Elucidating the Metabolic Role of Macrophage Janus Kinase 2

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Laboratory Medicine and Pathobiology
University of Toronto

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2017

Abstract

During obesity, macrophages can infiltrate metabolic tissues and contribute to chronic low-grade inflammation as well as mediate insulin resistance and diabetes. Our study addresses the essential role of macrophage JAK2, a key mediator downstream of various cytokines, hormones and growth factors, in the pathogenesis to obesity and insulin resistance. Macrophage-specific JAK2 knockout (M-JAK2\(^{-/-}\)) mice are protected from HFD-induced systemic insulin resistance compared to littermate control M-JAK2\(^{+/+}\) mice. We observed decreased adipocyte size and enhanced expression of certain adipogenic genes in visceral adipose tissue (VAT) of HFD-fed M-JAK2\(^{-/-}\) mice. There was reduced tissue inflammation in HFD-fed M-JAK2\(^{-/-}\) mice, as observed through decreased macrophages in VAT along with reduced gene expression of certain macrophage markers and chemokines in liver and VAT. Leptin has a dose-dependent effect on augmenting chemokine expression in RAW 264.7 macrophage cell line while Jak2 knockdown suppresses chemokine expression. Collectively, our findings show that macrophage JAK2 deficiency improves systemic insulin sensitivity and reduces inflammation in VAT and liver in response to metabolic stress.
Acknowledgments

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<th>Description</th>
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<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Arg1</td>
<td>Arginase 1</td>
</tr>
<tr>
<td>AT</td>
<td>Adipose tissue</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose tissue macrophage</td>
</tr>
<tr>
<td>CCL</td>
<td>CC motif chemokine ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC motif chemokine receptor</td>
</tr>
<tr>
<td>CLS</td>
<td>Crown-like structure</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive laboratory animal monitoring system</td>
</tr>
<tr>
<td>CX3CR1</td>
<td>CX3C motif chemokine receptor 1</td>
</tr>
<tr>
<td>CXCL</td>
<td>CXC motif chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC motif chemokine receptor</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>i. p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of nuclear factor-kappaB kinase-beta</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin tolerance test</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal protein kinase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Ly6C</td>
<td>Lymphocyte antigen 6C</td>
</tr>
<tr>
<td>Lyz2</td>
<td>Lysozyme 2</td>
</tr>
<tr>
<td>LysM</td>
<td>Lysozyme M</td>
</tr>
<tr>
<td>KC</td>
<td>Kupffer cell</td>
</tr>
<tr>
<td>M-Jak2−/−</td>
<td>Macrophage-specific Jak2 knockout</td>
</tr>
<tr>
<td>M-Jak2+/+</td>
<td>Macrophage-specific Jak2 wildtype controls</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophage</td>
</tr>
<tr>
<td>M2</td>
<td>Alternatively activated macrophage</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemotactant protein</td>
</tr>
<tr>
<td>MGL</td>
<td>Macrophage galactose-type C-type lectin</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MRC</td>
<td>Mannose receptors C-type</td>
</tr>
<tr>
<td>nAChR</td>
<td>Nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NCD</td>
<td>Normal chow diet</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffer saline with Tween-20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PM</td>
<td>Peritoneal macrophage</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase – quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous adipose tissue</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline solution with Tween-20</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TPO</td>
<td>Thrombopoietin</td>
</tr>
<tr>
<td>TYK2</td>
<td>Tyrosine kinase 2</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
</tr>
<tr>
<td>VCO₂</td>
<td>Volume of carbon dioxide production</td>
</tr>
<tr>
<td>VO₂</td>
<td>Volume of oxygen consumption</td>
</tr>
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</table>
Chapter 1
Introduction

1.1 Inflammation

The term *inflammation*, originates from the Latin word *inflammare* (to set on fire). The word was first used 2000 years ago by the Roman encyclopedist Aulus Cornelius Celsus, who documented the four cardinal signs of inflammation, which included *rubor* (redness), *tumor* (swelling), *calore* (heat) and *dolore* (pain) (Rocha e Silva, 1994). Two centuries later, the Greek physician Galen identified inflammation (*phlogosis*) as a reaction of the body against injury (Rocha e Silva, 1994). He is also credited with introducing a fifth sign of inflammation, *functio laesa* (the loss of function in the affected tissue) (Rather, 1971). This general description of inflammation appears to have served clinical medicine well for most of the 2000 years until the middle of the nineteenth century.

In 1871, Rudolf Virchow, who is recognized as one of the founders of scientific medicine, viewed inflammation as inherently pathological (Heidland et al., 2006). This view was a dramatic departure from the traditional view of disease as an imbalance of the four humors (blood, yellow bile, black bile, and phlegm), which had dominated medicine since the time of Hippocrates. In the last few decades, it has become increasingly clear that inflammation could be considered a root cause of the pathogenesis of metabolic abnormalities such as obesity, diabetes, atherosclerosis, and cancer among other diseases.

Early clinical reports were noted from over a century ago in which high dose of anti-inflammatory drug salicylates was used appeared to decrease glycosuria in individuals with diabetes (Williamson, 1901). Decades later in the 1950s, clinical observations suggested that the use of high-dose aspirin in individuals with diabetes resulted in marked improvement in glycemia and, in at least one case, in the discontinuation of insulin (Reid et al., 1957; Shoelson et al., 2006). These clinical studies did not elucidate the mechanism behind these effects as their investigation focused on insulin secretion. Importantly, these studies hinted towards inflammation as a potential contributor to diabetes. The association between insulin resistance and immune responses has been recognized in many diseases with active inflammatory responses such as sepsis, hepatitis C, HIV, and rheumatoid arthritis, which display insulin resistance as a feature (Gregor and Hotamisligil, 2011).
The specific link between inflammatory cells and metabolic responses was not made till 2003, but a decade prior, a remarkable discovery revealed increased levels of the proinflammatory cytokine tumor necrosis factor-α (TNF-α) in adipose tissue (AT) and in adipocytes themselves of obese mice (Hotamisligil et al., 1995; Hotamisligil et al., 1993). Elevated levels of TNF-α was associated with peripheral insulin resistance (Hotamisligil et al., 1993) and TNF-α attenuated insulin signalling by suppressing insulin-induced tyrosine phosphorylation of insulin receptor and insulin receptor substrate 1 (Feinstein et al., 1993). These initial reports on TNF-α were soon followed by numerous studies describing differences in the state of inflammation between obese and lean animals as well as humans. It is now appreciated that obesity is not only associated with elevated levels of TNF-α, but there is also increased circulating levels of coagulation factors (fibrinogen and plasminogen activator inhibitor 1 (PAI-1)), acute-phase proteins such as C-reactive protein and serum amyloid A, other proinflammatory cytokines interleukin (IL)-1β and IL-6, and chemokines such as CC motif chemokine ligand (CCL) 2 (Shoelson et al., 2006). These inflammatory mediators have been shown to contribute to insulin resistance and metabolic deterioration in obesity. In 2003, two seminal studies showed that compared with lean tissue, obese AT secretes inflammatory cytokines and that these inflammatory cytokines themselves can inhibit insulin signalling (Weisberg et al., 2003; Xu et al., 2003). They also showed that there is substantial infiltration of macrophages in the AT during obesity in both mice and humans, which can contribute to the establishment of the chronic inflammatory state, increased tissue cytokine expression, and metabolic dysfunction (Weisberg et al., 2003; Xu et al., 2003). Later studies have shown that there are other immune cells such as dendritic cells, mast cells, neutrophils, B cells, and T cells which are also increased in obese AT; while specific subsets of T cells (helper T cell type 2, regulatory T cell, and invariant natural killer T cell) and eosinophil numbers are decreased (Huh et al., 2014; Osborn and Olefsky, 2012; Winer et al., 2011; Winer et al., 2009). Together, these studies demonstrate the connection of immune and metabolic pathways and the detrimental effects of this synergistic relationship on metabolic homeostasis during obesity.

1.2 Obesity and diabetes

Obesity and its associated metabolic diseases are one of the greatest public health challenges in Canada and worldwide. There has been a drastic increase in the proportion of overweight and
obese Canadians. According to Statistics Canada, almost two-thirds of Canadian adults are now either overweight or obese and childhood obesity rates are also dangerously high (Ogilvie and Eggleton, 2016). Obesity is caused by increased food intake and decreased energy expenditure that results in a massive increase in AT that is generally harmful to health (Bray, 2004). Obesity contributes to the pathogenesis of comorbid conditions associated with obesity, such as insulin resistance, type 2 diabetes mellitus (T2D), dyslipidemia, nonalcoholic fatty liver disease, and atherosclerosis (Odegaard and Chawla, 2013). Now it is well understood that obesity is a chronic low-grade inflammatory state. Moreover, chronic inflammation can feed into the vicious cycles to exacerbate its associated diseases. Thus, obesity can lead to inflammation which in turn can promote obesity-associated diabetes in part by inducing insulin resistance (Hotamisligil, 2006). The link between insulin resistance and T2D stems from the work of H.P. Himsworth in 1936 where he recognized two different types of diabetes: one with normal insulin sensitivity but simply lacked insulin and those who did not lack insulin but were insensitive to its effects (Himsworth, 2011). Indeed, insulin resistance is a core defect in T2D with the inability of metabolic tissues such as muscle, fat and liver to respond normally to insulin, that is coupled with inability of pancreatic β-cells to secrete sufficient insulin to compensate the peripheral insulin resistance (Donath and Shoelson, 2011).

AT is one of the master regulators of energy balance and fuel homeostasis (Rosen and Spiegelman, 2014). Traditionally, adipocytes have been divided into two types: unilocular white adipocytes that make up the bulk of fatty tissue in most animals and brown adipocytes, which are highly specialized cells that dissipate stored energy in the form of heat (Rosen and Spiegelman, 2014). Insulin resistance in the white AT and dysfunctional lipid storage in adipocytes are sentinel events in the progression toward metabolic dysregulation with obesity. By storing excessive fuel, adipocytes experience endoplasmic reticulum stress and hypertrophy, causing the release of aberrant adipokines, proinflammatory cytokines, and several chemokines from the AT (Donath and Shoelson, 2011). Eventually, lipid overload may lead to adipocyte death, thereby further triggering an inflammatory response. Adipokines, which are bioactive peptides or proteins secreted by AT, such as TNF-α, IL-6, CCL2, resistin, PAI-1, visfatin, leptin and adiponectin, are markedly dysregulated during obesity and T2D (Shoelson et al., 2006). While adiponectin expression decreases with increased adiposity, expression of other inflammatory mediators is upregulated, causing local and systemic inflammation and interfering with insulin signalling...
Inflammation can also be triggered by local hypoxia caused by the rapid expansion of AT without sufficient vascular adaptation (Donath and Shoelson, 2011). The proinflammatory signalling also induces lipolysis and release of free fatty acids. Therefore, the excess AT is often a source of increased fatty acid (FA) supply in obesity, resulting in lipotoxicity through chronically elevated concentrations of FAs and excess ectopic lipid accumulation in tissues such as liver and muscle (Samuel and Shulman, 2012). This deposition of excess fat can also impair insulin signalling. Besides the increase in total adiposity with obesity, the pathogenic role of AT seems to be determined by its specific anatomic location. Indeed, visceral adipose tissue (VAT) is more strongly correlated with negative metabolic effects than subcutaneous adipose tissue (SAT) (Esser et al., 2014; Johnson and Olefsky, 2013). It should be pointed out that SAT and VAT distinction is simplified, as there appears to be clear distinctions between nominal visceral depots such as perigonadal, mesenteric and retroperitoneal fat pads (Rosen and Spiegelman, 2014).

The liver is another important insulin target tissue for metabolism and contributes to glucose homoeostasis. It is a major source of endogenous glucose production by gluconeogenesis and glycogen mobilization when blood glucose falls below normal concentrations, and can rapidly clear postprandial glucose from the portal vein. With hepatic insulin resistance, postprandial inhibition of glucose production and glycogenolysis (glycogen breakdown to glucose) is impaired. However, due to the rise in insulin levels which can still have a stimulatory effect on lipogenesis, insulin resistance results in increased lipid deposition in the liver (Perry et al., 2014). Therefore, an inadequate insulin-dependent suppression of hepatic glucose production, in turn, contributes to the development of hyperglycemia, and hepatic steatosis or nonalcoholic fatty liver disease. Steatosis is a characteristic feature of the liver in insulin-resistant states, and the evidence suggests that inflammatory mediators have an important role in this condition. Inflammatory cytokines induce lipogenic gene expression that promotes de novo lipogenesis (Lackey and Olefsky, 2016; Obstfeld et al., 2010).

Skeletal muscle, on the other hand, is the primary site of glucose uptake, accounting for around 80% of insulin-stimulated glucose disposal (Osborn and Olefsky, 2012). Therefore, decreased muscle insulin sensitivity in obesity has a profound effect on hyperglycemia in insulin-resistant state. With obesity, there is also increased fat accumulation within skeletal muscle, and these
intermuscular fat depots promote inflammation, which may cause insulin resistance in the muscle (Lackey and Olefsky, 2016; Schenk et al., 2008). In parallel with these inflammatory changes, alterations in FA metabolism can lead to the lipid accumulation in the liver and skeletal muscle. Obesity increases the release of free FA from the AT, which is taken up by muscle and liver that constitute two primary routes of FA disposal. These FAs can undergo β-oxidation in the mitochondria or they can be stored as triglycerides (Schenk et al., 2008). It has been hypothesized that FA disposal into the liver or muscle can lead to accumulation of various FA intermediates (i.e. diacylglycerol, ceramide, etc.) intracellularly as cytoplasmic lipid droplets (Schenk et al., 2008). Ectopic lipid accumulation can impair insulin receptor signalling, which can further exacerbate insulin resistance.

The pancreatic islets are at the heart of glucose homeostasis, given its role as the source of insulin. Under normal conditions, insulin produced by pancreatic β-cells binds to the insulin receptor on target cells to activate several downstream pathways to increase glucose uptake, inhibit gluconeogenesis, increase lipid synthesis, and decrease lipolysis (Cildir et al., 2013). However, chronic inflammation in metabolic tissues can lead to the unresponsiveness of metabolic tissues to the actions of insulin hormone, thereby causing the insulin resistant state underlying T2D. During the course of obesity, peripheral insulin resistance requires increased amounts of insulin to clear glucose from the circulation. This increased production results in stress on the pancreatic β-cells (Gregor and Hotamisligil, 2011). The chronic stress of pancreatic β-cells eventually leads to the decline in function resulting in chronic hyperglycemia which in turn can cause glucotoxicity. Other cytotoxic effects to the pancreatic β-cells that occur in T2D include chronic exposure to non-esterified FA (lipotoxicity), oxidative stress, inflammation, and amyloid formation (Tahrani et al., 2011). These effects can trigger β-cell apoptosis and dysfunction of pancreatic islets leading to reduced insulin secretion from decreased islet mass and function which contribute to the progression to diabetes (Lumeng et al., 2007).

Inflammation like that induced by obesity in peripheral insulin-sensitive tissues also occurs in some areas of the brain. For instance, diet-induced obesity can activate inflammatory signalling pathways in the hypothalamus, resulting in increased food intake and nutrient storage (Lumeng et al., 2007). Under such conditions, FA intermediates accumulate in the hypothalamus and induce leptin and insulin resistance in the central nervous system (Johnson and Olefsky, 2013). Additionally, obesity in humans and rodents is associated with changes in the composition of the
intestinal microbiota (dysbiosis), which can impact on host metabolism (Lackey and Olefsky, 2016).

Inflammation is often a short-term adaptive response which is a crucial component of tissue repair. However, the long-term consequences of prolonged inflammation in metabolic diseases are not beneficial. The chronic inflammation is principally triggered by a surplus in fuel intake and results in increased circulating concentrations of proinflammatory cytokines and chemokines. These inflammatory mediators can lead to the activation of several kinases that regulate inflammation, including c-Jun NH2 terminal kinase (JNK) and Inhibitor of nuclear factor-kappaB kinase (NF-κB)-beta (IKKβ) which can interfere with insulin action in multiple tissues including adipocytes, hepatocytes and myocytes (Shoelson et al., 2006). These inflammatory signalling pathways promote phosphorylation of insulin signalling proteins such as insulin receptor substrates, thereby reducing their signalling activity (Osborn and Olefsky, 2012). As a result, insulin receptor signalling that normally occurs through a tyrosine kinase cascade is inhibited by inhibitory serine/threonine phosphorylation. For instance, stimuli such as pro-inflammatory cytokines, fatty acids or hyperglycemia, can stimulate JNK, IKK or mitogen-activated protein kinase (MAPK), to phosphorylate inhibitory serine/threonine residues on insulin receptor substrate proteins (Boucher et al., 2014). This results in impairment in downstream signalling events by leading to the inactivation of proteins involved in insulin signalling such as phosphatidylinositol 3-kinase (PI3K)/AKT signalling (Taniguchi et al., 2006). The JNK and IKKβ pathways appear to regulate both immune and metabolic functions to mediate inflammatory response and insulin resistance in the development of obesity and T2D.

1.3 Macrophages

Macrophages are present in almost all tissues including skin (Langerhans cells), liver (Kupffer cells), brain (microglia), pancreas, lung (alveolar macrophages) and kidney (Epelman et al., 2014). They serve crucial roles in the development, regeneration, metabolism, and maintenance of tissue homeostasis, as well as play an integral part in mediating inflammation (Sica and Mantovani, 2012; Wynn et al., 2013). As early as the late nineteenth century, Elie Metchnikoff had emphasized the beneficial aspects of inflammation through the discovery of phagocytosis and the theory of cellular immunity (Tauber, 2003). He pointed out the key role of macrophages and
microphages (neutrophils) both in host defense and in the maintenance of tissue homeostasis (Tauber, 2003). Indeed, macrophages are professional phagocytic cells that function as immune sentinels in nonspecific host defense against pathogens or environmental challenges (Davies et al., 2013). They are one of the most active secretory cells in the body and release hundreds of mediators such as cytokines and chemokines that can regulate cytotoxicity, inflammation and proliferation.

Macrophage functions are driven largely by tissue-derived and pathogenic microenvironmental stimuli that help them adapt to changing conditions within tissues and tailor an appropriate response (Wynn et al., 2013). For instance, tissue-resident macrophages are extremely heterogeneous reflecting the heterogeneity in the origins and influence of the tissue environment in which they reside (Davies et al., 2013). Transcriptional profiling of resident macrophages by the Immunological Genome Project show that these populations have high transcriptional diversity with minimal overlap, suggesting that there are many unique classes of macrophages (Gautier et al., 2012). In fact, tissue-resident macrophages may be specialized sentinel cells that sense not only injury and infection but also other types of noxious conditions such as hypoxia and metabolic stress (Medzhitov, 2010). These tissue-resident macrophages are not all derived from circulating monocyte precursors, but fate mapping studies reveal that many of them are established during early embryonic development and are maintained during adulthood independently of an influx of blood monocytes (Davies et al., 2013; Gautier and Yvan-Charvet, 2014). Additionally, in the context of inflammation, monocyte can be recruited from the circulation, which can be differentiated to macrophages within the tissue. Proliferation in tissues of resident macrophages, recruited monocytes and monocyte-derived macrophages is another mechanism by which monocyte and macrophage cell numbers are controlled within the tissue during inflammation (Epelman et al., 2014).

Within the tissue, macrophages can adapt various phenotypes to carry out differential functions and elicit divergent effects on surrounding cells. The heterogeneity of macrophages has resulted in their classification into subtypes based on their phenotype and function. One major classification, based on function, is classically activated, proinflammatory macrophages (M1) and alternatively activated, anti-inflammatory macrophages (M2) (Sica and Mantovani, 2012). M1 macrophages upregulate expression of CD11c as well as proinflammatory cytokines and chemokines including TNF-α, IL-6, IL-1β, IL-12, inducible nitric oxide synthase (iNOS), and CCL2, and they increase
the production of reactive oxygen species and nitrogen intermediates (Mosser and Edwards, 2008; Sica and Mantovani, 2012). M2 macrophages upregulate expression of scavenger and mannose receptors C-type (MRC1, MRC2), macrophage galactose-type C-type lectins (MGL1, MGL2), FIZZ1, the IL-1 receptor antagonist, arginase-1 (Arg1), the chitinase family protein Chi3L3, and IL-10 while the production of proinflammatory cytokines are down-regulated (Gordon, 2003; Lumeng et al., 2007; Mosser and Edwards, 2008; Sica and Mantovani, 2012). Macrophages are polarized to the M1 state in vitro by exposure to cytokines such as IFN-γ and GM-CSF, and in the presence of bacterial products such as lipopolysaccharide (LPS). In contrast, M2 macrophages are polarized by stimulation with cytokines such as IL-4 and IL-13, as well as M-CSF (Lawrence and Natoli, 2011). Depending on the microenvironment, macrophages can acquire distinct function. M1 macrophage polarization is associated with inflammation and tissue destruction, whereas the M2 macrophages are associated with anti-inflammatory and homeostatic functions linked to wound healing and tissue repair (Price and Vance, 2014). Macrophage polarization is shown to be plastic since macrophages can switch phenotypes during the progression of an inflammatory response.

The binary classifications of macrophage activation as M1 and M2 are derived historically to reflect two extremes of a dynamic state of macrophage phenotypes under in vitro experimental conditions. Importantly these do not represent the phenotypic heterogeneity of macrophages within the complex in vivo environment where numerous cytokines and growth factors interact to modulate and define the final differentiated state of macrophages. Functional phenotypes of macrophages have been observed to mirror the canonical M1 and M2 activation states under some conditions such as parasite infections, allergy, and many tumor types (Sica and Mantovani, 2012). However, in other settings such as atherosclerosis, obesity and cancers, macrophage population express a mixed phenotype (Biswas and Mantovani, 2010; Kadl et al., 2010; Shaul et al., 2010). The spectrum of macrophage phenotypes that span between classic M1 and M2 definitions further casts doubt on the utility of M1 and M2 in defining macrophage subsets. Recent studies have classified monocytes/macrophages as inflammatory or resident (Epelman et al., 2014). The resident macrophage populations in tissues are phenotypically variable and distinct from inflammatory monocyte-derived macrophages (Ginhoux and Guilliams, 2016). Mouse monocytes can be divided into two main subsets on the basis of their expression of receptors that include lymphocyte antigen 6c (Ly6C), CC motif chemokine receptor (CCR) 2 and CX3C motif
chemokine receptor 1 (CX3CR1) (Auffray et al., 2009). Ly6C\textsuperscript{hi} monocytes are inflammatory macrophage precursors and Ly6C\textsuperscript{−} monocytes are known to patrol the luminal surface of endothelium (Geissmann et al., 2010; Ginhoux and Guilliams, 2016). These monocytes also express distinguishing markers that account for their different immunological functions. For example, Ly6C\textsuperscript{hi} monocytes express high levels of CCR2, while Ly6C\textsuperscript{−} monocytes lack CCR2 expression. Another immunological marker that distinguishes the two monocyte populations from each other is CX3CR1, which is expressed in the reverse pattern to CCR2, namely it is expressed by Ly6C\textsuperscript{−} but not by Ly6C\textsuperscript{hi} monocytes (Geissmann et al., 2010; Ginhoux and Guilliams, 2016). Ly6C\textsuperscript{hi} monocytes are rapidly recruited to sites of inflammation and sites of tissue remodelling, where they extravasate and can give rise to monocyte-derived macrophages (Ginhoux and Guilliams, 2016; Wynn et al., 2013). On the other hand, Ly6C\textsuperscript{−} monocytes can play an important role in the resolution of inflammation, and these cells may serve as precursors for alternatively activated macrophages (Auffray et al., 2009).

1.3.1 Role of macrophages in obesity and diabetes

Macrophages are central players in both the innate and adaptive immune systems and orchestrate a number of immune and inflammatory responses. While macrophages serve an adaptive function during infection or tissue injury to resolve acute inflammation, they can also contribute substantially to the progression of chronic diseases such as obesity. The studies related to obesity-induced tissue macrophage infiltration have demonstrated a close association of fuel homeostasis with immunity (Odegaard and Chawla, 2008; Olefsky and Glass, 2010). For instance, adipose tissue macrophages (ATMs) play a significant role in regulating AT function during health and disease (Boutens and Stienstra, 2016). However, it is important to note that specific differences in inflammatory profile exist between visceral versus the subcutaneous AT of obese individuals, with more macrophages and inflammatory cytokines in the VAT compared to SAT, suggesting that VAT has a more important role in the development of obesity-related insulin resistance (Esser et al., 2014).

During obesity, the AT becomes “inflamed” as a consequence of increased infiltration of macrophages (Weisberg et al., 2003; Xu et al., 2003). ATMs play an important role in the establishment of the chronic inflammatory state and metabolic dysfunction that is associated with
obesity (Hotamisligil, 2006). As well as increasing in number, ATMs change their localization and inflammatory features during obesity (Gericke et al., 2015; Lumeng et al., 2007; Lumeng et al., 2008). ATMs in obese AT can be observed histologically as crown-like structures (CLSs) surrounding dying adipocytes, particularly in obese visceral AT; their uneven distribution has been attributed to clustering around dead or dying adipocytes (Cinti et al., 2005). The presence of CLSs in AT has been directly linked with insulin resistance (Cinti et al., 2005; Murano et al., 2008).

Contrary to the obese state, ATMs in the lean state are distributed throughout the AT exhibiting a limited state of inflammation (Gericke et al., 2015; Lumeng et al., 2007; Lumeng et al., 2008). It has been observed that ATMs from lean organisms express M2 markers, whereas obesity leads to a reduction of these markers and an increase of genes associated with the M1 phenotype (Fujisaka et al., 2009; Lumeng et al., 2007; Lumeng et al., 2008). While M1 ATMs cause local and systemic insulin resistance, M2 macrophages contribute to improved insulin sensitivity due to their ability to attenuate inflammation and promote tissue repair and angiogenesis (Chawla et al., 2011; Sun et al., 2012). ATMs during obesity release proinflammatory cytokines, TNFα, IL-6 and IL-1β, that act both locally on adipocytes and vascular cells, and circulate to distal tissues to stimulate intracellular proinflammatory pathways (Chawla et al., 2011; Osborn and Olefsky, 2012). In a feed-forward cycle, these cytokines can also stimulate ATMs to secrete chemokines that promote the recruitment and infiltration of additional monocytes/macrophages into AT (Osborn and Olefsky, 2012; Romeo et al., 2012). These combined actions result in cell-autonomous insulin resistance in adipocytes, exacerbation of the inflammatory state and subsequent systemic insulin resistance (Osborn and Olefsky, 2012; Romeo et al., 2012).

ATMs execute numerous functions crucial for maintaining AT homeostasis. In addition to ATMs contributing to the development of obesity-induced inflammation, they also serve an important role in lipid trafficking. For example, macrophages buffer lipolysis by taking up and storing excessive amounts of adipocyte-released lipids, thereby allowing gradual lipid release into the bloodstream (Boutens and Stienstra, 2016). In obesity, ATMs take up triglycerides released from overburdened adipocytes and function to buffer fatty acid release into circulation through activation of the lysosome-dependent mechanism of lipid catabolism (Xu et al., 2013). However, the lipid buffering capacity of ATMs appears to be insufficient in obese states. Macrophages can also regulate adipogenesis in obese AT, but whether ATMs promote or suppress adipogenesis is still debated. During obesity, ATMs have been found to populate adipogenic clusters, which are
formed at sites away from CLSs, and facilitate angiogenesis and adipogenesis (Cho et al., 2007; Nishimura et al., 2007). In contrast to studies postulating a role for macrophages in adipogenesis, several studies used cell culture to demonstrate that proinflammatory macrophages inhibit both proliferation and differentiation of adipogenitor cells (Constant et al., 2008; Lacasa et al., 2007; Maumus et al., 2008). Reduced adipogenesis would promote hypertrophy of adipocytes to allow for storage of the excess amounts of lipids entering the AT, which is in turn linked to metabolic dysfunction such as adipocyte insulin resistance (Gustafson et al., 2015; Wang et al., 2014a).

Specific adipokines and chemokines produced by AT can function as chemoattractants for recruiting circulating monocytes, which is one important mechanism in populating AT with macrophages, which is enhanced in the presence of obesity (McNelis and Olefsky, 2014). In addition to recruitment during obesity, recent evidence suggests that there are local regulation and proliferation of ATM pools independently of the influx of blood monocyte precursors (Amano et al., 2014; Okabe and Medzhitov, 2014; Zheng et al., 2016). Moreover, enhanced ATM numbers during obesity may be implicated in reduced migration capacity and reduced apoptosis of resident ATMs (Hill et al., 2015; Ramkhelawon et al., 2014). It has not yet been deciphered whether the number of ATMs is primarily regulated at the level of the bone marrow or is determined by local factors in the AT.

Extensive research has been carried out over the past two decades to better understand obesity-associated inflammation in AT. However, inflammatory activation of macrophages is not limited to AT, but have also been reported in liver, muscle and pancreatic islets (Osborn and Olefsky, 2012). Many studies have shown that obesity induces hepatic inflammation associated with a substantial increase in liver macrophages (Lanthier et al., 2011; Obstfeld et al., 2010). The liver is populated with resident macrophages, the Kupffer cells (KCs), which are long-lived and relatively abundant in the liver (Tang et al., 2013). KCs are predominantly localized in the liver sinusoids and play an important role in tissue homeostasis, clearing foreign and harmful particles that enter the liver (Baffy, 2009). KCs have been implicated in the pathogenesis of steatosis and insulin resistance, through potential KC activators such as gut-derived toxins, antigens and ligands carried along by the portal flow (Baffy, 2009). The number of KCs does not increase with obesity, but their activation state changes (Cai et al., 2005; Huang et al., 2010; Morinaga et al., 2015). Interestingly, as observed in ATMs, M2 activation phenotype of KCs appears to ameliorate insulin resistance and to delay the progression to steatosis (Huang et al., 2010). In contrast to KCs,
recruited hepatic macrophages are short lived and enter the liver in increased numbers during obesity, and their proinflammatory potential is enhanced through changes in a variety of gene expression (Morinaga et al., 2015; Obstfeld et al., 2010; Oh et al., 2012). A recent study showed that in high-fat diet (HFD) fed mice, resident KCs (which express low levels of CCR2) secrete increased amounts of CCL2, which in turn serves as a migration signal to recruit monocytes into the liver, which eventually mature into differentiated macrophages (expressing high levels of CCR2 and low levels of CCL2) (Morinaga et al., 2015). The factors secreted by recruited hepatic macrophages can augment hepatic inflammation and insulin resistance (Morinaga et al., 2015). Chemical ablation of phagocytic cells in the liver, including KCs and recruited macrophages, protects mice from HFD-induced insulin resistance, demonstrating the importance of these cells in the development of chronic hepatic inflammatory state and associated metabolic dysfunction (Lanthier et al., 2011; Neyrinck et al., 2009). Collectively, these studies demonstrate that hepatic inflammation and insulin resistance during obesity is accompanied with increased recruitment of macrophages and activation of KCs.

Tissue inflammation has also been detected in the pancreatic islets as shown by the presence of amyloid deposits, fibrosis, increased β-cell death and infiltration of macrophages along with increased levels of proinflammatory cytokines and chemokines (Donath and Shoelson, 2011). Increased proportion of islets is infiltrated by macrophages in patients with T2D and in various animal models of T2D and insulin resistance (Ehses et al., 2008; Jourdan et al., 2013; Richardson et al., 2009). The increase in pancreatic expression of inflammatory cytokines and macrophage infiltration occurs in parallel with the onset of glucose intolerance (Ehses et al., 2008). Particularly the expression and local release of the proinflammatory cytokine IL-1β are increased in pancreatic islets of T2D individuals (Maedler et al., 2002). It is suggested that production of IL-1β through activation of inflammasomes within macrophages present in pancreatic islets can promote β-cell apoptosis and decreased insulin secretion (Lackey and Olefsky, 2016; Westwell-Roper et al., 2014). Interestingly, a recent study noted the beneficial effects of IL-1β by demonstrating that postprandial elevation in glucose can drive secretion of IL-1β from macrophages to promote secretion of insulin by pancreatic β-cell, thereby decreasing glycemia (Dror et al., 2017).

Muscle is another major site of insulin resistance in obesity and T2D. Recent reports have shown an infiltration of macrophages within skeletal muscles and increased muscle inflammatory gene in both obese mice and human, particularly in the intermuscular adipose depots (Fink et al., 2014;
Fink et al., 2013; Hevener et al., 2007; Nguyen et al., 2007; Weisberg et al., 2003). Similar to AT, skeletal muscle macrophages exhibit a proinflammatory M1 phenotype and the inflammatory factors released from these intramuscular macrophages can exert paracrine effects to cause local insulin resistance (Nguyen et al., 2007). Some studies have also reported an increase in macrophage number in obese and T2D subjects (Bruun et al., 2006; Tam et al., 2012).

Given that macrophage infiltration is one of the hallmarks of obesity, considerable progress has been made toward understanding the mechanisms of macrophage function and in determining its effects on the function of metabolic tissues. While increased macrophage infiltration in metabolic tissues impairs insulin sensitivity, depleting tissue macrophages using in adult mice using a Cre-inducible diphtheria toxin receptor disrupts energy homeostasis and disturbs immune balance (Lee et al., 2014). Macrophage depletion notably reduced body fat mass due to reduced food intake and robustly increased proinflammatory cytokines expression and their levels in circulation (Lee et al., 2014). Moreover, depletion of macrophages using liposome clodronate has not provided conclusive results. Some studies show that depletion of macrophages significantly improves insulin resistance and glucose tolerance in HFD-fed and ob/ob mice, while other studies have reported no beneficial effect on systemic glucose homeostasis in obese models (Boutens and Stienstra, 2016). The development of gene-targeted knock-out mice for different inflammatory molecules, such as cytokines, chemokines, and their receptors, have also proven to be immensely useful in the dissection of molecular mechanisms of macrophages inflammation in vivo. For example, the deletion of M1 marker genes such as TNF-α and CCR2 resulted in the normalization of insulin sensitivity in models of insulin resistance (Uysal et al., 1997; Weisberg et al., 2006). Additionally, the deletion of macrophage-specific genes of various inflammatory signalling components has revealed the contribution of macrophage functions in obesity-induced insulin resistance. Various groups have used Cre-mediated recombination under the control of lysozyme M (LysM) promoter to drive genetic deletion in myeloid cells of mice of components involved in the inflammatory or insulin pathways such as IKKβ, JNK or insulin receptor. Ablation of these factors can protect from obesity-induced impairments to glucose homeostasis and the development of insulin resistance, supporting their involvement in systemic metabolic regulation (Arkan et al., 2005; Han et al., 2013; Mauer et al., 2010). On the other hand, macrophage-specific knockout using LysM-cre mouse model or bone marrow deficiency of peroxisome proliferator-activated receptor (PPAR)γ or PPARδ displayed insulin resistance with reduced number and impaired
function of M2 macrophages (Hevener et al., 2007; Kang et al., 2008; Odegaard et al., 2007; Odegaard et al., 2008). Multiple signalling cascades within macrophages have been linked to insulin resistance and the immune response.

1.3.2 Role of chemokines in obesity and diabetes

Dysregulated expression of chemokines and their receptors is involved in the development of many human diseases, including autoimmune and chronic inflammatory diseases like obesity as well as immunodeficiency and cancer (Charo and Ransohoff, 2006; White et al., 2013). Chemokines are a family of low-molecular-weight (8-10 kDa) cytokines that play central roles in the migration of leukocytes to lesions and areas of inflammation, as well as activation of specific leukocyte population subsets (Xu et al., 2015). They are inducible and secreted proteins that comprise the largest mammalian cytokine superfamily that function to maintain homeostasis or induce inflammation (Mantovani et al., 2004). Chemokines are distinguished by overall sequence homology and classified into the following four subfamilies: CXC, CC, C, and CX3C, where X is any amino acid residue and C is a cysteine residue. The CC-chemokines, which are the largest group of chemokines, and include CCL2 (also known as monocyte chemoattractant protein (MCP)-1), CCL3 (or macrophage inflammatory protein (MIP)-1α), CCL4 (or MIP-1β), CCL5 (or RANTES), CCL7 (or MCP-3), CCL8 (or MCP-2) and many others (Xu et al., 2015). CC-chemokines induce their effects by binding to CC chemokine receptors (CCR1-CCR10), which are seven-transmembrane-spanning G protein-coupled receptors (GPCRs) on target cells that are expressed on a wide range of leukocytes among other cells in the body (White et al., 2013). In general, chemokines/chemokine receptors exhibit promiscuity, being able to bind multiple receptors/ligands. There is the inherent redundancy of the chemokine system; while some chemokines have a specific receptor but most chemokine ligands share the receptor (Mantovani et al., 2004). For example, four chemokine ligands—CCL2, CCL7, CCL8 and CCL13 bind to the receptor CCR2 while chemokine ligands—CCL3, CCL4, CCL5 and CCL8 bind to CCR5. CC motif chemokines act on different leukocyte populations—monocytes, eosinophils, basophils, natural killer cells, and different lymphocyte subpopulations to mediate chronic inflammation (Charo and Ransohoff, 2006). Given that chemokines are expressed and regulated in different cells and tissues, their roles may vary based on the conditions and diseases.
High levels of multiple CC-chemokine ligands (CCL2, CCL3, CCL5, CCL7, CCL8, CCL11) and receptors (CCR1, CCR2, CCR3, CCR5) have been observed in AT of obese subjects and are associated with increased inflammation (Huber et al., 2008). Similarly, another study also reported that expression of numerous chemokines (CCL2, CCL5, CCL7, CCL19, CXCL1, CXCL5, CXCL8, CXCL10) is increased in AT of obese subjects compared to lean subjects (Tourniaire et al., 2013). Thus, these studies suggest the possibility that loss of one chemokine may be compensated by other chemokines.

There is strong evidence suggesting a role for the CCL2-CCR2 axis in adipose and hepatic inflammation and obesity. Levels of CCL2, both systemically and in white AT, are increased in HFD fed mice and plasma CCL2 levels correlate with body weight (Takahashi et al., 2003). CCL2 may have a direct role in insulin resistance because CCL2 added to mature adipocytes inhibits insulin-stimulated glucose uptake and expression of several genes required for adipogenesis (Sartipy and Loskutoff, 2003). In AT, inflammation is caused in part by the recruitment and activation of CCR2-positive ATMs (Lumeng et al., 2007; Weisberg et al., 2006). Consistently, mice overexpressing CCL2, the primary ligand for CCR2, in AT accumulate more ATMs and are insulin resistant (Kamei et al., 2006; Kanda et al., 2006), whereas a deficiency of CCL2 or its receptor CCR2 reduces proinflammatory macrophage accumulation in AT and provides protection from insulin resistance as well as hepatic steatosis (Kanda et al., 2006; Lumeng et al., 2008; Weisberg et al., 2006). Interestingly, during HFD-induced obesity, enhanced hepatocyte expression of CCL2 leads to hepatic recruitment of CCR2-positive myeloid cells, distinct from KCs, that promote hepatic steatosis (Obstfeld et al., 2010). Studies in humans have also shown that hepatic expression of CCL2 is increased in obesity and non-alcoholic fatty liver disease (Westerbacka et al., 2007). However, additional studies have produced conflicting results and indicated greater complexity than initially suggested. Loss of CCL2 neither attenuates obesity-associated monocyte influx into AT nor improves metabolic function (Inouye et al., 2007). Furthermore, Ccr2-deficient mice fed HFD had fewer macrophages in AT; however, lack of CCR2 did not decrease ATM content to the levels detected in lean mice (Inouye et al., 2007; Kirk et al., 2008). These conflicting results indicate that ATM infiltration in obese mice, in addition to the CCL2-CCR2 axis, can also be regulated by other chemokine-chemokine receptor interactions.

Another chemokine with a known role in obesity, adipose and liver inflammation, and diabetes is CCL5. Both CCL5 and one of its receptors CCR5 were found to be upregulated at the mRNA
level in AT of male HFD fed mice (Wu et al., 2007). CCL5 expression was increased further in VAT of morbidly obese patients and correlated with expression of the T cell marker CD3 and the macrophage marker CD11b (Wu et al., 2007). Serum levels of CCL5 were also found to be elevated in cohorts of individuals with either impaired glucose tolerance or T2D compared with control subjects (Herder et al., 2005). Studies in obese human AT suggests that a potential mechanism for the action of CCL5 may be to both recruit and promote the survival of macrophages (Keophiphath et al., 2010). Studies in mice showed that Ccr5 deficiency and chimeric mice lacking CCR5 only in myeloid cells are protected from insulin resistance, hepatic steatosis and diabetes, and demonstrate a robust increase in CCR5-positive ATMs in response to HFD feeding (Kitade et al., 2012). However, a later study showed that CCR5 ablation impairs systemic glucose tolerance as well as AT and muscle insulin signalling (Kennedy et al., 2013). They demonstrated that CCR5 only has a minor role in regulating infiltration of M1 ATMs but increases the influx of CD4-positive T cells into AT (Kennedy et al., 2013). However, CCL3 deficiency, which is another ligand for CCR5, did not impact ATM accumulation or other metabolic parameters in Western diet (containing 0.15% cholesterol)-fed mice (Surmi et al., 2010). Besides CCL2 and CCL5, other chemokines such as CXC motif chemokine ligand (CXCL) 5, CXCL14 and CX3CL1 are also involved in ATM infiltration and obesity-induced insulin resistance (Yao et al., 2014). Because of the potent biological actions of chemokines CCL2 and CCL5, efforts have also focused on identifying the mechanisms that activate and limit the chemokine gene expression during obesity associated inflammation.

1.4 JAK-STAT pathway

Cellular responses to dozens of cytokines and growth factors are mediated by the evolutionarily conserved Janus kinase-signal transducers and activators of transcription (JAK-STAT) signalling pathway. These responses include proliferation, differentiation, migration, apoptosis, host defence, cell growth, and cell survival, depending on the signal, tissue and cellular context (Rawlings et al., 2004). JAK-STAT signalling is essential for numerous developmental and homeostatic processes, including hematopoiesis, immune cell development, stem cell maintenance, mammary gland development, organismal growth, and inflammation (Ghoreschi et al., 2009; O'Shea and Plenge, 2012). JAKs are involved in inflammatory and immune disorders in which cytokines play crucial
roles including chronic inflammatory diseases like cancers and obesity (Ghoreschi et al., 2009; Gurzov et al., 2016; Pesu et al., 2008; Tefferi, 2008; Valentino and Pierre, 2006; Wunderlich et al., 2013). Mammalian JAKs represent a family of four non-receptor tyrosine kinases: JAK1, JAK2, JAK3, and Tyrosine kinase 2 (TYK2), while STATs comprise a family of seven structurally and functionally related proteins: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Harrison, 2012). JAKs mediate the downstream signalling of more than 50 cytokines and peptide hormones. While JAK1, JAK2 and TYK2 are ubiquitously expressed, the expression of JAK3 is restricted to hematopoietic cells (Rane and Reddy, 2000). At the cellular level, JAKs can be found in the cytosol when they are experimentally expressed in the absence of cytokine receptors; however, because of their intimate association with cytokine receptors, they ordinarily localize to endosomes and the plasma membrane, along with their cognate receptors (Yamaoka et al., 2004). Understanding of JAK-STAT signalling has revealed that both cell-type specific transcription, and core or stereotypic mRNA profiles are induced by activated cytokine receptors in different cell types (Murray, 2007).

The major action of JAKs is to promote gene transcription by activating STAT proteins. The cytokine binding to their corresponding receptor results in close apposition of the kinase domains of two membrane proximal receptor-associated JAKs within the cell. This promotes JAK activation through the reciprocal interaction of two juxtapositioned JAKs and auto- and/or trans-phosphorylation of tyrosine residues of the JAK kinase domain (O'Shea and Plenge, 2012). JAKs are constitutively associated with the membrane-proximal regions of cytokine receptors, although in some cases interaction between the JAK and the receptor is increased upon ligand binding (Yamaoka et al., 2004). JAK activation is followed by tyrosine phosphorylation within the receptor cytoplasmic domain which generates docking sites for the SH2 domain containing proteins such as STAT proteins, so that the docked protein is phosphorylated and activated (Levy and Darnell, 2002). Once activated, phosphorylated STAT proteins form homodimers or heterodimers and translocate to the nucleus, where they activate gene transcription through binding to specific promoter response elements (Levy and Darnell, 2002) (Figure 1). The JAK-STAT pathway can be negatively regulated by suppressor of cytokine signalling proteins family (SOCS1-7 and CIS), protein tyrosine phosphatases (such as SHPs, CD45, PTP1B) as well as protein inhibitor of activated STAT (PIAS), each has varied roles in dampening down the JAK-STAT mediated cytokine response (Yoshimura et al., 2007). Although the canonical JAK-STAT
pathway is simple and direct, pathway components regulate or are regulated by members of other signalling pathways, including those involving the extracellular signal-regulated kinase (ERK), MAPK, PI3K, and others (Li, 2008).
Janus kinase-Signal transducer and activator of transcription (JAK-STAT) signalling pathway, is activated by a wide variety of cytokines and growth factors. Binding of the ligand to the receptor activates the JAKs, which phosphorylates specific tyrosine residues within itself and the associated receptor, forming high-affinity docking sites for subsequent recruitment of other signalling molecules. These signalling proteins include the SH2 domain containing STAT family of transcription factors. Once recruited, STATs are phosphorylated by JAK kinases, they homo- or hetero-dimerize and translocate to the nucleus where they bind to response elements on DNA and modulate transcription of downstream target genes.

**Figure 1: Schematic of JAK-STAT signalling**
1.5 Metabolic role of JAK2

JAK2 acts as a central mediator in regulating a wide variety of physiological processes including lactation, erythropoiesis, myelopoiesis, thrombopoiesis, regulation of metabolism, and inflammatory response (Rawlings et al., 2004; Roskoski, 2016). The activity of JAK2 is crucial for the induction of numerous genes, including cytokines and inflammatory mediators. JAK2 integrates signals from various cytokines and growth factors including growth hormone (GH), prolactin (PRL), erythropoietin (EPO), thrombopoietin (TPO), and the family of cytokines that signal through the IL-3 receptor such as IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) (Rawlings et al., 2004). JAK2 is also important for cytokines that use the gp130 receptor (IL-6, leptin, oncostatin M) and IFN-γ (Rawlings et al., 2004).

Since each JAK transduces signals from multiple cytokines and receptors, gene knockout models have been generated in an attempt to better understand physiological functions of JAK2 in vivo. Germline deletion of Jak2 causes embryonic lethality at embryonic day 12.5 by deficient erythropoiesis (Neubauer et al., 1998; Parganas et al., 1998). This was confirmed by Wagner and his team using an alternate approach; they created Jak2 null mutation by achieving excision of the promoter and the first coding exon of Jak2 through Cre-mediated recombination (Krempler et al., 2004). Conditional knockout of Jak2 in adult mice using tamoxifen-inducible Cre-estrogen receptor under the control of either a hematopoietic stem and progenitor or ubiquitous promoter was lethal as it abrogates erythropoiesis and induces anemia and thrombocytopenia (Grisouard et al., 2014; Park et al., 2013). These studies highlight the significance of JAK2 signalling for steady-state hematopoiesis. Moreover, the specific requirement for JAK2 in different terminally differentiated myeloid lineages, has only been clarified for platelets. Jak2 deletion in platelets and megakaryocytes leads to thrombocytosis due to dysregulated TPO turnover and non-autonomous expansion of committed megakaryocytic progenitors (Anderson et al., 2013; Meyer et al., 2014). Additionally, activating mutations of JAK2 can contribute to various hematopoietic malignancies. For instance, activating JAK2 fusion proteins evoke lymphoid and myeloid leukemia while mutations in the JAK2 gene were found with high incidence in patients with myeloproliferative neoplasms and various leukemia (myeloid, acute lymphoblastic leukemia and acute megakaryoblastic) (Valentino and Pierre, 2006).
Recent studies using JAK2 conditional knockout mice show its importance for the growth and homeostasis of a variety of tissues. JAK2 plays key roles in mammary gland development, fertility, maintaining normal heart function, the suppression of fatty liver disease and the regulation of AT homeostasis in adult mice. Shillingford and colleagues transplanted cells and tissue fragments from JAK2 knockout embryos into adult mice to demonstrate the requirement of JAK2 in mammary gland for epithelial cell proliferation and formation of secretory alveoli (Shillingford et al., 2002). Later, JAK2 was selectively ablated in mammary epithelial cells resulting in impaired alveolar development and maintenance (Wagner et al., 2004). They showed that JAK2 in the mammary gland is essential for PRL signalling through STAT5 (Wagner et al., 2004). Likewise, conditional knockout of Jak2 in gonadotrophin releasing hormone impaired fertility and reproductive development in female mice (Wu et al., 2011). A more recent study showed that loss of Jak2 in cardiac myocytes causes the development of severe cardiac defects including hypertrophy, dilated cardiomyopathy and progressive left ventricular dysfunction as well as early mortality (Gan et al., 2015).

Several studies have also employed conditional knockout models to study tissue-specific functions of JAK2 in the context of obesity associated abnormalities. Choi and colleagues used pancreatic β-cell specific JAK2 knockout to show EPO signalling through this kinase has protective effects against diabetes development (Choi et al., 2010). However, JAK2 does not appear to play a major role regulating the compensatory increase in β-cell mass in response to HFD suggesting that pancreatic JAK2 may have a redundant role in the β-cell expansion (Choi et al., 2011). On the other hand, hepatocyte-specific deletion of JAK2 leads to profound fatty liver and hepatic insulin resistance spontaneously (Shi et al., 2012; Sos et al., 2011) with a paradoxical protection against glucose intolerance and systemic insulin resistance (Shi et al., 2012). The effects observed due to liver-specific loss of JAK2 could be alluded to the impaired hepatocellular GH signalling (Shi et al., 2012; Sos et al., 2011), but deletion of JAK2 in adipocytes was sufficient to prevent liver lipid accumulation (Nordstrom et al., 2013). On the other hand, JAK2 deficiency in adipocytes promotes adiposity with reduced energy expenditure and impaired lipolysis and insulin sensitivity (Nordstrom et al., 2013; Shi et al., 2014). Mice with adipose-specific JAK2 disruption were also not able to activate HFD- or cold-induced thermogenesis (Shi et al., 2016). Collectively, whole body and tissue-specific knockout models of Jak2 to date have helped address important biological functions of JAK2 in tissue homeostasis. However, the function of JAK2 in other
immune cells such as lymphocytes or other myeloid cells such as macrophages remains to be elucidated.

Table 1: Cytokine and growth factor stimulation of JAK2-STAT signalling.

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<th>Cytokine/hormone</th>
<th>Downstream JAKs b</th>
<th>Downstream STATs b</th>
<th>Few biological functions b</th>
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<td><strong>Type I common β-chain cytokines</strong></td>
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<td>GM-CSF</td>
<td>JAK2</td>
<td>STAT3, STAT5</td>
<td>Development of macrophages and granulocytes; differentiation of stem cells</td>
</tr>
<tr>
<td>IL-3</td>
<td>JAK2</td>
<td>STAT3, STAT5</td>
<td>Proliferation and differentiation of stem cells; regulates activation of myeloid cells</td>
</tr>
<tr>
<td>IL-5</td>
<td>JAK2</td>
<td>STAT3, STAT5</td>
<td>Stimulates B cell growth and immunoglobulin secretion; regulate eosinophil functions</td>
</tr>
<tr>
<td><strong>Type I common γ-chain cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-13</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT6</td>
<td>Anti-inflammatory actions, suppress the production of proinflammatory cytokines</td>
</tr>
<tr>
<td><strong>Type I gp130 cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3</td>
<td>Proinflammatory cytokine; role in acute phase response and various chronic inflammatory diseases</td>
</tr>
<tr>
<td>IL-11</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3</td>
<td>Induces megakaryocyte colony formation and maturation; anti-inflammatory roles</td>
</tr>
<tr>
<td>IL-27</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT2, STAT3, STAT4, STAT5</td>
<td>Regulation of T cell response and monocyte activation</td>
</tr>
<tr>
<td>OSM</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Induces fibroblast proliferation, inhibits tumor cell proliferation, plays a role in immune regulation</td>
</tr>
<tr>
<td>CT-1</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Induces cardiac myocyte hypertrophy</td>
</tr>
<tr>
<td>CNTF</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Survival factor for various neuronal cell types</td>
</tr>
<tr>
<td>LIF</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Regulates various functions including proliferation and differentiation of cells during development</td>
</tr>
<tr>
<td><strong>Type I heterodimeric cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>JAK2, TYK2</td>
<td>STAT4</td>
<td>Promote Th1 immune response and induces IFN-γ production in different immune cells</td>
</tr>
<tr>
<td>IL-23</td>
<td>JAK2, TYK2</td>
<td>STAT1, STAT3, STAT4, STAT5</td>
<td>Proinflammatory cytokine; induce proliferation and regulate T cell function</td>
</tr>
<tr>
<td><strong>Type I, hormone-like cytokines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPO</td>
<td>JAK2</td>
<td>STAT5</td>
<td>Promotes survival and terminal differentiation of erythrocytes</td>
</tr>
<tr>
<td>TPO</td>
<td>JAK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Differentiation of megakaryocytes and platelets</td>
</tr>
<tr>
<td>G-CSF</td>
<td>JAK1, JAK2, TYK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Regulates the proliferation, differentiation of stem cells and granulocytes</td>
</tr>
<tr>
<td>Hormone</td>
<td>JAK/STAT</td>
<td>Response</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>----------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>GH</td>
<td>JAK2</td>
<td>STAT1, STAT3, STAT5</td>
<td>Regulates post-natal body growth; immunomodulatory and metabolic effects</td>
</tr>
<tr>
<td>Leptin</td>
<td>JAK2</td>
<td>STAT3, STAT5, STAT6</td>
<td>Coordination adipose tissue mass and energy balance, roles in regulating immune function</td>
</tr>
<tr>
<td>PRL</td>
<td>JAK2</td>
<td>STAT3, STAT5</td>
<td>Mammary gland development</td>
</tr>
</tbody>
</table>

**Type II IFN family cytokines**

| IFN-γ   | JAK1, JAK2 | STAT1, STAT3 | Inflammatory and antiviral effects; regulates T cell and monocyte/macrophage activation |

**Growth factors**

| PDGF    | JAK1, JAK2 | STAT1, STAT3, STAT5 | Proliferation, migration, and enhanced survival of endothelial cells |
| EGF     | JAK1, JAK2 | STAT1, STAT3, STAT5 | Induces proliferation, differentiation, and survival of various cells |

**G-protein coupled receptors**

| α-Thrombin | JAK2 | STAT1, STAT3 | Components of the coagulation cascade, activate platelets |
| AT        | JAK2, TYK2 | STAT1, STAT2, STAT3, STAT5 | Vascular smooth muscle cell proliferation, vasoconstriction |
| Serotonin | JAK1, JAK2 | STAT1, STAT3 | Modulatory neurotransmitter in the central nervous system |

*a Abbreviations: AT angiotensin, CNTF ciliary nerve growth factor, CT-1 cardiotropin-1, EGF epidermal growth factor, EPO erythropoietin, G-CSF granulocyte colony-stimulating factor, GH growth hormone, GM-CSF granulocyte macrophage colony-stimulating factor, IFN-γ interferon-γ, IL interleukin, LIF leukemia inhibitory factor, OSM oncostatin M, PDGF platelet-derived growth factor PRL prolactin, TPO thrombopoietin |

*b Adapted from references (Cell Signaling Technology, 2013; Fauci et al., 2015; R&D Systems, 2017; Rawlings et al., 2004; Roskoski, 2016).
1.6 Role of JAK2 in monocytes/macrophages

The cytokines that promote polarization of macrophage phenotypes signal predominantly through the JAK-STAT pathway, leading to the activation of transcription factors that dictate M1/M2 polarization. Indeed, various studies have demonstrated that distinct signals can activate JAK2 within macrophages, an essential mediator downstream of various cytokines and growth factors, providing further insight into its dynamic function in mediating inflammatory response. Lee et al. (2006) have shown that phosphatidic acid (PA), an important metabolite that is involved in phospholipid biosynthesis and membrane remodeling, induces activation of the JAK2 resulting in the production of proinflammatory cytokines, IL-1β and IL-6. Treating RAW 264.7 cells, a murine macrophage cell line, with the JAK2 inhibitor AG490 or knockdown of JAK2 by small interfering RNA (siRNA) abolished PA-induced IL-1β and IL-6 release but not TNFα production (Lee et al., 2006). PA-induced γ-interferon activating sequence (GAS), a known STAT target gene, was also significantly suppressed by AG490 (Lee et al., 2006).

Similarly, another group previously demonstrated that lipopolysaccharide (LPS), a bacterial endotoxin, stimulation induced tyrosine phosphorylation of JAK2 which was important in the production of IL-1β and IL-6 (Okugawa et al., 2003). Both the JAK2 inhibitor AG490 and the kinase-deficient JAK2 protein significantly inhibited the production IL-1β and IL-6 in RAW 264.7 cells. More specifically, they demonstrated that LPS induced tyrosine phosphorylation of JAK2 via toll-like receptor 4 (TLR4), because cells pretreated with an anti-TLR4 neutralizing antibody (MTS510) inhibited LPS-induced phosphorylation of JAK2 (Okugawa et al., 2003). Furthermore, JAK2 was shown to regulate the phosphorylation of JNK mainly through activation of PI3K, since phosphorylation of JNK and PI3K was decreased in cells treated with AG490 or transfected with kinase-deficient JAK2 (Okugawa et al., 2003). Another study by Marrero et al. (1998) highlighted the positive role of macrophage JAK2 in LPS induced expression of iNOS. In RAW 264.7 cells, protein expression of iNOS upon LPS and IFN-γ stimulation is blocked by AG490 (Marrero et al., 1998). It has been known that IFN-γ causes tyrosine phosphorylation of STAT1α by the IFN-γ receptor-associated JAK1 and JAK2 (Darnell et al., 1994). Furthermore, IFN-γ and LPS are suggested to function synergistically in mouse macrophages in the transcriptional induction of the mouse iNos gene through direct involvement of Stat1α (Gao et al., 1997).
The JAK2 pathway is the most studied cascade in GH signalling since JAK2 associates with the GH receptor upon GH binding. GH also induced phosphorylation of JAK2 in murine peritoneal macrophages (PMs) but this effect was inhibited when macrophages were pretreated with AG490 (Tripathi and Sodhi, 2009). Pre-treatment of macrophages with AG490 also significantly reduced GH-induced production of proinflammatory cytokines TNF-α, IL-1β, IFN-γ and IL-12 (Tripathi and Sodhi, 2009). GH also leads to activation of PI3K, protein kinase C (PKC), ERK1/2, and JNK, which are components of prominent pathways other than JAK2 that are involved in GH-induced macrophage activation (Tripathi and Sodhi, 2009). It clearly establishes that JAK2 lies at the hub and initiates most of the signalling cascades involved in the production of GH-induced cytokines in macrophages.

IL-3 is a growth and survival factor for immature myeloid progenitor cells and IL-3 binding to its receptor also activates JAK2. Using IL-3 dependent, immature myeloid cell line 32D, Kovanen and colleagues showed that PKC isoforms α and δ induce monocytic differentiation by inhibiting IL-3 signalling through specific abrogation of JAK2 activation resulting in growth arrest (Kovanen et al., 2000). The interaction of PKC with JAK2 shows that communication of JAK2 with other pathways is also essential in regulating various cellular functions.

Interestingly, acetylcholine (ACh), an important vagal neurotransmitter, inhibited LPS-induced metalloproteinase 9 (MMP-9) production and macrophage migration through the JAK2/STAT3 signalling pathway (Yang et al., 2015). MMPs can mediate macrophage migration and can contribute to the development of many inflammatory diseases (Khokha et al., 2013). ACh inhibited MMP-9 expression and activity, and cell migration in a concentration-dependent manner after LPS administration in RAW 264.7 cells (Yang et al., 2015). ACh mediates this by enhancing JAK2/ STAT3 phosphorylation and attenuating NF-κB activation. These effects were abolished when cells were treated with AG490 or stattic (a STAT3 inhibitor) (Yang et al., 2015). Moreover, de Jonge and coworkers demonstrated that nicotine, which is a nicotinic acetylcholine receptor (nAChR) agonist, exerts its anti-inflammatory effect on PMs via Jak2 and STAT3 signalling in vitro and in vivo (de Jonge et al., 2005). In PMs, JAK2 was recruited to the α7 subunit of the nAChR and was phosphorylated after nicotine binding (de Jonge et al., 2005). They further showed that JAK2-STAT3 signalling contributes to the cholinergic inhibition of macrophage activation in vivo on the occurrence of post-surgical intestinal inflammation in a mouse model of postoperative ileus (de Jonge et al., 2005). Additionally, adipokine visfatin also increases the
levels of iNOS mRNA and protein and nitric oxide production in RAW 264.7 macrophages (Kang et al., 2013). Treatment with visfatin increased the phosphorylation of JAK2 and STAT3 along with IκBα, ERK1/2 and JNK. However, the enhancement of iNOS expression was prevented by treating the cells with inhibitors of the JAK2, NF-κB, ERK1/2, and JNK pathways (Kang et al., 2013). The diversity of factors that activate JAK2 within monocytes/macrophages further implicates the importance of JAK2 signalling in these cells.

1.6.1 Chemokines

The variety of cytokines and growth factors that signal through JAK2 in monocytes/macrophages can also regulate chemokine expression within these cells. GM-CSF is important for growth and differentiation of committed myeloid progenitor cells (Shi et al., 2006). It can also act as a proinflammatory cytokine as it is produced by inflammatory cells, including T cells, monocytes/macrophages, and vascular endothelial and smooth muscle cells (Shi et al., 2006). GM-CSF enhances CCL2 expression in human monocytic cell line U937 via JAK2-STAT5 signalling pathway (Tanimoto et al., 2008). However, AG490 pre-treatment decreased GM-CSF-stimulated CCL2 expression at both protein and mRNA levels (Tanimoto et al., 2008). Another study showed that IFN-γ-induced activation of JAK1/2 can also synergistically enhance TNF-α-induced production of CXCL10 (also known as interferon inducible protein-10) by increasing NF-κB activation via IκBα pathway in the human monocytic THP-1 cell line (Qi et al., 2009).

Interestingly, IFN-γ-enhanced degradation of IκBα is reversed by the inhibition of JAK pathways by JAK inhibitor I (JAK1/2/3 inhibitor) or AG490 in TNF-α-stimulated cells (Qi et al., 2009).

There is now emerging evidence that GPCRs are also capable of activating these JAK/STAT signalling pathways. The chemokine receptors, CCR2 and CCR5, belong to the superfamily of GPCRs. In human monocytic Mono Mac-1 cell line, CCL2-triggered CCR2 receptor activation resulting in CCR2 tyrosine and JAK2 phosphorylation, and STAT3 binding (Mellado et al., 1998). Both Ca²⁺ mobilization and cell migration were blocked by tyrphostin B42, a specific JAK2 kinase inhibitor (Mellado et al., 1998). This latter observation along with the rapid association (within seconds) of JAK2 to the CCR2 suggests a role for this tyrosine kinase in early receptor phosphorylation following ligand stimulation. Similarly, CCL3 activation of CCR5 also leads to activation of JAK2, as shown by increased tyrosine phosphorylation in THP-1 cells (Mueller and
Co-immunoprecipitation studies confirmed the interaction between CCR5 and JAK2. CCR5 activation also leads to independent activation of G protein G12 (Mueller and Strange, 2004). Additionally, CXC motif chemokine receptor (CXCR) 4 is another GPCR that activates JAK-STAT pathway upon selective binding of the stromal cell-derived factor 1 (SDF-1, also known as CXCL12) (Vila-Coro et al., 1999). SDF-1 induced the transient association of JAK2 with CXCR4, leading to the tyrosine phosphorylation and activation of the transcription factor STAT3 (Ahr et al., 2005; Vila-Coro et al., 1999). These studies further highlight the importance of cytokines that signal through JAK2 to regulate chemokine expression as well as other players including chemokine receptors that interact with JAK2.

1.6.2 Leptin

Leptin, a 16-kDa peptide hormone encoded by the Ob gene derived primarily from adipocytes, is secreted into the circulation and has multiple physiological roles. Leptin is also expressed in the gastric wall, vascular wall, placenta, ovary, skeletal muscle, and the liver (Singla et al., 2010). Its primary function is to activate neurons in the hypothalamus that reduce food intake and energy expenditure (Leshan et al., 2006). Leptin appears to be a key molecule not only in energy homeostasis but also in immune responses (Fernández-Riejos et al., 2010). Leptin is structurally similar to the family of helical cytokines that includes IL-2, IL-12 and GH, and signals similar to class I cytokine superfamily (Fernández-Riejos et al., 2010). Following leptin binding to its receptor, JAK2 is one of the downstream mediators activated (Frühbeck, 2006). Functional receptors of leptin are widely distributed throughout the body in many different kinds of cells including monocytes and macrophages among other immune cells (Fernández-Riejos et al., 2010).

Leptin expression and circulating leptin levels are dramatically increased under inflammatory conditions. Studies show that leptin has a proinflammatory effect on various immune cells including monocytes and macrophages, by inducing production of inflammatory mediators. Leptin can enhance several proinflammatory mediators such as iNOS and cyclooxygenase-2 in macrophage cell line J774A.1 (Raso et al., 2002). Leptin also induces TNF-α, IL-6, and IL-12 production in PMs (Loffreda et al., 1998). Leptin also induces IL-18 secretion in THP-1 cells and implicated the involvement of JAK2 in this signalling (Jitprasertwong et al., 2014). Moreover, human leptin stimulated proliferation in a dose-dependent manner and functionally activates
human circulating monocytes *in vitro*, by inducing the production of cytokines such as TNF-α and IL-6 (Santos-Alvarez et al., 1999). Leptin also induces the secretion of chemokines such as CXCL10 in THP-1 monocytic cells (Meier et al., 2003), as well as CCL3, CCL4 and CCL5 in RAW 264.7 macrophages (Kiguchi et al., 2009). Kiguchi et al. (2009) showed that leptin-induced upregulation of CCL3, CCL4 and CCL5 mRNA expression in a dose- and time-dependent manner. Treatment of AG490 had no significant effect on basal CCLs mRNA expression, but the addition of leptin to cells pretreated with AG490 significantly suppressed the leptin-induced upregulation of CCL mRNA expression. JAK2 inhibition also completely suppressed the leptin-induced increase in phosphorylation of STAT3 (Kiguchi et al., 2009). These studies indicate that leptin can induce inflammatory responses through cytokine and chemokine upregulation.

Leptin is also reported to be a potent chemoattractant for monocytes and macrophages. Leptin has been shown to increase adhesion and transmigration of blood monocytes in a concentration-dependent manner (Curat et al., 2004). Leptin induced migration of human THP-1 monocytes at concentrations as low as 1 pg/mL (Gruen et al., 2007). However, inhibition of JAK2 resulted in the prevention of migration to leptin. Inhibition of PKC and phospholipase C, which are downstream of the leptin receptor, also resulted in a complete absence of leptin-induced monocyte migration (Gruen et al., 2007). MAPK and PI3K pathways involved in CCL2 signalling were also implicated in leptin-induced THP-1 migration (Gruen et al., 2007). The study highlights the role of JAK2 as a mediator of canonical signalling pathway required for leptin in monocyte/macrophage motility and chemotaxis.

In order to determine the involvement of leptin in the phenotype characterization of ATMs, these cells were cultured with leptin (Acedo et al., 2013). These authors observed that macrophages maintained with this adipokine expressed M2 phenotype surface markers, but they were able to produce cytokines and chemokines typically synthesized by M1 cells, suggesting the contribution of leptin to the observed phenotype of macrophages found in AT (Acedo et al., 2013). Another study showed that incubation of human adipocytes with chemokines IL-8, CCL2 or CCL3 induces leptin production (Gerhardt et al., 2001). The acute effects of chemokines on leptin production were likely through a post-transcriptional mechanism (Gerhardt et al., 2001). These results suggest that leptin secretion can be influenced by changes in the AT microenvironment and leptin can also influence other cells in the vicinity such as macrophages.
While in vitro studies have provided valuable insight into the inflammatory role of leptin, in vivo studies examining the role of leptin receptor on immune and inflammatory function have also been examined. It is well-established that ob/ob mice (lacking leptin secretion) and db/db mice (lacking leptin receptor) are hyperphagic, obese and insulin-resistant. These mice also have other hormonal abnormalities, and many of these problems are corrected by treatment with exogenous leptin (Campfield et al., 1995; Pelleymounter et al., 1995). These strains also have altered immune system function (Conde et al., 2010). Claycombe and team showed that the ob/ob mouse have a nearly normal proportion of myeloid cells compared to C57BL/6 lean wild-type controls but exhibited a reduction in absolute numbers of granulocytes and monocytes (Claycombe et al., 2008). Seven days of provision of recombinant leptin promoted myelopoiesis such that the bone marrow of the obese mice contained normal numbers of monocytes and granulocytes (Claycombe et al., 2008).

The first line of evidence in the involvement of leptin in macrophage recruitment to AT was demonstrated in ob/ob and db/db mice, which have lower numbers of ATMs accompanied by less inflammatory gene expression in AT than might be expected based upon their body weight (Xu et al., 2003). However, subsequent studies to further elucidate the role of leptin on macrophage function in AT have not been conclusive. The work by Dib et al. (2014) suggests that leptin may be a key mediator of macrophage recruitment to AT. They show that mice transplanted with bone marrow from db/db mice (WT/db), when challenged with HFD, have reduced weight gain and adiposity, decreased macrophage infiltration, and subsequently diminished AT inflammation compared to their littermate controls (WT/WT) (Dib et al., 2014). They demonstrated that gonadal AT displayed a twofold lower expression of the inflammatory genes Tnfa, Il6 and Ccl2, and contained significantly fewer CLS compared with WT/WT. In addition, most of their ATMs expressed MGL1 and were CCR2-negative, indicative of an anti-inflammatory phenotype, which could lead to the greater insulin sensitivity in WT/db mice when compared to WT/WT mice (Dib et al., 2014). On the other hand, Gutierrez and the team used an identical bone marrow transplantation technique and showed that recipients of db/db bone marrow were not protected from weight gain, insulin resistance and inflammatory macrophage recruitment into AT (Gutierrez and Hasty, 2012). Only noted difference was a significant decrease in the percentage circulating Ly6C<sup>hi</sup> monocytes in WT/db mice (Gutierrez and Hasty, 2012). Hasty and Gutierrez suggested that a number of genetic and environmental factors such as duration of HFD feeding (16 vs 12
weeks), the age of recipient mice at time of bone marrow transplantation (5 versus 8 weeks), use of littermate controls and gut microbiota could be taken into account to explain the conflicting data on effects of leptin on macrophage recruitment and function in the AT (Hasty and Gutierrez, 2014). Overall, studies suggest that leptin can activate proinflammatory cells, mediate the production of the other proinflammatory cytokines, and its levels can be upregulated by proinflammatory signals.

1.7 Conditional gene targeting in monocytes/ macrophages

The use of Cre recombinase for site-specific DNA recombination has become an invaluable tool to generate tissue and temporal specific gene knockouts (Nagy, 2000). The target gene is flanked directly by repeated 34 base pair loxP DNA sequences (“floxed”). Expression of Cre recombinase in the same cell leads to specific deletion of the floxed sequence. This tool is useful for designing mouse models to answer a variety of research questions, especially in cases where complete gene knockout cause embryonic or perinatal lethality. Since the discovery of Cre recombinase over 25 years ago, there has been huge effort to generate floxed mouse strains as well as generating Cre-expressing mouse strains. However, researchers must be critical of interpreting results for these mouse models (Schmidt-Supprian and Rajewsky, 2007). There are several approaches to generate Cre-expressing strains, using either a transgene that includes a specific promoter or a “knock-in” approach that uses endogenous regulatory sequences. Neither of these approaches guarantees the desired Cre expression pattern. In addition, loxP-flanked target genes can differ dramatically with respect to their sensitivity to Cre-mediated recombination (Schmidt-Supprian and Rajewsky, 2007). Thus, to be sure about the specificity of a given conditional mutagenesis experiment, one ideally must analyze deletion efficiency of the chosen target gene in various tissues of mice expressing the Cre recombinase construct and the loxP-flanked target sequences.

There are close to three dozen myeloid-Cre-driven strains available, they vary in their specificity of Cre expression (Abram et al., 2014). Various Cre strains target macrophages (CD68-cre, CSF-1R-cre, F4/80-cre. Cx3cr1-cre, CD11b-cre, LysM-cre to name a few), but many of these strains use promoters which have been reported to have non-cell type-specific Cre expression or only deletes in a fraction of macrophage population analyzed. To assess the efficiency and specificity of Cre-mediated deletion in a variety of mouse strains expressing Cre recombinase under the
control of different myeloid-specific promoters, each Cre strain was crossed to ROSA-EYFP mice. In peripheral monocytes and peritoneal and splenic macrophages, LysM-cre and Cx3cr1-cre strains show comparable deletion efficiency. However, Cd11b-cre strain shows relatively lower deletion in these populations, while the F4/80-cre only exhibit deletion in a fraction of peritoneal macrophages. LysM-cre mice show deletion also in neutrophils and some deletion in these cells was also seen in Cd11b-cre mice. On the other hand, the Cx3cr1-cre strain shows deletion in CD11b+ dendritic cells along with mast and natural killer cells. Minimal non-specific deletion in lymphoid cells is observed only for CD11b-cre strains (Abram et al., 2014). Therefore, comparison of different myeloid-Cre strains may be helpful when planning experimental design involving genetic manipulations in monocytes/macrophages.

Given the heterogeneous nature of cell populations such as monocytes and macrophages, deletion in these cells may be problematic (Hume, 2011). The LysM-cre strain developed by Clausen and colleagues is widely used by researchers to ascertain the role of monocytes and macrophages in mouse models as it offers efficient deletion in macrophages (Clausen et al., 1999). However, this strain also results in complete deletion of the gene in the neutrophil granulocyte population and partial deletion in CD11c-positive dendritic cells (Clausen et al., 1999; Faust et al., 2000). The Cre recombinase cassette is recombined at the translation start site (Clausen et al., 1999). Consequently, the lysozyme 2 (Lyz2-cre) allele was expected to closely resemble the native locus in terms of regulated expression; and inactivation of both copies of the Lyz2 gene developed normally and were fertile (Faust et al., 2000).

The LysM-cre mouse has provided a tool for functional studies of genes in myeloid cells. Investigators have conditionally deleted many genes using this Cre strain, some examples include PPARγ, IKKβ, IL-4R, SOCS1, STAT3, TNF-α, leptin receptor, insulin receptor, and IL-10 (Hume, 2011). In the present work, using the LysM-cre line we generated conditional mutant mice of JAK2 to assess important biological functions of Jak2 in mature macrophages. To generate JAK2 conditional knockout mice, Wagner and his team created Jak2 floxed mice by placing loxP sites around the first coding exon of the Jak2 locus (Krempler et al., 2004). Selective deletion of macrophage JAK2 allowed us to study its role in the pathogenesis of inflammation and insulin resistance. We hypothesized that loss of macrophage JAK2 will attenuate macrophage activation and therefore reduce macrophage infiltration into adipose and other metabolic tissues, thereby suppress systemic inflammation and improve insulin sensitivity.
Chapter 2
Materials and Methods

2.1 Animals

Macrophage-specific Jak2 knockout mice were generated by breeding mice with the Jak2 gene flanked by loxP sites \(\text{Jak2}^{\text{fl/fl}}\) (Krempler et al., 2004; Wagner et al., 2004) with mice expressing Cre recombinase under the control of the Lyz2 promoter. JAK2 floxed mice were generously provided by Kay-Uwe Wagner (University of Nebraska Medical Center, Omaha, NE, USA) and LysM-cre mouse was purchased from Jackson Laboratory (Bar Harbor, ME, USA). The resulting \(\text{LyzM-cre}^+\text{Jak2}^{+/+}\) mice were intercrossed to generate \(\text{LysM-cre}^+\text{Jak2}^{+/+}\) (herein referred to as M-JAK2\(^{+/+}\)) and \(\text{LysM-cre}^+\text{Jak2}^{0/0}\) (herein referred to as M-JAK2\(^{-/-}\)) mice. M-JAK2\(^{+/+}\) served as littermate controls, and mice were maintained on a mixed 129\(\times\)1/SvJ and C57BL/6 background. Mice were housed in a pathogen-free facility at the Toronto Medical Discovery Tower (Toronto, ON, Canada) with a 12 hr light–dark cycle and free access to water standard irradiated rodent chow diet (5% energy from fat; Teklad LM-485; Envigo, Madison, WI, USA). Starting at 6-7 weeks of age, a cohort of mice was randomly selected to receive high-fat diet (60% fat, 24% carbohydrates and 16% protein based on caloric content; F3282; Bio-Serv, Flemington, NJ, USA) for 12 weeks. All animal experimental protocols were approved and performed in accordance with animal license guidelines and regulations established by the Toronto General Research Institute Animal Care Committee.

2.2 Polymerase chain reaction (PCR) genotyping

Genotyping of \(\text{Lyz2-cre}\) allele was performed using information Jackson Laboratory with the following primers: common forward: \(5'-\text{CTTGGGCTGCCAGAATTTCTC}-3'\); wild-type reverse: \(5'-\text{TTACAGTCGGCCAGGCTGAC}-3'\); and mutant reverse: \(5'-\text{CCCAGAAATGCCAGATTACG}-3'\). For the PCR amplification protocol, the melting temperature was 94°C for 20 s, annealing temperature was 65°C (with 0.5°C decrease each cycle) for 10 s, and the primer extension phase was 68°C for 10 seconds, for 10 cycles; then melting temperature was 94°C for 15 s, annealing temperature was 60°C for 15 s, and the primer extension phase was 72°C for 10 second, for 28 cycles. The final PCR product was 350 bp for the wild-type allele and ~700 bp for the mutant allele with separate reaction performed for each allele. Genotyping of Jak2 was performed using the following primer sequences: forward: \(5'-\text{ATTCTGAGATTCCAGGTCTGAGC}-3'\) and reverse:
5′-CTCACAACCATCTGTATCTCAC-3′. For the PCR programme, the melting temperature was 95°C for 30 s, the annealing temperature was 57°C for 30 s, and the primer extension phase was 72°C for 45 s, for 38 cycles. The final PCR product was 230 bp for the wild-type allele and ~310 bp for the floxed allele. All primers were synthesized by Eurofins MWG Operon (Huntsville, AL, USA). PCR products were separated on 1% agarose gels, stained with 0.3 μg/mL ethidium bromide and visualized by ultraviolet light.

2.3 In vivo metabolic analyses

Body weight measurements were performed biweekly. All overnight fasts were 15 to 16 hr in duration. All blood glucose levels were determined from tail venous blood with an automated glucose monitor (One Touch II; Lifescan, Inc., Milpitas, CA). Random blood glucose was measured between 9 and 10 a.m. and fasting blood glucose at 9 am after overnight fasting. Glucose tolerance tests were performed on overnight-fasted animals between 9 and 10 a.m., utilizing a glucose dose of 1.0 g/kg of body weight injected intraperitoneally (i.p.) and measurements of glucose levels done at 0, 15, 30, 60, and 120 min after the injection. Insulin tolerance tests were performed on 4 hr-fasted animals between 12:30 and 1:30 p.m. by i.p. injection using insulin lispro (B28Lys,B29Pro human insulin; Humalog, Eli Lilly, Scarborough, ON) at a dose of 0.75 units per kg body weight, and blood glucose levels were measured at 0, 15, 30, 45, 60 and 120 min after the injection. Male and female data are combined for weight and glucose homeostasis as they showed similar trends when examined separately. For insulin signalling experiments, mice fasted overnight were injected i.p. with insulin lispro (5 units/ kg). Tissues were removed 10 min later and snap-frozen in liquid nitrogen.

To measure food intake, energy expenditure, oxygen consumption (VO₂), carbon dioxide production (VCO₂), respiratory exchange ratio (RER), and activity level, mice were individually housed in a comprehensive laboratory animal monitoring system (CLAMS; Columbus Instruments, Columbus, OH, USA) with free access to food and water. After 24 hr acclimation to the apparatus, data for 24 hr were collected. The mice were weighed prior to each trial. The analyses were performed using the CLAX software as supplied by the manufacturer. Light cycle runs from 6 am to 6 pm while the dark cycle runs from 6 pm to 6 am the following day. Energy intake was calculated using following conversions according to the product information supplied by manufacturer: 3.1 kcal/g for normal chow diet and 5.49 kcal/g for high-fat diet. The RER was
calculated as $\frac{VCO_2}{VO_2}$ and heat production was calculated as $VO_2 \times (3.815 \pm 1.232 \times RER)$.

### 2.4 Analysis of serum hormones and cytokines

Overnight-fasted mice were anesthetized and blood was collected by cardiac puncture. Serum insulin levels were measured using a mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA); serum PAI-1 total and Resistin using Milliplex Adipokine kit (Millipore, Billerica, MA, USA); and serum TNF-α, IL-1β, IL-6, IL-10, IFNγ, CCL2, IL-12p70 and IL-13 levels using Milliplex Cytokine Kit (Millipore) and the Luminex 100 Instrumental System (Luminex, Austin, TX, USA) as per manufacturer’s instructions.

### 2.5 Immunohistochemistry and morphological analyses.

Liver, pancreas and visceral adipose tissue (VAT) were isolated, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4) for two days and then processed to paraffin blocks. For each sample, at least 3 levels of tissue sections were obtained at 150 μM intervals (UHN Pathology Research Program, Toronto, ON). Tissue sections were stained with hematoxylin and eosin (H & E). Briefly, sections were dewaxed and rehydrate in xylene twice for 5 min, then 100%, 95%, 90% and 70% ethanol for 4 min each followed by running tap water for 30 s. Next, sections were stained with hematoxylin (Sigma Aldrich, St. Louis, MO, USA), then destained for 30 s in 1% HCl (Sigma-Aldrich) with 70% ethanol, then rinsed in running tap water for 15 s followed by eosin (Sigma-Aldrich) staining for 2 min and rinse with tap water for 40 s. Sections were dehydrated as follows: 70%, 90%, 95% and 100% ethanol for 4 min each then xylene twice for 5 min; and coverslips were applied to slides using Permount (Fisher Scientific, Waltham, MA, USA). Adipocyte area and diameter were determined on H & E-stained sections in 9 to 10 fields at 200× using CellSens software (Olympus, Tokyo, Japan). Adipocyte numbers were determined in 5 fields at 200×.

Pancreas sections were immunostained for insulin (Dako, Glostrup, Denmark). Insulin immunostained slides were scanned by ScanScope XT System (UHN AOMF, Toronto, ON) at 20X magnification and analyzed using ImageScope version 12.1 software (Aperio Technologies, Vista, CA, USA) for β-cell area which was measured over the total pancreatic area. For Oil Red O staining, frozen liver sections were prepared using tissue frozen in O.C.T. compound (Tissue-Tek, Torrance, CA, USA) prior to staining (UHN Pathology Research Program).
ATMs were detected in VAT by immunostaining for F4/80. VAT tissue sections were dewaxed and rehydrated as above for 5 min in each solution followed by H₂O₂ block (10 min) (30% vol/vol in dH₂O), then rinsed in dH₂O twice (5 min) and PBS with 0.1% Tween-20 (PBST, 5 min). Antigen retrieval for F4/80 staining was done in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween-20, pH 9.0) using a pressure cooker. After antigen retrieval, cooled slides were placed in dH₂O (10 min) and then rinsed with PBST. Blocking was performed using Avidin/Biotin Blocking Kit (Invitrogen, Carlsbad, CA, USA): Solution A (10 min), rinsed with PBST (3 times), Solution B (10 min) and then rinsed with PBST (3 times). Sections were further blocked with 5% (in PBST) goat serum for 30 min. Sections were incubated with F4/80 (Santa Cruz Biotechnology) at 1:200 dilution for 2 hr at room temperature (RT) and then rinsed with PBST (3 times). Primary antibodies were detected using goat anti-rat IgG-biotinylated (Santa Cruz Biotechnology) at 1:200 dilution with 30 min incubation at RT followed by rinse with PBST (3 times). Secondary antibodies were detected using streptavidin horseradish peroxidase (HRP) (Vector labs, Burlingame, CA, USA) for 30 min at RT followed by rinse with PBST (3 times), followed by colour development using Vector NovaRED Peroxidase (HRP) Substrate Kit (Vector labs) as per manufacturer’s instructions. Rinsed slides in dH₂O (3 times) were stained with hematoxylin for 30 s, then rinsed in cool dH₂O, then quick dip in 0.2% (v/v) HCl (in dH₂O), then rinsed in dH₂O and then a quick dip in PBS. Sections were then dehydrated as described previously for 5 min in each solution and then xylene twice for 5 min; and coverslips were applied to slides using Permount. CLSs were quantified per 100× power field in at least 15 fields using Leica DM1000 microscope (Wetzlar, Germany) with Olympus DP72 camera (Waltham, MA, USA).

2.6 Hepatic lipid analysis

Lipids were extracted from livers and triacylglycerol (TAG) content and FA composition were quantified as previously described (Kitson et al., 2016). Briefly, lipids were extracted from livers using 2:1:0.8 chloroform: methanol: 0.88% KCl (v/v/v) following the addition of internal standard for TAG quantitation (triheptadecanoate, Nuchek Prep, Elysian, MN, USA). Thin-layer chromatography was used to isolate TAGs using silica G plates (silica gel 60, Millipore) and a mobile phase of 60:40:2 heptane: diethyl ether: glacial acetic acid (v/v/v). Lipid classes were visualized by spraying resolved plates with 0.1% (w/v) 8-anilino-1-naphthalenesulfonic acid and identified in relation to the mobility of a reference standard mixture containing lipid classes of...
interest. TAG bands were collected from TLC plates. FA methyl esters were prepared by heating samples at 100°C for 1 hr in 14% boron trifluoride in methanol, and FAMEs were resolved on a Varian 430 gas chromatograph- FID (Varian, Lake Forest, CA, USA) with a DB-23 capillary column (30 m x 0.25 mm interior diameter x 0.25 µm film thickness; Agilent Technologies, Mississauga, ON). Peaks were identified in relation to a reference standard mixture (GLC 455 mixed with GLC-48 and supplemented with 8:0, 10:0, and 12:0 fatty acid methyl esters, Nuchek Prep), and concentrations of fatty acids were determined in relation to peak area of internal standard.

2.7 Thioglycollate preparation and peritoneal macrophage isolation

Thioglycollate medium (BD Difco, Sparks, MD, USA) was dissolved in ddH₂O using heat to prepare 4% (w/v) solution. Solution was autoclaved at 121°C for 15 min, kept overnight in a loosely-capped bottle and then stored in a tightly closed bottle for two months protected from light at RT. The solution was use immediately or was kept at 4°C for long-term storage away from light.

1 ml of 4% thioglycollate was injected i.p. into each mouse at 8 to 10 weeks of age. 4 days after injection, mice were euthanized with CO₂, their abdomens were washed with ice-cold PBS containing 1% fetal bovine serum (FBS; Gibco, Burlington, ON) to harvest elicited PMs and centrifuged at 1250 rpm for 5 min. Cells were frozen at -80°C prior to subsequent analysis.

2.8 In vitro experiments

2.8.1 Cell culture

RAW 264.7 murine macrophage cells (TIB-71, American Type Culture Collection (ATCC), Manassas, VA, USA) were a kind gift from Dr. Tianru Jin (University Health Network, Toronto, ON). Cells were cultured as per manufacturer’s protocol in Dulbecco’s modified eagle’s medium (DMEM; 4.5 g/L D-glucose; Gibco) supplemented with 10% (v/v) FBS, 100 units/mL penicillin and streptomycin (Gibco) at 37°C with 5% CO₂ in humidified air. Briefly, cells were passaged each day using cell scraper and seeded with a splitting ratio of 1:3. Cells were frozen at an earlier passage in culture media with 5% dimethyl sulfoxide and stored in liquid nitrogen.
2.8.2 Leptin stimulation

RAW 264.7 cells were plated at a density of 1.0 x 10^6 per 22-mm 6-well plate with growth media supplemented with varying concentrations of recombinant murine leptin (450-31; PeproTech, Rocky Hill, NJ, USA) or vehicle (0.1% bovine serum albumin) for 6 hr before collecting cells for subsequent analysis. Three independent experiments were performed in triplicates.

2.8.3 siRNA transfection

RAW 264.7 cells were plated at a density of 3.5 x 10^5 per 35-mm 6-well plate and then cultured for 24 hr in its respective media at 37°C under standard conditions. For silencing experiments, cells were transfected with 80 nM of either siRNA Silencer® Select targeted against the mRNA of Jak2 (s68540, ThermoFisher Scientific, Waltham, MA, USA), or a scramble siRNA sequence as control. Transfections were performed using Lipofectamine™ RNAiMAX transfection reagent (Invitrogen) based on the manufacturer’s protocol in the absence of antibiotics. More specifically, the siRNA was diluted in the appropriate amount of Opti-MEM® I Reduced Serum Medium (250 µL/well) (Invitrogen). Lipofectamine™ RNAiMAX (6 µl/ well) was then diluted and incubated for 5 min at RT in the appropriate amount in Opti-MEM® I Reduced Serum Medium (250 µL/ well). After the 5 min incubation, the diluted siRNA was combined with the diluted Lipofectamine™ RNAiMAX. This was incubated and mixed gently for 20 min at RT to allow the complex formation to occur. During the 20 min, the wells were replaced with 1.25 µl of DMEM without FBS. After the 20 min, 500 µL of the appropriate solutions were added to the appropriate wells. After 4 hr, the remaining 0.25 ml/well of medium was added which contained the appropriate amount of FBS to establish a final concentration of 10% FBS to each well. The wells of the silencing experiments were replaced with fresh DMEM containing 10% FBS the morning of the following day. Cells were either collected 24 hr after transfection for subsequent analysis or treated with vehicle or leptin prior to collection. Four independent experiments were performed in triplicates.

2.9 RNA isolation and reverse transcriptase-quantitative PCR (RT-qPCR)

Total RNA from PMs was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's protocol. Total RNA from tissues or RAW 264.7 cells was isolated using Trizol reagent (Ambion, Carlsbad, CA, USA) as per manufacturer’s protocol with following changes.
After isopropanol addition, the suspension was stored at -20°C overnight before RNA wash. Next day, the pellet is washed for an additional time with 75% ethanol. 1μg RNA was treated with 1U DNase I (Invitrogen) with 10X DNase I Reaction Buffer (1X final concentration; Invitrogen), incubated for 15 min at RT and then inactivated at 75°C for 5 min. Subsequently, 0.5 nM dNTP and 200 ng of random primers (Invitrogen) were added to the reaction mixture, incubated at 65°C for 5 min followed by quick chill on ice. Then 5X First-Strand Buffer (1X final concentration; Invitrogen), 0.01 M DTT (Invitrogen) and 40U RNase OUT (Invitrogen) were added to the reaction mixture and incubated at 37°C for 2 min. RNA was then reverse-transcribed with 200 U of M-MLV enzyme (Invitrogen) using the following protocol: 25°C for 10 min, 37°C for 50 min, 70°C for 15 min and held at 4°C. qPCR was performed under standard conditions using SYBR Green master mix on a 7900HT Fast-Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA). Each sample was run in triplicate in 10μL volume. The relative mRNA abundance of each gene was normalized to the expression level of the housekeeping gene 18S and calculated by a standard curve. Primer sequences are listed in Table 2.
**Table 2: Primer sequences for quantitative RT-qPCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’ to 3’)</th>
<th>Reverse (5’ to 3’)</th>
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<tr>
<td>18s</td>
<td>AGTCCCTGCCCTTTGTGACACA</td>
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<tr>
<td>Adipoq</td>
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<tr>
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<td>AGGAACCTGCATCTAGGACACATC</td>
</tr>
<tr>
<td>Ccl2</td>
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<td>TGCTTGAGGTGTTGTTGGAA</td>
</tr>
<tr>
<td>Ccl3</td>
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<td>CTGGAATCTCCGAGGTGTAG</td>
</tr>
<tr>
<td>Ccl4</td>
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<td>CTGCTGTCTCCCTTTGGTCAG</td>
</tr>
<tr>
<td>Ccl5</td>
<td>CCCTCACCATCATCTCCTACT</td>
<td>AGGAGTACGCAAAGCAGCAG</td>
</tr>
<tr>
<td>Ccl7</td>
<td>AAGAAGGCGAGGAGCTCTGCTG</td>
<td>TCAAGGGCTTTGGAGTTGGG</td>
</tr>
<tr>
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<tr>
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<td>CAGGAGTGGTCTTGGATGT</td>
</tr>
<tr>
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<td>TTGGACCTGAGCCATAATC</td>
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2.10 Western blotting

Tissues or cells were mechanically homogenized in ice-cold lysis buffer in RIPA buffer (Sigma-Aldrich) with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA) and centrifuged for 10 min at 14000 g and 4°C. 25 to 30 µg of total protein from tissue or cell lysate was run on a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a running buffer solution containing 25 mM tris base, 192 mM glycine and 0.1% SDS. After electrophoresis, proteins were transferred from the gel to a methanol-hydrated PVDF (polyvinylidene fluoride) membrane for 105 min in transfer buffer (25 mM Tris base, 192 mM glycine and 20% methanol). To prevent nonspecific binding, the PVDF membrane was then incubated in 5% non-fat dry milk in tris-buffered saline solution containing 20 mM Tris-Cl, 136 mM NaCl with 0.1% Tween-20 (TBST) for 1 hr at RT. Membranes were probed overnight at 4°C with a primary antibody against the protein of interest: phosphorylated AKT (S473; 1:1000 dilution), total AKT (1:1000) total JAK2 (1:1000), tubulin (1:1000) and GAPDH (1:5000) (Cell Signaling Technology, Danvers, MA, USA). After overnight incubation, membranes were washed three times with TBST for 10 min and then incubated for 1 hr at RT with a secondary horseradish-peroxidase (HRP)-conjugated polyclonal antibody against the primary antibody with HRP-conjugated polyclonal goat anti-rabbit antibody (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 5% non-fat dry milk in TBST at RT. Following incubation, membranes were washed with three times with TBST for 10 min. Membranes were then incubated in chemiluminescent ECL-plus reagent (PerkinElmer Inc., Waltham, MA, USA) at RT and then imaged using MicroChemi 4.2 (DNR Bio-Imaging Systems, Mahale HaHamisha, Jerusalem, Israel). Protein loading was confirmed by probing for tubulin or GAPDH. The quantification of the Western blots was performed by densitometry using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.11 Statistical analysis

All data are presented as mean ± standard error of mean (SEM). Values were analyzed by two-tailed Student’s t-test or one-way ANOVA with Holm-Sidak post-hoc test, as appropriate, using GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). A value of p < 0.05 was considered statistically significant.
Chapter 3
Results

3.1 Generation of a myeloid specific JAK2 deficient mouse model

To investigate the essential role of macrophage JAK2 in obesity-related insulin resistance and inflammation in vivo, we conditionally disrupted Jak2 in mice by homologous recombination in myeloid cells. We generated a mouse model with myeloid-specific JAK2 deficiency (referred herein as M-JAK2<sup>−/−</sup>), which expresses the Cre recombinase under the control of the Lyz2 promoter, which is expressed specifically in cells of the myeloid lineage (Clausen et al., 1999), and is homozygous for loxP-flanked Jak2 alleles (Figure 2a). Briefly, the targeted insertion of loxP sites around the first coding exon (exon 2) creates a conditional knockout allele of Jak2 that can be excised upon Cre-mediated recombination. The excision results in a Jak2 null allele. The M-JAK2<sup>−/−</sup> mice were compared with littermate controls with wildtype Jak2 alleles (referred herein as M-JAK2<sup>+/+</sup>). M-JAK2<sup>−/−</sup> mice were born in the expected Mendelian ratio and showed no gross abnormalities. M-JAK2<sup>+/+</sup> and M-JAK2<sup>−/−</sup> mice were genotyped through allele-specific PCR analysis to confirm the correct insertion of the outermost loxP site into the endogenous JAK2 locus (Figure 2b). The amplification yielded a 230 bp PCR fragment without the loxP site (wild-type allele; JAK2<sup>+/+</sup>) and a band of 310 bp in size with the loxP site (floxed JAK2 allele; JAK2<sup>fl/fl</sup>). To verify the efficiency of Jak2 deletion, we measured Jak2 mRNA levels using RT-qPCR in thioglycollate-elicited PMs and observed that they were decreased by approximately 80% in M-JAK2<sup>−/−</sup> mice compared with control M-JAK2<sup>+/+</sup> mice (Figure 2c). Moreover, JAK2 protein expression in PM lysates as analyzed by Western blot was reduced by about 75% in M-JAK2<sup>−/−</sup> mice compared to M-JAK2<sup>+/+</sup> mice (Figure 2d). The JAK2 protein expression in other tissues we examined including liver, visceral adipose tissue (VAT), skeletal muscle and hypothalamus were similar between M-JAK2<sup>+/+</sup> and M-JAK2<sup>−/−</sup> mice (Figure 2e). Thus, macrophages from M-JAK2<sup>−/−</sup> showed specific ablation of JAK2 mRNA and protein.
Figure 2: Generation of M-JAK2+/+ and M-JAK2−/− mice.

(a) Diagram of the Cre-dependent conditional knockout strategy for Jak2: loxP sites (green triangles) flank exon 2, which is removed following Cre-mediated recombination. The floxed and knockout allele are depicted. Adapted from Wagner et al. (2004). (b) Representative PCR genotyping for ear DNA samples indicates the presence of the wildtype (~230 bp) and/or floxed (~310 bp) allele in order to determine heterozygous (fl/+ or fl/fl) and homozygous mutants (fl/fl) as well as wildtype controls (+/+). (c) mRNA expression for Jak2 normalized to 18S mRNA and (d) protein band intensity for JAK2 normalized to tubulin in thioglycollate-elicited peritoneal macrophages (PMs) from M-JAK2+/+ and M-JAK2−/− mice; n = 6-7. (e) Immunoblotting for JAK2 and tubulin in lysates of PMs, liver, visceral adipose tissue (VAT), muscle, and hypothalamus from M-JAK2+/+ and M-JAK2−/− mice. All results are mean ± SEM; *p < 0.05, **p < 0.01 and ***p < 0.001.
3.2 Macrophage-specific JAK2 deficient mice show decreased adipocyte hypertrophy during HFD feeding

To assess the role of macrophage JAK2 in regulating body mass, we fed M-JAK2<sup>−/−</sup> and M-JAK2<sup>+/+</sup> mice either a normal chow diet (NCD) or a high-fat diet (HFD) and monitored body weight biweekly from 6 weeks of age to 18 weeks of age (Figure 3a). A lesser degree of weight gain for M-JAK2<sup>−/−</sup> mice was observed transiently compared with M-JAK2<sup>+/+</sup> mice during HFD feeding (Figure 3b); however, these differences in body weight were absent for M-JAK2<sup>−/−</sup> and M-JAK2<sup>+/+</sup> mice at 6 weeks of age and after 18 weeks of NCD feeding (Figure 3b). In our study, M-JAK2<sup>−/−</sup> and M-JAK2<sup>+/+</sup> mice on an HFD started showing a significant difference in body weight after 4 weeks of HFD feeding, although the difference was not statistically significant after 10 weeks on HFD.

To investigate what could account for the slight differences in weight in HFD-fed M-JAK2<sup>−/−</sup> mice, we assessed organ weights at necropsy in mice fed NCD or HFD. The representative photos of mice fed NCD or HFD are depicted in Figure 3c. We examined weights of various organs including pancreas, spleen, liver, kidney, heart, and lung as well as different visceral adipose depots, SAT and brown adipose tissue. We did not observe any significant differences in absolute weights of fat pads or other tissues in M-JAK2<sup>−/−</sup> compared to controls at 6 weeks of age (Figure 3d,e) or at 18 weeks of age after NCD or HFD feeding (Figure 3f, g). There was a slight decrease in absolute weight of liver and white adipose depots in HFD fed M-JAK2<sup>−/−</sup> mice compared to control, but when the weights of tissues were expressed relative to total body weight, there was no significant difference between genotypes (Figure 3h, i). In addition, considering the difference in body mass, we sought to examine metabolic changes in M-JAK2<sup>−/−</sup> compared with that of M-JAK2<sup>+/+</sup> during NCD or HFD feeding. Thus, we performed metabolic analysis on these mice using CLAMS prior to sacrificing them. There were no overall differences in the parameters between genotypes during NCD or HFD feeding for food intake, oxygen consumption, carbon dioxide production, energy expenditure, respiratory exchange ratio, and activity level (Figure 4a-e).

Interestingly, histological analyses of VAT performed on the HFD fed cohort revealed increased proportion of smaller adipocytes in H & E stained VAT sections from M-JAK2<sup>−/−</sup> mice (Figure 5a). When we measured the adipocytes in the stained sections, we found reduced mean adipocyte area (Figure 5b) and diameter (Figure 5d) compared to those of M-JAK2<sup>+/+</sup> littermates. The distribution
of analyzed adipocytes also showed increased proportion of adipocytes with a smaller area (Figure 5c) and diameter (Figure 5e) in HFD fed M-JAK2−/− mice compared to M-JAK2+/+ mice. We also counted the number of adipocytes in specified fields, which indicated a significantly increased number of adipocytes per section (Figure 5f). The differences in adipocyte size led us to examine mRNA expression in VAT for genes involved in adipogenesis, a process for adipocyte turnover or renewal. In support of increased adipogenesis and maturation of adipocytes, we found that M-JAK2−/− mice fed a HFD had upregulated expression of *Adipoq* and *Pparγ* (Figure 5g). There was no difference in gene expression of other factors involved in adipocyte differentiation such as *Leptin*, *Cebpα*, *Cebpδ* and *aP2* (Figure 5g). We also performed histological analyses of SAT from HFD fed M-JAK2+/+ and M-JAK2−/− mice. H & E stained SAT sections did not show any morphological differences in adipocyte size (Figure 6a). Upon quantification of adipocyte size in HFD fed SAT we did not find any statistical differences in average adipocyte area (Figure 6b,c) or diameter between genotypes (Figure 6d-e). Taken together, our results reveal that during HFD feeding, VAT from macrophage-specific JAK2 deficient mice displayed decreased adipocyte hypertrophy and enhancement of some adipogenic gene expression compared to control littermates.
Figure 3: Characterization of M-JAK2<sup>+/−</sup> and M-JAK2<sup>/−</sup> mice fed NCD or HFD.

(a) Schematic overview of experimental study design. (b) Body weight measurements of M-JAK2<sup>+/−</sup> and M-JAK2<sup>/−</sup> mice fed NCD (n = 22-24) or (c) HFD (n = 24-27). (c) Representative photograph of M-JAK2<sup>+/−</sup> and M-JAK2<sup>/−</sup> mice at 18 weeks of age after NCD or HFD feeding. Absolute tissue weights of pancreas (pan.), spleen, liver, kidney, heart and lungs; and (d) adipose depots: perigonadal (peri.), retroperitoneal (retro.), mesenteric (mes.), inguinal (ing.), and interscapular brown adipose tissue (BAT) in M-JAK2<sup>+/−</sup> and M-JAK2<sup>/−</sup> at (d,e) 6 weeks of age (n = 6-10) and (f,g) 18 weeks after NCD (n = 10-14) or 12 weeks of HFD (n = 17-20) feeding. (h,i) Tissue weights relative to total body weight for M-JAK2<sup>+/−</sup> and M-JAK2<sup>/−</sup> mice fed NCD or HFD. All results are mean ± SEM; *p < 0.05.
Figure 4: Metabolic measurements of M-JAK2+/+ and M-JAK2−/− mice fed NCD or HFD. (a) Energy intake, (b) energy expenditure, (c) volume of oxygen consumption (VO2), (d) volume of carbon dioxide production (VCO2), (d) respiratory exchange ratio (RER), and (e) activity level measured during light and dark cycles for M-JAK2+/+ and M-JAK2−/− mice fed NCD (n = 11-13) or HFD (n = 14-16), respectively. All results are mean ± SEM; *p < 0.05.
Figure 5: Decreased adipocyte hypertrophy in VAT of HFD fed M-JAK2−/− mice.
(a) Representative micrographs of hematoxylin and eosin stained visceral adipose tissue (VAT) sections from HFD fed M-JAK2+/+ and M-JAK2−/− mice; scale bar, 50 μm. Quantitative analysis of adipocytes in HFD fed VAT sections: (b) average adipocyte area, (c) adipocyte area distribution, (d) average adipocyte diameter, (e) adipocyte diameter distribution, and (f) number in 5 fields at 200X; n = 7-9. (g) mRNA expression normalized to 18S mRNA of genes involved in adipogenesis in HFD fed VAT; n = 7-11. All results are mean ± SEM; *p < 0.05 and **** p <0.0001.
Figure 6: Histological analysis of SAT in HFD fed M-JAK2⁻/⁻ and M-JAK2⁺/⁺ mice.
(a) Representative micrographs of hematoxylin and eosin stained subcutaneous adipose tissue (SAT) section from HFD fed M-JAK2⁺/⁺ and M-JAK2⁻/⁻ mice; scale bar, 50 μm. Quantitative analysis of adipocytes in HFD fed SAT sections: (b) average adipocyte area, (c) adipocyte area distribution, (d) average adipocyte diameter, and (e) adipocyte diameter distribution; n = 5-7. All results are mean ± SEM.
3.3 Lack of macrophage JAK2 results in protection from HFD induced systemic insulin resistance

Next, we investigated alterations in glucose homeostasis after prolonged HFD feeding. First, we performed i.p. GTT and i.p. ITT at 6 weeks of age before subjecting mice to HFD challenge. We did not find any difference in glucose tolerance or insulin sensitivity in the young mice (Figure 7a,b). Fasting and random glucose levels were also similar at this age (Figure 7c). At 18 weeks of age, M-JAK2−/− mice and M-JAK2+/+ control cohort fed NCD had no significant difference in the glucose profile during the GTT or ITT (Figure 7d, e). Interestingly, HFD fed M-JAK2−/− mice showed comparable glucose tolerance, but enhanced glucose lowering during the ITT, suggesting improved systemic insulin sensitivity compared to M-JAK2+/+ control mice (Figure 7g, h). M-JAK2−/− and M-JAK2+/+ mice fed NCD or HFD showed no differences in fasting blood glucose levels (Figure 7f, i). However, HFD fed M-JAK2−/− exhibited reduced random blood glucose levels than their M-JAK2+/+ counterparts (Figure 7f, i). Next, we measured fasting insulin levels and found M-JAK2−/− fed HFD had a trend towards lower fasting serum insulin concentrations compared to their M-JAK2+/+ littermate controls, but the circulating insulin levels were similar between NCD fed M-JAK2−/− and M-JAK2+/+ (Figure 8a). We also assessed AKT signalling in the liver and VAT upon insulin challenge in HFD fed mice through Western blot analysis. We did not find a difference in insulin-induced tyrosine phosphorylation of AKT at serine position 473 in either VAT or liver of M-JAK2−/− mice compared with M-JAK2+/+ cohorts (Figure 8b, c). We also measured β-cell area on insulin-immunostained pancreas sections of HFD mice and this was also not different in M-JAK2−/− mice compared to M-JAK2+/+ littermates (Figure 8d, e). Together, these observations show that M-JAK2−/− mice demonstrate protection from HFD-induced systemic insulin resistance without differences in AKT signalling in liver or VAT, or in pancreatic β-cell area compared to M-JAK2+/+ controls.
**Figure 7: Increased systemic insulin sensitivity in HFD fed M-JAK2⁻/⁻ mice.**

Intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test (ITT) performed on M-JAK2⁺/⁺ and M-JAK2⁻/⁻ mice at (a,b) 6 weeks of age \((n = 22-23, n = 19-21)\) and at 18 weeks after (d,e) NCD \((n = 14-17, n = 14-16)\) or (g,h) 12 weeks of HFD \((n = 20-23, n = 16-21)\) feeding. Area under the curve (AUC) measured for ITT using baseline glucose values. Fasting and random blood glucose levels measured at (a,b) 6 weeks of age \((n = 16-19, n = 18-20)\) and at 18 weeks after (d,e) NCD \((n ≥ 14-17, n = 14-17)\) or (g,h) 12 weeks of HFD \((n = 20-23, n = 18-22)\) feeding. All results are mean ± SEM; \(^* p < 0.05\) and \(^{**} p < 0.01\).
Figure 8: Assessment of fasting insulin levels and insulin-stimulated AKT phosphorylation in liver and VAT of HFD-fed mice.

(a) Fasting serum insulin levels in NCD (n = 9-11) or HFD (n = 8-10) fed M-JAK2+/+ and M-JAK2−/− mice. (b) Western blots showing phosphorylated AKT (pAKT), total Akt (tAKT) and GAPDH in visceral adipose tissue (VAT) and liver lysates from HFD fed mice injected with insulin. (c) Protein band intensity quantified for pAKT normalized to tAKT (n = 5-7). (d) Representative image of pancreas sections immunostained for insulin in HFD-fed M-JAK2+/+ and M-JAK2−/− mice; scale bar, 200 μm. (e) β-cell area per total pancreatic area was determined on insulin-immunostained pancreatic sections from HFD fed M-JAK2+/+ and M-JAK2−/− mice (n = 4-5). All results are mean ± SEM; *p < 0.05.
3.4 Macrophage JAK2 ablation attenuates HFD-induced inflammation in VAT and liver

Macrophage infiltration of white AT is implicated in the metabolic complications of obesity. A notable feature of AT in obese mice is the presence of unique clusters of proinflammatory macrophages that surround dying adipocytes called CLSs (Cinti et al., 2005). The high prevalence of CLSs is highly correlated with AT inflammation and metabolic disorder. To examine CLSs, we immunostained VAT sections with macrophage marker F4/80 (Figure 9a), which revealed a significant reduction in the presence of CLSs in M-JAK2−/− mice in comparison to M-JAK2+/+ mice after HFD feeding (Figure 9b). These findings were supported by mRNA expression of inflammation and macrophage markers in VAT. HFD fed M-JAK2−/− mice had reduced expression of macrophage-specific gene Cd68, but we did not observe significant differences in mRNA expression of another macrophage marker F4/80 or M1-related markers we examined such as Tnfa, Itgax, Il6 or iNos (Figure 9c). We also observed a reduction of some M2 specific genes, Mgl1, Mrc1 and Mrc2, but not others such as Arg1, Chi3l3, Fizz1 and Mgl2 in M-JAK2−/− mice (Figure 9d). Chemokines are another set of important cytokines implicated in tissue macrophage function. Therefore, we evaluated the mRNA expression of chemokine receptor CCR2 and its ligands (CCL2, CCL7, and CCL8), as well as CCR5 receptor and its ligands (CCL3, CCL4, CCL5, and CCL8), which are highly expressed within macrophages (Mantovani et al., 2004). In VAT from HFD-fed M-JAK2−/− mice, we observed a significant down-regulated expression for Ccl4 with some others such as Ccl3 and Ccl5 and their associated receptor Ccr5 which trended in the same direction (Figure 9e). Together, our histological analysis and gene expression indicate that macrophage JAK2 deficient mice appear to have dampened VAT inflammation.

HFD-induced obesity also leads to inflammatory changes in the liver that is associated with insulin resistance and steatosis; and macrophages can contribute to the hepatic inflammation signalling (Lackey and Olefsky, 2016). We performed histological analysis on liver sections from HFD-fed mice. We did not observe morphological differences in H & E stained sections between controls and M-JAK2−/− mice (Figure 10a). When we compared lipid accumulation in livers from HFD-fed cohort by Oil Red O, which stains neutral lipids, we observed no apparent differences suggesting less lipid accumulation in M-JAK2−/− mice (Figure 10a). We confirmed this by analyzing total hepatic TAGs levels (Figure 10b) or examining its FA composition, which showed no significant differences in HFD fed M-JAK2−/− mice (Figure 10c). To assess the effect on hepatic
inflammation with the lack of macrophage JAK2, we measured mRNA levels of inflammatory markers and macrophage-related genes including M1- and M2-associated markers. The gene expression of the cytokine Tnfa and M1-related marker Itgax were reduced with macrophage JAK2 deficiency. We did not see significant differences in expression for F4/80, Cd68, Tnfa, Il6, iNos or Il1β (Figure 10d). For M2-related markers, Chi3l3 was down-regulated in M-JAK2−/− mice while expression of Mgl1, Mgl2, Mrc1, Mrc2, Arg1, and Fizz1 was comparable in liver from M-JAK2−/− and M-JAK2+/+ during HFD (Figure 10e). Similar to VAT, we also examined hepatic chemokine expression in M-JAK2−/− and M-JAK2+/+ after HFD. We observed decreased expression of Ccr5, Ccl2 and Ccl7 with a trend towards decreased expression of Ccr2, Ccl3 and Ccl4. (Figure 10f).

Next, we measured levels of various cytokines including adipokines and chemokines in circulation in fasted HFD fed M-JAK2−/− and M-JAK2+/+ mice. Of the pro- and anti-inflammatory cytokines and chemokines we measured by ELISA, only IL-1β was significantly increased with no differences observed in serum levels of IL-6, TNF-α, IL-12p70, IFNγ, CCL2, IL-10 and IL-13 between the two HFD-fed experimental groups (Figure 11a-c). Circulating levels of adipokines, such as resistin and PAI-1 were comparable between genotypes (Figure 11d). Taken together, these findings suggest that macrophage JAK2 affects inflammatory changes at the tissue-specific level as demonstrated in liver and VAT but not at a systemic level.
Figure 9: Decreased CLS density and inflammation in VAT of HFD-fed M-JAK2+/− mice.

(a) Representative micrographs of F4/80 immunostained visceral adipose tissue (VAT) sections from HFD-fed M-JAK2+/+ and M-JAK2−/− mice; crown-like structure (CLS) is indicated by arrowhead; scale bar, 50 μm. (b) Quantification of CLSs from F4/80 stained sections in at least 15 fields at 100X; n = 7-8. mRNA expression normalized to 18S mRNA in VAT from HFD-fed M-JAK2+/+ and M-JAK2−/− mice for (c) macrophage (ϕ) and M1-associated markers, (d) M2-associated markers, and (e) chemokine receptors and its associated ligands; n = 8-11. All results are mean ± SEM; *p < 0.05.
Figure 10: Reduced hepatic inflammation in HFD fed M-JAK2−/− mice.

(a) Representative micrographs of hematoxylin and eosin (H & E) and Oil Red O stained liver sections from HFD-fed M-JAK2+/+ and M-JAK2−/− mice; scale bar, 50 μm. (b) Quantification of triacylglycerol (TAG) levels and (c) composition of different fatty acids (FA) in liver from HFD-fed mice: saturated (SFAs), mono-unsaturated (MUFAs), polyunsaturated (PUFAs) and highly unsaturated (HUFAs), Omega-6 (N-6) and Omega-3 (N-3); n = 14-18. mRNA expression normalized to 18S mRNA in liver from HFD-fed M-JAK2+/+ and M-JAK2−/− mice for (d) macrophage (ϕ) and M1-associated markers, (e) M2-associated markers, and (f) chemokine receptors and its associated ligands (n = 7-8). All results are mean ± SEM; *p < 0.05, **p<0.01.
Figure 11: Cytokine and adipokine levels in circulation.

Fasting serum analysis for (a, b) proinflammatory cytokines and chemokines ($n = 8$), (c) anti-inflammatory cytokines ($n = 8$) and (d) adipokines ($n = 5$). All results are mean ± SEM; *$p < 0.05$. 
3.5 JAK2 and leptin regulate chemokine expression in macrophages

To better understand the molecular mechanisms underlying reduced tissue inflammation with macrophage JAK2 deficiency, particularly, the suppressed HFD-induced chemokine expression in VAT and liver, we first elucidated what could induce chemokine expression in macrophages. It has been shown that leptin, an adipokine primarily secreted by AT, can induce proinflammatory cytokine and chemokine production in macrophages (Acedo et al., 2013). Since leptin receptors are abundant on the surface of macrophages (Gainsford et al., 1996) and JAK2 is known to be a main downstream mediator in leptin signalling (Frühbeck, 2006), we explored whether leptin might act to influence chemokine expression in macrophages. Indeed, upon leptin stimulation for 6 hours, expression of Ccl2, Ccl3, Ccl4, Ccl5 and Ccl7 were upregulated in a concentration-dependent manner in macrophage cell line RAW 264.7 (Figure 12). The expression of Ccl8 was not detected during our qPCR analysis.

Next, we assessed whether JAK2 plays a role in regulating chemokine expression in macrophages. We used a siRNA approach to effectively knockdown Jak2 in RAW 264.7 and we achieved over 60 percent reduction in Jak2 expression (Figure 13a). The Jak2 mRNA expression does not change upon leptin induction (Figure 13a). The Jak2 knockdown had no effect on the expression of the chemokine receptors Ccr2 and Ccr5 (Figure 13b). Interestingly, Jak2 knockdown significantly reduced basal mRNA levels of Ccl2, Ccl3, and Ccl4 by about 30 to 50 percent but did not influence the expression of the chemokines Ccl5 and Ccl7 (Figure 13c). Next, we evaluated chemokine expression after Jak2 knockdown and in response to leptin. We used two different concentrations, 50 nM and 100 nM, of leptin-based on our previous results suggesting that at these concentrations chemokine expression was significantly upregulated. We observed continued significant down-regulation in mRNA levels of Ccl2, Ccl3, and Ccl4 in RAW 264.7 macrophages with Jak2 knockdown (Figure 13c). There was a significant difference in Ccl2 and Ccl4 expression between RAW 264.7 cells transfected with scramble siRNA compared to cells transfected Jak2 siRNA at both 50 nM and 100 nM concentrations of leptin, but the differences in Ccl3 expression between the two groups was only seen at 100 nM leptin induction (Figure 13c). It was also noted that chemokine expression of Ccl2-5 increased in cells treated with leptin after Jak2 knockdown (Figure 13c). While chemokine expression in response to leptin was dampened in RAW 264.7 macrophages after Jak2 knockdown, when we compared their induction as
measured by fold change in chemokine expression comparing leptin to the vehicle treated cells was similar for RAW 264.7 cells transfected with scramble or Jak2 siRNA (Figure 14). Our in vitro work show that leptin upregulates chemokine expression. While knockdown of Jak2 diminishes chemokine expression, JAK2 does not appear to be essential in the induction of chemokines in response to leptin.

**Figure 12: Chemokine expression increased with leptin stimulation in RAW 264.7 cells.** mRNA expression of various chemokines normalized to 18S mRNA in RAW 264.7 after a 6 hr-stimulation with vehicle or varying concentrations of leptin. Three independent experiments were performed in triplicates. All results are mean ± SEM. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.
Figure 13: Chemokine expression decreased after Jak2 knockdown in RAW 264.7 cells. mRNA expression normalized to 18S mRNA for (a) Jak2, (b) chemokine receptors and (c) chemokines in RAW 264.7 cells transfected with scramble control (Scr) or Jak2 siRNA (siJak2) after vehicle treatment. mRNA expression of (a) Jak2 and (c) chemokines in RAW 264.7 cells transfected with Scr or siJak2 after treatment with 50 nM and 100 nM of leptin. Four independent experiments were performed in triplicates. All results are mean ± SEM. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.
Figure 14: Fold change difference in chemokine expression in transfected RAW 264.7 cells after leptin stimulation.

Fold change of mRNA expression of chemokines in scramble control (Scr) or Jak2 siRNA (siJak2) transfected RAW 264.7 cells after leptin treatment compared to vehicle treated cells. All results are mean ± SEM.
Chapter 4
Discussion

The biological role of JAK2 in obesity and insulin resistance appears to be tissue-specific, as demonstrated by genetic ablation in the pancreas, liver and AT (Choi et al., 2010; Nordstrom et al., 2013; Shi et al., 2014; Shi et al., 2012; Shi et al., 2016; Sos et al., 2011). It was previously not known whether macrophage JAK2 contributes to the development of obesity and insulin resistance. Various *in vitro* studies have demonstrated that distinct signals can activate JAK2 within macrophages, providing further insight into its dynamic function in mediating inflammatory response. Inhibition of JAK2 in murine macrophage cell lines by JAK2 specific inhibitor, AG490, suppresses the production of IL-6, IL-1β and iNOS upon PA or LPS stimulation (Lee et al., 2006; Okugawa et al., 2003). Several cytokines and hormones, for example, GH and GM-CSF signal through JAK2 to further mediate expression of various other cytokines and chemokines such as TNF-α, IL-1β, IL-12, and CCL2 (Tanimoto et al., 2008; Tripathi and Sodhi, 2009). In addition, a variety of other cytokines (IFN-γ, IL-6, IL-3 and IL-13) have been demonstrated to signal through JAK2 to regulate macrophage function (Rawlings et al., 2004). The diversity of factors that activate JAK2 within macrophages further implicates the importance of JAK2 signalling in these cells. Knowing that macrophages play a central role in orchestrating inflammatory responses and the involvement of JAK2 in regulating macrophage function, our work reveals that JAK2 deficiency in macrophage can have beneficial effects for reducing inflammation and improving systemic insulin resistance induced by HFD. Indeed, our findings indicate that mice lacking JAK2 in macrophages had decreased mRNA levels of certain inflammatory genes in VAT and liver of HFD-fed mice (Figure 15).

Chemokines can also mediate the complex intracellular signalling that control inflammation (Xu et al., 2015). We found that lack of macrophage JAK2 leads to lower expression of CCR2 and CCR5 and its associated ligands in liver and VAT. Interestingly, AT of obese subjects has elevated expression of these chemokines (Huber et al., 2008). Deficiency of chemokine receptors, CCR2 or CCR5, in mice leads to protection against HFD-induced AT macrophage accumulation (Kitade et al., 2012; Lumeng et al., 2007). Previous studies have also suggested that CCR2 and CCR5, which belong to the superfamily of GPCRs, are capable of activating JAK2 in monocytes (Mellado et al., 1998; Mueller and Strange, 2004). In addition, various
cytokines and growth factors such as GM-CSF and leptin can signal through JAK2 in macrophages to induce production of these chemokines (Gruen et al., 2007; Kiguchi et al., 2009; Tanimoto et al., 2008). Since chemokine expression in liver and VAT could be mediated by both parenchymal cells and macrophages, it is likely that the reduced chemokine expression detected in HFD-fed M-JAK2−/− mice is a consequence of both a primary macrophage defect and a secondary response to improved insulin sensitivity. Moreover, JAK2 deficiency represses chemokine expression by macrophages in vitro, supporting our in vivo findings in regards to the importance of JAK2 function in chemokine expression of Ccl2-4. However, the expression of chemokine receptors was not affected by Jak2 knockdown as seen at the tissue level, perhaps due to the lack of receptor activation during cell culture.

Macrophages within metabolic tissues can release proinflammatory cytokines and chemokines that act both locally on adipocytes and also circulate to distal tissues to stimulate intracellular proinflammatory pathways (Lackey and Olefsky, 2016). These combined actions result in cell-autonomous local and systemic insulin resistance and exacerbation of the inflammatory response. The dampened inflammation in liver and VAT of macrophage JAK2 knockout mice could explain the improvement of insulin sensitivity systemically. It could also help elucidate the reduced adipocyte hypertrophy and enhanced adipogenesis after HFD-induced obesity. The increased expression of adipogenic markers in VAT is consistent with the notion that reduced adipogenesis correlates with obesity and insulin resistance (Gustafson et al., 2015; Hajer et al., 2008). Alternatively, adipocyte hyperplasia may present a mechanism for healthy fat storage capacity. PPARγ and adiponectin are both important regulators of differentiation and renewal of adipocytes (Lefterova and Lazar, 2009; Trujillo and Scherer, 2005) and also play a role in maintaining AT and systemic insulin sensitivity (Peraldi et al., 1997; Yamauchi et al., 2001), but inflammation can downregulate these factors (Guilherme et al., 2008). Thus, disrupting JAK2 signalling in macrophages could preserve AT function through regulating adipocyte expansion and enhancing adipogenesis during HFD challenge.

There is evidence that paracrine interaction between macrophages and adipocytes establishes a vicious cycle that augments inflammatory changes in AT during the development of obesity (Suganami et al., 2005). Several studies have shown that secreted factors derived from the murine macrophage cell line or human monocyte-derived macrophages increase inflammatory responses, suppress expression of adipogenesis genes and differentiation, and reduce insulin
responsiveness of 3T3-L1 adipocytes and human preadipocytes (Constant et al., 2006; Lacasa et al., 2007; Permana et al., 2006; Suganami et al., 2005). The convergence of macrophages on dying adipocytes during obesity suggests that their presence in AT might be predominantly for clearance purposes (Cinti et al., 2005). The death of adipocytes might be a primary event enhanced by obesity, or secondary to inflammation and macrophage infiltration. Thus, it might reflect an attempt to limit the expansion of these cells. The decreased CLSs in macrophage JAK2 deficient mice suggests that the intercellular communication between adipocytes and ATMs may be altered. We proposed that adipokine leptin, which activates JAK2 as its main signalling pathway, is a key modulator in this interaction since it is recognized as a proinflammatory cytokine as it is known to stimulate the production of inflammatory mediators such as cytokines (TNF-α, IL-6, iNOS, IL-12) (Acedo et al., 2013; Loffreda et al., 1998; Santos-Alvarez et al., 1999) as well as chemokines (CCL3-5, CXCL10) (Acedo et al., 2013; Kiguchi et al., 2009; Meier et al., 2003) in monocytes and macrophages. We have shown that leptin induces expression of CC-chemokine ligands, CCL3, CCL4 and CCL5 as well as CCL2 and CCL7 in RAW 264.7 cells. Activation of JAK2 could be an important pathway to modulate chemokine expression in macrophages considering the opposite effects of JAK2 knockdown and leptin on the chemokine mRNA levels.

It is reported that incubation of human AT with chemokines such as CCL2 and CCL3 markedly decreased PPARγ protein expression while increasing leptin secretion by adipocytes (Gerhardt et al., 2001). On the other hand, PPARγ ligands like rosiglitazone could reduce chemokine expression in adipocytes and chemokine receptor expression in stromal vascular fractions (containing immune cells) (Nguyen et al., 2012). Our results combined with previous reports suggest that leptin can induce inflammatory responses through chemokine upregulation, which in turn can inhibit adipocyte differentiation. However, our work showed that there was a comparable fold change difference upon leptin after Jak2 knockdown compared to control. This could possibly be explained by residual Jak2 expression after the knockdown, or that leptin signals through a non-canonical pathway such as MAPK and/or PI3K to regulate chemokine expression with Jak2 knockdown (Fruhbeck, 2006; Gruen et al., 2007). The effects of leptin on inflammatory chemokine gene expression that have been found in vitro suggests that in vivo leptin likely complements effects on chemokine production by inflammatory stimuli such as LPS.
and/or other cytokines during the pathogenesis of obesity-related inflammation. Together, these findings shed light on the importance of communication between macrophages and adipocytes.

Macrophages can promote insulin resistance by interfering with insulin signalling in peripheral tissues (Shoelson et al., 2006). However, it was surprising that lack of macrophage JAK2 dampened inflammation at a tissue-specific level in liver and VAT without improvements in insulin signalling in these tissues. Given that macrophage JAK2 deficiency showed improvement in systemic glucose homeostasis suggests that an alternative mechanism could account for the protection from HFD-induced insulin resistance. During HFD feeding, macrophage JAK2 deficient mice had reduced circulating insulin levels without difference in pancreatic β-cell compensation, therefore it would be worth investigating glucose-stimulated insulin secretion in these mice. We also cannot rule out the important role of macrophage activation influencing other aspects of glucose homeostasis such as hepatic glucose output, muscle glucose uptake and lipid metabolism in peripheral tissues, which can affect the development of insulin resistance (Gregor and Hotamisligil, 2011; Samuel and Shulman, 2012). Additionally, our future work would warrant investigation on why VAT showed downregulation of different macrophage markers or chemokine genes compared to liver. This may be due to the distinct tissue environments or the competing effects of cytokines which signal through JAK2 such as IFNγ and IL-13, which can activate M1-like versus M2-like phenotype, respectively. Alternatively, the activation of other signalling pathways could have compensatory effects due to macrophage JAK2 ablation.

In addition to the well known expression of LysM promoter in macrophages, it is also shown to be expressed in neutrophils (Clausen et al., 1999). Therefore, LysM-Cre mediated recombination may also lead to JAK2 deletion in these cells (Avalos et al., 1997). Emerging evidence suggests that neutrophil infiltration of AT is involved in the development of obesity and obesity-associated insulin resistance. In HFD-fed mice, AT was infiltrated with neutrophils after 3 days of HFD feeding (Elgazar-Carmon et al., 2008), and neutrophil infiltration was maintained throughout the 90-day study period (Talukdar et al., 2012). However, the percent of neutrophils in VAT is considerably lower than macrophages after HFD-induced obesity (Wang et al., 2014b). While we did not focus on the role of neutrophil JAK2 in obesity-induced inflammation, the role of neutrophil JAK2 contributing to the phenotype in our LysM-Cre+Jak2fl/fl mice may also be considered.
Lastly, it is important to make certain considerations when designing and analyzing immuno-metabolic study such as this one. The background strain is important, because investigators have noted strain specific difference in metabolic parameters such as glucose metabolism, weight and inflammation. (Berglund et al., 2008; Fengler et al., 2016; Montgomery et al., 2013). The mixed background of the mice used in our study could be a possible explanation for the variability in glucose tolerance, weight gain and insulin resistance we observed. Thus, we used littermate controls to make comparisons between the genotypes. It is also worth noting that limiting metabolic studies to a single genetic background can be an issue when extrapolating the findings to the human population, which is extremely heterogenous by nature (Rivera and Tessarollo, 2008). Moreover, experimental and control mice were cohoused to account for the influence of gut microbiota on systemic metabolic responses (Turnbaugh et al., 2006). In our work, the experimental and control groups were not matched for body weight at the start of HFD. We did not see significant differences in weight between genotypes at 6 weeks of age, but it is important to take into account that small weight differences at baseline can also affect weight gain as it affect the inherent susceptibility to weight gain during HFD. The mouse strain and environmental factors should be considered when interpreting gene-phenotype associations during metabolic studies.

Overall, the study highlights the important role of macrophage JAK2 in peripheral metabolism. We show that genetic disruption of macrophage JAK2 reduces VAT and liver tissue inflammation and improves systemic insulin sensitivity. Our study suggests that stimulatory effect of leptin on chemokine production by macrophages can mediate inflammation in AT and liver. Developing strategies to target macrophage JAK2 could potentially have a beneficial effect in attenuating inflammation.
Figure 15: Proposed model on the regulatory role of macrophage JAK2 on inflammation.
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