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Adipose tissue macrophages in the Development of Obesity-induced Inflammation, Insulin Resistance and Type 2 Diabetes

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Abstract

It has been increasingly accepted that chronic subacute inflammation plays an important role in the development of insulin resistance and Type 2 Diabetes in animals and humans. Particularly supporting this is that suppression of systemic inflammation in Type 2 Diabetes improves glycemic control; this also points to a new potential therapeutic target for the treatment of Type 2 Diabetes. Recent studies strongly suggest that obesity-induced inflammation is mainly mediated by tissue resident immune cells, with particular attention being focused on adipose tissue macrophages (ATMs). This review delineates the current progress made in understanding obesity-induced inflammation and the roles ATMs play in this process.

Insulin resistance is a disease that is characterized by the failure of normal insulin levels to regulate glucose homeostasis (Biddinger and Kahn, 2006). As a result, much higher insulin levels are needed to properly maintain euglycemic conditions. Furthermore, insulin resistance is not only a major predisposing factor for the development of Type 2 Diabetes, but also a major component of the metabolic diseases, including cardiovascular diseases (Reaven, 1988). Although insulin resistance is a systemic disease, it occurs locally in the insulin-responsive tissues. The liver, muscle, fat and pancreas are the classical insulin-responsive tissues, but the brain and gut were also found recently to participate in glucose homeostasis. At the cellular level, it is generally accepted that the impairment of insulin signaling in parenchymal cells of insulin-responsive tissues (namely, hepatocytes, adipocytes, myocytes, and β cells) causes insulin resistance in these tissues. While the molecular mechanisms that underlie this process are not fully understood and are still being investigated extensively, many different possible mechanisms that are supported by strong evidence have been proposed. These include ER stress, lipid/glucose toxicity, abnormalities in metabolites (e.g., branched amino acids or lipids), dysregulation of hormones, particularly adipokines (e.g., adiponectin and resistin), and inflammation. All of these potential causes may interact with each other or work in coordination. However, this review will focus on the recent progress that has been made in understanding obesity-induced inflammation, especially the roles adipose tissue macrophages (ATMs) play in the development of obesity-induced insulin resistance.

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Inflammation

Inflammation is a cascade of immunological responses that serve to defend the host from infection with foreign pathogens. It has been recognized since ancient times, with the first written record about inflammation being by Celsus (ca 30 BC–38 AD) in the Roman era: he described the symptoms of inflammation as dolor (pain), calor (heat), rubor (redness), and tumor (swelling) (Celsus, 1971). Recent research on the molecular mechanisms that lead to inflammation has now shown that inflammation is also an important pathogenic mediator of various diseases, including rheumatoid arthritis, asthma, cancer, and metabolic diseases such as atherosclerosis and Type 2 Diabetes. Inflammation is mainly regulated by various immune cells and how these immune cells induce and resolve inflammation has been studied intensively.

On infection, inflammation is the primary defense system that serves to kill and remove pathogens and infected cells and to repair the cells and tissues that are damaged by infection. After viruses and microorganisms invade the host, neutrophils are the first immune cells to detect these pathogens at the sites of infection (Silva, 2010, Sadik *et al.*, 2011, Amulic *et al.*, 2012). Unlike most circulating immune cells, which are immunologically naïve, neutrophils are already activated when they egress from the bone marrow. As a result, when they patrol the circulation and then detect danger signals emanating from the site of infection, they can migrate readily to the site. The neutrophils mainly recognize the pathogens by using pattern recognition receptors (PPRRs) such as TLRs, which recognize pathogen-associated molecular patterns (PAMPs) such as LPS of gram-negative bacteria. When the PPRRs on a neutrophil bind to PAMPs, the TLR/MyD88/IKKβ/NFκB signaling pathway is initiated in the neutrophil and induces its production of chemokines or leukotrienes, which in turn recruit the second wave of immune cells, particularly monocytes, from the circulation. After migration to the inflamed site, the monocytes differentiate into macrophages. Thereafter, the differentiated macrophages and neutrophils together produce another set of chemoattractants that recruit other immune cells, particularly effector lymphocytes such as T and NKT cells. The coordinated migration and activation of these and other immune cells results in the sequential production of many different cytokines that regulate the proliferation, differentiation, and activation of other immune cells. As a result, the different types or subpopulations of immune cells can be identified on the basis of their unique cytokine profiles. The cytokine signatures of the CD4 helper T cell (Th) subtypes are particularly well characterized: Th1, Th2, regulatory T cells (Tregs), and Th17 cells can be distinguished from each other by their specific production of IFNγ, IL-4 (and IL-13), IL-10, and IL-17, respectively (Zhu *et al.*, 2010). With regard to the Th1 and Th2 T cell subsets, their signature cytokines are also required for their own differentiation; while these cytokines are produced by differentiated T cells, they are also generated by macrophages too. In addition, macrophages produce IL-12, which induces T cell differentiation. Conversely, IFNγ and IL-4 can regulate macrophages so that they differentiate into M1 and M2 phenotypes, respectively; these phenotypes are associated with distinct functions as they regulate inflammatory and immune functions, respectively (see “Macrophages in Classical Immunity”). Inflammasome pathways are also important in regulating inflammation, as they mediate the post-translational production of IL-1β and IL-18 (Davis *et al.*, 2011).

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Macrophages that infiltrate the site of inflammation also play an important role in removing pathogens. This is mediated by phagocytosis, which is guided by PPRRs as well as other molecules such as Fc receptors. Infected cells must also be killed, which can be achieved by phagocytosis as well. In addition, cytolytic lymphocytes, including CD8 T cells and NK cells, can kill infected cells directly by receptor-mediated cytotoxicity.

After the pathogens and infected cells are removed, the inflammation must be resolved (Serhan *et al.*, 2008, Soehnlein and Lindbom, 2010). This is achieved by the same immune cells that participated in the inflammatory cascade, albeit using different subtypes. For example, Tregs suppress the proinflammatory immune cells, namely Th1 T cells and macrophages, by producing anti-inflammatory cytokines such as IL-10 and TGFβ. Th2 T cells also suppress inflammation by producing IL-4 and IL-13. In addition, neutrophils play an important role in resolving inflammation by producing lipid mediators such as resolvin. Finally, B cells are recruited to the local inflammatory site, and this mediates the transition from innate immunity to adaptive immunity.

Thus, the inflammation cascade that is initiated by the invasion of a pathogen is a complex process that involves the well-coordinated production of cytokines by different immune cells at the right time. Any dysregulation of this process can lead to uncontrolled inflammation that could result in unwanted outcomes, such as septic shock.

It should be noted that, like pathogens, self-antigens can also induce an inflammatory cascade that involves a similar sequence of molecular and cellular events. In this case, the inflammatory cascade is initiated by cell necrosis that releases danger (or damage)-associated molecular pattern molecules (DAMPs) such as dsDNA.

One of the most important mediators that regulate inflammation is NFκB (Fig. 1). NFκB is a central integrator of proinflammatory signals and a master regulator of the genes that are involved in inflammation, innate and adaptive immunity, and apoptosis. In the basal state, NFκB is sequestered in the cytoplasm by IκBα. A large number of stimuli can activate the IκB kinase (IKK) complex, which then phosphorylates IκBα at Ser 32 and Ser 36 (Baeuerle and Baltimore, 1996, DiDonato *et al.*, 1997). This leads to the ubiquitination and subsequent proteasomal degradation of IκBα, which releases NFκB and exposes its nuclear localization sequence (NLS). NFκB then translocates into the nucleus, where it mediates the transcription of multiple target genes. These genes activate various biological responses.

The stimuli that can activate the IKKβ/NFκB pathway include proinflammatory cytokines, bacterial cell wall components, viral products, dsDNA, mitogens, engagement of CD40/CD40L, and activation of T and B cell receptors (TCR and BCR, respectively). However, the outcomes of these different stimuli are very diverse. For example, while proinflammatory cytokines and the activation of TLR signaling pathways mainly promote innate immunity by producing proinflammatory mediators and cytokines, the activation of TCR and BCR generates adaptive immunity, namely T and B cell activation, expansion, and effector function induction. In addition, the engagement of CD40 and CD40L generates humoral and cell-mediated immune responses, including the priming and expansion of antigen-specific CD4 T cells, peripheral CD8 T cell tolerance, activation of APCs that

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upregulates their co-stimulatory activity and cytokine production, and activation of macrophages and ECs. It should be noted that in the case of the activation of NFκB by TCR and CD40/CD40L signaling, cell-to-cell interaction is required.

The diversity of the signaling outputs of NFκB activation may also relate to the fact that NFκB activation is necessary but not always sufficient for NFκB-dependent gene expression. For example, IL-6 gene expression requires not just NFκB activation but also AP-1 site-binding by a dimer of c-Jun and c-Fos, both of which are also NFκB genes (Yan *et al.*, 1997, Hoffmann *et al.*, 2003, Hess *et al.*, 2004). Therefore, while IL-6 expression is dependent on NFκB, it will be induced only after c-Jun and c-Fos are expressed by NFκB. In another example, IFN-β gene expression requires NFκB, IRF-3 and -7, and a dimer of ATF-2 and c-Jun for the AP-1 site (McWhirter *et al.*, 2005). Thus, the activation of NFκB cannot increase IFN-β gene expression in the absence of the activation of IRF-3 and -7 by other signaling cascades.

History of the Discovery of the Role of Inflammation in Insulin Resistance and Type 2 Diabetes

It is increasingly being understood that obesity-induced chronic inflammation plays an important role in the development of obesity-induced insulin resistance and Type 2 Diabetes. The earliest modern experimental evidence that supports the notion that inflammation is a mediator of insulin resistance came from a series of studies showing that the treatment of 3T3-L1 adipocytes with TNFα decreases their levels of insulin-signaling components, including the insulin receptor, IRS-1 and Glut4, *via* transcriptional regulation (Stephens and Pekala, 1992, Stephens *et al.*, 1997). Since TNFα strongly activates the NFκB pathway, these studies quickly led to experiments that tested whether the activation of the NFκB pathway impairs insulin signaling *in vitro*. However, most of these studies failed to support this premise, which suggested that the NFκB pathway in adipocytes does not participate in their TNF-α-impaired insulin signaling (Ruan *et al.*, 2002). The role of TNFα in the development of insulin resistance was tested subsequently *in vivo* in obese murine models, first by using a neutralizing TNFα antibody and then by using TNFα receptor knockout mice (Hotamisligil *et al.*, 1993, Uysal *et al.*, 1997, Uysal *et al.*, 1998). These studies together showed strongly that TNFα plays an important role in the development of obesity-induced insulin resistance in murine models by impairing insulin signaling, particularly in adipocytes. This further led to several clinical studies that tested the ability of anti-TNFα antibody to improve Type 2 diabetes. However, the general consensus was that the treatment did not improve glycemic control in Type 2 Diabetes or only had very mild effects. In contrast, the same approach markedly improved rheumatoid arthritis in a highly convincing manner (Romeo *et al.*, 2012). However, these studies mainly focused on the effects of the cytokine TNFα on insulin signaling rather than on inflammation *per se*. Indeed, none of those studies, including our own at the time, seriously examined the regulation by obesity of entire inflammatory pathways.

A decade later, a series of epidemiological studies, particularly those led by the Ridker group, showed that the development of insulin resistance and T2D is associated with increased circulating levels of proinflammatory markers, including WBC, IL-6, plasminogen

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activation inhibitor-1 (PAI-1), serum amyloid A (SAA-1), and C-reactive protein (CRP) (Schmidt *et al.*, 1999, Festa *et al.*, 2000, Yudkin *et al.*, 2000, Barzilay *et al.*, 2001, Pradhan *et al.*, 2001, Ford, 2002, Freeman *et al.*, 2002, Vozarova *et al.*, 2002, Barzilay and Freedland, 2003, Duncan *et al.*, 2003, Pradhan *et al.*, 2003, Pickup, 2004). These studies strongly suggested that these markers, either alone or combined with other inflammatory markers, are risk factors for the development of insulin resistance and T2D in humans. Interestingly, many of these markers were also risk factors for the development of cardiovascular diseases. This led to the common soil hypothesis that was proposed by Stern (Stern, 1995). However, these epidemiological studies could not show whether these inflammatory mediators directly cause the development of insulin resistance and Type 2 Diabetes.

Independently of the epidemiological studies, we and others found that obesity and hyperlipidemia activate inflammation in animal models *via* the IKKβ/NFκB pathway, thereby inducing the expression of its target genes, such as those encoding TNFα and IL-6 (Yuan *et al.*, 2001, Tripathy *et al.*, 2003, Aljada *et al.*, 2004, Cai *et al.*, 2005, Bhatt *et al.*, 2006, Shi *et al.*, 2006). Moreover, pharmacological or genetic inhibition of the NFκB pathway in animal models was found to improve their obesity- and FFA-induced insulin resistance (Kim *et al.*, 2001, Yuan *et al.*, 2001, Arkan *et al.*, 2005, Shi *et al.*, 2006, Herrero *et al.*, 2010). The inhibition of this pathway in humans also improves Type 2 Diabetes with concomitant inhibition of NFκB activity (Ghanim *et al.*, 2001, Hundal *et al.*, 2002, Goldfine *et al.*, 2008, Goldfine *et al.*, 2010). We and others then showed that NFκB-dependent proinflammatory cytokines, including TNFα and IL-6, mediate obesity/diet-induced insulin resistance, and that anti-inflammatory cytokines such as IL-10 counter-regulate inflammation-induced insulin resistance (Hotamisligil *et al.*, 1993, Cai *et al.*, 2002, Klover *et al.*, 2003, Shoelson *et al.*, 2003, Dandona *et al.*, 2004, Kim *et al.*, 2004, Cai *et al.*, 2005, Boden, 2006, Hotamisligil, 2006, Neels and Olefsky, 2006, Shoelson *et al.*, 2006, Tilg and Moschen, 2006, Lumeng *et al.*, 2007a). Of particular interest was when we used salicylate, an anti-inflammatory drug whose discovery dates back to ancient times, to inhibit the IKKβ/NFκB pathway: treatment with a high dose of salicylates effectively improved insulin resistance and Type 2 Diabetes in both animal models and humans (Kim *et al.*, 2001, Yuan *et al.*, 2001, Goldfine *et al.*, 2008, Goldfine*et al.*, 2010, Herrero *et al.*, 2010). These studies led to the hypothesis that chronic subacute inflammation plays an important role in the development of obesity-induced insulin resistance and T2D.

The next important question was, "Which are the tissues that are inflamed by obesity and diet and that go on to cause systemic insulin resistance?" Of the three insulin-responsive tissues, namely muscle, liver and fat, it is now clear that inflammation in the muscle, especially that mediated by the IKKβ/NFκB pathway, does not affect the metabolic phenotype of animals; rather, it regulates muscle wasting (Cai *et al.*, 2004, Karin, 2006, Mourkioti *et al.*, 2006). It has been shown that obesity and diet also induce inflammation in the liver and adipose tissue (AT) (Yuan *et al.*, 2001, Cai *et al.*, 2002, Rohl *et al.*, 2004). However, these tissues are complex organs that consist of many different cell types, including immune cells, precursor for the parenchymal cells, and endothelial cells. Since immune cells, especially macrophages, are well-known to regulate inflammation, the

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hypothesis that infiltrating immune cells may be responsible for obesity/diet-induced AT inflammation was reasonable. The two seminal papers by the Chen and Ferrante groups directly tested this possibility with regard to ATs. Both papers showed strongly that it is the ATMs, not adipocytes, which express most of the proinflammatory cytokines that are induced by obesity (Weisberg *et al.*, 2003, Xu *et al.*, 2003). Other immune cells in AT have also been shown to play critical roles in the development of obesity-induced inflammation and insulin resistance (Weisberg *et al.*, 2003, Xu *et al.*, 2003, Feuerer *et al.*, 2009, Liu *et al.*, 2009, Nishimura *et al.*, 2009, Winer *et al.*, 2009, Winer *et al.*, 2011). Recently, it was also shown that liver-infiltrating immune cells in animal models, particularly Kupffer cells and monocytes/macrophages, play an important role in the regulation of obesity-induced inflammation in the liver and hence mediate the development of obesity-induced insulin resistance (Huang *et al.*, 2010, Obstfeld *et al.*, 2010). These results together suggest strongly that obesity-induced inflammation is mainly mediated by tissue-residing immune cells. At present, most of the research in this field has focused on the ATMs, which are also the topic of this review.

Macrophages in Classical Immunity

Macrophages have been studied extensively in the general immunology field and it will be helpful if their general characteristics are reviewed first. Macrophages are a type of myeloid cell that are derived from bone marrow hematopoietic stem cells (HSCs) (Fig. 2). After their generation from HSCs, they differentiate into common myeloid progenitors (CMPs) and then into monocytes. After the monocytes are fully differentiated, they egress from the bone marrow into the circulation. When monocytes in the circulation detect danger signals, they migrate into the local inflammatory sites. This migration is mainly mediated by chemoattractants. The cells then differentiate into macrophages or DCs.

Since macrophages and DCs belong to the same lineage and have overlapping functions, macrophages are often compared to DCs (Rosenbauer and Tenen, 2007). Classically, macrophages are defined in flow cytometry analyses as CD11b^{+} and F4/80^{+}, while DCs are defined as CD11c^{+}. At the functional level, macrophages are considered to be important players in innate immunity due to their production of cytokines in response to PAMPs and DAMPs. In contrast, DCs are thought to be critical in adaptive immunity due to their presentation of antigens to CD4 T cells *via* MHC II molecules. However, the two cell types can also play the roles of the other cell, albeit much less well. These overlapping functions make it very difficult to clearly differentiate the functions played by these two immunologically disparate cell types.

Another complication is that macrophages themselves are very complex (Gordon and Taylor, 2005, Gordon, 2007). The macrophages in different tissues exhibit very different characteristics and are therefore labeled with different names: the lung has alveolar macrophages, the liver has Kupffer cells, neural tissue has microglia, and bone has osteoclasts. Even within a single tissue, the macrophages can often be classified into many different subtypes based on their surface markers and functions. The best example of this is splenic macrophages (see the review (Taylor *et al.*, 2005)). The surface marker heterogeneity of macrophages is also matched by great functional diversity. These functions

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include phagocytosis of infected or dead cells, the production of proinflammatory cytokines to kill infected cells and activate other immune cells, wound healing/tissue remodeling, and angiogenesis. However, macrophages are often categorized functionally according to the dichotomous M1 and M2 phenotypes, which was originally developed on the basis of *in vitro* analyses (Table 1). Recent studies have further divided M2 macrophages into two categories, namely alternatively activated and anti-inflammatory macrophages. The M1 macrophages are also known as classically activated macrophages. Their phenotype is based on characterizations of bone-marrow derived macrophages (BMDM) that were stimulated with IFNγ and LPS. Hence, they express high levels of pro-inflammatory cytokines such as IL-6 and TNF-α. The alternatively activated M2 macrophage phenotype is based on characterizations of BMDM that were stimulated with IL-4. When BMDM are stimulated with IL-10 instead, they are specifically called anti-inflammatory macrophages as they are thought to have anti-inflammatory functions. However, the characteristics of this anti-inflammatory macrophage phenotype have not yet been fully determined. Although the M1/M2 system was established by *in vitro* analyses, it is often also used to classify macrophages *in vivo* due to its simplicity and the fact that many studies have shown that it describes the macrophages in various diseases with reasonable accuracy. For example, in proinflammatory diseases such as atherosclerosis, the predominant macrophage phenotypes are often classified as M1 macrophages because they produce proinflammatory cytokines. In contrast, TAMs are often considered to be M2 macrophages because they help the proliferation of cancer cells by producing anti-inflammatory cytokines that protect them from immune cells and that induce angiogenesis. However, the M1/M2 system should be used with caution as it may be too simplistic and may overlook much more refined and complex differences in macrophage functions. Supporting this is recent studies of the genomic profiles of the residential macrophages and DC from different tissues, as well as circulating monocytes, which revealed that the myeloid cells from different tissues and even different subpopulations from the same tissue have very different genetic signatures (Gautier *et al.*, 2012, Miller *et al.*, 2012).

Since macrophages are mainly derived from circulating monocytes, the regulation of monocytes can also affect the numbers and functions of macrophages in local inflammatory tissues. Circulating monocytes are very heterogeneous and can also be divided into various subpopulations on the basis of their surface markers, although it should be noted that these subpopulations remain to be fully defined (Gordon and Taylor, 2005). In general, mouse monocytes are $CD11b^+$ cells and can be divided into two subpopulations depending on their Ly6C expression, namely $Ly6C^{hi}$ monocytes ($CD14^{hi}$ $CD16^-$ monocytes in humans), which are known to be inflammatory monocytes and macrophage precursors, and $Ly6C^-$ monocytes ($CD14^+$ $CD16^+$ cells in humans), which are considered to be less inflammatory and can differentiate into DCs. They also express distinguishing molecules that account for their different immunological functions. For example, $Ly6C^{hi}$ monocytes express high levels of CCR2, while $Ly6C^-$ monocytes lack CCR2 expression. CCR2 is a receptor for MCP1 and is important for the migration of immune cells into inflammatory sites after the delivery of an immunological insult. Hence, in response to MCP1 release, $CCR2^+$ monocytes (i.e., $Ly6C^{hi}$ and $CD14^{hi}$ $CD16^-$ monocytes) may be able to infiltrate inflammatory sites, such as atheromatous plaques, where they differentiate into inflammatory macrophages. This

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scenario is supported by many experimental observations (Ziegler-Heitbrock *et al.*, 1993, Grage-Griebenow *et al.*, 2001, Geissmann *et al.*, 2003, Gordon and Taylor, 2005, Gordon, 2007, Ziegler-Heitbrock, 2007). Notably in atherogenesis studies, it has been shown that hypercholesterolemia expands the Ly6C$^+$ monocyte population and that these Ly6C$^+$ monocytes (i.e., CCR2$^+$ monocytes) infiltrate atheromatous plaques more efficiently than Ly6C$^-$ (CCR2$^-$) monocytes (Swirski *et al.*, 2007, Tacke *et al.*, 2007). A very recent study also showed that Ly6C$^+$ monocytes are important for the induction of inflammation in a myocardial infarction mouse model (Swirski *et al.*, 2009). Another immunological marker that distinguishes the two monocyte populations from each other is CX_3CR1, which is expressed in the reverse pattern to CCR2, namely it is expressed by Ly6C$^-$ (CD14$^+$ CD16$^+$) but not by Ly6C^{hi} (CD14^{hi} CD16$^{+/-}$) monocytes. CX_3CR1 is required for the migration of DCs from the gut lumen to the sub-endothelium, where they remove and present exogenous antigens to other immune cells (Niess *et al.*, 2005). Indeed, it has been shown that Ly6C$^-$ (CD14$^+$ CD16$^+$) monocytes migrate into the sub-endothelium and differentiate into DCs *in vitro* and *in vivo* (Geissmann *et al.*, 2003, Angeli *et al.*, 2004). Furthermore, Ly6C$^-$ monocytes play an important role in the resolution of inflammation (Nahrendorf *et al.*, 2007). These studies strongly suggest that circulating monocytes may regulate the number and fate (i.e., functions) of macrophages after they move into the local inflammatory sites and differentiate into the final effector cells (macrophages or DC). It should also be noted that tissue-residing macrophages can proliferate. In particular, a recent study showed that M2 macrophages, but not M1 macrophages, can proliferate (Jenkins *et al.*, 2011). Hence, macrophage numbers in the local sites can be potentially regulated by the influx of monocytes/differentiation of macrophages and the proliferation of macrophages at the local sites.

Adipose Tissue Infiltration by Adipose Tissue Macrophages (ATMs)

Since the two seminal papers by the Chen and Ferrante groups first reported that it is the infiltrating macrophages rather than the adipocytes that express most of the proinflammatory cytokine genes that are induced by obesity and diet (Weisberg *et al.*, 2003, Xu *et al.*, 2003), numerous papers have supported this premise. It is clear that AT macrophage infiltration increases in mice and humans as they become more obese (Lee *et al.*, 1999, Takahashi *et al.*, 2003, Curat *et al.*, 2004, Liang *et al.*, 2004, Cancello *et al.*, 2005, Cinti *et al.*, 2005, Di Gregorio *et al.*, 2005). Conversely, the loss of body weight by laparoscopic Roux-en-Y bypass surgery or improvements in insulin resistance due to TZD treatment decreases AT macrophage infiltration (Cancello *et al.*, 2005, Di Gregorio*et al.*, 2005).

The question then is, “How does obesity increase macrophage recruitment”? One of the best-known chemoattractants for macrophage recruitment is MCP1, which is the chemoattractant that is recognized by the Ly6C$^+$ monocyte subset. Indeed, gene deletion or inhibition of MCP-1 or gene deletion of its receptor, CCR2, not only decreases AT macrophage infiltration, it also improves insulin resistance, although the improvement is mild (Kanda *et al.*, 2006, Weisberg *et al.*, 2006). Conversely, over-expressing MCP-1 in adipocytes has the opposite effect, namely it increases macrophage infiltration and insulin resistance (Kamei *et al.*, 2006, Kanda *et al.*, 2006). Although the latter studies should be interpreted carefully since MCP-1 is normally mainly expressed by macrophages, not

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adipocytes (Weisberg *et al.*, 2003), they remain the only studies in which the intervention (i.e., the specific upregulation of MCP-1 in AT) specifically modulates the ATMs, as indicated by the fact that the numbers of only these cells, not other tissue-residing macrophages, are upregulated. In addition, a recent study showed that deletion of CX_3CR1, which is expressed by the $Ly6C^-$ monocyte subset, does not affect ATM numbers (including obesity-induced ATM infiltration) or insulin resistance (Morris *et al.*, 2012). Thus, these studies suggest that $Ly6C^+$ monocytes are the primary source of the obesity-induced recruitment of ATMs from the circulation and that this event occurs in an MCP-1/CCR2-dependent manner.

Recently, an interesting new concept regarding ATM recruitment emerged. It is well-known that FFA induces inflammation *via* TLR4 (Shi *et al.*, 2006). Since obesity is associated with dysregulation of lipid homeostasis, which increases circulating TG and FFA levels, it is plausible to propose that obesity-induced inflammation is mediated by the FFA/TLR4/inflammation pathway. However, a recent paper has suggested that FFA may also play a different role in obesity-induced inflammation (Kosteli *et al.*, 2010). This report shows that the FFA that is released from adipose tissue by lipolysis can recruit macrophages into AT, which suggests that FFA in obesity may also be a chemoattractant, potentially one that is specific to AT in obesity. An extreme case of this role of FFA may be a lipodystrophic mouse model, namely adipocyte-specific transgenic mice that express nSREBP1c (Herrero *et al.*, 2010). These mice do not have significant fat and yet have increased insulin resistance. The lipodystrophy is thought to be caused by the increased death of the adipocytes in the fat. Surprisingly, the epididymal fat of these mice is highly inflamed in terms of macrophage infiltration and the expression of proinflammatory genes by AT. More interestingly, when this inflammation was suppressed by using high dose of salicylates or by deleting IKKβ in macrophages, the insulin resistance did not improve: only fat transplantation improved it. However, fat transplantation did not appear to reduce the macrophage infiltration into the endogenous fat pads. Hence, this study suggests that adipocyte cell death and the FFA that may be released by this may be a recruiting signal for macrophages. In the immunology field, this is called sterile inflammation, namely inflammation that is induced without infection (Chen and Nunez, 2010). This is also characterized by the increased infiltration of immune cells, especially macrophages, and the induction of proinflammatory mediator expression. The causes of sterile inflammation are often cell death (mainly due to necrosis), tissue wounding, and crystals such as uric acid. In atherosclerosis, it has been shown that crystals of oxidized cholesterol induce sterile inflammation *via* the inflammasome and autophages (Duewell*et al.*, 2010, Razani *et al.*, 2012). It may be that, in obese AT, the continuous expansion of AT increases adipocyte death and their release of lipids (including FFA) and/or endogenous DAMPs such as HMGB. This in turn induces sterile inflammation, which results in increased macrophage infiltration into AT and the production of inflammatory mediators.

Role of ATMs in Inflammation

The roles ATMs play in regulating obesity-induced inflammation and insulin resistance have mainly been studied by using genetic mouse models. Since mouse lines that specifically target ATMs do not exist, whole-body knockout mice, chimeric mice that are the result of

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bone marrow transplantation (BMT), and myeloid-specific knockout mice have been often used for these studies.

However, all of these approaches bear intrinsic technical limitations, although all are very useful and informative. Obviously, whole-body knockout mice cannot be used to identify the role played by specific target tissues and cells. In contrast, BMT is very useful for this, as it can differentiate the roles played by immune cells from the roles played by parenchymal cells. However, it cannot be used to specifically target a particular type of immune cells (e.g., macrophages) since it replenishes the entire immune cell compartment with cells in which the expression of the target gene is modulated. This is especially significant because many different immune cell types, including macrophages, B cells, T cells, Tregs, and mast cells, have been shown to play an important role in the development of inflammation and insulin resistance (Weisberg *et al.*, 2003, Xu *et al.*, 2003, Feuerer *et al.*, 2009, Liu *et al.*, 2009, Nishimura *et al.*, 2009, Winer *et al.*, 2009, Winer *et al.*, 2011). Thus, chimeric mice will show the effects of the gene modulation on all immune cells, not just the macrophages. In contrast, LysM-Cre mice can be used to specifically target myeloid cells, including monocytes, DCs, neutrophils and macrophages. Again, however, the mutant mice generated by using LysM-Cre mice will show the effects of gene deletion in all of these myeloid cells, most of which have been shown to play a role in the development of insulin resistance. Thus, even if the most specific LysM-Cre mice are used for gene deletion, the resulting data should be interpreted carefully, as it is possible that the gene deletion affects not only ATMs but also a much broader range of cell types in many other tissues. Nonetheless, it should be emphasized that, given the limited ability of the existing technology to specifically target ATMs, these studies have provided valuable knowledge about the role that ATMs, or at a minimum myeloid cells, play in obesity.

Another interesting, potentially problematic, phenomenon that has been observed in many knockout mice is that the modulation of the inflammatory pathway in myeloid cells or whole immune cells often affects their body weight, mainly because of changes in obesity. This phenomenon should be considered carefully because, in most cases, the direction of the body weight changes is opposite to the direction of the changes in insulin resistance. In other words, decreased body weight (obesity) associates with improved insulin resistance, and *vice versa*. This raises a conundrum: is it the change in body weight or inflammation *per se* that is the primary cause of improvements in insulin sensitivity? The molecular mechanisms by which the modulation of inflammation affects body weight, especially in obesity, remain poorly studied. Thus, the answer to this conundrum is not yet known.

The following studies have helped to elucidate the roles played by ATMs in obesity-induced inflammation and insulin resistance. Since more detailed descriptions of these knockout studies can be found in other excellent reviews (Solinas and Karin, 2010, Chawla *et al.*, 2011, Osborn and Olefsky, 2012), these studies will only be described briefly here.

The first example of this line of studies is the analysis of whole-body or myeloid-specific IKKβ knockout mice, whose macrophages do not respond to inflammatory stimuli such as LPS. These mice show better insulin resistance when fed a high fat diet (HFD) than the wild-type mice (Yuan *et al.*, 2001, Arkan *et al.*, 2005). However, the effect of these

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knockouts on ATM numbers and functions, or AT inflammation, has not yet been studied. Other notable mutations in the inflammatory pathway include the RP105/MD-1 complex, Map4k4, AIM, JNK, SOCS1, TNFα and its receptor, and TLR4(Hirosumi *et al.*, 2002, Jaeschke *et al.*, 2004, Nguyen *et al.*, 2007, Tsukumo *et al.*, 2007, Aouadi *et al.*, 2009, Saberi *et al.*, 2009, Westcott *et al.*, 2009, Kurokawa *et al.*, 2011, Sachithanandan *et al.*, 2011, Watanabe *et al.*, 2012). Overall, effects of these deletions improve obesity-induced insulin resistance. With regard to TLR4, it is known to act as a FFA sensor in the development of obesity-induced insulin resistance. Interestingly, however, deletions of MyD88, an adaptor molecule that is exclusively used in all TLR signaling pathways, or TLR2 induce insulin resistance (Hosoi *et al.*, 2010, Caricilli *et al.*, 2011). This suggests that the role of the TLR signaling pathway in the regulation of obesity-induced inflammation is more complex than was thought initially. Myeloid cell-specific KLF4 knockout mice also exhibit insulin resistance along with a suppression of M2 macrophage phenotypes in AT; however, these mice also show a significant increase in obesity (Liao*et al.*, 2011). In contrast, whole-body deletion of MGL1, a classical M2 marker, improves obesity-induced insulin resistance without changing either ATM infiltration or ATM polarization towards the M1 phenotype. In addition, the inflammatory gene expression of AT is not changed, although the levels of some circulating mediators are changed (Westcott *et al.*, 2009). Several reports have also shown that the inflammasome pathway that regulates IL-1β and IL-18 production plays an important role in the development of obesity-induced insulin resistance (Stienstra *et al.*, 2011, Vandanmagsar *et al.*, 2011, Wen *et al.*, 2011, Stienstra *et al.*, 2012). Although these latter studies did not directly test whether ATMs or macrophages regulate this pathway in obesity, many immunological studies have shown strongly that macrophages are the primary cells that regulate inflammasome activity in terms of controlling inflammation. Therefore, it is reasonable to hypothesize that macrophages or ATMs are also the primary cell types that regulate the inflammasome in an insulin resistance-promoting manner. However, this notion should be tested.

It has been shown that macrophage recruitment factors also contribute to ATM infiltration and obesity-induced inflammation. These factors include MCP-1 and its receptor CCR2, CXCL5 and its receptor CXCR2, BLT1, and osteopontin. All of the mice that have deletions in these genes exhibit improved insulin sensitivity, and this is frequently associated with a decrease in ATM infiltration (Kanda *et al.*, 2006, Weisberg*et al.*, 2006, Nomiyama *et al.*, 2007, Chavey *et al.*, 2009, Spite *et al.*, 2011). However, it should be also noted that, in general, macrophages are the primary source of chemokines.

Other molecules that are involved in insulin signaling and lipid metabolism have been specifically deleted in myeloid cells or hematopoietic cells, and the effect of this on the development of insulin resistance has been studied. These molecules include CAP, GPR120, insulin receptor, CD36, PPARγ, and PPARδ (Liang *et al.*, 2004, Hevener *et al.*, 2007, Lesniewski *et al.*, 2007, Odegaard *et al.*, 2007, Kang *et al.*, 2008, Oh *et al.*, 2010, Nicholls *et al.*, 2011). The PPARγ, and PPARδ also regulate the polarization of ATMs (see the next section).

Another notable study is when $CD11c^+$ cells were depleted by using CD11c-DFTR mice (Patsouris *et al.*, 2008). These mice show an improvement in obesity-induced insulin

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resistance along with decreased ATM infiltration and AT inflammation. However, since CD11c is the classical marker for DCs, the DCs in other tissues were affected by the deletion along with the ATMs. This is supported by the fact that the $CD11c^{+}$ cell-depleted mice also exhibited decreased systemic inflammation, including in the liver, muscle and circulation.

Many of these studies show predictable results, namely that decreased inflammation in ATMs associates with improvements in obesity-induced insulin resistance, or *vice versa*. However, some studies yielded unexpected results, such as those involving MGL-1 and CD36 deletions. Nevertheless, these studies overall show that myeloid cells play an important role in regulating obesity-induced inflammation and insulin resistance, although it should be emphasized that these data should be interpreted with caution (see "Limitations and Future Studies").

ATM Heterogeneity

As discussed earlier, macrophages are very heterogeneous, which suggests that ATMs may also be very heterogeneous (Taylor *et al.*, 2005, Gordon, 2007). Therefore, recent studies on ATMs focused on their heterogeneity and functions. The first evidence of ATM heterogeneity came from immunohistological analyses of AT from obese animal models that detected "crown-like structures " (CLSs), namely clusters of macrophages surrounding dying or dead adipocytes (Weisberg *et al.*, 2003, Xu *et al.*, 2003, Robker *et al.*, 2004, Cancello *et al.*, 2005, Cinti *et al.*, 2005, Di Gregorio *et al.*, 2005, Kamei *et al.*, 2006, Kanda *et al.*, 2006, Weisberg *et al.*, 2006, Lumeng *et al.*, 2007a). CLSs are rarely observed in AT from lean control animals. Moreover, the macrophages in CLSs express proinflammatory mediators such as TNF-α, which suggests that these macrophages may be the inflammatory ATMs that regulate obesity-induced inflammation. Another interesting feature of CLSs is that many of their macrophages contain intracellular lipid droplets and thus resemble the foam cells observed in atheromatous plaques (Lumeng *et al.*, 2007b, Kosteli *et al.*, 2010, Prieur *et al.*, 2011). However, since CLSs can at present be only identified by immunohistology, it remains difficult to isolate the macrophages in CLSs for comparison with non-CLS macrophages at the molecular level.

An *in vivo* ATM labeling experiment revealed two other categories of ATMs, namely newly recruited macrophages and their local counterparts, the residential macrophages (Lumeng *et al.*, 2007b). Newly recruited ATMs are distinct from residential ATMs in that they are positive for CD11c, which is a typical DC surface marker; they also express higher levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Nguyen *et al.*, 2007). In contrast, the residential ATMs express higher levels of MGL-1, which participates in tissue remodeling. These differences match well with M1 and M2 phenotypes. Indeed, genetic analyses of ATMs show that the newly recruited macrophages express many typical M1 markers, whereas residential macrophages are polarized towards the M2 phenotype. Furthermore, when the polarization of *in vivo* macrophages toward the M1 phenotype is induced by the macrophage-specific deletion of PPARγ or δ, insulin resistance is enhanced (Odegaard *et al.*, 2007, Kang *et al.*, 2008). This suggests that the polarization of ATMs towards the M1 phenotype may promote obesity-induced insulin resistance. However, these studies should

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be interpreted with some caution because other studies have shown that ATMs can simultaneously express both M1 and M2 phenotypes (Zeyda *et al.*, 2007). Moreover, it has been shown that M2 phenotypes, not M1 phenotypes, correlate with BMI, which suggests that M2 phenotypes associate with obesity (Bourlier *et al.*, 2008). Furthermore, a recent genetic analysis of human ATMs shows that ATMs are similar to tumor-associated macrophages (TAMs), which resemble M2 macrophages closely (Mayi *et al.*, 2012). These contradictions may be resolved when our understanding of ATM heterogeneity improves, as we may find that particular ATM subpopulations function differently. For example, it is possible that an ATM subpopulation with an M1 phenotype coexists with another ATM subpopulation that bears an M2 phenotype, and that it is the overall balance between these two ATM subpopulations that shapes obesity-induced inflammation. However, the possibility that the M1/M2 system may not be appropriate for classifying ATMs should also be considered.

Limitations and Future Studies

1. Lack of specificity regarding gene targeting in ATMs

Many studies have now consistently made it very clear that obesity increases ATM numbers. This phenomenon is potentially mediated by classical chemoattractants such as MCP1 and BLT1 as well as atypical chemoattractants, possibly including AT-specific FFA. In addition, many genetic studies support the notion that inflammatory pathways in myeloid cells regulate obesity-induced inflammation. However, given the complex nature of myeloid cells, this conclusion should be considered carefully. All of myeloid cell-specific knockout mice have been generated by using LysM-Cre mice. However, since all systemic myeloid cells are targeted in the LysM-Cre mice, the gene of interest is not deleted in only the ATMs of these mice. A possible solution to this limitation is suggested by a recent study that used BMT with transgenic mice that overexpressed DGAT *via* the aP2 promoter (Koliwad *et al.*, 2010). The aP2 promoter has been traditionally used to engineer adipocyte-specific gene deletion or overexpression. However, it is also expressed in ATMs, especially those in obese mice. Therefore, knockout mice generated with aP2-Cre mice or transgenic mice using the aP2 promoter will also exhibit modulation of the target gene in ATMs. However, since only ATMs express aP2 promoter-mediated genes, only the ATMs can express the target genes after BMT; other macrophages will be unable to express the target gene. Clearly the best way to resolve the issue of lack of ATM specificity in terms of gene deletion is to develop an ATM-specific Cre mouse line, which has not yet been reported.

2. ATMs have other functions apart from promoting inflammation

The second consideration is that macrophages play various roles: they can have anti-inflammatory functions as well as serve to promote inflammation, and they also participate in tissue remodeling and wound healing, including angiogenesis. However, the current paradigm in the field is that obesity categorically polarizes the ATMs into M1 macrophages and downregulates polarization towards M2 phenotypes. Thus, the focus in the field is generally on the proinflammatory functions of ATMs. However, it is also well known that obesity increases the gene expression levels of IL-10, which is a typical anti-inflammatory cytokine. Hence, it is very possible that obesity regulates ATMs in more than one direction.

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Indeed, the effect of obesity on ATM molecular and functional phenotypes may be much more heterogeneous than can be expressed by the dichotomous M1/M2 system. To test this notion, ATM heterogeneity should be characterized carefully and the functions of individual ATM subpopulations should be elucidated. In addition, the focus of future studies on ATMs should expand from their inflammatory roles to other possible roles. Recent studies have shown that ATMs also participate in other pathways, especially in lipid metabolism (Koliwad *et al.*, 2010, Kosteli *et al.*, 2010). Since macrophages play such diverse roles in many immunological and physiological functions, it remains possible that macrophages contribute to the development of obesity-induced insulin resistance *via* other pathways. This should be explored more extensively.

3. The relationship between ATMs and other cells

Another important consideration is the relationship between ATMs and neighboring cells, including adipocytes and other immune cells. Of particular interest are adipocytes, which are the first cells to respond to obesity by increasing their size and lipid contents (including FFA). Few studies have analyzed whether adipocytes can regulate ATMs, although several studies suggest indirectly that in obesity, adipocytes may express proinflammatory cytokines, notably IL-6 (Weisberg *et al.*, 2003). In addition, obese AT bears CLSs, which consist of subpopulations of adipocytes that are surrounded by macrophages. These data suggest that an adipocyte sub-population (namely, the adipocytes in CLSs) may attract and then strongly interact with macrophages, perhaps by producing macrophage attractants such as chemokines or other signals such as HMGB1 or FFA. It should be noted that most of the current studies on AT immune cells focus on how their modulation affects ATM phenotypes, especially in terms of M1 and M2 polarization. However, it should be remembered that one of the most important macrophage functions is the regulation of other immune cells *via* cytokine production. Again, however, the effect of ATMs on other AT immune cells has rarely been explored.

4. The mechanisms by which ATMs regulate obesity-induced systemic insulin resistance

Another question that appears to be overlooked currently is the mechanism by which obesity-induced inflammation of ATMs regulates the eventual development of insulin resistance in AT as well as in other tissues, including the liver and muscle. This mechanism would ultimately impair the insulin signaling in parenchymal cells such as adipocytes, hepatocytes, and myocytes, and may involve factors such as TNFα that are secreted by ATMs. However, it is also possible that multiple cytokines are involved, and that very different mediators, such as lipids, may play important roles in the impairment of peripheral cell insulin signaling.

5. Implications for human studies

The most important question is, can the inflammation in ATMs (or even the systemic inflammation caused by myeloid cells) cause insulin resistance and Type 2 Diabetes in humans? This notion is strongly supported by the fact that inflammation *per se* is associated with the development of insulin resistance and Type 2 Diabetes in humans. Furthermore, anti-inflammatory interventions, including salicylates and IL-1 antagonists, improve T2D

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(Donath and Shoelson, 2011). TZDs are also well known to suppress inflammation in both animal models and humans. It should be noted that none of these studies have provided any evidence that points to a key role of ATMs or even myeloid cells in this pathogenesis. However, this is mainly due to technical difficulties in obtaining tissue samples and modulating ATMs in humans. As the relevant technologies and knowledge develop, it will become increasingly easy to accurately assess the role ATMs may play in humans in the development of insulin resistance and Type 2 Diabetes.

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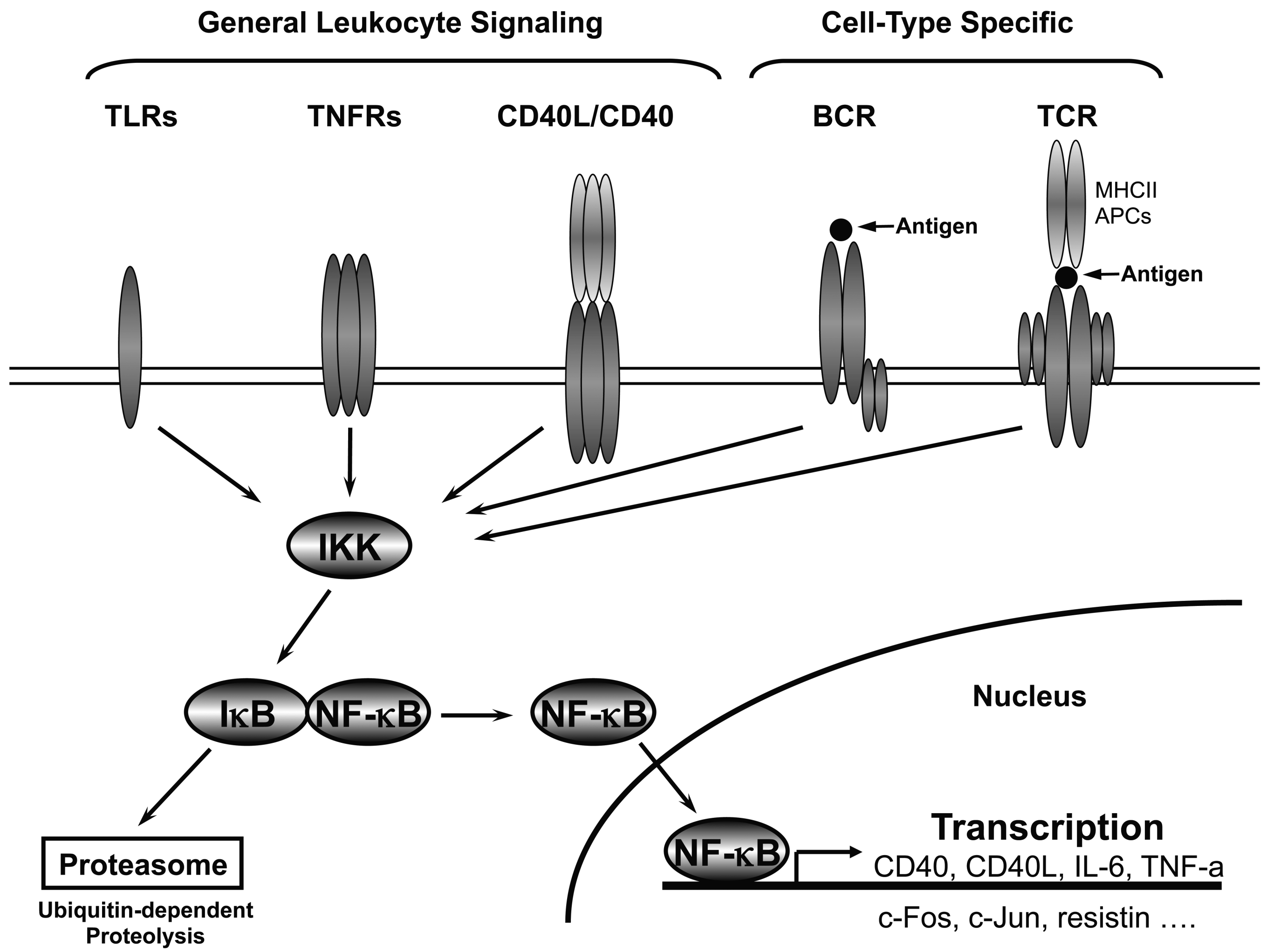

Fig. 1.
NFκB signaling pathway

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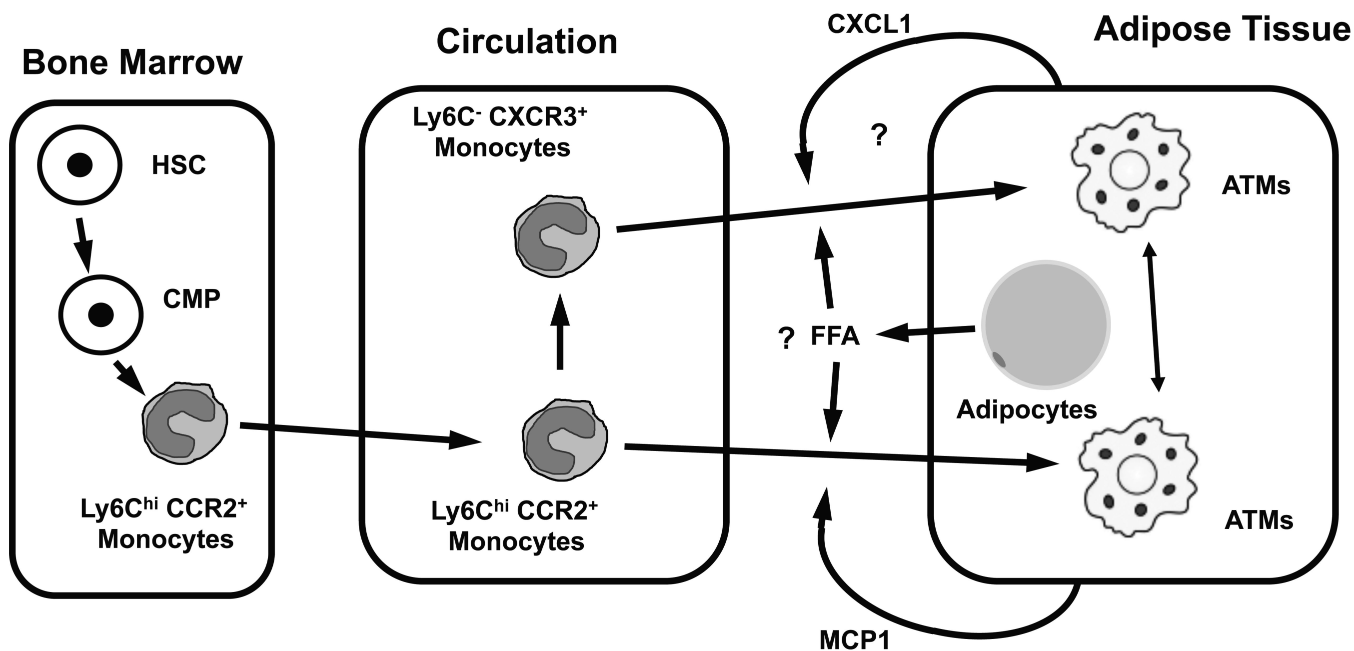

Fig. 2.

Monocyte synthesis and ATM infiltration. Monocytes are synthesized from HSC (hematopoietic stem cells) in bone marrow. HSC is developed to CMP (common myeloid progenitor) and then to monocytes as the Ly6C^{hi} subset. The Ly6C^{hi} monocyte subset egress from bone marrow and is transformed into the Ly6C^{-} monocyte subset in circulation. Adipose tissue in obesity recruits circulating monocytes in an MCP-1-dependent manner and migrated monocytes in adipose tissue are then differentiated into ATMs.

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Table 1

Characteristics of Ml and M2 Macrophages.

	M1	M2	
	Classically Activated	**Alternatively Activated**	**Anti-inflammatory**
Inducer	IFN$_{\gamma}$, LPS	IL-4, IL-13	IL-10
Biased T cell	Th1	Th2	Treg
Function	Pro-inflammatory	Wound Healing/Tissue Remodeling	Anti-inflammatory
Surface Marker	B7	CD206, CD209, CD301	?
Secretory Mediators	TNFα, IL-6, NO	Collagen, YM1, Fizz	IL-10, TGFβ
Internal Proteins	iNOS	Arginase	?