HEPATOCYTE GROWTH FACTOR REGULATES INFLAMMATORY MEDIATED DISEASES BY SUPPRESSION OF IL-6: IMPLICATIONS FOR TYPE 2 DIABETES

by

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HEPATOCYTE GROWTH FACTOR REGULATES INFLAMMATORY MEDIATED DISEASES BY SUPPRESSION OF IL-6: IMPLICATONS FOR TYPE 2 DIABETES

Gina Marie Coudriet, Ph.D.

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The generation of the pro-inflammatory cytokines IL-6 and TNF-α by macrophages recruited to adipose tissue facilitates obesity-induced inflammation resulting in insulin resistance and type 2 diabetes (T2D). Increased adipose tissue is associated with inflammation and expression of acute phase response (APR) proteins secreted by the liver. Proper homeostasis of the liver is regulated by IL-6-depdendent expression of Hepatocyte Growth Factor (HGF) upon cleavage to its active form (aHGF) by the urokinase-type plasminogen activator (uPA). Plasminogen Activator Inhibitor Type-1 (PAI-1) is a pro-thrombotic APR protein known to inhibit the function of uPA; however, since HGF's activation, interaction and signaling through its receptor, MET are dependent upon uPA, PAI-1 is also capable of regulating the function of hepatic HGF. *In vitro* data demonstrates that aHGF significantly suppressed IL-6 production by macrophages stimulated with LPS via an increase in phosphorylation of GSK3β, rendering it inactive. Phosphorylated GSK3β correlated with increased retention of the phosphorylated NF-κB p65 subunit in the cytoplasm and an enhanced interaction between CBP and phosphorylated CREB resulting in IL-10 cytokine production. These changes were a direct result of signaling through MET, as effects were reversed in the presence of a selective inhibitor of MET (SU11274) or when using BMM from macrophage-specific conditional MET knockout mice.

It is known that obese T2D patients present with an accumulation of PAI-1, which we hypothesize, results in the inactivation of HGF. The loss of HGF-MET signaling results in

increased active GSK3β and the progression to unchecked inflammation and disease progression. *In vivo* studies using male, C57BL6 mice on a high fat diet alongside control fed mice demonstrates move severe hepatic steatosis in obese mice at 44 weeks compared to control. Steatosis coincided with the decrease in aHGF and elevated levels of PAI-1 protein. These results demonstrate that elevated levels of PAI-1 inhibit aHGF, leading to unresolved chronic inflammation in obesity and T2D.

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PREFACE

Doctor of Philosophy, abbreviated Ph.D., for the Latin *philosophiae doctor*, meaning "teacher in the love of wisdom"

The experience of graduate school can be described as a journey; trying, frustrating, time consuming, and just plain hard. But the truth is that on the day of your defense, as you sit back and reflect on the last six, long, and arduous years of your life, the thought of those years no longer evokes feelings of such distress. Suddenly, as you realize that the end of the trek is only moments away, and you recognize the significance of what you have achieved, suddenly the only feeling you have is pure, unadulterated, bliss.

I'd like to first thank those that have been a source of information and guidance from the beginning of my graduate career: the Office of Graduate Studies. Led by Sandra Honick, the staff, including Cindy Duffy, Veronica Cardamone, Jennifer Walker, and Carol Williams has all been instrumental in getting us newbie graduate students acclimated to our new environment. Also I'd like to thank our dean at the time, Dr. Stephen Phillips whose dedication and commitment to mentoring students is greatly appreciated and will never be forgotten.

As a student in the Cellular and Molecular Pathology (CMP) Graduate Training Program, I would like to thank the director of our program, Dr. Wendy Mars. Not only is she my co-mentor and thesis committee chair, she is an unlimited source of information, direction and guidance to the entire CMP graduate population. Her commitment to mentoring students has

been underscored this year as she was named the 2010 Distinguished Mentor Award winner. Additionally, I would like to thank Shari Tipton, the CMP administrator, for keeping the students organized and well-informed about issues within the department.

I would now like to thank the Rangos Research faculty and staff. Patrick Hnidka, Darlene Noah, and Bernice Johns are always ready and willing to help with administrative issues with a smile on their face. The entire faculty is always ready and eager to help with scientific questions and the international community of post-docs, graduate students, and technicians make the everyday life of a research scientist much more bearable. I would like to celebrate the memory of Robert Johns. I wish he could be here so that I could thank him myself, but somehow, I know he hears me. Finally, I would like to give a heart-felt thank you to Dr. Massimo Trucco for endless support, guidance, and for giving me the opportunity to work with the entire Diabetes Institute research community.

Within the structural walls of the Rangos Research center are the open arms of my lab family: The Piganelli Lab. Past and present, the members of this family have been the support and encouragement that has brought me to the place I stand today. The first two graduate students to fly from the nest, Dr. Sheila Schreiner and Dr. Martha Sklavos, continue to be a source of friendship stretching over state lines. Dr. Hubert Tse, also a life long friend and mentor continues to be a source of scientific collaboration. Meghan Delmastro, currently a graduate student in our lab, has gotten me through perhaps the hardest parts of graduate school and life. I thank you for letting me vent my frustrations numerous times, for sharing in my successes, and I thank you for being my friend. The newest addition to our family is JT Coneybeer who I thank again and again for much needed comic relief! It is a joy to have you as a part of our lab. And finally to our loyal technician, Jen Profozich, I thank you for fulfilling our

every biological reagent and office supply need! And also, thank you for your support, encouragement, and friendship throughout my laborious journey of grad school.

Now, to the man who had made all of my success possible, my mentor and friend, Dr. Jon Piganelli. The first time you meet Jon, you can immediately see why people like him: he's funny. Not only is he funny, he's an excellent researcher, extremely approachable, and he is willing to give all that he can in order to collaborate and contribute to the field of science. When you join the Piganelli lab, you join the family, and as the head of the family, Jon will support you 100%. Even as graduate students, he listens to our ideas while fostering our creativity. First and foremost, Jon is a mentor, sitting on numerous thesis committees contributing his knowledge throughout the university. His passion for research and mentoring has brought me many successes throughout my graduate career and I thank him for that. I am honored to have trained in his lab, and also to continue my training and transformation into an independent researcher with him in the future.

In addition to Dr. Piganelli, my entire thesis committee: Dr. Dong, Dr. Giannoukakis, Dr. Liu, Dr. O'Doherty, and Dr. Mars have all been instrumental in getting me to this point. Jon and Wendy have been intimately involved with my training, as co-mentors and Henry and Nick have been especially helpful through daily interactions at Rangos. Youhua, thank you for all of your prior work on HGF and MET upon which I built my thesis and I thank Rob for your expertise in metabolism and for always pushing me to be the best that I could be. I thank you all for your invaluable guidance and support in making my thesis what it is today.

I'd be remiss if I forgot to thank these folks: my family. I've been fortunate for not only my immediate family, but for my married family. Luckily, these two families have become one and serve as a tremendous source of support and inspiration. I thank my siblings: Diane, Philip,

and Sally as well as Michelle and Ashley for unconditional love. I also want to thank my niece and nephews: Ricky, Anthony, Sal, Isabella and Carmen who continue to make me smile as I watch them grow up into little adults. Many thanks to my parents Salvatore and Linda, and my mother-in-law and father-in-law Posey and Larry: I couldn't ask for a better set of parents! To say that my mother has supported me is an understatement. She listens unconditionally giving me invaluable advice, always supporting and believing in me. She is a true inspiration, always putting herself before others. I love you mom!

Finally, to my other half Gregory, I thank you more than words can express. Your love and support are immeasurable. But most of all, you take me as I am, the good and the bad, exhibiting patience like none other. We have already built a lifetime of memories, and I am honored to live out a lifetime of more with you. Having you by my side throughout this journey has truly inspired me to drudge through the rough times so that we could celebrate the good. For all of this, I thank you. I love you.

1.0 INTRODUCTION

1.1 INNATE IMMUNITY

Our immune system equips us with a set of biological mediators to fight infection and disease and eventually regain homeostasis. The immune system is divided into two arms: the innate and the adaptive. Innate immunity, present in almost all multicellular organisms, is the more primitive of the two components and acts as the first line of defense against invading pathogens[1]. How the host senses these microbes through receptor-ligand interaction is a complex and critical event in host-pathogen recognition and clearance. Cells of the innate immune system include granulocytes, macrophages, dendritic cells, mast cells, neutrophils, eosinophils, and natural killer cells[2]. These cells possess germ-line encoded pattern recognition receptors (PRRs), which have been evolutionarily selected to recognize conserved pathogen-associated molecular patterns (PAMPs)[3]. The PRRs allow the innate immune system to quickly recognize foreign molecules and begin to mount a potent immune response via activation of intracellular signaling cascades including 1) generation of reactive oxygen and nitrogen species, 2) production of cytokines, chemokines, adhesion molecules, and acute phase proteins, and 3) upregulation of costimulatory molecules as signals for T cell activation[3]. Therefore, the hallmark of innate immunity resides in a select group of highly conserved microbial antigens resulting in an immediate effector response against infection.

On the other hand, adaptive immunity is comprised of specialized lymphocytes including CD4+ and CD8+ T cells (MHC class II and MHC class I restricted, respectively) and antibody producing B cells[4]. The receptors of adaptive immune cells are generated somatically, giving them structural uniqueness; however, these receptors remain exclusive to the host and cannot be passed on to their progeny[1]. The large and diverse population of lymphocytic antigen receptors capable of specific antigen recognition and immunological memory are what set the two arms of the immune system apart. As a result, each lymphocyte has a unique receptor and participates in antigen-specific clonal expansion, which can take from 3 to 5 days and is necessary in order to mount an efficient immune response[1]. Consequently, the rapid induction of the innate immune system serves to restrict pathogen load until the adaptive immune system has expanded enough to exert specific clearance of the infectious agent (Figure 1).

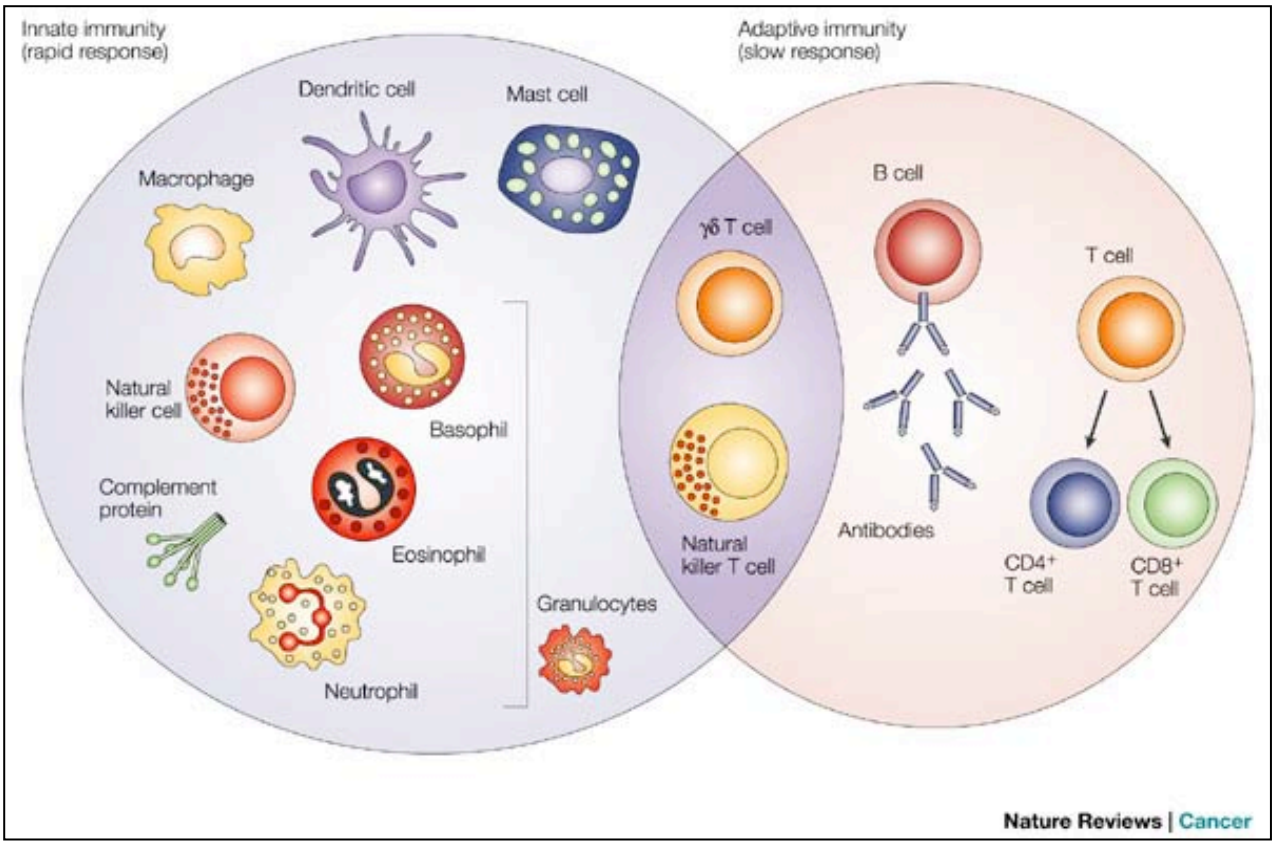


Figure 1. The Innate and Adaptive Immune Response

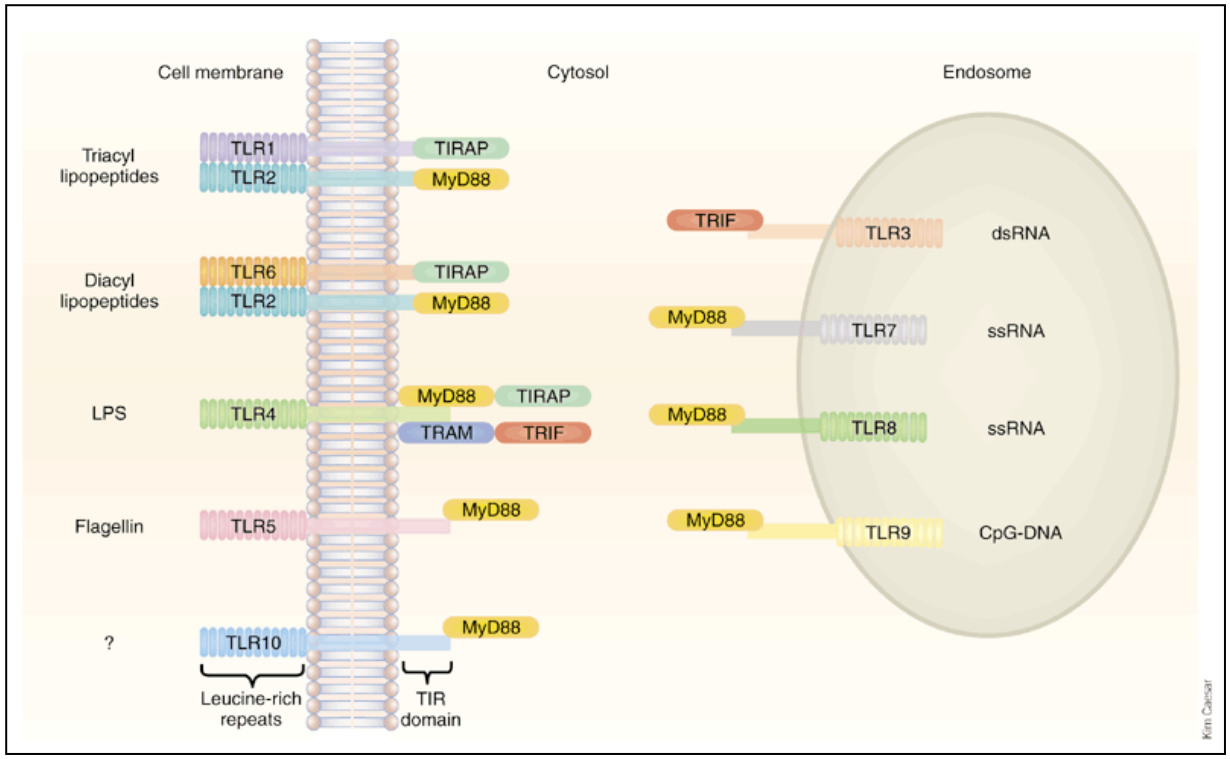
The innate immune response functions as the first line of defense against infection. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. Figure taken from[5].

1.1.1 Toll-like receptors

One group of PPRs, the Toll family of receptors, was first identified in Drosophila as contributing to the signaling cascade responsible for dorsoventral polarity in fly embryos[6]. This family of receptors, referred to as the toll-like receptors (TLRs), has been identified in mice and humans as playing a key role in inducing inflammation. TLRs are transmembrane proteins with large leucine-rich repeat extracellular domains. Their cytoplasmic domains are similar to the interleukin-1 receptor and therefore, induce nuclear factor-κB (NF-κB) activation to

perpetuate the innate immune response[6]. Ten TLRs have been identified in humans and 12 in mice, where TLR1 through TLR9 are conserved in both[7]. TLRs are more sophisticated than other PPRs because they are able to recognize and differentiate between various types of pathogens and require the recruitment of adapter proteins for signal transduction. TLR1, 2, 4, 5, 6, and 11 are expressed on the cell surface and recognize microbial membrane components including lipids, lipoproteins, and proteins. TLR3, 7, 8, and 9 recognize microbial nucleic acids and are present in intracellular vesicles such as the endoplasmic reticulum (ER), endosomes, lysosomes, and endolysosomes [7,8](Figure 2).

Figure 2. Human Toll-like Receptors



Schematic diagram of human Toll-like receptors showing adaptors, cellular orientation and examples of ligands. Figure taken from[8].

TLR2 and TLR4 both function as important receptors in innate immunity. TLR4, the receptor for lipopolysacharride (LPS), was the first TLR identified in humans[6]. The TLR4-LPS interaction induces NF-κB activation to perpetuate the innate immune response. LPS, a component of gram-negative bacteria, binds to the serum protein, LPS-binding protein, which transfers LPS to CD14, a macrophage receptor anchored to the cell surface by a glycosylphosphoinositol tail. MD-2 is another protein required for TLR4-mediated recognition of LPS. CD14 and MD-2 then facilitate binding of LPS to TLR4. Upon activation, TLR4 forms a homodimer and the adapter protein MyD88, which is associated with the serine-threonine protein kinase IL-1 receptor-associated kinase (IRAK), is recruited to the membrane. IRAK autophosphorylates and dissociates from the receptor complex, which recruits the TNF receptor-associated factor 6 (TRAF-6) adapter protein, leading to the activation of downstream signaling pathways, including NF-κB[1] (Figure 3).

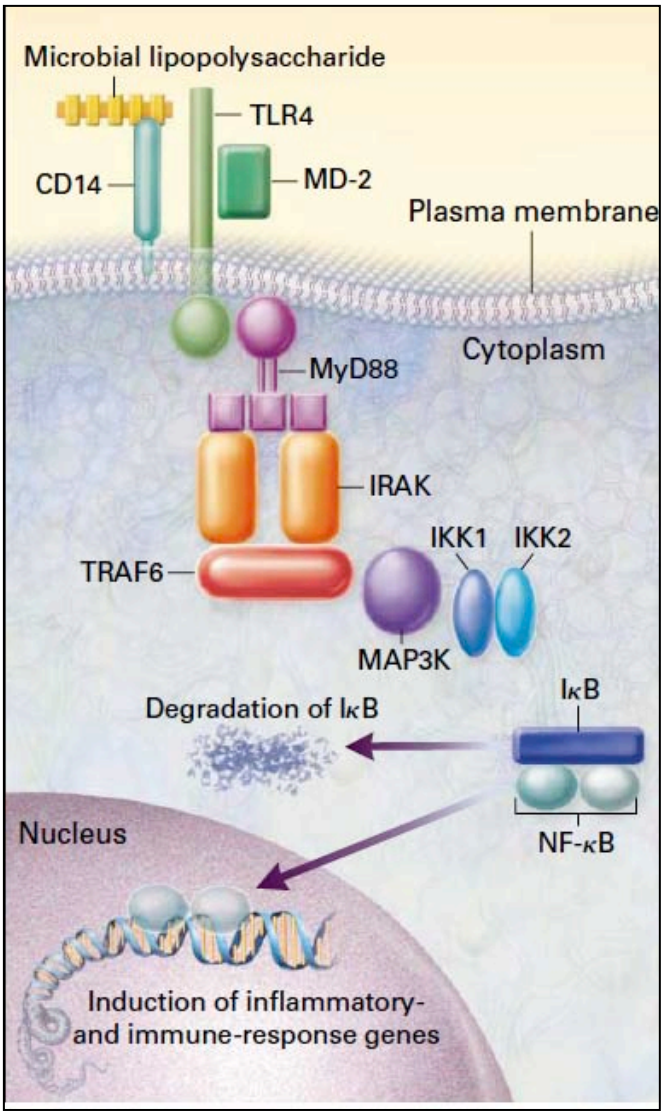


Figure 3. The Signaling Pathway of Toll-like Receptors

Schematic diagram of TLR signaling and activation of NF-κB. Figure taken from[1].

1.1.2 Nuclear Factor-κB activation

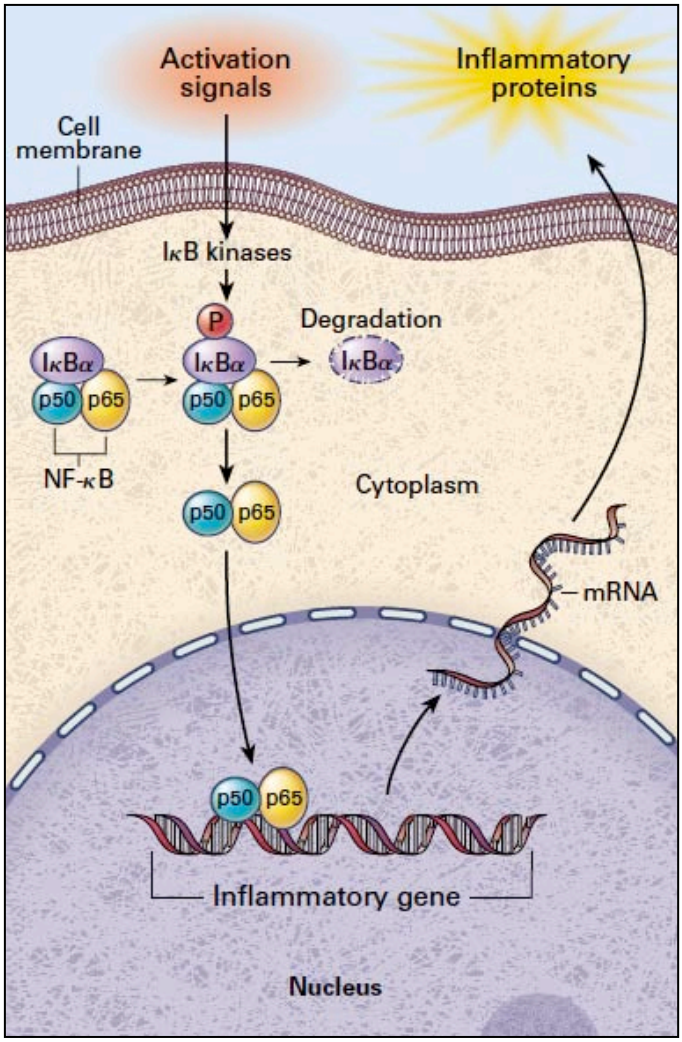
NF-κB was originally discovered in B cells and was thought to be a B cell specific transcription factor; however, it is now known to be present in several different cell types[9], acting as a major regulator of immune, inflammatory, apoptotic and pro-survival gene expression[3]. Microbial products, proinflammatory cytokines, T and B cell mitogens, and physical and chemical stresses

can activate NF-κB all in order to regulate the expression of activators such as cytokines, chemokines, and acute phase proteins[3]. The family of NF-κB transcription factors includes 5 members: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (p65), c-Rel and RelB, the latter three being characterized by their N-terminal Rel homology domain (RHD). This domain is responsible for DNA binding, dimerizing, and inhibitory protein interaction. The active form of NF-κB is a heterodimer clasically consisting of RelA and p50 subunits. Both homo- and heterodimers may form; however, in order for transcription to occur, RelA, c-Rel or RelB must be present whereas p50/p50 and p52/p52 homodimers repress transcription. Inhibitors of NF-κB, the IκB proteins, maintain negative regulation of NF-κB activation. These proteins function via ankyrin repeats which facilitate IκB binding and masking of the Rel proteins' nuclear localization sequences, retaining them in the cytoplasm[9].

Positive regulation of NF-κB is maintained by IκB kinase (IKK) trimers consisting of catalytic IKKα and IKKβ, and the regulatory IKKγ. These protein kinases are specific for IκB N-terminal regulatory serines[10,11]. In order for NF-κB activation to take place, the inhibitory IκB proteins in complex with NF-κB in the cytoplasm must be phosphorylated and degraded. For example, once phosphorylated at serines 32 and 26 at the N-terminus[12], IκBα becomes ubiquinated at lysines 21 and 22[13], resulting in proteasome-mediated degradation[14]. Once released from the inhibitors, NF-κB translocates to the nucleus where it may bind to the promoter of target genes[15] (Figure 4). Taken together, the initiation of innate immunity via TLRs induces NF-κB activation and resultant expression of various immune mediators including pro-inflammatory cytokine and chemokine production, enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules that recruit leukocytes to the site of inflammation[15]. Without sufficient innate immune function, the adaptive immune response is

blunted as the two systems act in concert in order to effectively clear infection and promote immunological memory for future contact with specific pathogens.

Figure 4. Activation of the NF-κB Signaling Pathway



Schematic diagram of NF-κB activation. Figure taken from[15].

1.1.3 Macrophages

Macrophages are phagocytic white blood cells derived from blood monocytes and are involved in many innate and adaptive immune processes[16]. The functions of macrophages include

phagocytosis, antigen presentation and cytokine production. Many tissues susceptible to infection and injury, including lung, liver and bone, contain fixed or resident macrophages (alveolar macrophages, Kupffer cells, and osteoclasts, respectively)[16,17]. The expression of many cell surface molecules identify cells as macrophages including CD11b, F4/80 and CD68[16]. TLR4 is also present on macrophages, contributing to their activation through the activation of NFκB.

Macrophages are one of the primary effector cells of the innate immune system. At the site of damage, resident macrophages become activated and start producing NF-κB dependent cytokines and chemokines. These mediators serve to chemotactically recruit additional cells to the site of injury. Circulating blood leukocytes participate in leukocyte extravasation whereby selectin and integrin receptor/ligand interactions mediate "rolling" of the cells, leading to eventual migration through the endothelium[18]. Once they arrive at the site of infection/injury, blood monocytes differentiate into macrophages, become activated and start to ameliorate the injury. When a bacterial infection is present, macrophages exert anti-microbial actions by killing bacteria via reactive oxygen species[19]. In most cases, this process of inflammation results in clearance of the injury, thus regaining homeostasis. However, when innate immunity cannot resolve the pathogen, macrophages work in concert with adaptive immunity. Through the process of phagocytosis, macrophages will present digested antigenic peptides in the context of the major histocompatibility complex (MHC) molecules to T cells via the T cell receptor (TCR)[2]. Upon MHC-TCR engagement and costimulatory molecule interaction, the T cell is activated and mediates further immune function to clear the pathogen.

Much like the way T lymphocytes are divided into phenotypic subsets (T_H1 and T_H2, for example) a new line of research is aimed at investigating the phenotypic switch of macrophages,

that depends on their cytokine profiles for characterization. The classically activated macrophages, termed 'M1', are induced by LPS and IFNγ and have a pro-inflammatory profile producing and secreting molecules including IL-6, TNF-α, MCP-1 and NO[20,21]. The alternatively activated macrophages, or 'M2', are induced by IL-4 and IL-13. M2 polarized macrophages are characterized by expression of CD206, arginase-1, Mgl1, and IL-10 and exhibit anti-inflammatory properties[20,22,23]. The phenotypic "switching" of macrophages, specifically those that reside in the adipose tissue, is of great interest in the progression of insulin resistance[24].

1.2 THE ACUTE PHASE RESPONSE

Innate immune activation and inflammation are accompanied by the acute phase response (APR) and production of acute phase proteins (APP) by hepatocytes. The APR orchestrates a number of endocrine and metabolic or neurological changes observed a short time after injuries, the onset of infections, and inflammatory processes. Derek Willoughby coined the term "pillars of inflammation" to include the tell tale signs of the inflammatory process as: heat, redness, swelling, pain, and loss of function[25] (Figure 5).

APPs are defined as "proteins whose plasma concentration increases (positive acute-phase proteins) or decreases (negative acute-phase proteins) by at lease 25% during inflammatory disorders"[26] (Figure 6). Examples of APP include: C-reactive protein, urokinase (uPA) and plasminogen activator inhibitor type-1 (PAI-1)[27]. Instances where significant changes in these proteins occur include infection, surgery, and advanced cancers, while moderate changes may occur after heatstroke and childbirth[26]. Pro-inflammatory cytokines, namely

interleukin-6 (IL-6), produced by macrophages promote the production of APPs and aid in the transition from neutrophilic to monocytic recruitment at the site of injury[28] via MCP-1[29]. However, the main goal of the APR is to ameliorate the stress and regain whole body homeostasis[26].

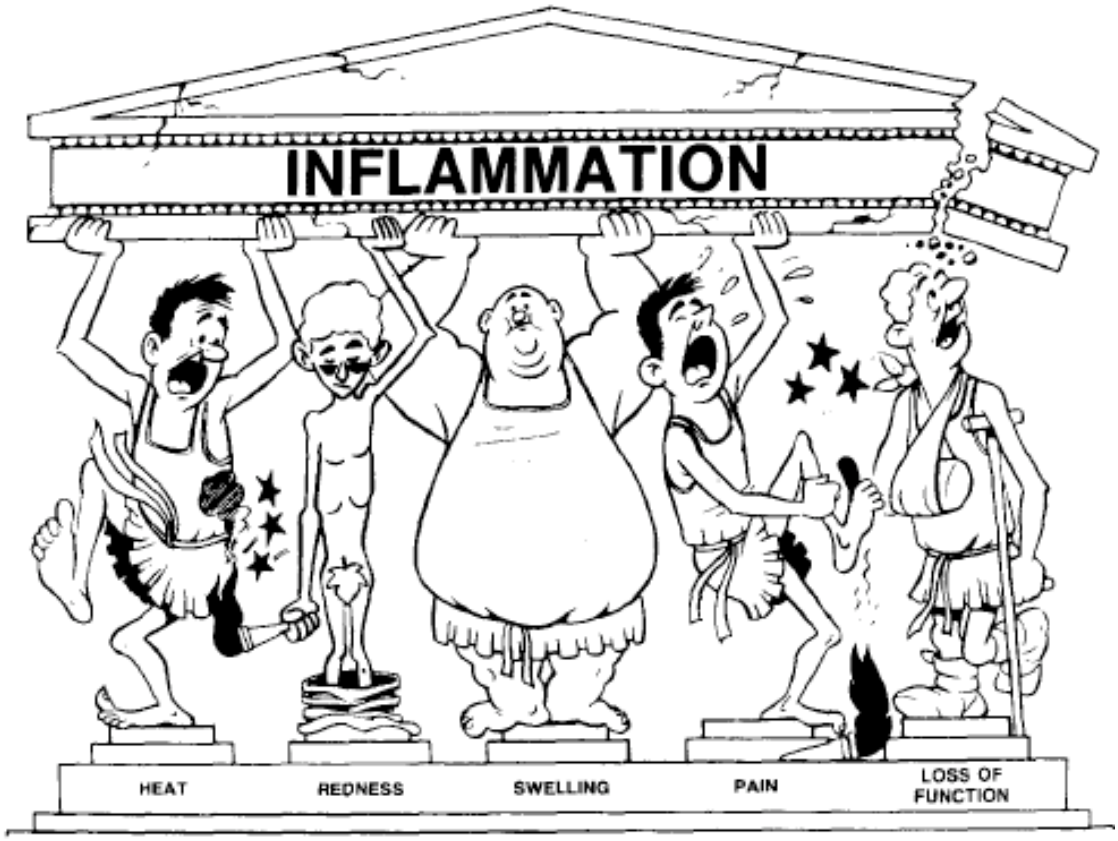


Figure 5. The Pillars of Inflammation

For years, the five "pillars of inflammation" figure provided the centerpiece for any major discussion in inflammation. Figure taken from[25].

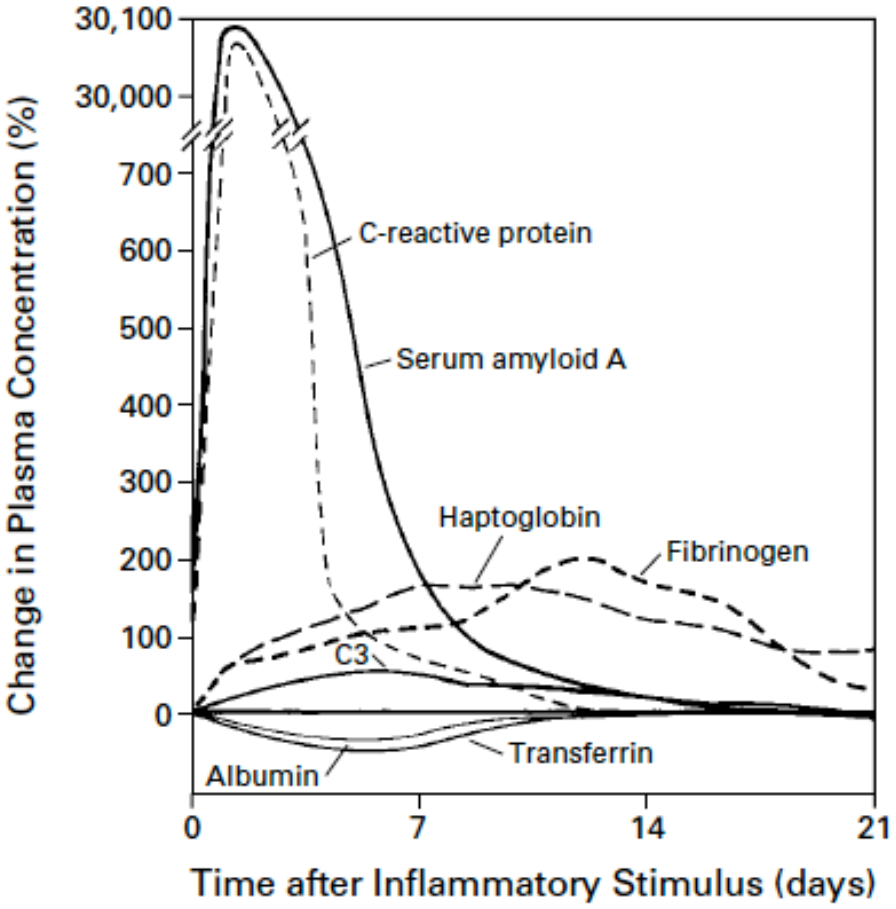


Figure 6. Acute Phase Proteins

Characteristic Patterns of Change in Plasma Concentrations of Some Acute-Phase Proteins after a Moderate Inflammatory Stimulus. Figure taken from[26].

1.2.1 Interleukin-6

IL-6 type-cytokines including IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and cardiotrophin (CT-1) are classified by a four-α-helix bundle structure and use gp130 for signal transduction with Janus kinase (Jak)/STAT activation. IL-6 is about 20 kDa in size with an N-terminal signal peptide and is N-glycosylated.

The IL-6R is a type 1 membrane protein of 80 kDa, a member of the cytokine receptor class 1 family and contains cytokine-binding modules (CBM) and an IgG-like domain. Residues located in the CBM are critical for biological activity, and ligand binding is contained to this region and the IgG-like domain[30]. Upon ligand binding, gp130 is recruited to the receptor complex and induces homodimerization[31]. IL-6 utilizes the Jak/STAT family as the major mediator of signal transduction. Jak1, Jak2, and Tyk2, all gp130 associated kinases, become activated upon stimulation of the receptor causing the cytoplasmic tail of gp130 to be phosphorylated. STAT3 and STAT1 are recruited to phospho-tyrosines of gp130. The STATs become phosphorylated, dimerize, and translocate to the nucleus to initiate transcription of target genes, including many of the APP such as C-reactive protein. Src homology region 2 domain-containing phosphatase-2 (SHP2), a tyrosine phosphatase, can bind gp130, leading to IL-6 mediated mitogen-activated protein kinase/ERK (MAPK/ERK) pathway activation[30] (Figure 7).

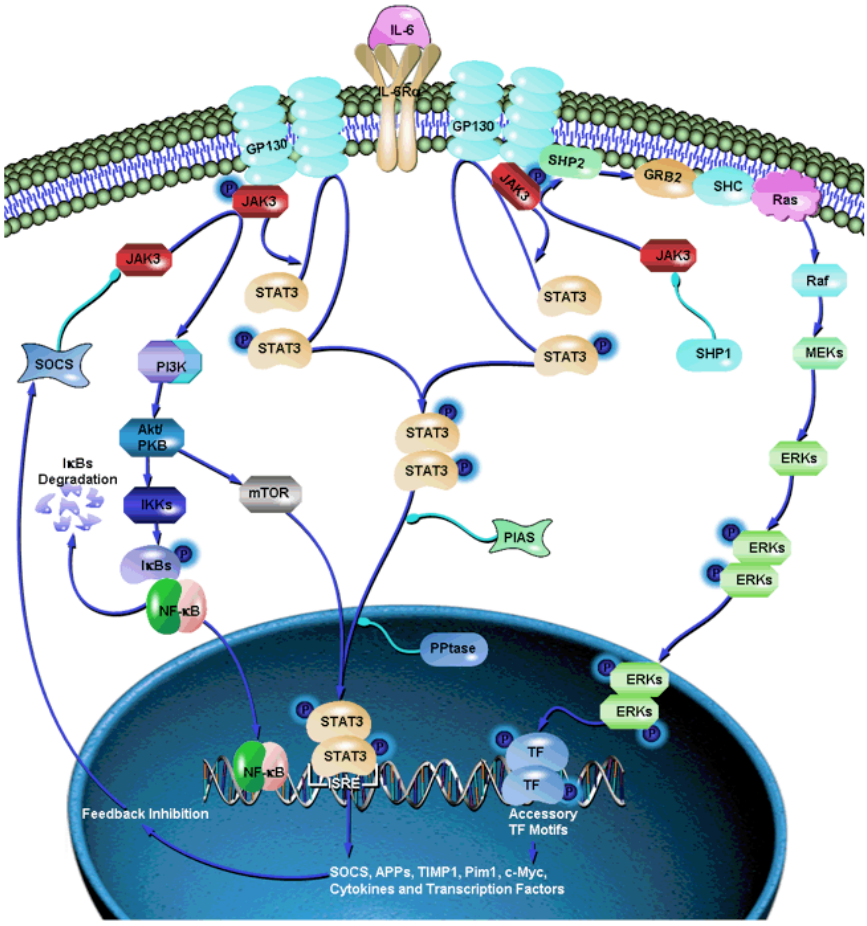


Figure 7. IL-6 Signaling Pathway

Interleukin-6 is a cytokine that provokes a broad range of cellular and physiological responses, including the immune response, inflammation, hematopoiesis, and oncogenesis by regulating cell growth, gene activation, proliferation, survival, and differentiation. Figure taken from[32].

IL-6 is an NF-κB dependent cytokine, and together with TNF-α and IL-1β, is critical in initiating a robust immune response. IL-6 deficient mice fail to mount a normal inflammatory response and induction of the APR is weakened in a turpentine model; however, when LPS is the stimulus, the presence of other NF-κB dependent cytokines may compensate for the loss of IL-6[27,33]. However, IL-6 plays an important role as an anti-inflammatory cytokine as well. IL-6

knockout mice have greater pro-inflammatory cytokine production with local endotoxic lung injury, and this systemic injury results in increased mortality with lethal endotoxin exposure[27]. This suggests a feedback loop in which IL-6 also aids in contraction of inflammation by promoting the generation of anti-inflammatory mediators such as the IL-1 receptor antagonist[34] and expression of the Suppressor of Cytokine Signaling (SOCS) genes, thus promoting homeostasis. It was found that resultant effects of IL-6 deletion could not be compensated for by other anti-inflammatory cytokines like IL-10, suggesting the importance of IL-6's anti-inflammatory role [27]. Taken together, cytokines are key mediators of innate immunity fueling the APR to fight infection, diminish stress and regain homeostasis.

1.3 INSULIN AND GLUCOSE

1.3.1 Insulin signaling

Insulin, a potent anabolic hormone, is responsible for growth and development, as well as proper metabolic control, including maintenance of normoglycemia and normolipidemia. Produced by pancreatic β-cells, insulin signaling increases energy storage into cells by stimulating the translocation of the glucose transporter, Glut4, concentrating it in the plasma membrane of fat and muscle cells where it facilitates glucose uptake[35]. Insulin signaling also maintains euglycemia by suppressing hepatic gluconeogenesis.

Insulin binds to the insulin receptor (IR), a receptor tyrosine kinase present in many insulin-sensitive tissues, including skeletal muscle, adipose, liver, and pancreas, to facilitate glucose uptake from the blood. Upon insulin binding, the cytoplasmic kinase domains

transphosphorylate Tyr 1158/62/63 in the catalytic loop of the kinase domain, followed by phosphorylation of the C-terminal Tyr 1328/34 and the juxtamembrane region Tyr 972, which is the major docking site for downstream interacting proteins[36]. These downstream proteins include the insulin receptor substrate (IRS) proteins. The phosphorylated tyrosines in these substrates serve as docking sites for proteins containing Src-homology-2 (SH2) domains, propagating the insulin-signaling cascade. Pleckstrin homology (PH) domains at the N-terminus in IRS proteins bring the phosphotyrosine binding (PTB) domains in close proximity to the cell membrane where they can bind to the IR at Tyr 960 in a NPXY motif of the juxtamembrane region[37] leading to insulin signaling within the cell. The phosphatidylinositol 3-kinases (PI3-K) pathway plays a critical role in metabolic and mitogenic actions of insulin signaling. PI3-K is translocated to the plasma membrane where its p85 regulatory and p110 catalytic subunits may bind IRS tyrosine phosphorylated YMXM and YXXM motifs via SH2 domains[38]. These interactions allow for the interaction between PI3-K and phosphatidylinositol (PtdIns) (4,5)P2, yielding the second messenger PtdIns(3,4,5)P3 and resulting in the binding of PH domains of many other signaling molecules including Akt. Overall, the signaling partners involved in PI3-K signaling alter their activation via phosphorylation or subcellular localization resulting in Glut 4 translocation, glycogen synthesis, and lipogenesis[36].

1.3.2 Glucose metabolism

Glucose is the energy source for humans and may be used directly or stored as glycogen. The liver is capable of de novo glucose production via gluconeogenesis. Glycogen is stored by the liver and muscle, therefore, they both are capable of breaking down existing glycogen stores into glucose by glycogenolysis, or in the event of excess glucose, can form glycogen from glucose

via glycogenesis[39]. As discussed above, insulin signaling is key to glucose metabolism. The liver, muscle and adipose tissues are insulin responsive and metabolize glucose, however skeletal muscle mediated glucose uptake accounts for 70 – 90% of glucose disposal[39]. Upon insulin signaling, glucose enters cells via Glut4 and is phosphorylated by hexokinase yielding glucose-6-phosphate (G6P)[38]. From here, G6P can either be utilized by the glycolytic pathway or stored as glycogen. Insulin-dependent PI3-K activation results in the regulation of many signaling molecules, however the phosphoinositide-dependent kinase 1 (PDK1) activation of Akt/PKB is best characterized. Akt has a PH domain that directly interacts with PtdIns(3,4,5)P3 resulting in insulin-dependent inactivation of glycogen synthase kinase 3 (GSK3)[36,38]. Akt thus decreases the rate of phosphorylation of glycogen synthase, thereby rendering it active and resulting in increased glycogen storage at peripheral sites[40]. The regulation of hepatic gluconeogenesis by insulin signaling is a hallmark to maintaining euglycemia. Under fasting conditions, hepatic glycogen stores are mobilized in order to maintain circulating glucose concentrations. In hepatocytes, insulin signals to inhibit the transcription of the gene encoding phosphoenolpyruvate carboxylase, the rate-limiting step in gluconeogenesis, and also decreases the transcription of other genes encoding gluconeogenic proteins, including the enzymes fructose-1,6-bisphosphate and glucose-6-phosphatase. Insulin increases the transcription of glycolytic enzymes such as glucokinase and pyruvate kinase and lipogenic enzymes such as fatty acid synthase and acetyl-CoA carboxylase[38]. There are a number of transcription factors and co-factors that partake in insulin-dependent decreases of gluconeogenesis, including sterol regulatory element binding protein (SREBP)-1, hepatic nuclear factor (HNF)-4, the forkhead protein family (Fox) and PPAR-γ co-activator 1 (PGC1).

1.3.3 Dysregulated insulin signaling

Although tyrosine phosphorylation is critical in activating the IR and IRS proteins to propagate the signaling cascade, serine phosphorylation is also important for contraction of insulin signaling, which may result from direct phosphorylation by serine/threonine kinases, cross talk from other signaling pathways, or by protein tyrosine phosphatases (PTPases)[38].

As described above, tyrosine phosphorylation occurs at the level of the IR and serves to propagate insulin signaling by the recruitment of other signaling molecules. Continual Tyr phosphorylation will drive positive feedback control; whereas controlled Ser/Thr phosphorylation will uncouple the IRS proteins from upstream and downstream effectors, resulting in negative feedback regulation, terminating the signal[41]. Serine phosphorylation also leads to degradation of the IRS proteins via the proteasomal degradation pathway, which also attenuates insulin actions[42]. It is important to note that Ser/Thr phosphorylation may occur both by insulin and agents that promote insulin resistance; therefore, a threshold serine-phosphorylation level is necessary for IRS protein function, and in fact phosphorylation of Ser 1177/78/82 located C-terminal to the IR kinase domain is required to achieve phosphorylation of IRS1 and IRS2[43]. It has also been shown that phosphorylation of Ser residues within the phospho-tyrosine-binding domain of IRS1 (by insulin-stimulated PKB) protects IRS proteins from PTPs, enabling IRS proteins to maintain their phospho-tyrosine active conformations[41]. However, inappropriate Ser/Thr phosphorylation and inactivation of the insulin signaling pathway may lead to insulin resistance, a pathological state and progression to diabetes. Dysregulation of Ser/Thr phosphorylation can result in negative metabolic outcomes because it:

- inhibits further Tyr phos of IRS proteins
- induces the dissociation of IRS proteins from the IR

- hinders phospho-Tyr sites
- releases the IRS proteins from intracellular complexes that maintain them in close proximity to the receptor
- induces IRS degradation
- turns IRS proteins into inhibitors of IRK[41]

Many of the serine/threonine kinase pathways activated by the innate immune system are responsible for the disruption in insulin signaling (Figure 8). For example, high fat diet-fed mice are protected from insulin resistance when serines 302, 307, and 612 of the muscle-specific IRS-1 are mutated to alanines preventing serine-phosphorylated inactivation of insulin signaling[44]. Therefore, inflammation is a major perpetrator in the development of insulin resistance and progression of Type 2 diabetes (T2D). The first signs of pathological abnormalities in glucose homeostasis due to insulin resistance are seen in muscle and adipose tissue as a delayed response to insulin results in increased circulating glucose and fatty acids[36]. This is followed by increased hepatic gluconeogenesis and hyperglycemia. Pancreatic β-cells try and compensate for the increases in circulating glucose by secreting more insulin, however when the insulin secretory response cannot compensate for the defects in peripheral insulin action, diabetes ensues[36,45].

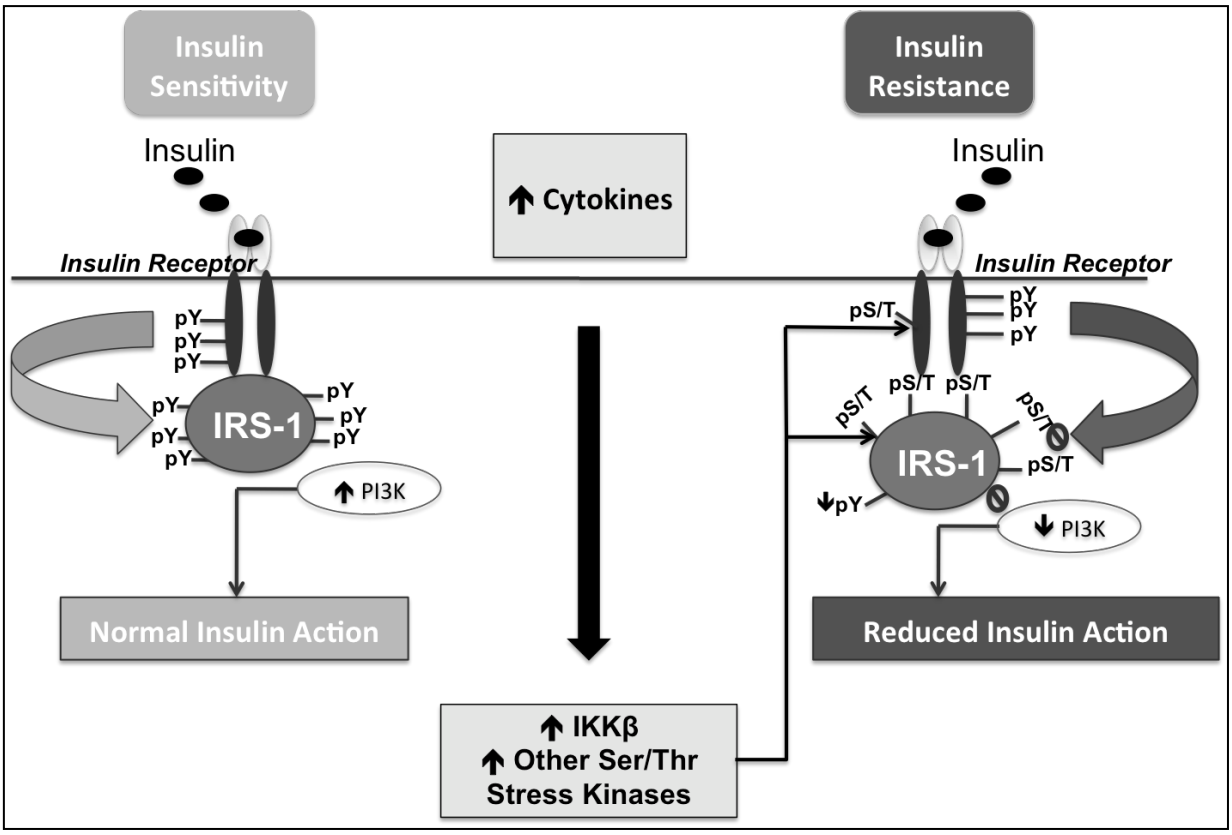


Figure 8. The Role of Serine Kinase Activation in Insulin Resistance

The activation of serine/threonine kinases lead to the phosphorylation of multiple targets, such as the IR and IRS proteins. Increased phosphorylation of IR or IRS proteins on discrete serine or threonine sites (pS/T) decreases the extent of insulin-stimulated tyrosine phosphorylation (pY). Consequently, the association and/or activities of downstream signaling molecules (e.g., phosphatidylinositol 3-kinase [PI3K]) are decreased, resulting in reduced insulin action (insulin resistance). Figure modified from[46].

1.4 TYPE 2 DIABETES

23.6 million people or 7.8% of the population is afflicted with diabetes. Of those individuals, 90-95% are diagnosed with Type 2 Diabetes (T2D) (2007 National Diabetes Fact Sheet). The first symptoms of T2D may include increased thirst, appetite and urination, blurred vision and fatigue. These symptoms are the results of the first pathological events of T2D including insulin resistance, hyperglycemia and glucose intolerance. Over time, glucotoxicity can lead to several T2D-related complications including: heart disease, stroke, blindness, kidney failure, leg and foot amputations, and pregnancy complications (The Obesity Society).

Traditionally called non-insulin-dependent diabetes mellitus (NIDDM), or adult-onset diabetes, T2D typically presents itself later on in life whereas juvenile onset or Type 1 Diabetes, an autoimmune disease, presents as young as 1 year. The risk factors for T2D, in addition to old age, include family history of diabetes, history of gestational diabetes, impaired glucose metabolism, race/ethnicity, and inactivity and obesity. However, since obesity rates are increasing world wide in adults and children, the overall prevalence of T2D in all age groups is on the rise, as roughly 90% of people with T2D are overweight (Figure 9).

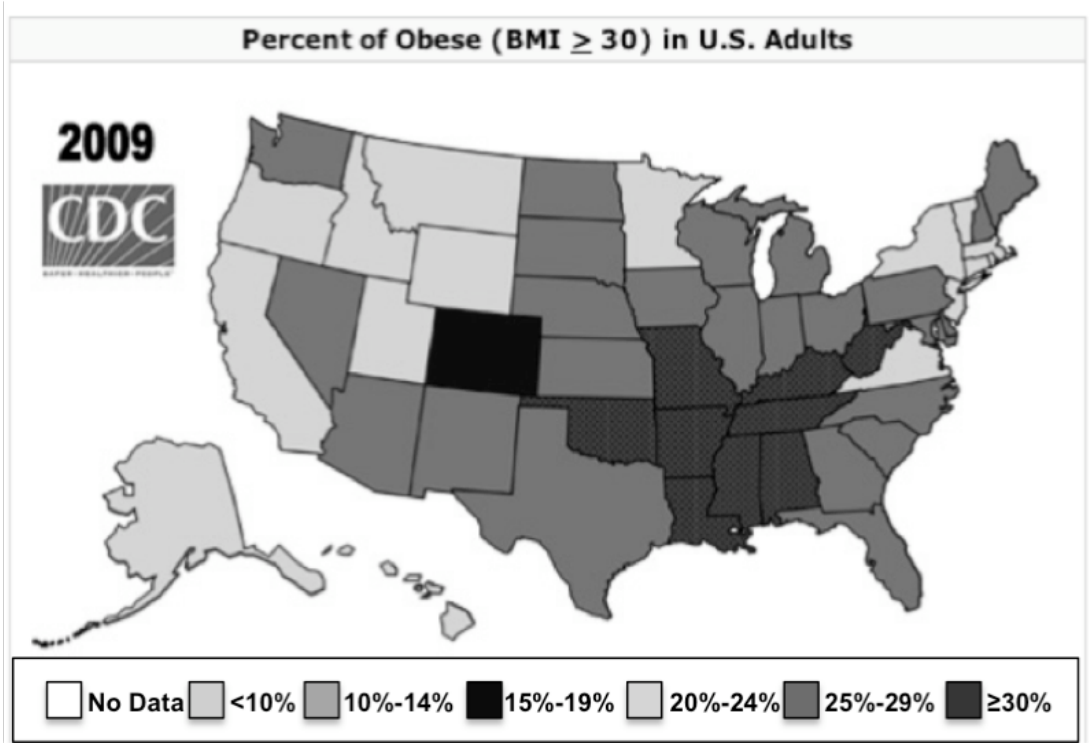


Figure 9. United States Obesity Prevalence in 2009

The data shown in this map was collected through the CDC's Behavioral Risk Factor Surveillance System (BRFSS). Figure taken from[47].

1.4.1 Obesity and inflammation

Obesity is a major risk factor for the development of T2D and is defined as an abnormal accumulation of body fat, usually 20% or more over an individual's ideal body weight (Figure 10). An adult is considered obese when he or she has a body mass index (BMI) greater than 30[48]. Obesity has become an epidemic with an estimated 1 billion overweight adults globally (BMI between 25 – 29.9), where at least 300 million of these individuals are considered obese[49].

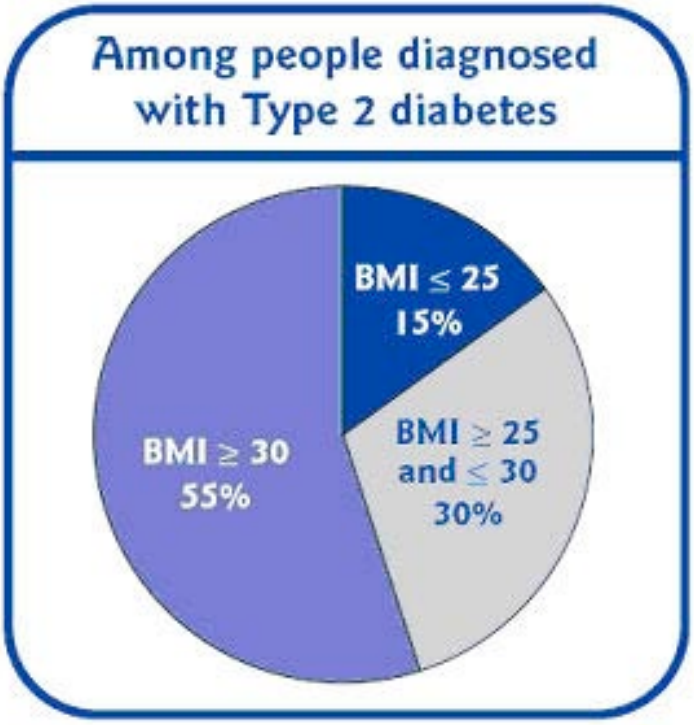


Figure 10. BMI of Individuals Diagnosed with Type 2 Diabetes

The CDC reports that among people diagnosed with Type 2 diabetes, 85 percent have a BMI > 25 (classified as falling within the overweight range) and 55 percent have a BMI > 30 (classified as obese)[50].

Additionally, substantial literature supports the hypothesis that obesity is linked to chronic inflammation via a currently unknown mechanism[51,52,53,54,55]. This low-grade inflammatory response is thought to stimulate a state of insulin resistance and impaired glucose tolerance that ultimately leads to diabetes. Significant advances have been made in understanding the roles of glucotoxiciy, lipotoxicity, and cellular nutrient excess and how the three contribute to the pathogenesis of T2D in obese individuals[54]. Nevertheless, a universally accepted and pathophysiologically conclusive explanation linking the three primary components of the disease: excessive adiposity, insulin resistance and insulin secretory dysfunction, has not been fully demonstrated[54].

It is well known that infection, tissue damage or other stressors lead to the activation of the innate immune response in order to mobilize the necessary cells to the affected target site to resolve the damage[1,2]. Pickup and Crook made the early observation that short-term innate immune activation is beneficial for not only controlling infection and tissue damage, but also to restore homeostasis after stress; however, in chronic states of inflammation, such as T2D, the innate and acute phase responses are sustained and therefore disease progression ensues[52]. The ungoverned response is likely a result of obesity fueling innate immune-derived pro-inflammatory cytokine synthesis through the continuous activation of the NF-κB pathway. NF-κB activation is not resolved in cases of continued accumulation of adipose tissue and therefore perpetuates the acute phase response to evolve into a chronic state resulting in inflammation induced insulin resistance.

To further ascribe a role for NF-kB's involvement in the unresolved inflammation, studies have revealed that salicylates, or aspirin, reduce the activation of NF-κB, an important transcription factor responsible for fueling inflammation[56]. This effects was shown to occur through the direct molecular targeting of IKK-β, the kinase that directly activates NF-κB, is a direct molecular target of the salicylates[57]. IKK-β contributes to insulin resistance by directly phosphorylating serine residues on IRS1[57,58]. Mice devoid of myeloid IKK-β develop global insulin sensitivity, and obese mice devoid of hepatic IKK-β are protected from insulin resistance[59]. Therefore, enhanced NF-κB activity is a key inducer of cytokine-induced insulin resistance.

NF-κB dependent pro-inflammatory cytokines, including TNFα, IL1β and IL-6, further promote serine phosphorylation either directly or by activation of other kinases. For example, TNFα was among the first molecular links between inflammation and insulin resistance,

impairing insulin-stimulated uptake of glucose as well as reducing tyrosine IRS-1 phosphorylation[60]. Furthermore, TNFα will activate c-Jun N-terminal kinase (JNK) that in turn phosphorylates IRS-1 at Ser 307[61], making JNK a crucial component of the biochemical pathway responsible for obesity-induced insulin resistance in vivo[62]. IL-6 has been shown to inhibit tyrosine phosphorylation of IRS-1, decrease the association of the p85 subunit of PI3-K with IRS-1, reduce the activation of Akt, and inhibit insulin-induced glycogen synthesis by 75%[63]. Systemic addition of a neutralizing IL-6 antibody improved insulin resistance in IKK-β transgenic mice[64]. Taken together, inhibition of NF-κB dependent pro-inflammatory cytokine production is key in ameliorating obesity-induced insulin resistance and progression of T2D.

Obesity results from the accumulation of adipose tissue, a metabolically active entity, characterized by abnormal cytokine production, increased acute-phase proteins, and inflammatory activation, and is a primary risk factor for developing T2D. In a resting individual, the adipose is responsible for producing 10 – 35% of IL-6, with production increasing as adiposity elevates[65]. In addition to IL-6, the adipose tissue of obese individuals expresses elevated levels of: TNF-α, iNOS, TGF-β1, C-reactive protein, soluble ICAM, MCP-1, PAI-1, tissue factor, and factor VII[55]. It has been described that the adipose-induced infiltration of macrophages is likely responsible for the increased generation of pro-inflammatory mediators[55] and that MCP-1 is a critical chemokine produced by adipose tissue responsible for the increase of macrophage content, as well as the T2D phenotype[51]. Furthermore, CSF-1 dependent F4/80+ cells, or bone marrow-derived adipose tissue macrophages, are present in obese mice[55] (Figure 11).

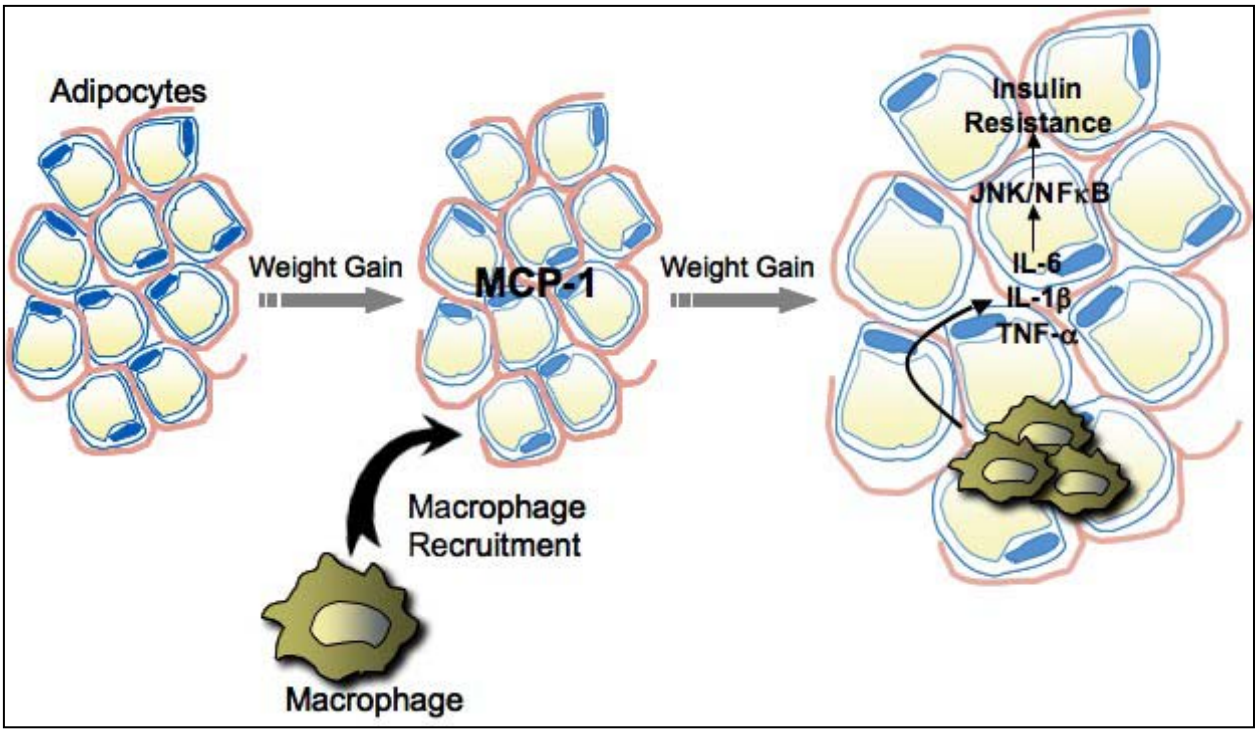


Figure 11. Macrophages Infiltration of Adipose Tissue

Obese adipose tissue is characterized by inflammation and progressive infiltration by macrophages as obesity develops. Changes in adipocyte and fat pad size lead to physical changes in the surrounding area and modifications of the paracrine function of the adipocyte. Figure modified from[66].

1.5 THE LIVER'S ROLE IN T2D

Increased adiposity requires the body to store excess fat in peripheral sites such as liver and muscle. Excess fat accumulation in the liver, or hepatic steatosis, further perpetuates chronic inflammation by increasing the production of glucose, VLDL, cytokines and acute phase proteins including CRP, PAI-1, fibrinogen and IL-6[67]. Given that not all insulin resistant subjects are

overtly obese and that constitutive IKK-β activity in hepatocytes induces NF-κB activity in the absence of adiposity, it appears as if steatosis alone is sufficient to induce subacute inflammation contributing to overall insulin resistance and T2D progression[64]. These observations suggest the important role the liver plays in the development of T2D.

1.5.1 Hepatocyte growth factor

Hepatocyte growth factor (HGF) is a potent mitogeneic, motogenic, and morphogenic cytokine and is the primary growth factor involved in liver development and repair. It is a heparin-binding glycoprotein secreted as a single-chain inert precursor, produced by hepatic stellate cells and Kuppfer cells, as well as several other tissues including the lung and kidney[68]. Since HGF is similar in structure to plasminogen, it becomes a suitable substrate for the urokinase-type and tissue-type plasminogen activators (u-PA and t-PA, respectively)[69]. HGF is cleaved from its inactive, single chain form by proteolytic digestion at the Arg-Val-Val (aa494-495) site[70] to the mature, two-chain bioactive form (Figure 12).

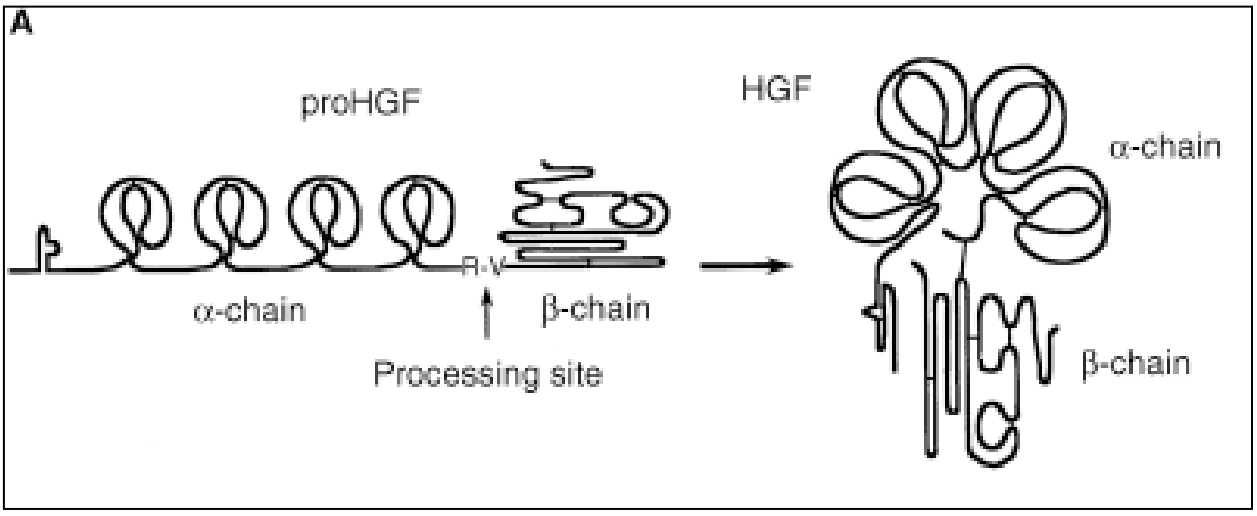


Figure 12. Pro-HGF and Mature HGF

The structures of proHGF and mature HGF after processing. Figure taken from[71].

Active HGF is a heterodimer comprised of an alpha and beta chain held together by a disulfide bond. The alpha chain contains a hairpin loop and four kringle domains, and the beta chain contains a serine protease-like structure; however, HGF harbors no known protease activity since the characteristic amino acids, Histidine and Serine at the catalytic site are replaced with Glutamine and Tyrosine[68]. The HGF gene promoter contains four IL-6 response elements; therefore, HGF expression is induced by inflammation and the acute phase response[72]. The receptor for HGF is the tyrosine kinase, MET, a disulfide linked extracellular α and transmembrane β chain heterodimer expressed in almost all tissues. Both the inactive and active forms of HGF protein may bind MET, however only the mature two-chain form of HGF exerts biological activity[73]. Before the discovery of HGF, the MET protooncogene was discovered for its ability to transform fibroblast cell lines, and it was found that the HGF-MET autocrine-loop enhances tumorigenecity in some lung cancers[74].

1.5.2 Plasminogen activator inhibitor type-1

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of plasminogen activators: urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) and plays a regulatory role in fibrinolysis by inhibiting the production of plasmin. PAI-1 is an acute phase protein normally expressed by adipocytes and endothelial cells and will become elevated during injury and inflammation. Macrophage-derived TNF-α promotes the expression of PAI-1 through the MAP kinase pathway[75], and as described above, obesity is a major perpetuator of inflammation. Therefore, the link is made between obesity, pro-inflammatory cytokine production and the acute phase response (TNF-α, IL-6 and PAI-1) in the progression of insulin resistance and T2D. The variety of plasminogen activator substrates introduces PAI-1's

regulatory role into other systems in addition to fibrinolysis. u-PA and t-PA are biological activators of HGF, cleaving the latent protein to the mature form[69]. Therefore, PAI-1 can negatively control the maturation of HGF and downstream signaling with the MET tyrosine kinase. The inactivation of HGF by PAI-1 is a critical event in maintaining metabolic and inflammatory homeostasis as HGF is not only the main growth factor involved in liver regeneration, but recently has been described as an anti-inflammatory agent (Figure 13).

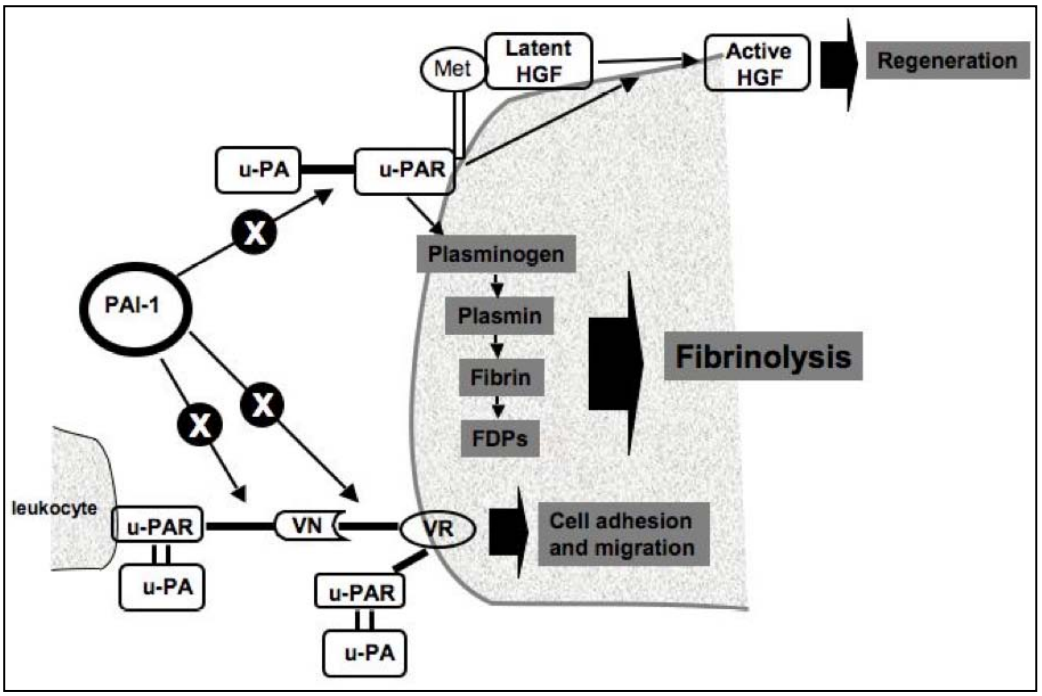


Figure 13. PAI-1 Signaling Cascade

The signaling pathway of PAI-1 by inactivation of uPA. Figure taken from WM Mars, unpublished.

1.5.3 HGF signaling

Recent studies demonstrate how HGF can ameliorate inflammation via inactivation of NF-κB gene transcription. In order to understand this phenomenon, the signaling events of HGF must

be examined. Upon ligand binding, MET undergoes tyrosine phosphorylation allowing for the recruitment of SH2-containing proteins including PI3-K. Particularly in the multidocking site, Tyr-1349 and Tyr-1356 are critical for recruiting adaptor molecules for downstream signaling[76] (Figure 14). Much like insulin signaling, HGF signals via PI3-K and Akt. As described above, GSK3β is a downstream target of Akt, and in addition to mediating glycogen storage, it has also been described as a powerful governor of inflammation.

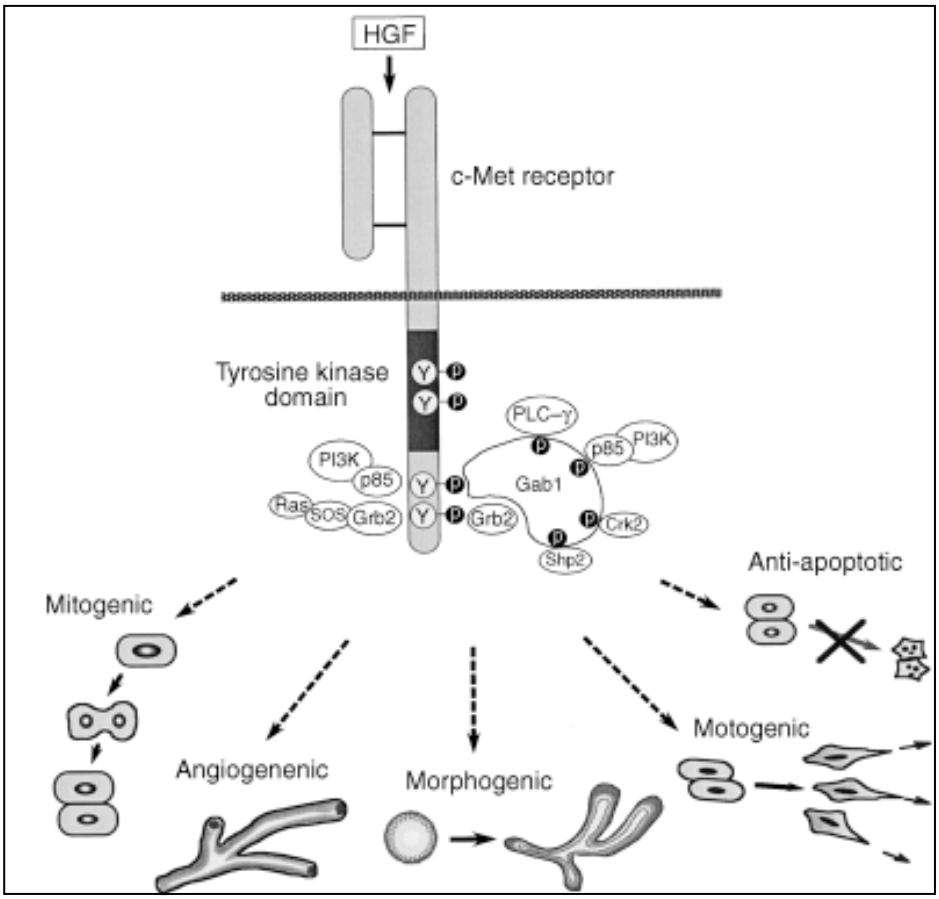


Figure 14. MET Kinase Signaling Cascade

Typical biological activities of HGF mediated by c-Met/HGF receptor and intracellular signal transducers which associate with tyrosine-phosphorylated c-Met. Figure taken from[71].

1.5.4 Glycogen synthase kinase 3β

GSK3β is a constitutively active serine/threonine kinase whose activity is reduced upon phosphorylation of an N-terminal serine at position 9, however phosphorylation of tyrosine 216 results in its activation[77]. GSK3β is the substrate for many protein kinases including Akt, thus it mediates regulation of a diverse set of signaling molecules including developmental (Wnt signaling), metabolic (insulin signaling) and inflammatory (TLR signaling) pathways. This kinase may be cytosolic as well as nuclear and is involved in promoting apoptosis as well as cell survival via regulation of a number of transcription factors including AP-1 and CREB. Substrates of GSK3β include glycogen synthase and β-catenin, whereas NF-κB activation can also be a GSK3β mediated event. NF-κB activation requires the interaction with the coactivator protein, CBP, for optimal function[78], including pro-inflammatory cytokine production. However, CREB also competes for association with CBP, which results in anti-inflammatory cytokine production, such as IL-10. Active GSK3β will inactivate CREB function; however, phosphorylating and inactivating GSK3β on serine 9 will allow CREB to freely associate with CBP, suppressing inflammation. It is these events that demonstrate GSK3β's potent regulation of inflammatory activation. Since Akt is a downstream target of TLR4 signaling, it has been postulated that an alternative TLR4 ligand will promote signaling to serine phosphorylate GSK3β, leading to anti-inflammatory cytokine production via CREB activation (Figure 15).

On the other hand, as described above, since innate immunity and the acute phase response must resolve in order to regain homeostasis, perhaps the accumulation of a certain acute phase reactant, such as HGF, acts as an internal rheostat in resolving inflammatory events.

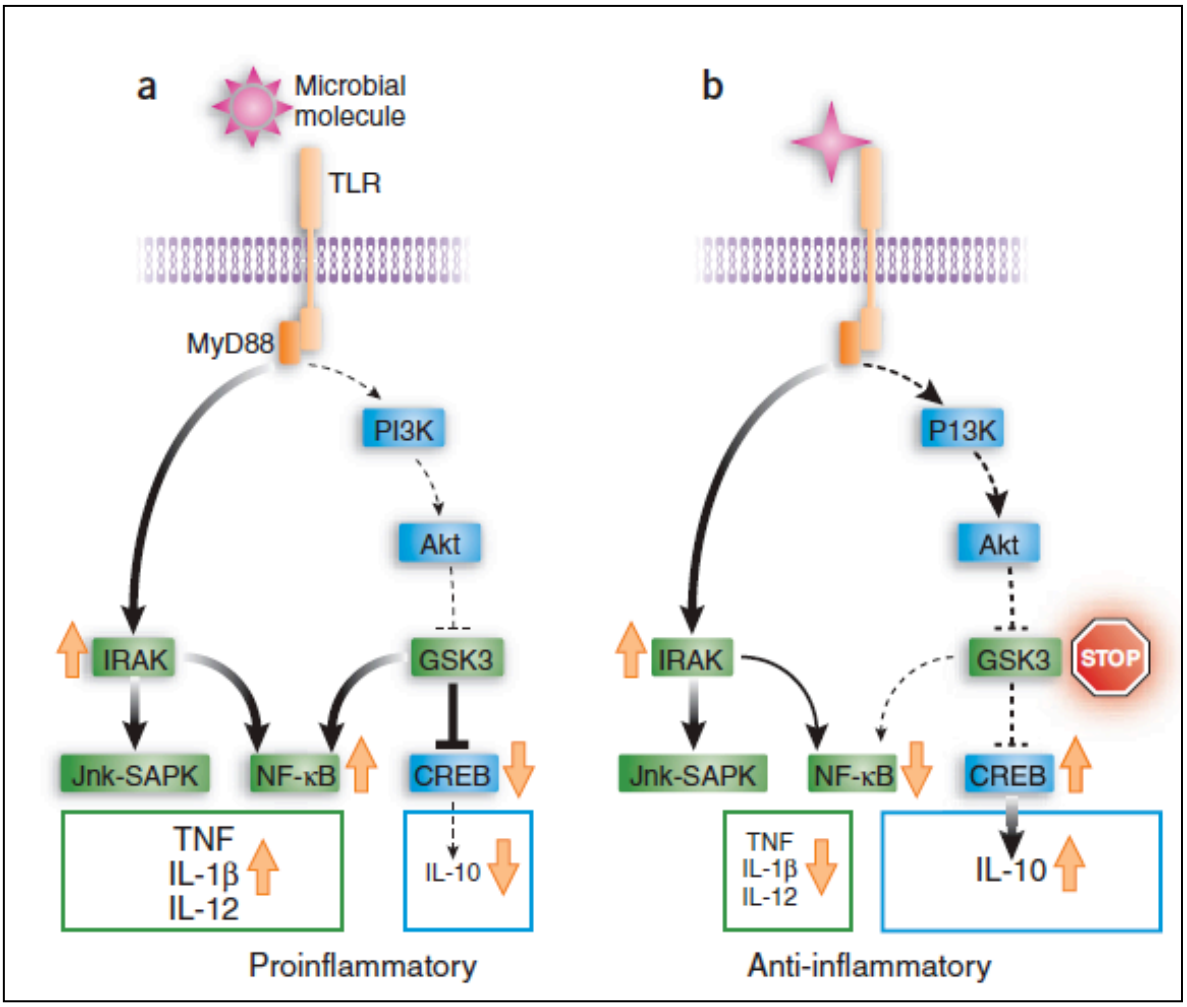


Figure 15. Alternative GSK3β Signaling Pathway

TLR activation induces multiple pathways that mediate proinflammatory responses. Figure taken from[79].

1.5.5 HGF as an anti-inflammatory agent

Many studies have demonstrated HGF's anti-inflammatory role. For example, HGF attenuated renal inflammation in vivo by reducing the expression of MCP-1 and RANTES, as well as in vitro by suppressing TNF-α induced expression of MCP-1 and RANTES in tubular epithelial cells[80]. HGF also inhibited TNF-α activated monocyte adhesion to an endothelial monolayer,

an NF-κB dependent event[81]. Furthermore, HGF gene transfer diminished expression of MCP-1 and RANTES while ameliorating renal inflammation[82]. Additionally, the serine phosphorylation of GSK3β by HGF signaling prevented GSK3β-mediated phosphorylation of NF-κB p65 on serine 468 to inhibit NF-κB activation and downstream inflammation[83]. Therefore, the mechanism whereby HGF acts in an anti-inflammatory nature might include HGF-MET signaling through PI3-K and Akt, resulting in inactivation of GSK3β, promoting the interaction of CBP with CREB and subsequent NF-κB inactivation. However, dysregulation of this system will occur in obesity-induced diabetes in the presence of persistent inflammation and acute-phase proteins. In the event of chronic obesity, steatosis will ensue, propagating hepatic inflammation including the production of PAI-1, u-PA, IL-6 and TNFα. IL-6 will fuel HGF production and the latent protein will be cleaved and activated by u-PA. However, as the levels of TNF-α increase, so will PAI-1 proteins, resulting in inactivation of u-PA, accumulation of inactive HGF and eventual loss of inflammatory control concomitant with progressing insulin resistance and hyperglycemia.

Experiments described herein will further demonstrate HGF's role in modulating cytokine production by macrophages mechanistically through GSK3β *in vitro.* Furthermore, HGF's anti-inflammatory effects play a role in obesity-induced inflammation *in vivo* as increasing amounts of hepatic PAI-1 protein prevent the activation of HGF. The loss of HGF signaling results in steatosis, accumulation of hepatic and adipose macrophages, and the progression of T2D. Therefore, we hypothesize that active HGF can ameliorate chronic stress induced by obesity, to reset metabolic function, by impeding persistent activation of NF-κB and inhibiting downstream inflammation.

2.0 HEPATOCYTE GROWTH FACTOR MODULATES INTERLEUKIN-6 PRODUCTION IN BONE MAROW DERIVED MACROPHAGES: IMPLICATIONS FOR INFLAMMATORY MEDIATED DISEASES

2.1 ABSTRACT

The generation of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β fuels the acute phase response (APR). To maintain body homeostasis, the increase of inflammatory proteins is resolved by acute phase proteins via presently unknown mechanisms. Hepatocyte growth factor (HGF) is transcribed in response to IL-6. Since IL-6 production promotes the generation of HGF and induces the APR, we posited that accumulating HGF might be a likely candidate for quelling excess inflammation under non-pathological conditions. We sought to assess the role of HGF and how it influences the regulation of inflammation utilizing a well-defined model of inflammatory activation, lipopolysaccharide (LPS)-stimulation of bone marrow derived macrophages (BMM). BMM were isolated from C57BL6 mice and were stimulated with LPS in the presence or absence of HGF. When HGF was present, there was a decrease in production of the pro-inflammatory cytokine IL-6, along with an increase in the anti-inflammatory cytokine IL-10. Altered cytokine production correlated with an increase in phosphorylated GSK3β, increased retention of the phosphorylated NFκB p65 subunit in the cytoplasm, and an enhanced interaction between CBP and phospho-CREB. These changes were a direct result of signaling

through the HGF receptor, MET, as effects were reversed in the presence of a selective inhibitor of MET (SU11274) or when using BMM from macrophage-specific conditional MET knockout mice. Combined, these data provide compelling evidence that under normal circumstances, HGF acts to suppress the inflammatory response in macrophages.

2.2 INTRODUCTION

As a first line of defense in response to infection, tissue injury and stress, macrophages generate the NFκB-dependent pro-inflammatory cytokines, TNF-α, IL-1β and IL-6[15]. The expression of these pro-inflammatory cytokines serves to facilitate the expeditious infiltration of immune cells by rapidly leading to an increase in blood flow and permeability in capillaries. The immune response is tightly regulated and dependent on signaling through ligands binding to Toll-like receptors (TLRs) on the surface of macrophages[1,2]. Receptor/ligand interaction initiates a signaling cascade that involves the multifaceted enzyme, GSK3β that can then further modulate NFκB activity to transition between the generation of pro- and anti-inflammatory signals[84,85,86,87]. Although necessary and beneficial during infection and tissue injury, the pro-inflammatory cytokine response must be resolved in order to reset the homeostatic threshold and subsequently repair affected tissues in the absence of excess inflammatory mediators[26,27,88].

IL-6 induction serves a dual role in the transition between propagation of the inflammatory response and initiation of the APR[27]. The APR serves to reset homeostasis after the ensuing inflammation by mediating the production of acute phase proteins from hepatocytes[26,89,90]. These proteins include plasminogen activator inhibitor type 1 (PAI-1)

and urokinase plasminogen activator (uPA), both of which are involved in the regulation of hepatocyte growth factor (HGF) activity[90]. IL-6 also promotes HGF transcription and generation of the latent protein[72,91,92]. Since IL-6 production both promotes the increased generation of HGF and induces the APR, we hypothesized that accumulating HGF may act to resolve inflammation after stress.

To investigate this hypothesis we sought to assess the role of HGF and its cognate receptor, MET, with regard to innate immune activation of LPS-stimulated BMM. Our results demonstrate that in the presence of HGF there is a significant decrease in the secreted levels of IL-6, suggesting that HGF suppresses inflammation after injury. The suppression of IL-6 is achieved through HGF-dependent inactivation of GSK3β, a powerful governor of inflammatory signaling. This inactivation of GSK3β enhances the anti-inflammatory pathway by promoting the interaction of phospho-CREB with CBP and, occurs in concert with an overall decrease of phospho-p65 (Ser 276) and elevated levels of the anti-inflammatory cytokine IL-10[93,94,95,96,97]. Hence, our results indicate that HGF is a potent anti-inflammatory agent.

2.3 MATERIALS AND METHODS

2.3.1 Mouse strains

C57BL/6J and B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J mice were purchased from The Jackson Laboratory and the MET floxed mice were a gift from Dr. Snorri Thorgeirsson[76]. B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J were crossed with MET floxed mice to produce CRE^{lysZ+} MET$^{+/+}$ animals. Male mice 6 – 8 week of age were used as a source of bone marrow-derived macrophages. All animals were

housed under specific pathogen-free conditions in the Animal Facility at the University of Pittsburgh. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Assurance Number A3187-01).

2.3.2 Isolation of mouse bone marrow derived macrophages

Bone marrow derived macrophages (BMM) were cultured as previously described[98]. Femurs and tibias were dissected from sacrificed mice. Bone marrow cells were flushed from the bones using a 26 g needle until the bone is clear. The cells were then centrifuged and filtered through a cell strainer. Cells were cultured in 10% L929 conditioned media, with a media change every two days. Cultured cells were harvested and stained with the macrophage specific marker F4/80 to assess purity 7 days after isolation. Cells were plated on 24-well tissue culture plates at 1 x 10^6 cells/well for supernatant analysis or on 100 cm tissue dishes at 2.5 x 10^7 cells/dish for protein analysis.

2.3.3 Preparation of samples for ELISA

BMM plated in 24-well plates were pretreated with recombinant mouse HGF (R&D Systems) for 24 h at 37°C prior to stimulation with 1 μg/ml lipopolysacharride (LPS) from *Escherichia coli* (055:B5) (Signa Aldrich) for 24 h. In assays using the MET kinase inhibitor, SU11274 (Calbiochem), BMM were pretreated with 1 μM of the inhibitor for 2 h at 37°C followed by the

24 h HGF incubation and subsequent LPS stimulation. Cell culture supernatants were collected from triplicate wells, pooled and stored at -20°C for further analysis.

2.3.4 Preparation of protein lysates

BMM were prepared as above and cultured in 100 mm dishes. At 15 minutes post-LPS stimulation, the BMM were washed with ice cold PBS and cells were harvested with gentle scraping and centrifugation. Cellular extracts were harvested using RIPA buffer supplemented with phosphatase and protease inhibitor cocktails (Roche) and 1 mM PMSF (Sigma-Aldrich). After the addition of 0.3 ml of lysis buffer, the macrophages were incubated on ice for 20 min, vortexed 3 times and centrifuged for 5 min at maximum speed at 4°C. The supernatant (whole cell lysate) was collected and the protein concentration of the lysates was determined by the bicinchonninic acid (BCA) protein assay according to the manufacturer's instruction (Pierce).

2.3.5 Enzyme linked immunosorbent assay (ELISA)

Supernatants from BMM pretreated with HGF and/or SU11274 and stimulation with LPS were collected at 24 h. IL-6 and IL-10 cytokines secreted by BMM were measured by ELISA using purified capture and biotinylated detection antibody pairs (BD Pharmingen). The ELISA plates were read using a SpectraMax M2 plate reader, and the data was analyzed using SoftMax Pro software (Molecular Devices).

2.3.6 Western immunoblotting

Protein lysates were assayed by Western blot as previously described, with minor exceptions Tse, 2004 #34. Anti-phospho-GSK3β (Ser 9), anti-phospho-CREB (Ser 133) and anti-CREB used at 1:1000 from Cell Signaling. Anti-GSK3β was used at 1:1000 from Santa Cruz. Secondary antibodies were used from either Cell Signaling or Jackson ImmunoResearch. Blots were analyzed using the Fujifilm LAS-4000 imager and Multi Gauge software (Fujifilm Life Science).

2.3.7 Immunoprecipitation

1 μg of CBP antibody (Santa Cruz) was added to 100 mg protein from whole cell lysates and incubated at 4°C overnight. The following day, Protein G-conjugated sepharose beads were added to the protein/antibody complex tube and incubated for 2 h. Immunoprecipitates were collected by centrifugation, washed twice with PBS, and then boiled in Laemmli sample buffer. Western blot was performed as described above.

2.3.8 Cytospins

BMMs were washed with PBS and gently scraped from multi-well tissue culture dishes. Cells were collected into centrifuge tubes and spun at high speed for 10 min. Cell pellets were resuspended to $1x10^3$ cells per ml and cytospins were prepared with 100 μl of cells per slide. Slides were dried and stored at -80°C until staining.

2.3.9 Phospho-NFκB p65 (Ser276) staining

Cytospin slides were fixed in 2% paraformaldehyde for 5 minutes and blocked with 20% non-immune normal goat serum for 1 hour at room temperature. After immuno-staining with primary antibody (Rabbit anti-Phospho-NFκBp65 (Ser276) 1:100, (Cell Signaling Technology), slides were washed 5 times in PBS and incubated with secondary antibodies Cy3-conjugated goat anti-rabbit (Jackson ImmunoResearch). Nuclear staining (Hoechest staining, Molecular Probes) was performed. Images were viewed at 40x magnification and captured by using a Nikon confocal microscope (Nikon D-ECLIPSE C1, Japan).

2.4 RESULTS

2.4.1 HGF suppresses inflammation in bone marrow-derived macrophages

In order to determine that HGF plays a role in regulating the APR to suppress inflammation, we utilized a well-defined *in vitro* model of acute inflammation: LPS stimulation of BMM. BMM were cultured with various physiological concentrations of HGF and then stimulated with LPS. Figure 16 demonstrates that 10 pg and 10 ng of HGF exhibit a significant suppression of IL-6 production in LPS-stimulated BMM after 24 hours.

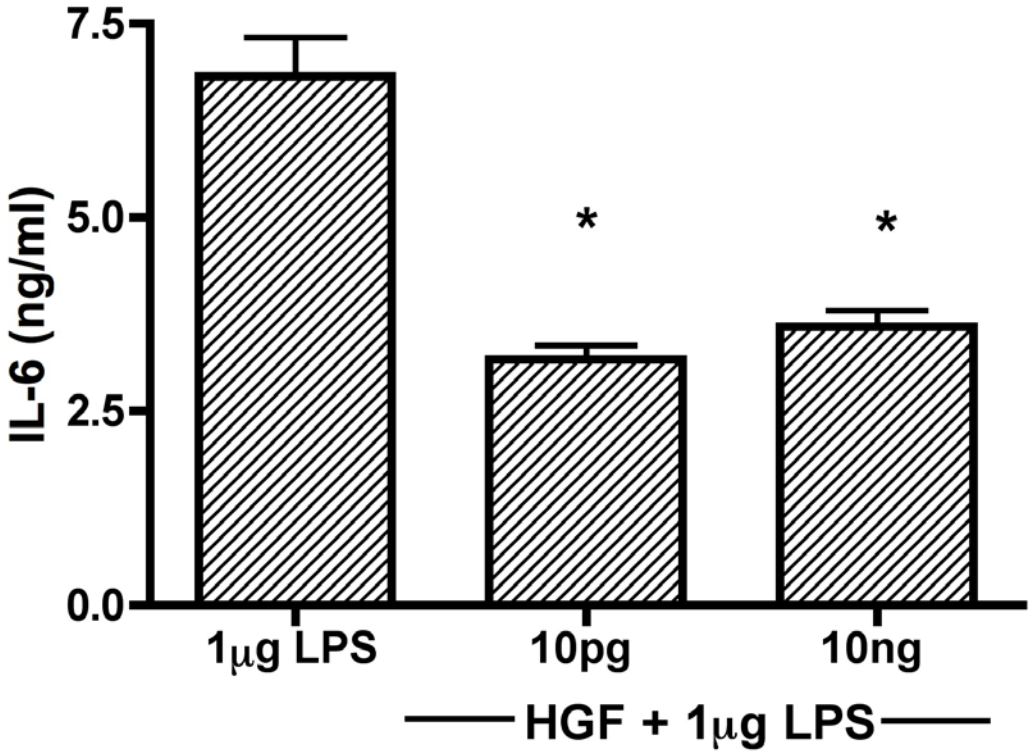


Figure 16. HGF Modulates IL-6 Production in LPS Stimulation Macrophages

*BMM derived from C57BL6 mice were pretreated with or without 10 pg and 10 ng HGF for 24 hours and stimulated with 1 μg/ml LPS. Cell culture media was collected (24 h) and IL-6 levels were measured by ELISA. Results are representative of the mean (± SEM) of three independent experiment done in triplicate, * indicates <0.001.*

2.4.2 Pharmacological inhibition of HGF-MET signaling abolished HGF's suppressive effects in BMM

To further confirm that the inhibition of IL-6 production was a result of HGF signaling, we repeated the *in vitro* model of acute inflammation, this time in the presence of SU11274, a specific MET inhibitor. An optimal concentration of 1 μM was chosen for the inhibition of signaling in BMM. SU11274[99,100] was added to BMM cultures 2 hr prior to the addition of HGF and then cultures were stimulated with LPS. The results demonstrate that incubation with the MET inhibitor abolished the inhibitory effect induced by HGF on IL-6 production in BMM stimulated with LPS (Figure 17).

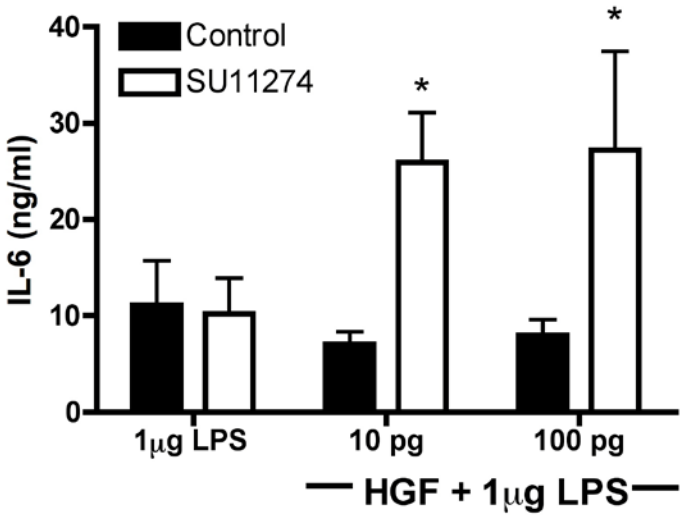


Figure 17. A MET Kinase Inhibitor Abrogates HGF Suppression of IL-6

*Using an optimal dose of the MET inhibitor SU11274 (1 μg), BMM were pretreated for 2 hours before an overnight incubation with 10 and 100 pg HGF and 24 hour stimulation with LPS. Results are representative of the mean (± SEM) of three independent experiments done in triplicate. *, p = 0.02 vs. the respective control group.*

2.4.3 Conditional deletion of MET-receptor on BMM confirms pharmacoloigical data demonstrating HGF's suppressive effects in BMM

To rule out any offsite pharmacological effects by the MET inhibitor as the cause for the IL-6 inhibition and to further study the important role HGF plays in tempering the acute inflammatory response, conditional MET flox mice specific for the macrophage lineage were generated. Figure 17 demonstrates that BMM isolated from MET conditional knockout mice fail to suppress IL-6 production in response to LPS as compared to their wild type littermate controls. Use of the knockout animals confirmed the results demonstrated with the pharmacological MET inhibitor whereby the suppressive effect of HGF on IL-6 production was significantly reduced as compared with cultures not treated with the inhibitor (Figure 18). Taken together, these results clearly illustrate the interaction of HGF and MET suppresses IL-6, further supporting the important role HGF plays in tempering the acute inflammatory response.

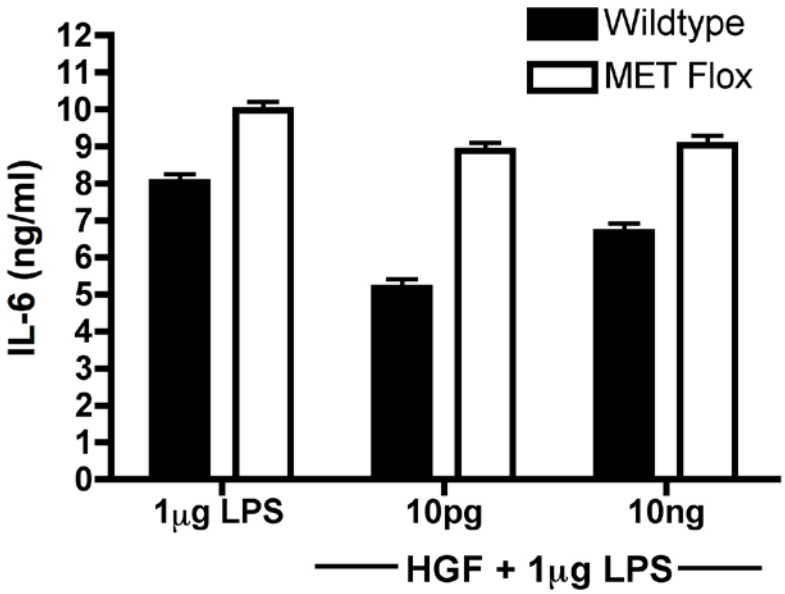


Figure 18. Deletion of the HGF Recepotor MET Deomstrates a Reversal in the Effects of HGF on LPS Simultated BMM

BMM derived from either macrophage specific MET conditional knockout mice ($MET^{fl/fl}$:$cre^{lysZ+/-}$) or their wild type littermate controls ($MET^{fl/fl}$:$cre^{lysZ-/-}$) were pretreated with or without 1, 10 and 100 pg HGF for 24 hours and stimulated with 1 μg/ml LPS. Cell culture media was collected (24h) and IL-6 levels were measured by ELISA. Results are representative of two independent experiments done in triplicate.

2.4.4 HGF suppresses inflammation through GSK3β

To further understand the mechanism in which HGF suppresses the inflammatory response, we looked downstream of HGF-MET signaling at potential regulatory targets. One target, GSK3β, is known to regulate inflammation through activation of NFκB, resulting in pro-inflammatory cytokine production[77,79,83,85,86,101]. When GSK3β is in its inactive state (phosphorylated), its influence over NFκB activation is limited and therefore pro-inflammatory cytokine production is quantitatively lessened[85]. GSK3β is known to be a downstream target of

HGF[81,82,83]. We found that protein lysates prepared from BMM isolated from C57BL6 mice cultured with 10 ng HGF demonstrated an increased in phosphorylated, or inactive GSK3β (Figure 19), supporting the idea that HGF-MET interactions lead to inactivation of GSK3β.

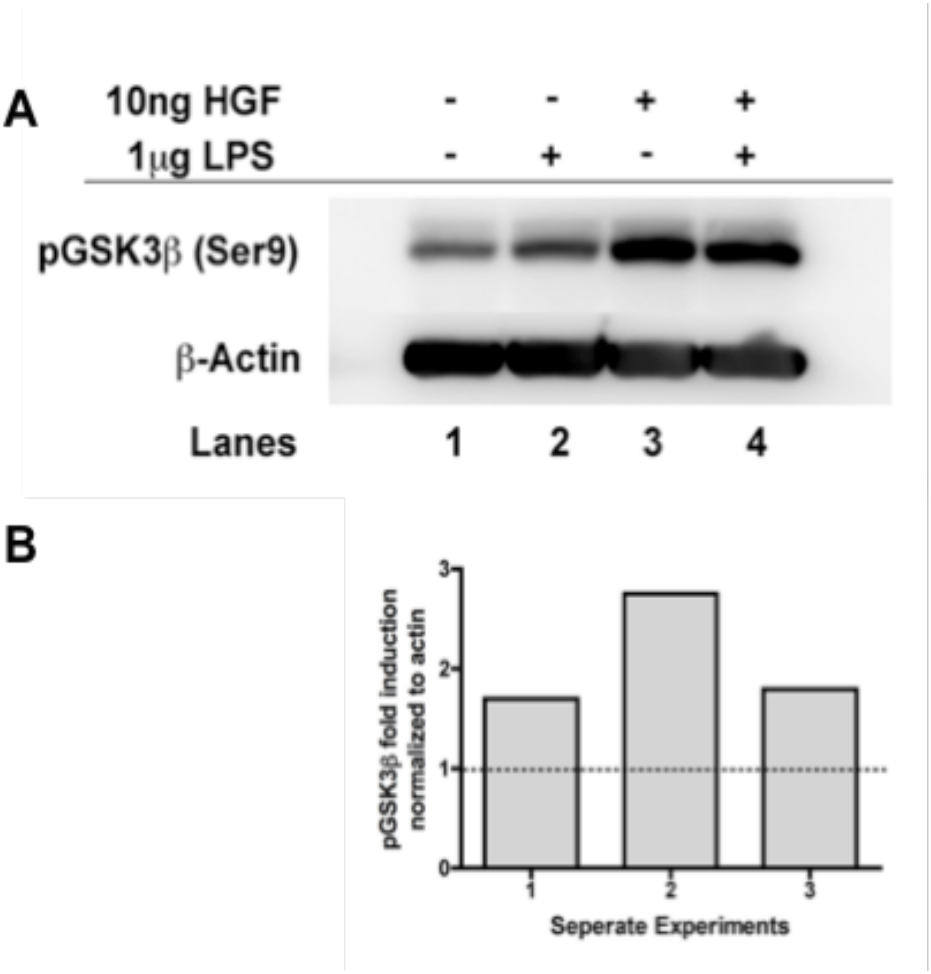


Figure 19. Tretament with HGF Leads to Increased GSK3β Phosphorylation

(A). Cytoplasmic lysates prepared from BMM were incubated with 10 ng HGF for 24 hours prior to stimulation with LPS (1μg/ml) for 15 minutes. The lysates were separated by SDS-PAGE and probed with a phospho-specific GSK3β antibody before re-probing for β-actin. (B). Densitometric analysis for phospho-GSK3β fold induction normalized to β-actin is shown for 3 separate experiments. Note that all experiments show induction greater than 1 when HGF is present.

2.4.5 HGF signaling leads to the interaction of CBP with phospho-CREB by GSK3β

To further determine the downstream signaling that results from HGF's regulation of GSK3β, we investigated the interaction of NFκB with the CREB-binding protein, CBP. Interactions between NFκB and CBP are facilitated by activated GSK3β when there is promotion of pro-inflammatory cytokine production[79,86]. Our data, however, shows that the inhibition of GSK3β following HGF treatment is associated with an increased interaction between CBP and phospho-CREB. Stimulation of BMM with LPS in the presence of HGF led to an increase of phosphorylated (inactive) GSK3β (Ser 9) (Figure 19), which correlated with an increase in CBP-phospho-CREB (Ser 133) interaction (Figure 20A) followed by an increase in the levels of IL-10 production (Figure 20C).

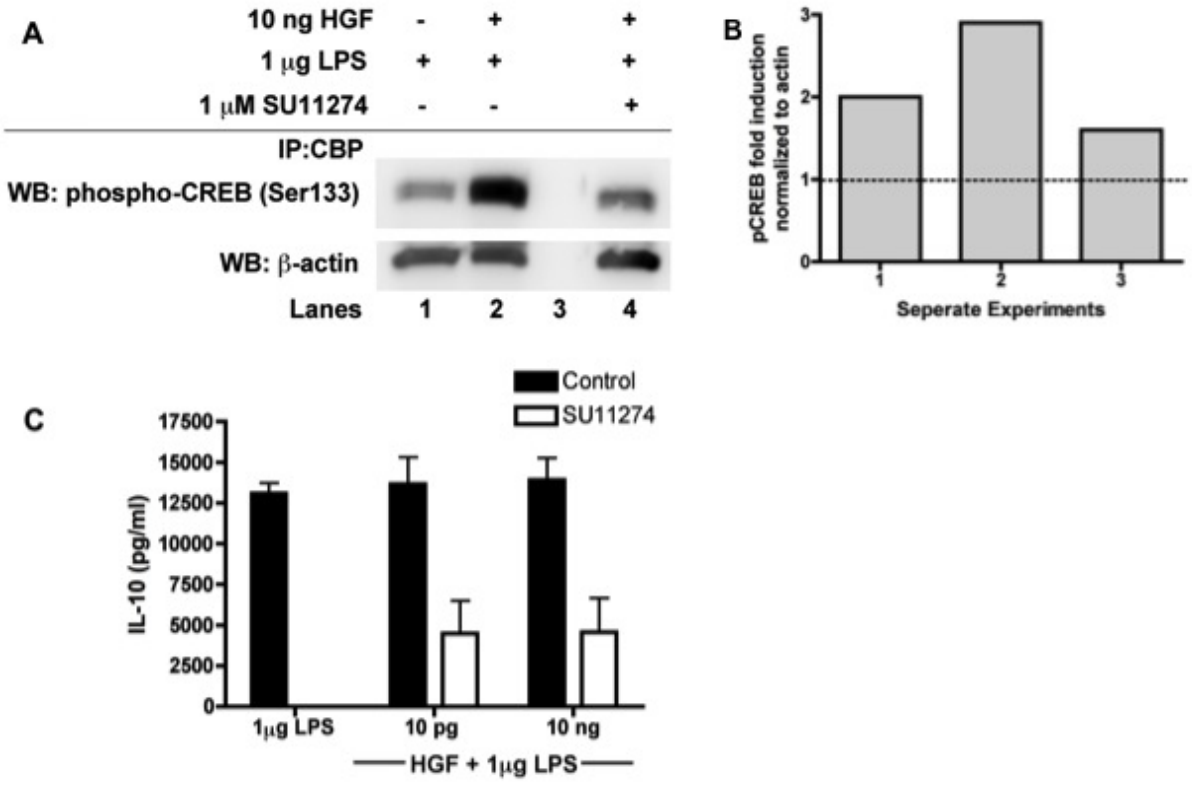


Figure 20. HGF Promotes the Interaction of Phosphorylated CREB with CBP Along with an Increased Production of IL-10

(A). Whole cell lysates prepared from BMM were incubated overnight with HGF (10 ng) prior to stimulation LPS (1μg/ml) for 15 minutes, then subjected to immunoprecipitation with a CBP antibody. The lysates were separated by SDS-PAGE and probed with a phospho-specific CREB antibody before re-probing for β-actin. (B). Densitometric analysis for phospho-CREB fold induction normalized to β-actin. Is shown for 3 separate experiments. Note that all experiments show induction greater than 1 when HGF is present. (C). BMM were pretreated with or without 1 μM of the MET kinase inhibitor (SU11274) for 2 h prior to incubation with HGF (1, 10 and 100 pg) for 24 hours followed by stimulation with 1 μg/ml LPS. Cell culture media was collected (24 h) and IL-10 levels were measured by ELISA. Results are representative of two independent experiments done in triplicate.

2.4.6 HGF inhibits the phosphorylation of Ser 276 on p65 of NFκB

The enhanced interaction between CBP and phospho-CREB during the HGF-induced anti-inflammatory response suggests CBP is being sequestered away from NFκB. Normally, the transcriptional activity of NFκB occurs through the phosphorylation of p65 at Serine 276 whereby a conformational change takes place that allows its nuclear association with CBP/p300 [78]. This interaction allows for NFκB-dependent transcription of pro-inflammatory cytokines such as IL-6. Figure 21 demonstrates abundant phosphorylation of p65 at Ser 276 in both the nucleus and cytoplasm upon stimulation with LPS alone (Figure 21B). However, with the addition of HGF (10 pg) the overall phosphorylation of p65 is reduced with nuclear localization essentially absent (Figure 21C). Again, the effects of HGF are abrogated when SU11274 is added to the cultures, indicating the suppression is mediated via the HGF receptor, MET (Figure 21D)

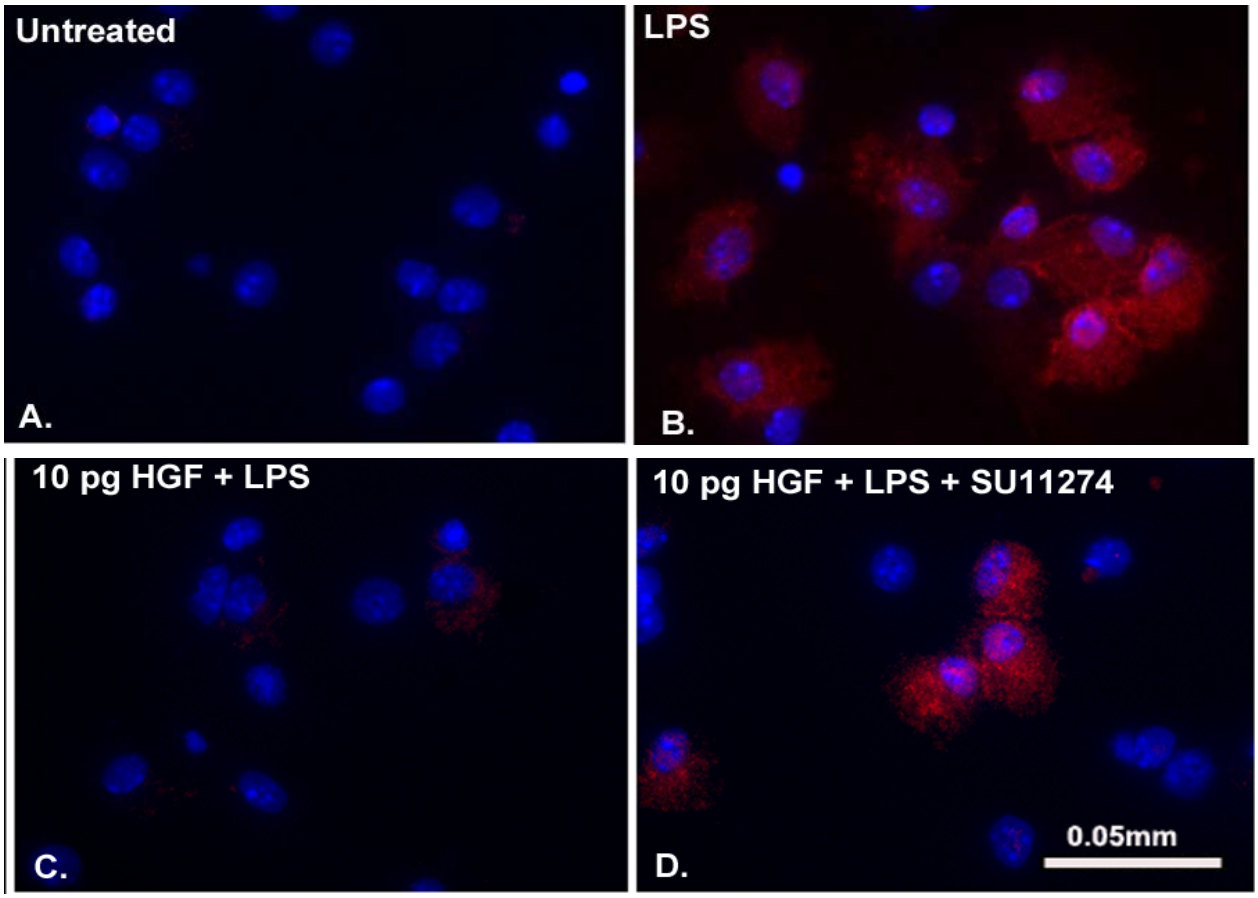


Figure 21. HGF Prevents the Nuclear Translocation of Phosphorylated p65

BMM were (A). untreated (B). stimulated with LPS (1μg/ml) (C). treated with 10 pg HGF and stimulated with LPS (1μg/ml) or (D). treated with the MET kinase inhibitor, SU11274, 10 pg HGF and stimulated with LPS (1μg/ml). Cytospin preparations were then stained by immunoflourescence for phosphorylated p65 (Ser 276) and for nuclei with DAPI. The blue staining indicates nuclei, red staining indicates phosphorylated p65, and purple staining indicates colocalization of phospho-p65 within the nucleus.

2.5 DISCUSSION

IL-6, a key pro-inflammatory cytokine, is upregulated "as a defense mechanism" in order to promote the APR and to initiate homeostasis as quickly as possible after an acute injury. In cases of chronic stress; however, IL-6 changes its role by modulating the leukocytic repertoire resulting in a chronic inflammatory state[27,89]. Although the presence of IL-6 is imperative during early injury and acute inflammation, contraction of the inflammatory process must occur in order for the system to regain normal homeostasis and to initiate repair. As a result, IL-6 is also posited to possess anti-inflammatory properties. Evidence to support this latter hypothesis is shown by the enhanced inflammatory responses induced following endotoxin exposure to IL-$6^{-/-}$ mice[27], although, the authors never suggest a biochemical mechanism to explain why this unanticipated phenomenon occurs. HGF, a cytokine widely known for perpetuating liver regeneration, has also been described as having anti-inflammatory properties in cases of persistent inflammation[80,81,82,83,102]. Importantly, the expression of HGF is induced by IL-6 while its regulation is controlled via acute phase proteins (urokinase and PAI-1) that are also induced following IL-6 stimulation. Hence, we posited a feedback loop wherein the pro-inflammatory properties ascribed to IL-6 are exhibited through induction of the APR and the anti-inflammatory properties are mediated via HGF that is produced in response to IL-6 stimulation.

Using LPS stimulated primary BMM cell cultures as a source of IL-6, we demonstrate that addition of HGF is in fact anti-inflammatory (Figure 16). Furthermore, we are able to confirm that the HGF-MET interaction propagates the suppression of cytokine production by using the pharmacological inhibitor of MET, SU11274 (Figure 17). Other studies have focused on using inhibitors of the PI3K and Akt signaling cascade[86,87], that are down-stream of HGF-

MET signaling; however, by using a direct MET kinase inhibitor as well as the macrophage -specific MET floxed mice (Figure 18), we demonstrate that HGF-MET signaling is capable of suppressing inflammation.

Traditionally, GSK3β is known for regulating glycogen synthase and the storage of glycogen into peripheral sites[103,104], but recent evidence suggests this kinase may also function as a key player in modulating inflammation[86,94]. It is well known that the activation of NFκB through TLR signaling leads to the transcription of pro-inflammatory cytokines; however, recently data was published indicating that at a more general level, cytokine production is regulated through GSK3β, which in turn regulates NFκB activity[86,87]. Hence, GSK3β appears to be a pivotal kinase that serves as a nodal point for both the generation and resolution of the inflammatory response[82,83,94]. Our data demonstrates that treatment BMM with HGF leads to increased phosphorylation and inactivation of GSK3β (Ser 9) (Figure 19) and that this response is sustained, even in the presence of LPS.

Pharmacological inhibitors of PI3K, Akt, and GSK3β induce inactivation of GSK3β. Inactive GSK3β can then promote the association of phospho-CREB (Ser 133) with CBP and sequester the CBP away from NFκB p65 (Ser 276). These signaling changes are associated with a switch from a pro- to anti-inflammatory pathway with a resultant increase in IL-10 production[86]. Our data shows that using HGF in place of those inhibitors gives similar results. BMM treated with both LPS and HGF demonstrated an increased CBP-phospho-CREB interaction, which was reduced in the presence of the MET kinase inhibitor (Figure 20). Furthermore, there was an increase in the production of IL-10 (Figure 20C) and, a reduction in the nuclear translocation phosphorylated p65. Combined, the data suggest that during an

inflammatory response, active HGF may be key in switching the cellular response from a pro- to an anti-inflammatory pathway.

In addition to the canonical pathway, TLR signaling has been shown to weakly activate PI3K. This then leads to anti-inflammatory events by altering the cytokine repertoire[86]. Hence, it has been postulated that PI3K is the point at which TLR signaling is differentiated from a pro-inflammatory to an anti-inflammatory condition. Our data indicates that in the absence of HGF, TLR signaling promotes the phosphorylation of NFκB along with its translocation to the nucleus and that this correlates with the production of the pro-inflammatory cytokine IL-6. In contrast, in the presence of HGF, GSK3β is phosphorylated (inactive) and TLR stimulation leads to production of the anti-inflammatory cytokine, IL-10. Notably, HGF signaling is well known to signal through the PI3K pathway[82,83,105]. Hence, we propose that under normal circumstances, induction of IL-6 through pro-inflammatory stimuli leads to the eventual production of HGF[72,92,106]. HGF-MET interactions then ultimately result in phosphorylation of GSK3β and in the continued presence pro-inflammatory stimuli, facilitates an increased association of phospho-CREB with CBP. This then suppresses NFκB's transcriptional activity and results in the resolution of the inflammatory response (Figure 21). Our data describe the intimate interaction between IL-6 and HGF in regulation of inflammation. Hence we propose that HGF acts as an internal rheostat regulating the complex cascade of induction and resolution of inflammation (Figure 22).

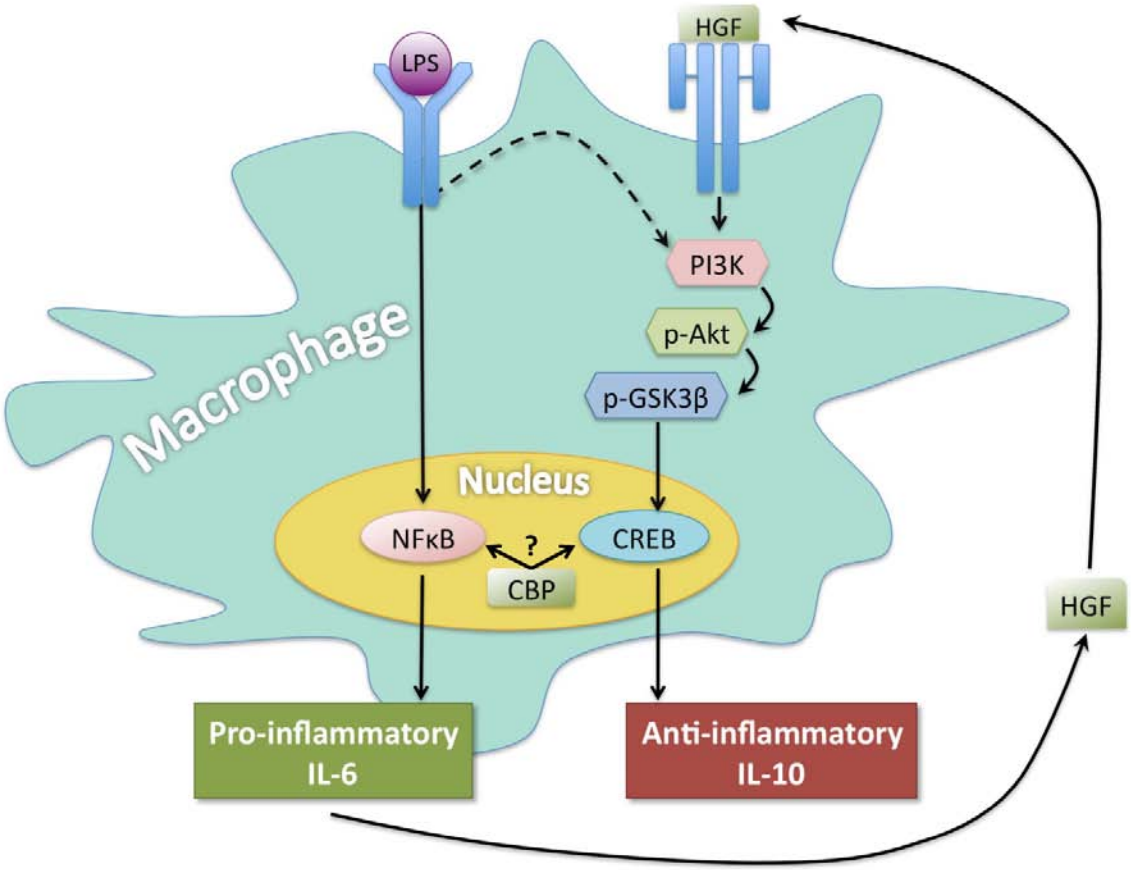


Figure 22. Proposed Mechanism of HGF-Mediated Suppression

The canonical signaling pathway of LPS-TLR engagement leads to NFκB dependent pro-inflammatory cytokine production through the interaction of CBP with NFκB. However, TLR signaling has also been shown to weakly activate alternative signaling through PI3K, resulting in phosphorylation and inactivation of GSK3β, subsequent sequestration of CBP from NFκB to phospho-CREB, and resultant anti-inflammatory (IL-10) production [86]. Our results show that the presence of HGF enhances the IL-10 pathway. We postulate that in order to resolve inflammation, the generation of IL-6 by LPS-TLR signaling leads to the production of HGF, ultimately leading to the inhibition of inflammation. Hence, we propose HGF acts as an internal rheostat for resolving acute phase inflammatory responses.

3.0 THE ROLE OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 IN OBESITY-INDUCED DIABETES

3.1 ABSTRACT

Increased adipose tissue is associated with chronic inflammation and expression of acute phase response (APR) proteins, including elevated levels of PAI-1. Hence obesity is considered to be a major risk factor toward the progression to T2D; aberrant expression of the cytokines TNF-α and IL-6 can initiate insulin resistance and lead to T2D. On the other hand, the liver APR functions to ensure that the pro-inflammatory cytokines are down regulated, restoring homeostasis. Hepatocyte Growth Factor (HGF) is a key protein responsible for resetting the hepatic homeostatic threshold after injury and is cleaved to its active form (aHGF) by the urokinase-type plasminogen activator (uPA). PAI-1, an inhibitor of uPA and anti-fibrinolytic agent, has been linked to increased cardiovascular events in obese patients. Since HGF's activation, interaction and signaling through its receptor MET is dependent upon uPA, by default elevated PAI-1 also regulates HGF's function. We hypothesized that elevated PAI-1 inhibits HGF's ability to regulate inflammation *in vivo*, augmenting insulin resistance and hepatic steatosis. Mice fed a high fat diet (HFD) were significantly overweight, glucose intolerant, hyperinsulinemic, and steatotic displaying increased macrophage recruitment to the liver and adipose tissue. Importantly, HFD fed mice showed a decrease in aHGF and elevated levels of PAI-1 protein.

The results demonstrate that inhibition of HGF by PAI-1 may play a role in unresolved inflammation seen in obesity and T2D.

3.2 INTRODUCTION

Obesity is a leading cause for the development of type 2 diabetes (T2D). Over nutrition leads to increased caloric intake and dysfunction in metabolic homeostasis with the overwhelming presence of adipose tissue resulting in obesity-induced T2D[54]. The dysregulation in metabolism leads to a state of chronic inflammation, characterized by elevated pro-inflammatory cytokine production and the activation of the acute phase response (APR), ultimately leading to insulin resistance and dysregulation in hepatic glucose metabolism[52,54,107]. The generation of pro-inflammatory cytokines by circulating macrophages recruited to adipose tissue facilitates obesity-induced inflammation, resulting in insulin resistance and T2D[51,55,59,64].

Increased adiposity is associated with chronic inflammation and expression of APR proteins that are secreted by the liver[52,54,107]. These proteins reflect the presence and intensity of inflammation and are part of how the liver attempts to maintain body homeostasis during stress, infection and/or injury[27,89]. On the other hand, proper homeostasis of the liver itself is regulated by a protein known as Hepatocyte Growth Factor (HGF), a mitogen for liver repair and maintenance that is transcribed in response to the pro-inflammatory cytokine IL-6[72]. Among its other physiological roles in liver, HGF also plays a key role in resetting resetting the hepatic homeostatic threshold after injury and is cleaved to its active form (aHGF) by the urokinase-type plasminogen activator (uPA)[73,92,108]. Plasminogen activator inhibitor type-1 (PAI-1), an acute phase protein, inhibitor of uPA and anti-fibrinolytic agent, is upregulated in

obesity and has been linked to increased cardiovascular events in obese patients[109,110,111,112]. Since HGF's activation, interaction and signaling through its receptor MET are dependent upon uPA[90,113], and because PAI-1 inhibits the function of u-PA, by default elevated PAI-1 also regulates HGF's function.

We have shown that aHGF is palliative in chronic inflammatory conditions by suppressing IL-6 production in a GSK3β dependent manner[114]; therefore in T2D, aHGF is a likely candidate for controlling excess inflammation. Early cytokine and APR protein production promotes the generation of aHGF, leading to suppression of inflammation and inflammatory associated pathologies. However over time, increasing adiposity generates pro-inflammatory cytokines, such as TNF-α, leading to accumulations of PAI-1 resulting in increased levels of inactive HGF, chronic inflammation and progression to T2D.

3.3 MATERIALS AND METHODS

3.3.1 Animals and diet

Early diabetes: Male C57BL/6J mice were purchased from Jackson Laboratory. At 6 weeks of age, the mice were divided into two groups. One group (n=4) was fed a high fat diet (HFD) consisting of 60% kCal fat (Research Diets, Inc) and the other group (n=5) was fed the standard chow diet used as controls. The animals were fed the diet for 12 weeks. *Late diabetes*: Male C57BL/6J mice (n=18) were fed the HFD and housed at the Jackson Laboratory through the diet-induced obesity service. The animals were fed for 40 weeks before being transferred to the

University of Pittsburgh animal facility. Age matched retired male breeder C57BL/6J mice (n=16) purchased from Charles River fed the standard chow diet were used as controls.

All animals were housed under specific pathogen-free conditions in the Animal Facility at the University of Pittsburgh. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh (Assurance Number A3187-01).

3.3.2 Biological parameters

Body weight was recorded weekly up until termination of the experiment. At the time of sacrifice, whole body composition was evaluated by quantitative nuclear magnetic resonance scans using the EchoMRI system. The scans result in body composition including lean and fat mass per animal. Fasting serum insulin was evaluated for the long term-HFD fed animals (43 weeks). Blood samples were collected and serum insulin levels were measured with a mouse insulin enzyme-linked immunosorbent assay (ELISA) kit (Mercodia). The ELISA plates were read using a SpectraMax M2 plate reader, and the data was analyzed using SoftMax Pro software (Molecular Devices).

3.3.3 Intraperitoneal glucose tolerance test (IPGTT)

A 20% glucose solution (2.5 g/kg) was intraperitonealy administered to fasted mice at 9 weeks of HFD feeding (short term) and 44 weeks of HFD feeding (long term). Blood samples were taken

at 0 (before glucose injection), 30, 60 and 120 minutes after glucose injection. Blood glucose levels were measured using a glucometer (Ascensia Breeze, Bayer).

3.3.4 Histology

Livers and adipose tissue of sacrificed animals fed the standard or HFD were harvested and either fixed in 10% formalin and embedded in paraffin or frozen in OCT freezing medium. Cut paraffin sections were stained with hematoxylin and eosin and an F4/80 antibody. Frozen sections were cut and stained with Oil Red O.

3.3.5 Tissue lysates preparation

Protein lysates were prepared from snapfrozen tissue using a detergent-free lysis buffer (10 mM Tris-HCl, pH 7.5) with inhibitors as previously described[113,115,116]. Amiloride and aprotinin were used in the lysis buffer as they specifically inhibit u-PA and plasminogen activities, respectively. Briefly, frozen chunks of tissue were weighed, homogenized and centrifuged for 3 h in a refrigerated microfuge at 21,000 x g. The supernatant was removed and save and the pellets were further solubilized in 1% SDS with inhibitors. The Bicinchoninic Protein Assay (Pierce, Rockford, Il) will be used to determine the protein concentrations. Supernatants are enriched for cytosolic proteins; pellets represent a nuclear-, matrix- and membrane-enriched fraction.

3.3.6 Western blot

Lysates (preparation described above) were separated electrophoretically on a 10% SDS gel. The gel was then transferred onto a PVDF membrane, blocked and probed with a HGF or PAI-1 antibody. In order to distinguish between latent HGF and aHGF, these lysates will be assayed under reduced conditions (addition of DTT) since the HGF isoforms are different sizes (scHGF: ~92kD, hcHGF: ~60kD, lcHGF: ~33kD). Alternatively, non-reducing (without DTT) conditions will provide the level of total HGF protein in the sample. Densitometry will be used in order to quantitate the differences in protein levels between each group of mice.

3.3.7 Statistics

Prior work from our laboratory has demonstrated that group sizes ≥ 5 are sufficient for generating statistically meaningful results with significance is set at a p value <0.05. For the hepatic studies, we propose to use 6 animals (6 male) per time point unless differences related to sex are noted. If so, for statistical purposes we will increase the test cohort to 10. Comparisons between groups will be evaluated by analyses of variance (ANOVA) with post-analyses performed using the Tukey multiple-comparison test. Either the InStat program (GraphPad Software) or JMP statistical package software (SAS) will be used.

3.4 RESULTS

3.4.1 Weight gain and body composition of C57BL6 mice fed a high fat diet

In order to test our hypothesis that aHGF is lost due to the accumulation of PAI-1 as a result of obesity-induced diabetes, we placed C57BL/6 mice on a 60% kCal high fat diet for 12 (early diabetes) and 44 (late diabetes) weeks. Mice fed a control diet were also monitored for the duration of the study. Figure 23 shows the body weight composition of both cohorts of control and HFD fed mice. As expected, the body weight of HFD fed mice (early and late) was significantly increased due to the presence of fat mass as compared to mice fed the control diet.

Body Weight and Composition

Early Diabetes (11 Weeks)

	Control	HFD	*p*
Weight, g	27.1 ± 0.54	34.1 ± 1.26	0.0002
Lean Mass, g	21.27± 0.81	19.13± 0.35	NS
Fat Mass, g	3.40± 0.13	10.15± 1.46	0.0092

Values are mean ± SEM; n=9/group for weight, control and HFD; n=5/group for lean and fat mass, control; n=4/group for lean and fat mass, HFD. HFD, High Fat Diet; NS, not significant.

Late Diabetes (44 Weeks)

	Control	HFD	*p*
Weight, g	33.2 ± 0.85	50.0 ± 1.50	<0.0001
Lean Mass, g	24.01 ± 0.47	26.97 ± 0.50	<0.0001
Fat Mass, g	5.72 ± 0.76	19.60 ± 1.64	<0.0001

Values are mean ± SEM; n=16/group for weight, lean and fat mass, control; n=18/group for all HFD values. HFD, High Fat Diet; NS, not significant.

Figure 23. Body Weight and Composition of Control and HFD Fed Mice

3.4.2 HFD-fed mice are glucose intolerance and hyperglycemic

In an effort order to determine that the HFD was indeed leading to advanced metabolic dysfunction and glucose intolerance, we performed an intraperitoneal glucose tolerance test (IPGTT). The early cohort of diabetic mice fed the HFD presented with only mildly glucose intoleranance peaking at 30 minutes and then returning to normal blood glucose levels (Figure 23). However the mice fed HFD for 44 weeks were significantly glucose intolerant and demonstrated elevated blood glucose levels near 600 mg/dL for the full 2 hrs post initial challenge (Figure 25).

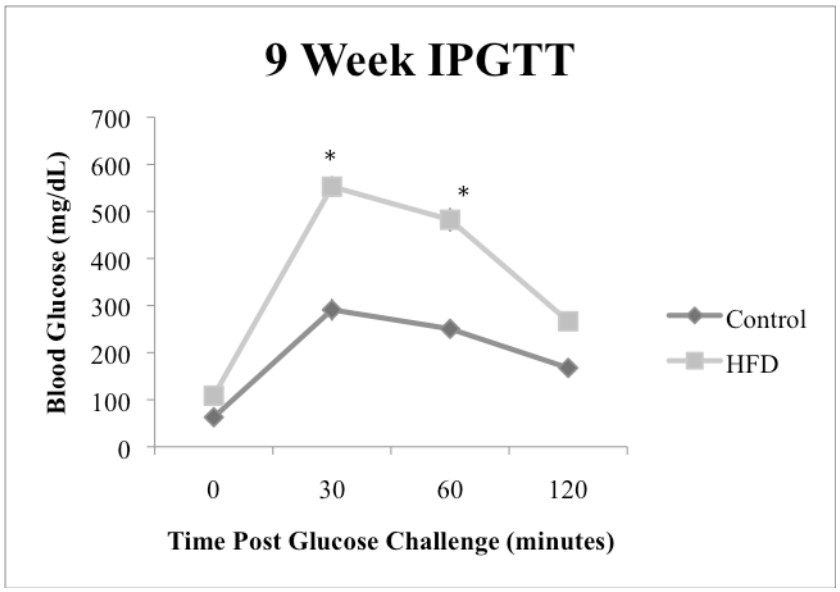


Figure 24. IPGTT for Mice Fed HFD for 9 Weeks

*Mice fed HFD or standard chow for 9 weeks were subjected to an IPGTT. Animals were fasted overnight and given a dose of glucose (2.4 g/kg) IP. Blood glucose levels were recorded at 0, 30, 60 and 120 minutes post-glucose challenge. Values are mean ± SEM post glucose challenge; n=5 per group for 0 minutes control; n=9 per group for 30 and 120 minutes control; n=8 per group for 60 minutes control and all HFD time points. * indicates p < 0.0001.*

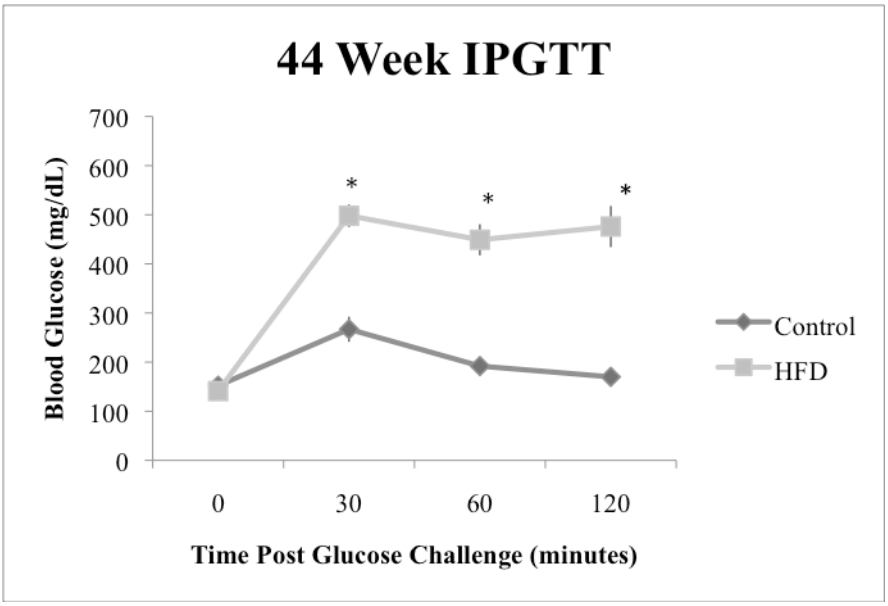


Figure 25. IPGTT for Mice Fed HFD for 44 Weeks

*Mice fed HFD or standard chow for 44 weeks were subjected to an IPGTT. Animals were fasted overnight and given a dose of glucose (2.4 g/kg) IP. Blood glucose levels were recorded at 0, 30, 60 and 120 minutes post-glucose challenge. Values are mean ± SEM post glucose challenge; n=17 per group for all control time points; n=10 per group for all HFD time points. * indicates p < 0.0001.*

In addition, the late stage diabetic mice (44 weeks) were significantly (p <0.0001) hyperinsulinemic compared to the control fed mice. Fasting insulin levels (pmol) for control vs. HFD fed mice measured 150.92 ± 22.32 vs. 806.94 ± 67.13, respectively (Figure 26). These results corroborate the glucose intolerance results.

Fasting Serum Insulin

Late Diabetes (43 Weeks)

	Control	HFD	*p*
Insulin, pmol	150.92 ± 22.32	806.94 ± 67.13	<0.0001

Values are mean ± SEM; n=12 per group for control; n=18 per group for HFD. HFD, High Fat Diet.

Figure 26. Fasting Serum Insulin

3.4.3 HFD feeding induces hepatic steatosis and macrophages infiltration

A histological examination of livers from mice in the HFD study illustrates lipid accumulation as seen by H & E staining (Figure 27A**)**. Whereas the early diabetic mice begin to show a slight hepatic lipid accumulation, the 44-week HFD fed animals present with a dramatic increase in the accumulation of lipid droplets. The elevated lipid accumulation in the 44 weeks HFD animals as compared to the standard chow fed animals was further confirmed by Oil Red O staining of liver sections (Figure 27B).

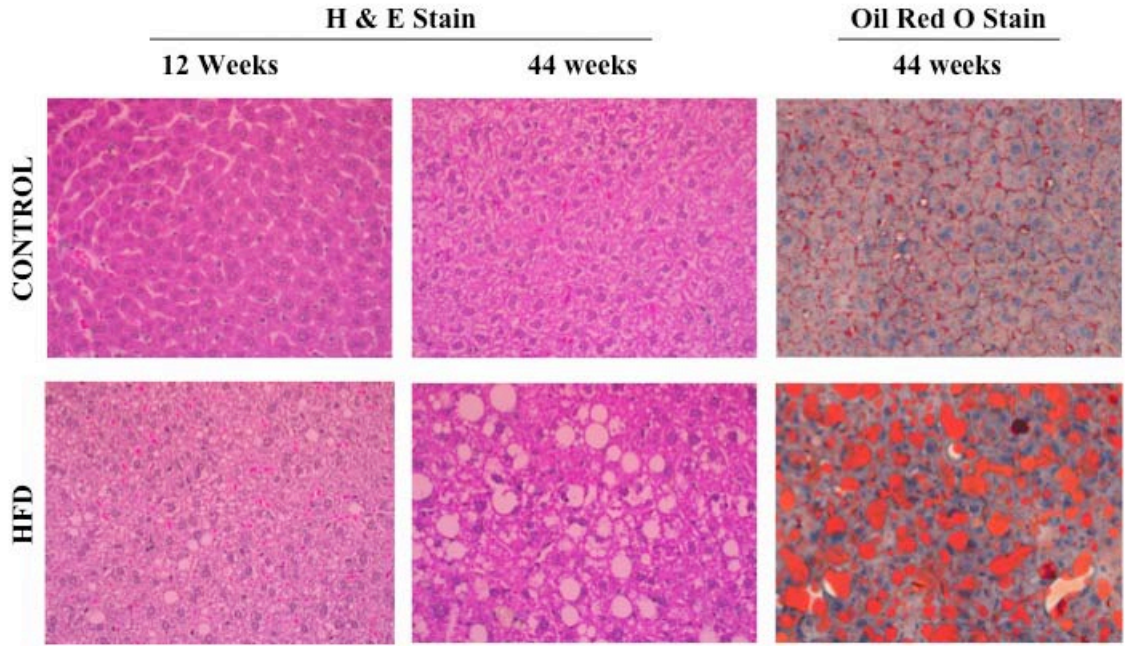


Figure 27. Histological Examination of Livers From Mice Fed HFD

(A) Paraffin sections of livers harvested from 12 week and 44 week HFD fed animals were stained with hematoxylin and eosin. Lipid droplets, indicative of hepatic steatosis, can be seen in the livers of the HFD fed mice with an increase in steatosis in the late stage diabetics. (B). These results are further confirmed using the lipid specific dye, Oil Red O, on frozen liver section of mice fed a HFD for 44 weeks. The red stain shows the presence of lipids within the liver tissue. Shown are single tissue sections representative of each group, 40x magnification.

In addition to hepatic lipid accumulation, immune cells such as macrophages will also be activated and recruited to peripheral sites including the liver and adipose tissues[55,66,117]. The presence of macrophages can perpetuate insulin resistance with the over production of NF-κB-dependent[59,64] pro-inflammatory cytokines such as IL-6 and TNF-α[60,63,118]. To determine the presence of macrophages, we stained both adipose and liver sections with an antibody specific to the F4/80 antigen present on macrophages; as resported in adipose tissue, macrophages formed a "crowning" pattern around adipocytes[119] (Figure 28A**).** In the liver the macrophages focused around lipid droplets (Figure 28B**)**.

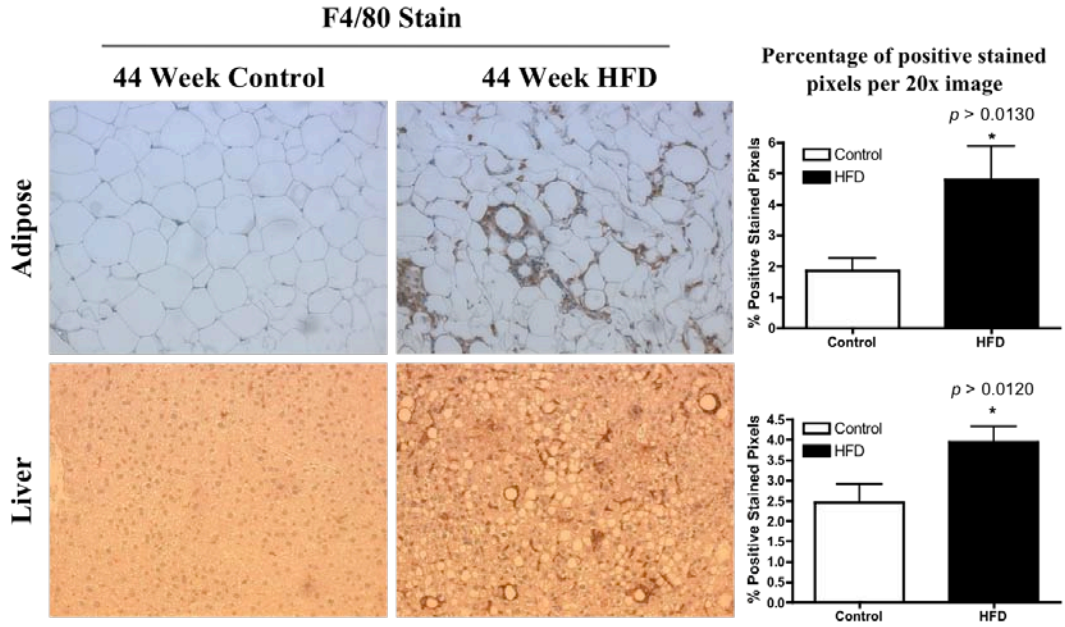


Figure 28. Histological Examination of F4/80 Positive Cells in Liver and Adipose Tissue

Adipose (A) and liver (B) tissue from mice fed a HFD for 44 weeks (Late Diabetes) were examined for the macrophage marker F4/80. Panel (A) shows significant F4/80$^+$ staining/accumulation, and characteristic crowning, in the adipocytes from HFD fed mice compared to control. Shown are single tissue sections representative of each group. 400x Magnification. Percent positive pixels were determined by Image J Analysis. (B) shows a significant increase of F4/80$^+$ cells in the mice fed HFD versus the control fed mice, indicative of infiltration. Note the characteristic 'crowns' of F4/80 staining appears circling accumulated

lipid droplets in the hepatic tissue. Three sections each were analyzed from three individual animals / group, HFD vs control to calculate mean ± SEM, followed by Student's T-test for significance.

3.4.4 Obesity leads to increases in PAI-1 protein resulting in decreases levels of aHGF

Recently we reported that HGF plays a key role in contracting the innate immune response by suppressing and shifting cytokine production[114] in models of inflammation. In the context of T2D, the presence of increased adipose tissue feeds chronic inflammation and the acute phase reaction resulting in insulin resistance, glucose intolerance and exacerbation of the disease (Figs 24, 25, 26). We propose that the increase of the acute phase protein PAI-1 by the adipose tissue negatively regulates the activation of HGF and therefore abolishes its anti-inflammatory effects. Using the diet induced obesity model of T2D we were able to further examine the expression and regulation of these proteins. At 12 weeks, HFD fed animals were obese and displayed an abnormal response to glucose challenge (Figure 24). Consequently, there is an increase the presence of aHGF in the cytosol indicative of its activation and signaling likely due to the stress of diet-induced obesity. (Figure 29A). At 12 weeks, there is also an increase in PAI-1 protein the presence of which indicates induction of the acute-phase response. By 44 weeks when the animals fed a HFD were fully insulin resistant (Figure 25), latent HGF was highly elevated on the cell surface, in conjunction with elevated surface PAI-1 (Figure 29B). These data support the hypothesis that elevated levels of obesity-induced PAI-1 on the cell surface will inactivate u-PA, leading to loss of aHGF.

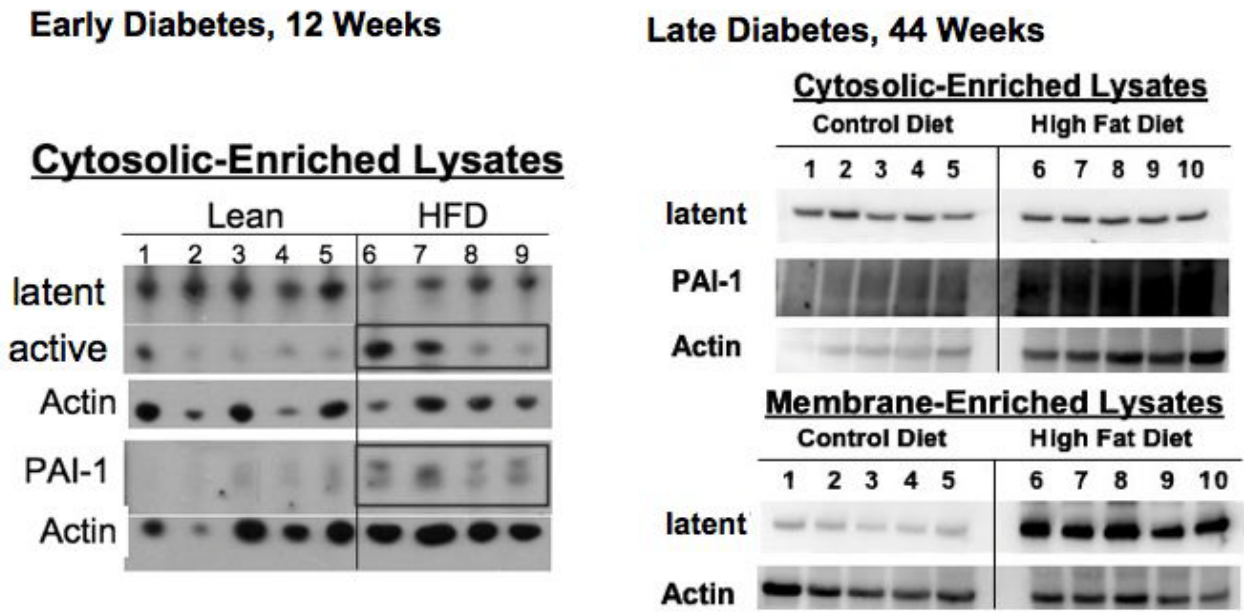


Figure 29. Effects of High Fat Diet on HGF and PAI-1

(A) Early diabetic mice (12 weeks) were sacrificed, cytoplasmic-enriched proteins were isolated, and lysates were subjected to western blot analyses for HGF and PAI-1. The HFD fed mice demonstrate an apparent increase in aHGF as well as PAI-1 compared to the lean controls. (B) Late diabetic mice subjected to the same assay demonstrate an apparent increase in the latent form of HGF in the membrane-enriched lysates as compared to the early diabetic mice.

3.5 DISCUSSION

The diet-induced obesity model is a standard approach to induce the T2D phenotype in male C57BL6 mice. We chose this model over the genetically predisposed mouse strains of obesity, the leptin deficient (ob/ob) or leptin receptor deficient (db/db) strains because the HFD model most likely reflects was is occurring in the human population: a polygenic and environmentally/lifestyle-induced disease. In our hands, diet-induced obesity was evident as early as 12 weeks of feeding and lasted into late stage diabetes (44 weeks of feeding). The

weight gain contributed to the glucose intolerance and was correlated with an increase in fat mass, not lean mass present in these animals (Figure 23). The early stage diabetic animals had only a slightly abnormal glucose intolerance test, however the presence and intensity of the fat mass over time (44 weeks) resulted in the obese mice failing the glucose tolerance test concomitant with hyperinsulinemia (Figures 23 - 26). These data strengthen the hypothesis that increases in obesity will perpetuate the metabolic syndrome leading to severe glucose intolerance.

Recent studies have demonstrated the significance of NFκB-dependent pro-inflammatory cytokines fueling inappropriate insulin signaling resulting in insulin resistance[53,59,64,120]. IL-6 and TNF-α promote serine phosphorylation of the insulin receptor substrates as well as JNK activation all of which result in abnormalities in glucose metabolism[54,60,62,121]. Cells of the innate immune system, including macrophages become activated and are the chief producers of these cytokines. Additionally, adipocytes are also described to be 'metabolically active' capable of producing cytokine and chemokines. As the caloric intake is exceeded in individuals by overnutrition and lack of exercise, surplus fat accumulates in peripheral sites, including the liver, resulting in hepatic steatosis. Increased hypertrophy of adipocytes as a result of overnutrition leads to hypoxia induced oxidative stress and the production of MCP-1[122,123]. MCP-1 produced by adipocytes will recruit circulating macrophage into the adipose tissue or any other tissue where adipocytes exist. As macrophages are also able to produce MCP-1, a paracrine loop forms and macrophage infiltrates occur within peripheral organs[51,55,66,124]. Overtime, an excess of pro-inflammatory cytokines exist and disrupts insulin signaling and progression to T2D ensues. Our data supports this mechanism as we see hepatic steatosis increasing in severity in animals on the HFD from 12 to 44 weeks (Figure 27)

as evidenced by H&E and Oil Red O staining. This occurs in concert with an increase in the presence of F4/80^{+} cells (Figure 28), indicative of macrophages within the liver and adipose tissue of obese versus lean mice.

Pro-inflammatory cytokines are responsible for the production of APR proteins from hepatocytes, including HGF, uPA and PAI-1[26,89]. Obesity, as a result of chronic HFD feeding stimulates inflammation activating the APR resulting in elevated levels of PAI-1[52,107,124]. In cases of acute stress, the acute phase proteins serve to clear the injury and return the system to homeostasis. We and others have demonstrated HGF's anti-inflammatory role in several incidences of inflammation[80,81,82,83,114]. Traditionally, uPA and PAI-1 have been associated with cardiovascular complications. Their alternate function however is in the regulation of HGF activation, as urokinase activators (uPA and tPA) cleave HGF from its latent form to the biologically active form[70]. Since PAI-1 inhibits the function of uPA, indirectly it negatively regulates HGF. Therefore we hypothesize that increasing amounts of obesity-induced PAI-1 inhibits the activation of GF thus negating its anti-inflammatory effects. Our data demonstrates that livers from HFD fed mice at 12 weeks show an increase in aHGF and PAI-1 protein as a result of the activation of the APR (Figure 29A). However, livers from mice fed HFD for 44 weeks continue to demonstrate increased levels of PAI-1, but show an accumulation of inactive HGF (Figure 29B). A proposed model for PAI-1's negative regulation of HGF is shown in figure 30.

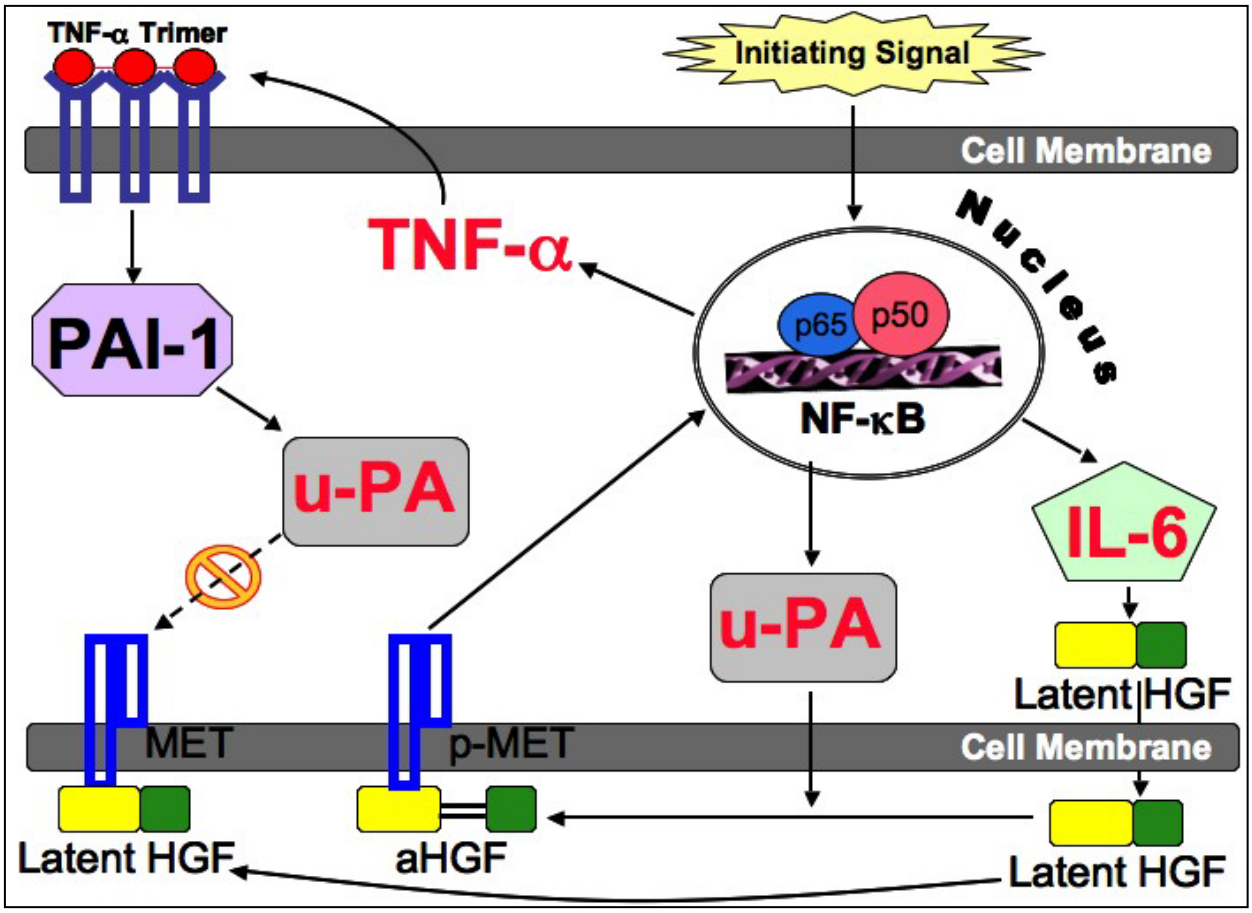


Figure 30. Hypothesized Model of Hepatocyte Growth Factor (HGF) Activation and Control of the Inflammatory Response

Stimulation of the transcription factor NF-κB leads to production of pro-inflammatory cytokines and acute phase proteins, including TNF-α, IL-6, urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor 1 type I (PAI-1). Production of TNF-α promotes production of PAI-1 and also leads to further activation of NF-κB. mRNA for HGF are transcribed in response to IL-6 resulting in enhanced translation of the protein through NF-κB activation. PAI-1 can inactivate u-PA, a protease that activates latent HGF to active HGF (aHGF). Therefore when high levels of aHGF are present, inflammatory signaling of cytokine production through the NF-κB pathway is inhibited

4.0 CONCLUSIONS AND FINAL REMARKS

The best treatment for obesity-induced diabetes is a balanced diet and regular exercise. Not only are BMIs of 30 and above a primary risk factor for a plethora of diseases including stroke, hypertension, and cancer, increase adiposity also feeds low-grade inflammation and perpetuates insulin resistance and T2D. Obesity is becoming an epidemic worldwide and childhood obesity rates are increasing at alarming rates, having tripled in the past 30 years[125]. It is this time, more than ever, that our youth requires early education on eating well and staying active. However, when diet and exercise are not enough, pharmaceuticals and even surgical procedures are implemented in order to combat obesity and diabetes. Traditionally aspirin therapy was utilized for T2D therapy, and presently there are 6 classes of oral medications to lower blood glucose including sulfonylureas (Glucotrol) and thiazolidinediones (Avanida) as well as injectable medications (Byetta) (diabetes.org). Additionally, weight loss or bariatric surgery is popular for severely obese individuals with a BMI of at least 40. However, with all of these options, risk factors are always a concern, particularly with diabetes medications being ‘pulled from the shelves’ (Avandia) and possible mortalites of surgical procedures. Therefore prevention is key in avoiding extreme and often times fatal treatment options.

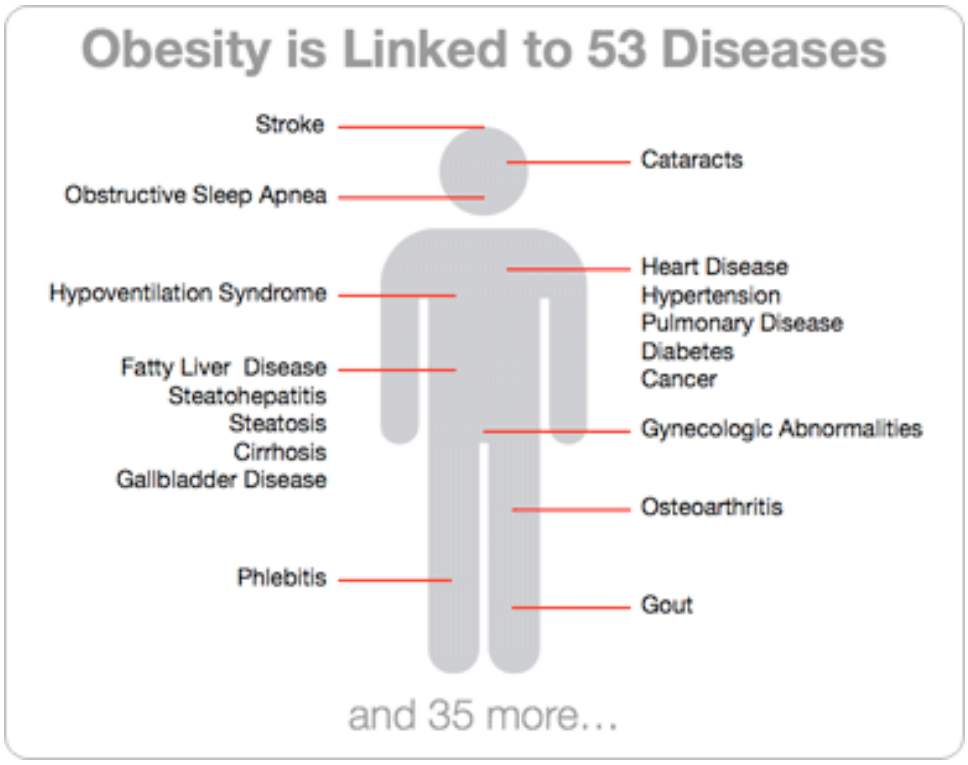


Figure 31. The Contribution of Obesity to Disease

Figure taken from[126].

One way to improve prevention strategies is to understand the mechanism of the disease. Luckily, as of 2010 we have a great understanding of how environmental stress, such as over nutrition and obesity fuels inflammation by interrupting insulin signaling resulting in insulin resistance and the deleterious effects of hyperglycemia on the body. Ultimately, suppressing inflammation was the primary focus of my research. The early observation that HGF acts to suppress pro-inflammatory cytokine production in bone marrow derived macrophages, one of the key cells involved in pressing the inflammatory cascade as a result of obesity, lead to unraveling the mechanism of HGF signaling through the MET kinase, thus inactivating GSK3β allowing for CBPs association with phospho-CREB skewing the cytokine profile from pro-inflammatory (IL-6) to anti-inflammatory (IL-10). In non-pathological conditions, the anti-inflammatory effects of HGF in concert with the acute phase response are enough to suppress inflammation enabling the

system to regain homeostasis. However, how the effects of HGF are lost in pathological, inflammatory mediated diseases is the greater question.

Realizing the redundancy of signaling pathways is the first step in understanding the intersection of inflammation and metabolic dysfunction. For example, GSK3β is not only a key enzyme in promoting glycogen storage (phosphorylating glycogen synthase), but it also key in embryonic development (Wnt pathway) as well as a potent regulator of inflammation (TLR signaling). More importantly, the fibrinolytic system parallels HGF activation. Since HGF and plasminogen are structurally similar, they are both substrates of u-PA. This becomes important in obesity as the inhibitor of u-PA, PAI-1 is upregulated in response to the production of TNF-α a proinflammatory cytokine. This leads to the inactivation and u-PA preventing the maturating of HGF and loss of its anti-inflammatory effects further contributing to insulin resistance. Therefore, it is no surprise that obesity not only leads to cardiovascular complication, but also T2D.

As a result of my research efforts, I see this pathway as a therapeutic avenue for exploit to control the aberrant inflammatory responses. For example, one potential therapeutic target is the use of MET kinase agonist that are not inhibited by elevated PAI-1, but act in the same manner as HGF in suppressing cytokine production. However the concern is tumor formation as HGF-MET signaling has been described in different cancer models. Nevertheless, the inactivation of HGF by PAI-1 can clearly be seen in the liver of mice fed HFD. This is accompanied by severe glucose intolerance and hyperinsulinemia as well as steatosis. Therefore, it is worth exploring as regaining the physiological effects of HGF signaling may ameliorate the progression of obesity-induced T2D. Dysregulation of metabolic functions leads to a complex and difficult syndrome to treat since many physiological systems become involved. It is clear

that the best treatment is one of proactive education of diet and exercise, however when these fail efforts to reset the APR and normal liver function may provide critical steps to ameliorate the ensuing obesity induced unchecked inflammation.

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