ$_2$ production in the presence of MSP (S. Figure 2B). However, we observed that MSP alone does not increase Pparg expression and MSP does not increase Pparg expression in LPS-skewed M1 macrophage (S. Figure 3 A and B). MSP also does not promote the DNA binding of PPARγ to the PPAR response elements (PPREs) in an *in vitro* luciferase assay (S. Figure 3 C). In addition, the ability of MSP increasing IL-12p40 production is not dependent on PPARγ, as the PPARγ inhibitor GW9662 did not abrogate the ability of MSP to inhibit IL-12p40 production (S. Figure 4). It suggests that MSP-induced PGJ$_2$ might serve its role in reducing inflammation in a receptor-independent manner. The PGJ$_2$ is a downstream product of cyclooxygenase 1 (Cox1) and 2 (Cox2). We observed that MSP does not significantly affect LPS-induced Cox1 and Cox2 expression, and the inhibition of IL-12p40 production by MSP does not depend on either Cox1 or Cox2 (S. Figure 5), but this inhibition of IL-12p40 production is abrogated when both Cox1 and Cox2 are inhibited (S. Figure 4). Subsequently, we observed that PGJ$_2$, together with other PGs and the product of EPA, in turn increases expression of Ron in primary macrophages (S. Figure 6). According to Matinspector, putative PPARγ binding sites are present in the Ron promoter region, suggesting the possibility that PGJ$_2$-induces Ron expression by binding to PPARγ, which subsequently translocates to the nucleus and initiates Ron transcription. However, it is also possible that these PGs increase Ron through some unknown mechanisms. Overall, our results suggest that Ron activated by MSP, together with PGs, could serve as a positive feedback loop in reducing inflammation.

The mechanism identified here provides evidence of how MSP regulates the target genes of TLR-4 signaling. Other than eliminating LPS-induced Th1-mediated inflammatory gene expression, including *Il12b, Il1b, Tnf*, and *Inos*, MSP reduces IL-4-induced Th2-mediated gene expression, including *Chi3l3, Mrc1*, and *Retnla*. It suggests that MSP/Ron can be a potential therapeutic target for not only Th1-mediated inflammatory diseases, such as bacterial infection.
and diet-induced chronic inflammation, but also Th2-mediated inflammation, such as helminth infection or asthma. Here, we mainly focus on the characterization of some mechanisms, through which MSP inhibits LPS-induced Th1-mediated immune responses.

In conclusion, we demonstrated that Ron receptor tyrosine kinase plays a critical role in attenuating inflammatory stimuli, such as LPS and IL-4, induced macrophage activation. We identified here that MSP/Ron signaling inhibits NF-κB-dependent inflammatory gene expression by targeting IKK and its downstream signaling. The ability of Ron in protecting animals from septic shock suggest that Ron is important in maintaining a balance between enabling innate immune system to promote immunity to infection, and preventing host tissue from inflammation-induced damage. Thus, Ron or its downstream signaling components are potential therapeutic targets for various inflammatory diseases.
Chapter 3

The Ron receptor tyrosine kinase attenuates obesity-associated diseases by regulating macrophage heterogeneity

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Abstract

Diet-induced metabolic diseases are leading causes of death worldwide. Obesity increases the likelihood of atherosclerosis, type 2 diabetes and fatty liver disease, which are caused largely by recruitment and activation of macrophages at sites of inflammation in arteries, adipose tissue and liver, respectively. Macrophages are classified as classically activated macrophages (M1), which are associated with increased production of cytokines, chemokines and cytotoxic molecules by immune cells recruited to inflammatory sites, and alternatively activated macrophages (M2), which are associated with inhibition of inflammation and tissue regeneration. A switch from M2 to M1 properties or the direct recruitment of M1 macrophages in the aorta and adipose tissue promotes the progression of atherosclerosis, fatty liver disease and insulin resistance. The Ron receptor tyrosine kinase, expressed predominantly on tissue-resident macrophages, inhibits hallmarks of classical macrophage activation and skews macrophage populations towards an M2 phenotype both in vitro and in vivo. We demonstrated here that Ron expression in aortic macrophages and Kupffer cells is associated with an M2 phenotype, and Ron expression in macrophages decreases with disease progression. Additionally, the absence of Ron results in increased severity of atherosclerotic lesions and liver injury when mice are fed on a high cholesterol diet. This phenotype is associated with elevated expression of inflammatory markers in atherosclerotic aortas and livers in ApoE -/- X Ron -/- (DKO) mice. Taken together, our results show that Ron plays a central role in limiting obesity-induced inflammation, and thus plays a protective role in the progression of atherosclerosis, fatty liver disease and obesity.
**Introduction**

Diet-induced metabolic disorders exhibit many hallmarks of chronic inflammatory diseases by manifesting increased expression of pro-inflammatory cytokines and mediators. Macrophages, key components of the innate immune response, regulate the progression of these diseases. Depending on cues present in the microenvironment, resident macrophages are activated through either a classical activation pathway or an alternative activation pathway. Classically activated macrophages (M1) express high levels of iNOS and low levels of Arg1 and are involved in the clearance of intracellular pathogens. Conversely, alternatively activated macrophages (M2) express the reverse pattern and not only develop in response to parasitic infections in a Th2 cytokine-dependent manner but also protect host tissue from inflammatory damage[119]. Excessive intake of a high fat diet causes disequilibrium of nutrient metabolism, including lipid dysfunction, which promotes classical macrophage activation or a shift from an M2 to an M1 phenotype. This pathogenic activation of M1 macrophages together with other immune components leads to the development of diet-induced metabolic disorders, including obesity[30][27], atherosclerosis[120][121] and fatty liver disease[122][50].

Macrophages with various phenotypes in tissues regulate chronic inflammatory disease progression and resolution. In healthy humans and mice, resident macrophages present in adipose tissue, liver and blood vessels express several markers of M2 activation, such as IL-10, Ym1, arginase I, and the mannose receptor. In the meanwhile, tissue resident macrophages isolated from obese individuals exhibit a pro-inflammatory M1 phenotype and overexpress IL-1β, IL-6, iNOS, and TNFα. These M1 macrophages not only produce various cytokines, but also express distinct cell surface markers. The expression of CD11c on pro-inflammatory macrophages is closely associated with the progression of inflammation in atherosclerotic lesions, oversized
adipose tissues and non-alcoholic fatty livers [123] [124] [125] [27]. Genetic manipulation of CD11c expression in macrophages shows improved systemic metabolic regulation, including attenuated insulin resistance and glucose tolerance [27, 30, 126]. Therefore, the balance of macrophage activation needs to be tightly regulated to control the progression of diet-induced metabolic disorders.

The Ron receptor tyrosine kinase, a member of the Met family of kinases, regulates immune responses by balancing M1 and M2 macrophage activation [68-71, 75, 103, 105]. Ron, is expressed on tissue-resident macrophages including peritoneal macrophages, Kupffer cells, alveolar macrophages, osteoclasts, mesangial cells and microglial cells. Ron deficiency promotes an M1 macrophage phenotype in tissues leads to enhanced inflammation in animal models of multiple sclerosis, acute lung injury and delayed-type hypersensitivity, while dampening the M2-like phenotype of tumor associated macrophages. Stimulation of Ron by its ligand, macrophage stimulating protein (MSP), suppresses M1 activation by inhibiting expression of iNOS, IL-12p40, and TNFα in primary macrophages stimulated with LPS and IFNγ [69] [105]. This suppression is due to the ability of Ron to inhibit NF-κB activation in response to LPS and inhibit Stat1 phosphorylation in response to IFNγ [66] [68]. In contrast, MSP stimulation of Ron promotes an M2 phenotype by enhancing arginase I expression and activity [70]. These results indicate that Ron regulates immune responses by inhibiting M1 macrophages while promoting an M2-like phenotype.

Taking into account the immunological nature of diet-induced metabolic disorders and the role of Ron in regulating immune responses, we hypothesized that Ron attenuates the progression of these diseases by reshaping the phenotype of macrophages located in pathological tissues and tipping the balance of macrophage activation away from an M1 and towards an M2 phenotype. Results from these studies could lead to the development of novel therapeutic
methods to limit the progression of diet-induced metabolic disorders by altering macrophage phenotypes.

Methods

Mouse Models. ApoE−/− (ApoE KO) mice on the C57BL/6 background were purchased from Jackson Laboratory. Ron−/− mice on the C57BL/6 background were described previously [71]. ApoE−/− and Ron−/− (Ron KO) mice were intercrossed to generate ApoE−/− X Ron−/− (DKO) mice. The DKO genotype was determined by genomic polymerase chain reaction. All mice were bred and maintained in Pennsylvania State University animal facility according to protocol approved by the Institutional Animal Care and Use Committee.

In the high fat diet accelerated model of metabolic disorder, male WT, Ron KO, ApoE KO and DKO mice were switched from normal chow to a high fat diet (60% calorie from fat, BioServ3282) at the age of 5 weeks and analyzed for plaque, cytokine expression, blood metabolites 9 to 23 weeks later. In the high cholesterol diet accelerated model of metabolic disorder, male WT, Ron KO, ApoE KO and DKO mice were switched from normal chow to a high fat diet (60% calorie from fat plus 1.25% cholesterol, BioServ6334) at the age of 5 weeks and analyzed for plaque, cytokine expression, blood metabolites 9 to 20 weeks later. In both models, the WT, Ron KO, ApoE KO and DKO mice were always treated and analyzed in parallel.

Antibodies and other reagents. For flow cytometry, PE-anti-F4/80, PECy7-anti-CD11c, Pacific Blue-anti-CD45, and the isotype controls for CD11c (PECy7 Armenian Hamster IgG) and F4/80 (PE Rat IgG2a,κ) were purchased from biolegend (San Diego, CA), PECy5-anti-CD11b was purchased from eBiosicence (San Diego, CA). Anti-mouse Ron was purchased from R&D (Minneapolis, MN), isotype control for Ron (goat IgG) was purchased from Santa Cruz
Biotechnology (Santa Cruz, CA), and the secondary antibody Alexa 647-anti-goat for Ron and Texas-red live/dead fixable stain were purchased from Molecular Probes (Eugene, OR). For immunohistochemistry, anti-Ron (C-20) antibody and isotype control for Ron (rabbit IgG) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-MSP was purchased from R&D and Alexa 488-anti-MOMA2 was purchased from Serotec (Raleigh, NC). AnnexinV apoptosis detection kit FITC was purchased from eBioscience.

**Cell culture.** Murine resident peritoneal macrophages were harvested and cultured as described previously[109]. Thioglycollate elicited macrophages were prepared by injecting 2 month old BALB/c mice with 2 mL of 3% aged thioglycollate media (Difco, Sparks, MD). On day 4, cells were harvested by lavage and macrophages were isolated and cultured as described for resident macrophages. Cells were maintained in complete cell culture medium (DMEM with L-glutamine, Non-essential amino acids, Sodium Pyruvate, ciprofloxacin, and 10% FBS). MS1 cells were purchased from ATCC (Manassas, VA) and were maintained in complete cell culture medium. MS1 cells were cultured for 24 h before being used for indicated experiments.

**Aorta cell isolation.** Aortas beginning at the root outside of the heart and stopping at the thoracic diaphragm were collected and flushed with PBS containing 10 U/mL heparin, and cut into ~1 mm pieces, and incubated for 1 hour at 37 C in PBS containing 20 mmol/L HEPES, 1.5% BSA, 0.1% collagenase type II (Gibco), 125 U/mL collagenase type XI, 450 U/mL collagenase type I, 60 U/mL hyaluronidase type I, 60 U/mL DNase I (Sigma-Aldrich). The isolated cells were counted and used for analysis. For flow cytometry, cells were stained with the proper combination of antibodies as indicated in the figures and analyzed on a flow cytometer BD LSR Fortessa. For flow sorting, cells were stained with the proper combination of antibodies and sorted on BD Influx sorter. Cells sorted from 2 to 3 mouse aortas were pooled together for real-time PCR analysis.
Kupffer cell isolation. Mouse Kupffer cells were isolated as previously described[127] with some modifications. Briefly, mouse livers were perfused with Buffer I (142 mM NaCl, 6.4 mM KCl, 9.6mM HEPES, 30 mM NaOH) followed by Buffer II (Buffer I supplemented with 4.76 mM CaCl2 and collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ) via the inferior vena cava, prior to excision. Perfused livers were then minced and suspended in Buffer III (Buffer I supplemented with 1% BSA) prior to passing through a 70 µm nylon cell strainer. The filtered cells were either used directly for FACS experiments or were further purified for cell culture. Filtered cells were centrifuged twice at 54g to remove hepatocytes and the resulting supernatants were further centrifuged at 900g. The cell pellet was then re-suspended in Buffer III and passed through a discontinuous Percoll gradient of 25% and 50% Percoll. The Percoll fraction containing the purified non-parenchymal cells was then washed once with Buffer III and the Kupffer cell population was enriched by selective adherence to non-treated tissue culture plates. Isolated Kupffer cells were cultured in RPMI medium containing L-glutamine (Mediatech Inc., Manassas, VA) and supplemented with 10% fetal bovine calf serum and 1% penicillin and streptomycin. The purity of isolated Kupffer cells was determined by their ability to phagocytize latex beads and their surface expression of F4/80.

Adipose tissue stromal vascular fraction isolation. Epididymal white adipose tissue was excised, weighed, minced into smaller than 10 mg pieces and places into equal volume of digestion media consisting of DMEM supplemented with 2.5% HEPES and 10 mg/ml fatty-acid free bovine serum albumin (Rockland) and Collagenase type II (0.3%, Sigma-Aldrich). Tissues were placed on a rotator in a 37°C incubator for 25 min and then filtered through a 70 um filter (BD, Franklin Lakes, NJ) to remove debris and centrifuged at 4°C at 300g for 8 min. The pellet, consisting of stromal vascular cells (SVCs), was washed with DMEM and centrifuged at 4°C at 300g for 8 min. The supernatant was discarded and erythrocytes were lysed by incubating the
SVCs in 1 mL of ACK buffer (NH₄Cl 8.024 g/L, KHCO₃ 1.001 g/L, EDTA Na₂•2H₂O 3.722 mg/L) for 1 min at room temperature before stopping the reaction with 5 mL DMEM. Cells were centrifuged again at 4°C at 300g for 10 min, suspended in certain amount of FACS buffer for FACS analysis. SVCs isolated from 2 to 3 RD mice were pooled together for FACS analysis.

*En Face Oil Red O Staining and Quantification of Plaque Formation.* Aortas beginning at the root outside of the heart and stopping at the thoracic diaphragm were collected, opened up longitudinally and stained with Oil Red O dye as described by manufacture’s instruction (American MasterTech, Lodi, CA). Sizes of total aortic arch and neighboring regions and the Oil Red O staining plaque area within the regions were calculated on the basis of digital pictures of the stained aortas with the use of ImageJ software. The extent of plaque formation was expressed as the percentage of Oil Red O staining area of the total area of the aortic arch and neighboring region.

*Immunohistochemical Staining.* 7 um cryosections were obtained by cross-sectioning the heart aortic root area or the liver. Cryosections of the aortic root were stained with either anti-Ron with an anti-rabbit Alexa 594 secondary antibody and Alexa 488-anti-MOMA2, or anti-Ron and anti-rabbit Alexa488 secondary antibody and anti-MSP with an anti-goat Alexa 594 secondary antibody. Cryosections of the liver were stained with H&E or Oil Red O. The pathological scores of H&E and Oil Red O-stained samples were given blindly by a pathologist.

*Real-Time Reverse Transcription Polymerase Chain Reaction Analysis.* RNA was isolated using Trizol reagent (Invitrogen). RNA was reverse transcribed into cDNA using Reverse transcription kit (Applied Biosystems). Each Real-time PCR reaction included 5 ul perfeCTa qPCR SuperMix Rox (Quanta BioSciences, Gaithersburg, MD), 0.5 ul TaqMan probe (Applied Biosystems), and 4.5 ul diluted cDNA. Real-time PCR was performed using applied
biosystems 7900HT Fast Real-time PCR system (San Francisco, CA). All of the Taqman probes used for Real-Time PCR were purchased from Applied Biosystems.

Metabolic assays. Mice were fed a HFD or a HCD as indicated for 13-18 weeks. Glucose tolerance tests were performed after fasting mice for 16 hours and challenging with 2g/kg body weight of glucose solution by IP injection. Blood glucose was determined before and after glucose being injected for various times as indicated. Insulin tolerance tests were performed on random fed mice by determining blood glucose before and after IP injecting 0.75 U/kg body weight insulin solution (Eli Lilly) for various times as indicated. Blood glucose was measured by tail vein bleeding with a glucometer.

Statistical Analysis. Data are presented as the mean ± standard error of the mean (SEM). Student T test or one-way ANOVA was used for statistical analysis with * P < 0.5, ** P < 0.01, *** P < 0.001 and **** P<0.0001 unless stated otherwise. All analysis was performed using GraphPad Prism 5.0 (San Diego, CA).

Results

Ron, associated with an M2 phenotype, is reduced in aortic macrophages of progressed atherosclerosis

Activation of the receptor tyrosine kinase Ron in tissue resident macrophages inhibits pro-inflammatory macrophage activation while promotes an anti-inflammatory macrophage phenotype in many acute inflammatory disease models. However, the role of Ron in regulating chronic inflammatory metabolic diseases induced by diet was unknown. Atherosclerosis, one of the most common metabolic diseases, together with other cardiovascular diseases, is the leading cause of death. In order to study the role of Ron in atherosclerosis progression, we first
investigated whether Ron is expressed in atherosclerotic plaques. Immunostaining of atherosclerotic plaques from ApoE KO mice maintained on a HCD for 18 weeks showed expression of Ron in lesional cells that express the macrophage marker MOMA2 (Figure 3-1 A). Expression of MSP was also observed in the plaque region where expressed Ron (Figure 3-1 B). To examine whether Ron expression is affected by plaque formation, we compared Ron expression in the aortic arch (AA) and thoracic artery (TA) isolated from WT mice and ApoE KO mice fed on a high fat diet (HFD) (Bio-serv F3283) for 13 weeks. We found that both the AA and TA from ApoE KO mice exhibited significantly lower levels of Ron expression compared with the AA and TA from WT mice (Figure 3-1 C).

To determine whether this decrease in Ron expression associated with plaque formation occurs in macrophages, we isolated the aortas from regular chow diet (RD) and high cholesterol diet (HCD)-fed ApoE KO mice, and assessed Ron expression in aortic macrophages by FACS analysis. The aortic leukocytes were gated on CD45+ fraction (Figure 3-1 D left), which was further analyzed for live/dead staining (Figure 3-1 D right). The gated live population was analyzed for Ron and F4/80 expression (Figure 3-1 E). The percentage of Ron+ F4/80+ fraction in the live CD45+ fraction was significantly decreased in aortas from HCD-fed mice compared to their counterparts, while the actual numbers of Ron+ aortic macrophages of a RD or a HCD do not significantly differ (Figure 3-1 E). In order to determine whether Ron is a marker for M2 cells in the aortic lesions, we sorted Ron- and Ron+ fractions in the live CD45+ F4/80+ cells isolated from HCD-fed mice and compared Arg1 and Inos gene expression in these two fractions. Interestingly, these Ron+ cells express significantly higher levels of Arg1 than Ron- cells (Figure 3-1 F).
Consistent with previous studies[128] [129], we observed an increase in the CD11c+ monocyte/macrophage population in atherosclerotic aortas from mice that were maintained on a HCD for 18 weeks. While the percentage of CD11c+ F4/80+ population in the live CD45+ cells was significantly increased in aortas from HCD-fed mice compared to RD-fed mice (Figure 3-2 A and C), the numbers of CD11c+ F4/80+ cells did not significantly differ (Figure 3-2 D).

Interestingly, the percentage of Ron+ cells in these live CD45+ F4/80+ CD11c+ cells was reduced (Figure 3-2 B and E) in HCD-fed mice, while the percentage of Ron+ cells in live CD45+ F4/80+ CD11c- population did not significantly differ (data not shown). Taken together, these findings showed that Ron expression is associated with M2 macrophage activation in atherosclerotic lesions, and the expression of Ron is impaired in progressed atherosclerotic lesions.
Figure 3-2: Expression of Ron is reduced in CD11c+ aortic macrophages of HCD-fed mice compared to RD-fed mice. A) FACS result of CD11c and F4/80 expression in the live CD45+ aortic cells isolated from ApoE KO mice were either maintained on a RD or a HCD for 18 weeks. The percentage of F4/80+CD11c+ cells in the live CD45+ fraction and the number of the live CD45+F4/80+CD11c+ cells per aorta were quantified as shown in C) and D), respectively. B) FACS result of Ron expression in the live CD11c+F4/80+CD45+ aortic cells as shown in A). The percentage of Ron+ cells in the live CD45+F4/80+CD11c+ cells was quantified as shown in E). RD n=9, HCD n=8. Values of C), D) and E) were expressed as mean ± SEM. Student’s T test was performed in with *P<0.05.
Absence of Ron accelerates atherosclerosis progression by promoting M1 activation and macrophage-mediated endothelial cell inflammation

In order to test our hypothesis that Ron plays a critical role in down-regulating diet-induced atherosclerosis, we generated ApoE-/- X Ron-/- (DKO) mice by crossing ApoE KO mice with Ron KO mice. For all experiments, mice were maintained on a HFD or a HCD starting at five weeks of age. The severity of atherosclerosis in mice on a HFD was scored and categorized at 18 weeks and 23 weeks. At both time points, DKO mice on a HFD developed more severe atherosclerosis compared to ApoE KO controls (Figure 3-3 A and B). In order to accelerate the progression of atherosclerosis, we switched mice onto a HCD (HFD+1.25% cholesterol, Bio-serv F6334). The severity of atherosclerotic lesions was quantified by Oil Red O staining of longitudinally opened aortas from mice administered a HCD for 13 and 18 weeks. The percentage of lesion area in the DKO aortas was slightly increased after 13 weeks on a HCD (Figure 3-3 C), and significantly increased after 18 weeks compared to ApoE KO aortas (Figure 3 D). Oil Red O staining of aortic root cross-sections confirmed the results that DKO mice develop more severe atherosclerosis associated with greater accumulation of lipids in atherosclerotic plaques compared to ApoE KO at 18 weeks (Figure 3-3 E). This accelerated progression of atherosclerosis in Ron deficient mice was not associated with changes in body weight, cholesterol levels or triacylglycerol levels (Figure 3-3 F, G and H).

To determine whether this increase in severity of atherosclerosis in DKO mice corresponds to enhanced inflammation in the aorta, we examined the expression of a panel of M1 and M2 associated genes using real-time PCR in aortic arches from ApoE KO and DKO mice administered a HFD for 23 weeks. Inflammatory genes, including Inos, Il12b, Tnf, Il1b, Il6, were all significantly increased in the aortas of DKO mice compared to ApoE KO mice, while the ArgI to Inos ratio, an indicator of anti-inflammatory to pro-inflammatory macrophage ratio, was dramatically decreased in DKO aortas (Figure 3-4 A). This observation suggests that Ron
Figure 3-3: Ron deficiency, attenuating M2 phenotype in aortas, potentiates severity of atherosclerosis in ApoE KO mice. A) Microscopic examination of representative aortic arches from ApoE KO and DKO mice that were maintained on a HFD for 16 weeks. Plaques are indicated by red arrows. B) The percentage of categorized atherosclerotic severity based on microscopic examination was quantified using aortas from ApoE KO and DKO mice that were maintained on a HFD for 18 weeks and 23 weeks. 18 weeks ApoE KO n=17, DKO n=12. 23 weeks ApoE KO n=15, DKO n=15. C) Representative Oil Red O staining of longitudinally-opened aortas of mice that were maintained on a HCD for 13 weeks. The percentage of Oil Red O stained lesion area out of the total aorta area was quantified. ApoE KO n=6, DKO n=10. D) Representative Oil Red O staining of longitudinally-opened aortas of mice that were maintained on a HCD for 18 weeks. The percentage of Oil Red O stained lesion area out of the total aorta area was quantified. ApoE KO n=7, DKO n=5. E) Representative Oil Red O staining of aortic root cryo-sections from ApoE KO and DKO mice that were maintained on a HCD for 18 weeks. Lesion size of aortas was quantified using ImageJ. ApoE n=7, DKO n=7. F) Body weight (ApoE KO n=10, DKO n=10), G) Cholesterol (ApoE KO n=7, DKO n=6) and H) Triacylglycerol levels of ApoE KO and DKO mice was measured after they were maintained on a HCD for a period as indicated. Values were expressed as mean ± SEM. Student’s T test was performed in with *P<0.05.
attenuates diet-induced atherosclerosis, at least in part, by down-regulating inflammatory gene expression. To assess whether aortic macrophages contribute to the elevation of inflammation in the DKO aortas, we sorted the F4/80+ fraction that was gated on live CD45+ cells from the ApoE KO and DKO aortas (Figure 3-4 B), and assessed the expression of pro-inflammatory genes using real-time PCR. *Il1b* and *Tnf* were significantly increased, while *Il12b, Il6 and Inos* were slightly increased in the DKO aortic macrophages (Figure 3-4 C). This result indicates that accelerated progression of atherosclerosis in the Ron-deficient aortas is due to, at least partially to, an increase in pro-inflammatory macrophages in the aortas. CD11c, a pro-inflammatory macrophage marker, is highly expressed by resident macrophages in inflamed tissues, including adipose tissue, liver and aorta. We next examined whether the absence of Ron contributes to an increase in the CD11c+ population in the aorta. FACS analysis of cells isolated from the aortas showed that CD11c+ macrophage numbers were not different between ApoE KO and DKO mice when they were fed a HCD (data not shown). This finding indicates that not all pro-inflammatory macrophages in the aortic lesions express CD11c, and that the use of other markers may be necessary to distinguish pro-inflammatory and anti-inflammatory macrophages.

Ron expression has been reported not only on tissue resident macrophages, but also on epithelial cells and endothelial cells[74, 130, 131]. Thus, the severe atherosclerotic phenotype in mice lacking Ron could be a result of deficiency of Ron in aortic endothelial cells. To address this question, we sorted CD45- CD31+ endothelial cells in the aortas from ApoE KO and DKO mice that were administered a HCD for 18 weeks, and examined gene expression in these sorted cells. We observed a slight increase in *Il1b, Il6, Tnf* and a significant increase of *Inos* in endothelial cells sorted from DKO aortas compared with ApoE KO (Figure 3-5 A). This increase in inflammatory gene expression in DKO aortic endothelial cells could due to either the direct
**Figure 3-4: Ron deficiency results in increased inflammatory cytokine expression in the aorta.**

A) Aortas from ApoE (n=5) and DKO (n=8) mice that were maintained on a HFD for 23 weeks were isolated. Gene expression of Inos, Il12b, Tnf, Il1b, Il6 and Arg1 to Inos ratio was quantified. B) F4/80+ fraction of live CD45+ aortic cells from ApoE KO (n=16) and DKO (n=18) mice that were fed a HCD for 18 weeks were sorted by FACS. C) Pro-inflammatory gene expression was assessed in FACS sorted F4/80+ fraction of live CD45+ aortic cells using real-time PCR. Values were expressed as mean ± SEM. Student’s T test was performed in with *P<0.05, **P<0.01 and ***P<0.001.
attenuation of endothelial inflammation by Ron, or the indirect effect of Ron mediated by macrophages surrounding endothelium. To test these possibilities, we employed MS1 cells - a pancreatic endothelial cell line, and confirmed cell surface expression of Ron on these cells (Figure 3-5 B). The expressions of a panel of inflammatory genes were examined in MS1 cells...
stimulated with or without MSP followed by LPS stimulation. MSP did not have significant
effect on LPS-induced inflammatory marker expression in MS1 cells (Figure 3-5 C). Next, we
tested inflammatory gene expression in MS1 cells after incubation with macrophage-conditioned
media (MCM), consisting cell culture supernatants from primary peritoneal macrophages treated
with or without MSP followed by LPS stimulation. The results showed that *Il6, Tnf* and *Inos*
expression were significantly reduced in endothelial cells that were incubated with MCM
collected from macrophages treated with MSP (Figure 3-5 D). Overall, these results suggest that
Ron might not directly affect endothelial cell responses to inflammatory stimuli, but decrease
endothelial cell inflammation through a macrophage-dependent manner.

**Ron is associated with M2 Kupffer cell activation in the liver**

Several studies have demonstrated that Ron plays an important role in regulating Kupffer
cell immune function in response to various toxic challenges, including acetaminophen and
endotoxin [74] [76] [75]. However, the role of Kupffer cells expressing Ron have not been
studied in the context of chronic inflammation in diet-induced fatty liver disease. In order to
determine whether Ron likewise marks M2 macrophages in the liver, we compared the phenotype
of Ron+ and Ron- Kupffer cells as well as CD11c+ and CD11c- Kupffer cells isolated from the
CD45+F4/80+ fraction in WT mice challenged with a HCD (Figure 3-6 A). Real-time PCR
analysis showed that Ron+ Kupffer cells expressed higher level of *Arg1* and lower level *Inos*
compared with Ron- Kupffer cells, and Ron+ Kupffer cells also exhibited higher ratio of
*Arg1*/*Inos*, as an indicator of M2 to M1 Kupffer cell population, suggesting Ron+ Kupffer cells
are closely associated with an M2 phenotype (Figure 3-6 B). As expected, CD11c+ Kupffer cells
expressed higher level of *Inos* and lower level of *Arg1*, accompanied with lower level of *Ron*,
suggesting CD11c+ Kupffer cells are associated with an M1 phenotype with reduced Ron
expression (Figure 3-6 C). Together, these results suggest that Ron is preferentially expressed by M2 rather than M1 Kupffer cells in the fatty liver.

**Figure 3-6:** Ron is preferentially expressed by the M2 Kupffer cells in the fatty liver. A) Livers were isolated from WT mice that were administered a HCD for 18 weeks and were digested using collagenase buffer. CD11c expression and Ron expression were further analyzed in the CD45+F4/80+ fraction. B) Gene expression of Arg1 and Inos was compared in Ron+ and Ron- Kupffer cells sorted from WT livers of mice as shown in A). N=4. C) Gene expression of Arg1, Inos and Ron was analyzed in CD11c- and CD11c+ Kupffer cells that were sorted from WT livers of mice as shown in A). Lines between two groups connect the samples isolated from the same liver. N=9. Values were expressed as mean ± SEM. Paired T test was performed in C) with *P<0.05, ***P<0.001.
Absence of Ron potentiates HCD-induced liver injury and inflammation

To further assess the role of Ron in regulating the severity of liver injury induced by HCD, we measured the levels of the liver injury indicators alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum of mice fed on a HCD for various times (Figure 3-7 A). The results showed that the serum of DKO mice contained significantly higher levels of ALT compared to their ApoE KO counterparts after the mice were administered a HCD for 18 weeks. This observation was in accordance with significant increase in liver weight in these animals (Figure 3-7 B). Oil Red O staining showed that DKO livers exhibited higher grade fat accumulation (Figure 3-7 C). This increased fat accumulation is also associated with increased inflammation as shown in H&E staining (Figure 3-7 D). To confirm this result, we assessed the expression of inflammatory markers in livers isolated from ApoE KO and DKO mice. DKO livers showed a significant increase in the pro-inflammatory cytokines Il12b and Tnf, and slight increase in Inos, while expressing significantly lower levels of the anti-inflammatory marker Arg1 (Figure 3-7 E). However, we failed to detect differences in the expression of inflammatory mediators in FACS sorted Kupffer cells from Ron KO mice compared with those from WT mice (Data not shown). Surprisingly, both the percentage of CD11c+ Kupffer cell in the live CD45+ fraction and the number of live CD45+F4/80+CD11c+ were reduced in Ron KO livers of mice administered a HCD for 26 weeks (Figure 3-8 A). Annexin V and PI staining suggests this might due to increased cell apoptosis in Ron KO livers as early as when mice were fed a HCD for 13 weeks (Figure 3-8 B). Our results suggest that Ron deficiency exacerbates diet-induced liver injury and lipid deposition associated with increased inflammation and elevated cell apoptosis in the liver.
Figure 3-7: Ron deficiency results in impaired fatty acid metabolism and severe liver injury and inflammation in ApoE KO mice. A) Liver injury markers ALT and AST were measured using sera samples taken from ApoE KO (n=10) and DKO mice (n=10) that were maintained on a HCD for indicated periods. B) The liver weight and the body weight of ApoE KO (n=10) and DKO mice (n=10) were measured after they were maintained on a HCD for various times. The percentage of liver weight over total body weight was calculated. C) Representative Oil Red O staining of cryo-sections of livers from ApoE KO (n=6) and DKO mice (n=5) that were maintained on a HCD for 18 weeks. The pathological scores were quantified. D) Representative H&E staining of livers from ApoE KO (n=6) and DKO (n=5) mice that were maintained on a HCD for 18 weeks. The pathological scores were quantified. E) Gene expression was analyzed with whole livers isolated from ApoE KO (n=6) or DKO (n=5) mice that were administered a HCD for 18 weeks. Values were expressed as mean ± SEM. Student’s T test was performed in with *P<0.05, **P<0.01.
Expression of Ron is reduced in adipose tissue macrophages with weight gain

Throughout weight gain, adipocytes gradually increase lipid storage and secrete adipokines, which attract and activate immune cells in junctions between adipocytes. Macrophages play a key role in regulating adipose tissue inflammation, resulting in glucose homeostasis dysfunction and insulin resistance[25, 132]. A dramatic increase in CD11c+ M1 macrophages has been demonstrated in obese mice and humans and is related to impaired insulin sensitivity. In order to determine whether Ron regulates macrophage phenotypes in the adipose

Figure 3-8: Ron deficiency reduces CD11c+ macrophages in the fatty liver. A) Livers from WT (n=3) and Ron KO (n=3) mice that were administered a HCD for 26 weeks were digested by a collagenase buffer. The liver cell suspension was used for FACS analysis. The representative FACS results show F4/80+ and CD11c+ expression in the live CD45+ fraction. The percentage of F4/80+ CD11c+ cells in the live CD45+ fraction and the number of live CD45+F4/80+CD11c+ cells were quantified. B) Livers from WT (n=5) and Ron KO (n=3) mice that were administered a HCD for 13 weeks were digested. Representative FACS results show Annexin V and PI staining of total liver cell suspension. The percentage of Annexin V+ PI- cells and the percentage of Annexin V+ PI+ cells were quantified. Values were expressed as mean ± SEM. Student’s T test was performed in with *P<0.05.
tissue, we isolated the stromal vascular fraction (SVF) consisting of components of adipose tissue immune cells and vasculature cells other than adipocytes from WT mice that were fed either a RD or a HFD for 9 weeks. We analyzed the macrophage phenotype in the SVF by FACS analysis.

Figure 3-9: Expression of Ron is reduced in adipose tissue macrophages with weight gain. A) The adipose tissue stromal fraction (SVF) of mice that were administered a HFD for 9 weeks was isolated, and the expression of CD11c and F4/80 was analyzed by FACS. B) Ron expression of F4/80+CD11c- fraction in A). C) Ron expression of F4/80+CD11c+ fraction in A). D) Correlation between the percentage of fat weight in total body weight and the percentage of Ron expression in F4/80+ adipose tissue macrophages of mice that were administered a RD or a HFD for 8 weeks and 13 weeks. n=12. E) Quantified FACS results of the percentage of Ron expression on F4/80+ adipose tissue macrophages from mice that were administered a RD or a HFD for 8 weeks and 13 weeks. N=3 for each group. A) B) C) and E) are representative results of two independent experiments. Values were expressed as mean ± SEM. Student’s T test was performed in with **P<0.01, ***P<0.001.
SVF isolated from HFD-fed mice contained a higher percentage of F4/80+ CD11c+ macrophages (Figure 3-9 A). Additionally, we observed that Ron expression decreased in both F4/80+ CD11c- fraction (Figure 3-9 B) and F4/80+ CD11c+ fraction (Figure 3-9 C) with weight gain. Furthermore, Ron expression in F4/80+ CD11c+ fraction was reduced compared with its expression in F4/80+ CD11c- fraction within individual SVF group (Figure 3-9 B and C). The percentage of fat weight in total body weight and Ron expression in F4/80+ macrophages exhibited a negative linear correlation (Figure 3-9 D), suggesting Ron expression in adipose tissue macrophages decreases with the fat weight gain. Ron expression in F4/80+ SVFs was significantly reduced in HFD-fed mice compared to RD-fed mice and the Ron expression was further reduced when the mice were administered these diets for longer period of time (Figure 3-9 E). These results suggest that Ron, preferentially expressed by CD11c- adipose tissue macrophages, is reduced with the weight gain.

**Ron Deficiency results in increased CD11c+ macrophage population in adipose tissue**

To assess whether Ron expression contributes to the CD11c+ macrophage accumulation in adipose tissue, we compared the F4/80+ CD11c+ population in WT and Ron KO adipose tissue collected from mice that were administered a HFD for 13 weeks. The results showed that Ron KO SVF contained a significantly higher percentage of CD11c+ macrophages than WT SVF (Figure 3-10 A). However, we failed to observe any differences in weight gain and insulin sensitivity reflected by insulin resistance test and glucose tolerance test in WT mice or Ron KO mice that were maintained on a HFD for 13 weeks (Figure 3-10 B, C and D). Taken together, these results suggest that Ron plays an important role in regulating activated macrophage phenotype in the adipose tissue.
Figure 3-10: Ron deficiency results in increased CD11c+ macrophages in the adipose tissue. 
A) FACS result of CD11c and F4/80 expression of the SVF from WT (n=3) and Ron KO (n=3) 
mice that were administered a HFD for 13 weeks. The percentage of CD11c+F4/80+ cells in the 
SVF was quantified. B) The body weight of WT and Ron KO mice was measured after the mice 
were maintained on a RD or a HFD for indicated periods. Each group n=6. C) Glucose tolerance 
test result of WT and Ron KO mice that were maintained on a HFD for 8 weeks. WT n=6, Ron 
KO n=6. D) Insulin resistance test result of WT and Ron KO mice that were maintained on a 
HFD for 13 weeks. WT n=6, Ron KO n=6. Values were expressed as mean ± SEM. Student’s T 
test was performed in with *P<0.05.
Discussion

Recent studies suggest that macrophages play an important role in regulating many chronic inflammatory diseases, including diet-induced obesity, atherosclerosis and fatty liver disease. In lean and healthy individuals, resident macrophages present in tissues are in a resting state or exhibit an M2 phenotype, which are important in maintaining immune function and homeostasis. Throughout accumulation of lipids from diet intake, tissue resident macrophages are activated and exhibit a pro-inflammatory M1 phenotype by producing inflammatory cytokines and mediators, such as iNOS, IL-12, IL-1, TNFα. A switch of a pro-inflammatory M1 to an anti-inflammatory M2 phenotype alleviates metabolic disorder progression. Ron expression and activation tilts the balance of macrophage activation towards an M2-like phenotype and away from M1 phenotype during a traditional Th1-mediated response elicited by LPS. Therefore, we tested the hypothesis that presence of Ron attenuates progression of metabolic disorders by regulating the heterogeneity of macrophage populations. We observed a strong positive correlation between Ron expression and an M2 macrophage phenotype in the atherosclerotic plaque and the fatty liver, and Ron expression is reduced in the CD11c+ pro-inflammatory macrophage in the adipose tissue. The presence of Ron in tissue resident macrophages significantly attenuates the progression of atherosclerosis by decreasing plaque size, lipid deposition, aorta inflammation and necrosis. Ron reduces fatty liver disease progression by eliminating lipid deposition, liver injury and inflammation. Ron also decreases the CD11c+ macrophage accumulation in the adipose tissue and potentially attenuates inflammation-induced insulin resistance (Figure 3-11).

However, Ron does not affect atherosclerosis severity or the liver weight gain in ApoE KO mice in the early and middle stages of atherosclerosis development at 5 weeks and 9 weeks on HCD (S. Figure 7). The fact that Ron affects atherosclerosis in the late stage of disease
Figure 3-11: Ron attenuates the progression of diet-induced metabolic diseases. Ron is preferentially expressed by M2 tissue resident macrophages when mice were challenged with a HFD or a HCD, and thus plays a protective role in the progression of diet-induced obesity, atherosclerosis and fatty liver disease. Ron deficient mice exhibit increased severity of atherosclerosis and fatty liver disease, and increased CD11c+ macrophage accumulation in the adipose tissue.
development suggests that the accumulative effect of Ron deficiency-induced inflammatory mediator expression promotes disease progression. Furthermore, the loss of Ron expression in macrophages might impair the development of anti-inflammatory or M2 macrophages facilitating resolution of inflammation, resulting in exacerbated diet-induced inflammation in WT animals. These evidences also suggest that Ron could be used as a M2 marker for tissue resident macrophages in studies of chronic inflammatory metabolic diseases.

A continuum of macrophage phenotypes expressing various cell surface markers manifests at different stages of inflammation. CD11c has been identified as a hallmark for pro-inflammatory macrophages, including adipose tissue macrophages, atherosclerotic macrophages, and Kupffer cells[123][124][125][27]. Our observation that a gradual reduction in Ron expressing macrophages with the progression of metabolic inflammatory diseases is accompanied by an increase in the percentage of CD11c+ macrophages in corresponding tissues. It is possible that the CD11c+ macrophage population that accumulates in the advanced stages of disease fails to up-regulate Ron expression. It is also possible that Ron inhibits CD11c+ macrophage accumulation in the later stages of diseases. However, Ron regulates the population of CD11c+ macrophages differently in various tissues. In the adipose tissue, absence of Ron promotes CD11c+ macrophage accumulation at 13 weeks when mice were fed a HFD; while in the aorta, Ron deficiency does not contribute to the overall expression of CD11c on macrophages when mice where fed a HCD for 18 weeks. It is also possible that absence of Ron promotes CD11c+ macrophage accumulation in the early stages, which is overridden by its pro-apoptotic role in the late stages of disease progression. Ron deficient obese mice with increased CD11c+ adipose tissue macrophages do not exhibit reduced insulin sensitivity when they were maintained on a HFD for 13 weeks. This does not exclude the possibility of an accumulative effect of these pro-inflammatory macrophages on insulin sensitivity in later stages of obesity. Overall, our results
showed a close correlation between the progression of diet-induced chronic inflammation and macrophage Ron expression. However, it is not clear whether the reduced expression of Ron in macrophages with disease progression is caused by infiltration of new macrophages that do not express Ron or a gradual decrease of Ron expression on resident macrophage population.

Receptor tyrosine kinases play an important role in inducing autophagy and preventing cell death [133, 134] [135]. The activation of Ron in macrophages prevents cell apoptosis and secondary necrosis induced by LPS [96]. Deficiency of the related Mer receptor, reduces atherosclerosis progression by decreasing cell apoptosis [100]. Here, this study finds that a higher percentage of apoptotic cells is present in Ron deficient livers of HCD-fed mice. TUNEL results showed that DKO aortas contain more acellular necrotic areas, while ApoE KO aortas display more apoptotic cells when the mice were maintained on a HCD for 18 weeks, suggesting DKO aortas are in a more advanced disease stage than ApoE KO aortas (S. Yu’s unpublished results). These results provide evidence that Ron attenuates chronic disease progression by decreasing cell apoptosis and subsequent secondary necrosis.

Other than limiting cell apoptosis, it is likely that Ron reduces atherosclerosis by regulating lipid metabolism and trafficking in macrophages. Our observation showed that MSP does not induce Pparg expression or PPARγ activation (S. Figure 3). MSP also does not affect the PPARγ-regulated expression of Mcad and Lcad, which encodes proteins that regulate lipid peroxisomal β-oxidation. This result is further confirmed in vivo by the observation that ApoE KO and DKO livers from HCD fed mice have a similar capacity to perform peroxisomal β-oxidation (S. Figure 8). We also fail to detect any difference in PPARγ endogenous ligand - PGJ2 serum levels in the ApoE KO and DKO mice that were administered a HFD (S. Figure 9). These observations suggest MSP/Ron signaling might not affect PPARγ-dependent fatty acid metabolism. Previously, we showed that MSP induces scavenger receptor-A (SR-A)[69] and
lipoprotein lipase (LPL) expression (S. Figure 10) in primary macrophages, which suggests that Ron might promote lipid retention in macrophages. This observation is further confirmed by primary macrophage Oil Red O staining result, which finds that Ron activation by MSP increases the lipid deposition in macrophages (S. Figure 10). These results together seem inconsistent with our observation in vivo that presence of Ron reduces lipid accumulation in the aortas and the livers. It is speculated that presence of Ron might attenuate lipid deposition in the aortas by reducing further monocyte recruitment or by alleviating ER-stressed foam cell death.

One of the challenges for drug development for treating chronic inflammatory diseases is to overcome the redundancy and compensation of many inflammatory cytokines and mediators. Inflammation is such a fine tuned process that inhibition of one critical component of inflammation may trigger another compensatory inflammatory response. Targeting one or a few mediators may not be enough[136]. We demonstrated in both primary macrophages and the diet-induced inflammation in a mouse model that the presence of Ron significantly reduces progression of diet-induced inflammation in various tissues by targeting a range of inflammatory mediators[70]. The ability of Ron to inhibit the expression of Il1b and Tnfα by aortic macrophages, which are clinical therapeutic targets for various chronic inflammatory diseases[39, 137-139], suggests that Ron and its signaling components are potential therapeutic targets for treating diet-induced chronic inflammatory diseases.
Chapter 4
Summary and Future Directions

Diet-induced metabolic diseases, including obesity, atherosclerosis and fatty liver disease, are world’s leading causes of morbidity and mortality. The increased consumption of food, which contains high percentage of calories from fat, leads to the development of these metabolic diseases. These diseases are associated with low-grade chronic inflammation as a result of an imbalance in energy homeostasis. Lipid dysfunction, a main characteristic in these diseases, leads to immune cell activation. Macrophages, as key players in regulating the inflammatory response and lipid metabolism in these diseases, are skewed towards different phenotypes, depending on the cues present in the microenvironment. The receptor tyrosine kinase Ron is expressed on tissue-resident macrophages and plays a protective role in animals of septic shock, acute liver injury, acute lung injury, EAE and type VI hypersensitivity. In this work, we demonstrated for the first time that Ron also plays a protective role in regulating diet-induced metabolic diseases, including atherosclerosis, fatty liver disease and obesity.

We previously showed that Ron suppresses the M1 phenotype in macrophages by decreasing iNOS and IL-12p40 expression [71] [66] [69] [68, 105], while promoting an M2 phenotype by increasing Arg1 expression [70]. The first objective in this study was to identify the mechanism by which Ron regulates the expression of pro-inflammatory genes. We studied the regulation of TLR signaling by Ron in primary peritoneal macrophages treated with LPS as a stimulus to induce inflammation. LPS-induced inflammatory gene expression is controlled by both MyD88/TIRAP and TRIF/TRAM pathways. This study finds that Ron and its ligand, MSP, modulate TLR4 signaling downstream of MyD88/TIRAP resulting in negative regulation of transcription of pro-inflammatory genes. MSP enhances phosphorylation of Stat3 and expression
of Bcl3 (S. Figure1), which promotes anti-inflammatory gene transcription. Downstream of TLR4, MSP promotes robust phosphorylation of MAPKs, which could be a mechanism of turning down LPS induced inflammation. While Ron does not regulate receptor proximal signal events downstream of TLR4, MSP impedes IκB degradation as a result of increased IKK activity and phosphorylation of IκB, enhancing sequestration of NF-κB by IκB in the cytosol, which results in less NF-κB binding to the DNA in the nucleus. Furthermore, the expression of IκBζ, a positive regulator of LPS-induced inflammatory gene expression, is reduced by MSP. These findings, at least partially, explain the observation that MSP limits LPS-mediated pro-inflammatory gene expression, including Il12b, Il1b, Tnf and Inos, which are all NF-κB-driven genes. These findings provide possible mechanistically explanation that Ron regulated macrophage activation plays a protective role in many acute and chronic inflammatory disease models, including septic shock, acute liver injury, acute lung injury, EAE and type VI hypersensitivity.

Given the role of Ron in regulating macrophage heterogeneity, the second objective of this study was to test the hypothesis that Ron alleviates the progression of diet-induced metabolic diseases by balancing the macrophage activation towards an anti-inflammatory phenotype and away from a pro-inflammatory response. The results showed that Ron is preferentially expressed by M2 compared to M1 in the atherosclerotic aorta, the fatty liver and the adipose tissue. The expression of Ron decreases in the corresponding tissue resident macrophages with the when the animals were maintained on a HFD or a HCD. These results suggest that Ron might be an important regulator in maintaining the M2 phenotype of tissue resident macrophages, which play an important role in balancing energy homeostasis and local immune responses. To further confirm these results, we demonstrated that mice with a global deletion of Ron exhibit more severe atherosclerotic lesions and liver injury, correlated with an increase in the M1 macrophage
population and overall increased inflammatory cytokine expression in the relevant tissues. The Oil Red O staining of both aortas and livers suggests that Ron deficient mice deposit more fat in the disease tissues, indicating that Ron might be a regulator of lipid metabolism. However, there is not significant difference in the total weight gain or the epididymal fat weight in Ron deficient mice when compared to control mice (S. Yu unpublished results). These results suggest that the regulation of lipid metabolism by Ron might be tissue specific.

Regardless of the finding that Ron does not alter the weight of epididymal fat and insulin sensitivity when the mice were maintained on a HFD for 13 weeks, the accumulation of CD11c+ macrophages in the adipose tissue is significantly higher in Ron knockout mice. However, we show the percentage of CD11c+ population in aortic lesions is not altered in Ron knockout mice when the mice were fed a HCD for 18 weeks, and CD11c+ population significantly decreases in the liver when the mice were fed a HCD for 26 weeks. These results imply two possibilities: 1) Ron expressed by these tissue resident macrophage population plays an anti-apoptotic role, and lose of functional Ron on these macrophages promotes apoptosis of these pro-inflammatory CD11c+ macrophage population with the progression of metabolic diseases; 2) Ron regulates the accumulation of CD11c+ macrophage population differently in various tissues. To address these questions, future investigators need to compare the accumulation of CD11c+ macrophages in various tissues at different time points. These results also suggest that CD11c might not be a universal M1 marker, and more macrophage cell surface markers need to be identified to distinguish macrophage subsets within the context of disease tissues.

M2 macrophages can be further divided into M2a (induced by IL-4 or IL13) and M2b (induced by immune complexes plus LPS), which exert immunoregulatory function and drives type II responses, and M2c (induced by IL-10 or transforming growth factor-β), which are related to suppression of immune responses and tissue remodeling. These different subsets of
macrophages express various cytokines and surface markers[140]. In order to identify Ron expressing macrophages are of an M2 phenotype, we compared the gene expression of Ron- and Ron+ macrophages from atherosclerotic aortas and fatty livers. In accordance with our previous findings that Ron+ macrophages are associated with an M2 phenotype in a tumor model, we find Ron+ macrophages from the aorta and the liver express robustly higher levels of Arg1 and lower levels of Inos. To identify whether Ron is expressed by all M2 macrophages or a specific subset, we compared Ron expression in M2a, M2b and M2c primary peritoneal macrophages that were induced by stimuli indicated above. The result showed that Ron is preferentially up-regulated in IL-4 induced M2a subset, not in M2b or M2c subsets (S. Yu unpublished results). Taking into account that Ron also promotes a range of markers expressed by M2a macrophages, including SR-A, mannose receptor, IL-1ra and polyamines and playing a role in both immune regulation and tissue remodeling, Ron could be used as a marker for identifying M2a macrophages. With the progression of metabolic diseases, CD11c+ M1 macrophage population in aorta, fatty liver and adipose tissue increases, while Ron expression in these cells decreases. Taken together, these findings suggest Ron, associated with an attenuated metabolic disease phenotype, could serve as a potential marker for M2 macrophage activation.

Despite these findings, many questions still need to be addressed to further confirm the function of Ron in these metabolic diseases. First, is the overall severe metabolic phenotype in Ron deficient mice attributed by the macrophages or endothelial cells that are lacking Ron expression? In atherogenesis, endothelium as the border between circulating blood cells and components of blood vessel is one of the key players in regulating the disease development. It allows transmigration of monocytes into the intima of the aorta where they differentiate into macrophages, which result in the formation of lipid-laden plaques. We demonstrated that Ron deficient aortic endothelial cells express higher level of Inos. In vitro cell culture model suggests
that MSP/Ron signaling decreases the expression of pro-inflammatory mediators in endothelial cells in a macrophage-dependent manner. The gene expression analysis of the sorted aortic macrophages shows that Ron expression in these macrophages alleviates the expression of M1 hallmarks, which contributes to, at least partially to, an attenuated phenotype of atherosclerosis. Since macrophages are not the only source of Ron expression, a more in-depth investigation of the role of macrophage-specific Ron expression will require employment of other mouse models, including a macrophage-specific Ron deficient mouse model or a macrophage-specific Ron transgenic mouse model. These mice can be crossed with ApoE KO mice to assess diet-induced metabolic diseases. Furthermore, the future investigators can perform bone marrow transplant using CD45.1 ApoE KO mice as recipients and either CD45.2 WT or Ron KO mice as donors. These mice can then be assessed for metabolic disorders’ progression upon being challenged with a HCD. These approaches will clarify whether hematopoietic stem cells are the source of Ron that mediates disease progression. However, the last approach might be problematic that tissue resident macrophages have a much slower turn over following irradiation than other cells of the immune system.

The second question arises if we observe phenotype differences between WT and macrophage-specific Ron deficient mice in an ApoE KO background, but fail to observe any differences in the phenotype of recipients received either WT or Ron KO bone marrow in the bone marrow transplant model – What is the alternative possible source of macrophages that expression Ron other than hematopoietic stem cells? It was reported that F4/80bright tissue resident macrophages are derived from yolk sac-derived precursors, which reside in the tissues during embryonic development. These precursors give rise to brain microglia, liver Kupffer cells, epidermal Langerhans cells and macrophages in kidney, lung, spleen and other tissues, and are maintained in adult tissues through local self-renewal [2] [3, 5] [6]. In addition, Sca1+ CD45+
progenitor cells, identified in aorta adventitia, give rise to CFU-M and further differentiate into tissue resident macrophages (Peter Psaltis’s unpublished results). It is possible that these progenitor cells in the adventitia are derived from yolk sac. It also is possible that Ron+ macrophages are derived from yolk sac instead of bone marrow, which could explain the reason why Ron is expressed on tissue resident macrophages, but not bone marrow-derived macrophages or monocyte-derived macrophages. To determine whether yolk sac is the origin of Ron expressing macrophages, future investigators may examine Ron expression in mice that are deficient in Myb, which is dispensable for yolk sac myelopoiesis but is required for the development of hematopoietic stem cells. It will also be interesting to determine whether Ron is expressed on macrophages isolated from E14.5 and E16.5 fetal organs, such as liver, and whether Ron is expressed by F4/80 bright yolk sac-derived macrophages instead of F4/80 low typical monocyte-derived macrophages in adult livers and other tissues.

The third question that needs to be addressed is whether Ron attenuates these diseases in a ligand dependent or independent manner. According to literatures, MSP, Ron’s ligand, is mainly made in the liver in a pro-MSP form and circulates to the inflammatory site where it is activated by being cleaved by serine proteases [141] [58]. However, how the level and activation of MSP is regulated mains unknown. The observation that MSP co-localizing with Ron in the athero-plaques interests us to find out the origin of MSP in athero-plaques. To address this question, the future investigators should examine the RNA expression of MSP in the liver as well as relevant disease tissues. It will also be interesting to know whether the circulating pro-MSP level and the activation of MSP is regulated by HFD or HCD. To examine Ron needs to be activated by its ligand to exert its function in attenuating metabolic diseases, we can examine whether MSP deficient mouse or blocking the interaction between MSP and Ron using a neutralizing antibody exacerbates progression of these diseases.
The fourth question is to identify whether Ron can be used as a marker of anti-inflammatory macrophage and what regulates Ron expression on these macrophages. Given the results there is a strong correlation between Ron expression and the existing macrophage subset markers as shown in this study and previous studies, Ron might be a potential marker indicating the inflammatory status of metabolic disease progression. The underlying mechanism of the regulation of Ron expression by the development of metabolic diseases needs to be unrevealed. On one hand, the observation that Ron expression decreases with an increased accumulation of M1 macrophage population in metabolic disease tissues might be explained by the components of HFD or HCD regulating Ron expression in these macrophages. It will be interesting to investigate whether saturated fatty acids or oxLDL inhibits Ron expression on tissue resident macrophages.

On the other hand, future studies should identify Ron expression pattern in tissue resident macrophages along the progression of diseases, including the onset of inflammation, development and progression of disease phenotype and resolution of inflammation upon therapeutic treatment. This could provide evidence whether Ron can be used as a marker for M2 macrophage activation in diseases. Metformin, a widely used anti-diabetic drug, induces expression of atypical orphan nuclear receptor SHP, which is a negative regulator of inflammation. Given the report that Ron down-regulates LPS-induced inflammation through inducing the expression of SHP [142], we tested whether metformin regulates SHP through a Ron-dependent manner. However, we and others detected both bone marrow-derived macrophages and primary peritoneal macrophages express very low levels of SHP, and the expression of SHP is not induced by Ron (S Yu’s unpublished results). Other than traditional pharmaceutical drug, diet supplemental regimens for treating diet-induced metabolic disorders are also popular research topics. Omega-3 fatty acids present in fish oil are identified to play an important role in reducing inflammation of cardiovascular diseases and prevention of cancer [143] [144]. Polyphenols found in cocoa and
grape seeds reduces macrophage-mediated inflammation associated metabolic diseases in various tissues, including the adipose tissue and the aorta [145] [146] [147]. Given the therapeutic potential of these compounds found in natural food products, it will be interesting to identify the correlation between Ron expression in local tissue resident macrophages and the regulation of metabolic diseases by omega-3 fatty acids and polyphenols.

This work provides evidence for a direct role of Ron in regulating pro-inflammatory gene expression in macrophages in vitro, and in modulating inflammation and disease progression of diet-induced metabolic disorders in vivo. Therefore, these studies add to the current understanding regarding the role of Ron receptor tyrosine kinase in balancing macrophage activation and regulating diet-induced immune responses. These results provide evidence that as a potential marker for M2 macrophage activation and an indicator for the stage of metabolic disease progression, Ron is a candidate of therapeutic treatment for diet-induced metabolic disorders.
Appendix: Supplementary information

Methods

Reagents. The following reagents were obtained from the indicated sources: Antibodies for Western blot against Bcl3 and c-Fos were purchased from Santa Cruz Biotechnology (Dallas, Texas). Chemicals used for cell culture, including U0126, SB203580, SP600125, SC560, Celecoxib, Indomethacin and GW9662, were purchased from Cayman Chemical (Ann Arbor, MI). Native LDL and oxLDL were purchased from Kalen Biomedical (Savage, MD). Δ^{12}-PGJ_{2}, Δ^{12}-PGJ_{3}, PGD_{2}, PGD_{3} and EPA-BSA were kindly provided by Dr. Sandeep Prabhu.

Luciferase assay. 293 cells stably were plated overnight in 24-well plates and then transfected using the Mirus transfection reagent (Mirus Bio, Madison, WI), according to the manufacturer’s recommendations. The cells were transfected with PPRE luciferase (a gift from Dr. Jack Vanden Heuvel, Penn State) and the indicated concentrations of MSCV-mRon-HA. 24 hours after transfection, the cells were stimulated for 24 h with 100 ng/ml MSP. Cell lysates were harvested and the luciferase assay was performed using a Turner Designs TD-20/20 luminometer (Sunnyvale, CA). Luciferase assay reagents were purchased from Promega (Madison, WI).

Measurement of liver Peroxisomal β-oxidation. Liver Peroxisomal β-oxidation was measured as described previously with some modifications[148]. Briefly, 100 mg liver was homogenized in 9 vol of cold 0.25 M sucrose. Samples were centrifuged at 600 g for 10 min, and the upper lipid layer was aspirated. 50 µl of a 10% Triton X-100 was added to each 450 µl sample supernatant. The samples were then assayed for peroxisomal β-oxidation in the presence of potassium cyanide. Which inhibits mitochondrial β-oxidation. The oxidation of palmitoyl-CoA was quantified spectrophotometrically by measuring the reduction of nicotinamide adenine
dinucleotide-positive at 340 nm. The rate of nicotinamide adenine dinucleotide-positive reduction is directly related to the rate of fatty acid oxidation.

Arginase activity assay. Cells were lysed with 100 µl of 0.1% Triton X-100 and incubated at room temperature on a shaker for 30 min. 100 µl of 25 mM Tris-HCl (pH 7.5) were added to the cell lysate. 10 µl of 10 mM McCl2 was added to 100 µl of the cell lysate mix. The enzyme is activated by heating at 56 °C for 10 min. Arginine hydrolysis was conducted by incubating the lystate with 100 µl of 0.5 M L-arginine (pH 9.7) at 37 °C for 60 min. The reaction was stopped with 800 µl of H2SO4 (96%)/H3PO4 (85%)/H2O (1/3/7, v/v/v). 40 µl of α-isonitrosopropiophenone was added to each reaction before being heated at 100 °C for 30 min. The urea concentration was measured at 550 nm.

ELISA. Cell culture supernatants were collected and centrifuged at 2000 rpm to pellet cell debris. Serum was collected by centrifuging the blood that was stored overnight at 4 °C. Protein production of 15d-PGJ2 was assessed with an ELISA kit as instructed by the manufacture’s instruction (Cayman Chemical, Ann Arbor, MI).

Semi-quantitative RT-PCR. RNA was extracted from cultured cells using Trizol (Invitrogen). 2 µg of RNA was used for reverse transcription using Applied Biosystems High Capacity TR Kit according to manufacture’s instructions. PCR for Mcad, Lcad, Lpl and actin was carried out in an Eppendorf Mastercycler Pro. Primer sequences are as follows: Mcad Forward primer 5’-GACTCCGGTCGCCGAACAC-3’, Mcad Reverse primer 5’-CCTCCGCGCATGGGATCCGC-3’, Lcad Forward primer 5’-GCCCTCCGCCCCATGTTCTC -3’, Lcad Reverse primer 5’- CTTCGCCCCGTCATCGG-3’, Lpl Forward primer 5’-CGCACGACGAGGCGAAGAG-3’, Lpl Reverse primer 5’-GCCAGCAGCATGGGCTCACA-3’.
Statistical Analysis. Data are presented as the mean ± standard error of the mean (SEM). Student T test, paired student T test or one-way ANOVA was used for statistical analysis with * P < 0.5, ** P < 0.01, *** P < 0.001 and **** P<0.0001. All analysis was performed using GraphPad Prism 5.0 (San Diego, CA).
**S. Figure 1: MSP induces Bcl3 expression.** Primary peritoneal macrophages were collected and stimulated with 100 ng/ml MSP. The cell lysates were collected at indicated times. Bcl3 expression was assessed by western blot. The results are representative of two independent experiments.
**S. Figure 2: MSP increases 15d-PGJ₂ protein production.** A) Primary peritoneal macrophages were collected and treated with 100 ng/ml MSP. Cell culture supernatant was collected at indicated times and 15d-PGJ₂ levels were assessed by ELISA. B) Primary peritoneal macrophages were collected and treated 100 ng/ml MSP overnight followed by 100 ng/ml LPS treatment. Cell culture supernatant was collected at indicated times and 15d-PGJ₂ levels were assessed by ELISA. The results are representative of two independent experiments.
S. Figure 3: MSP does not induce *Pparg* expression or PPARγ binding ability. A) Primary peritoneal macrophages were treated with 100 ng/ml MSP. Gene expression of *Pparg* was assessed by real-time PCR. B) Primary peritoneal macrophages were treated with or without 100 ng/ml MSP overnight before stimulation with LPS for various times. Gene expression of *Pparg* was assessed by real-time PCR. C) PPRE-luc plasmid was co-transfected with MSCV-STK plasmid into HEK293 cells. The cells were stimulated with or without MSP before being collected for luciferase activity assay. The results are representative of two independent experiments.
S. Figure 4: Ron inhibits LPS-induced IL12p40 production by macrophages in a COX1/2-dependent, but PPARγ-independent manner. A) WT and B) Ron−/− primary peritoneal macrophages were isolated by lavage and adherence to plastic. The cells were cultured overnight in the presence or absence of 100 ng/ml MSP together with the indicated inhibitors or vehicle DMSO followed by stimulation with 100 ng/ml LPS for 24 hours. The level of IL-12p40 in the supernatant was assessed by ELISA and normalized to the cell lysate protein content. The fold induction of IL-12p40 was obtained by normalizing the MSP treated group to the corresponding MSP non-treated group. The results are representative of two independent experiments.
S. Figure 5: MSP does not significantly affect LPS-induced Cox1 or Cox2 expression. Primary peritoneal macrophages were treated with or without 100 ng/ml MSP overnight before stimulation with LPS for various times. Gene expression of A) Cox1 and B) Cox2 was assessed by real-time PCR. The results are representative of two independent experiments.
S. Figure 6: $\Delta^{12}$-PGJ$_2$, other PGs and EPA increased expression of Ron. A) Primary peritoneal macrophages were treated with different PGs and EPA for various times. Gene expression of Ron was assessed by real-time PCR. B) Primary peritoneal macrophages were treated with PGs at various dosages for 10 hours. Gene expression of Ron was assessed by real-time PCR. C) Ron expression in the live F4/80+ peritoneal macrophages was analyzed by FACS after the cells were treated with $\Delta^{12}$-PGJ$_2$ or EPA-BSA for 24 h. The results are representative of two independent experiments.
S. Figure 7: Macrophage marker expression of aortas and liver weights of mice that were administered a HCD for 5 weeks and 9 weeks. Aortas were collected from WT, Ron KO, ApoE KO and DKO mice that were administered a HCD for A) 5 weeks and B) 9 weeks. Gene expressions of macrophage markers of aortas were examined. 5 weeks WT n=3, Ron KO n=9, ApoE KO n=5, DKO n=7. 9 weeks WT n=7, Ron KO n=7, ApoE KO n=7, DKO n=6. C) The percentage of Liver weight in total body weight of WT (n=2), Ron KO (n=4), ApoE KO (n=2), DKO mice (n=4) that were maintained on a HCD for 5 weeks was calculated. D) The percentage of Liver weight in total body weight of WT (n=7), Ron KO (n=8), ApoE KO (n=8), DKO mice (n=11) that were maintained on a HCD for 9 weeks was calculated.
S. Figure 8: Ron does not affect PPARγ controlled lipid peroxosomal β-oxidation in the liver. A) Primary peritoneal macrophages were treated with MSP for various times. Gene expression of *Lcad* and *Mcad* was determined by real-time PCR. The results are representative of two independent experiments. B) ApoE KO and DKO Livers were collected from mice that were maintained on a HCD for 13 and 18 weeks. Peroxisomal β-oxidation was performed by measuring the rate of NAD+ converting into NADH. 13 weeks ApoE KO n=6, DKO n=9. 18 weeks ApoE KO n=7, DKO n=5.
S. Figure 9: Ron does not regulate 15d-PGJ\textsubscript{2} levels in the serum of ApoE KO mice on a HFD. Serum was collected from WT (n=4), ApoE KO (n=7) and DKO (n=5) mice that were maintained on a HFD for 13 weeks. Serum 15d-PGJ\textsubscript{2} levels were measured by ELISA.
S. Figure 10: MSP induces $Lpl$ expression in the primary macrophages. Primary peritoneal macrophages were collected and treated with 100 ng/ml MSP for various times. Gene expression of $Lpl$ was assessed by semi-quantitative RT-PCR. The results are representative of two independent experiments.
S. Figure 11: MSP increases the oxLDL accumulation in primary macrophages. Primary peritoneal macrophages were isolated and treated with 100 ng/ml MSP overnight. Cells were incubated with either native LDL or oxLDL for 8 hours before they were fixed and stained with Oil Red O. The results are representative of two independent experiments.
S. Figure 12: MSP induces Arg1 expression in primary macrophages in a MAPK-dependent manner. A) Resident peritoneal macrophages were stimulated with DMSO, 10 µM U0126, 10 µM SB203580, or 20 µM SP600125 for 2 h followed by stimulation for 24 h with 100 ng/ml MSP or 5 ng/ml IL-4, and arginase activity was assessed. Results are the average of two independent experiments. B) Primary peritoneal macrophages were isolated and stimulated with either MSP or IL-4 for various times. Cell lysates were collected and C-Fos expression was assessed by western blot. The results are representative of two independent experiments. Values of A) was expressed as mean ± SEM, and results are the average of two independent experiments with duplicates in each real-time PCR experiment. *** P<0.001.
S. Figure 13: Gene expression of M1 and M2 macrophage markers in peritoneal macrophages that were collected from lean and fat ApoE KO mice. Peritoneal macrophages were collected from ApoE KO mice that were either administered a RD or a HFD and treated with 100 ng/ml MSP. Gene expression of macrophage markers was assessed by real-time PCR.
S. Figure 14: MSP affects expression of Matrix Metalloproteinases (Mmp) in primary macrophages. Primary macrophages were isolated and treated with MSP overnight followed by LPS stimulation for 4 hours. Gene expression of Mmp2, 9, 13, and 14 was analyzed by real-time PCR. The results are representative of three independent experiments.
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03/2012 Ron, a receptor tyrosine kinase, attenuates atherosclerosis and liver injury in hypercholesterolemic mice (Poster). Graduate exhibition. Pennsylvania State University.