Introduction

The concept that atherosclerosis is a chronic inflammatory disease is now widely accepted. Interestingly, leukocyte infiltration into atherosclerotic lesions had already been described by the middle of the 19th century, and Rudolf Virchow suggested that inflammation and/or infiltration by leukocytes may directly contribute to the pathogenesis of atherosclerosis. Moreover, it was more than a century ago that Williamson et al. reported that the administration of sodium salicylate reduces the urinary sugar level, thus suggesting that inflammation is also involved in diabetes. After these pioneering works, however, inflammation and the role of immune cells in cardiovascular and metabolic disease were largely neglected for decades. In contrast, today, chronic inflammation is recognized to be a key player in the pathogenesis of cardiometabolic disease, including both atherosclerosis and type 2 diabetes (T2D). Indeed, the emerging field of immunometabolism, which explores the interplay between immunological and metabolic processes, focuses on the pathological mechanisms underlying various noncommunicable diseases (NCDs), including Alzheimer’s disease, renal disease and cancer, in addition to cardiometabolic disease.

Clinically, the circulating levels of proinflammatory cytokines, such as tumor necrotic factor-α (TNF-α), interleukin-6 (IL-6), IL-1β and IL-18, are elevated in patients with chronic heart failure, positively correlating with the disease severity. Similarly, a high level of C-reactive protein (CRP) is an independent risk factor for cardiovascular disease, while elevated CRP, IL-6, fibrinogen and plasminogen activator inhibitor-1 (PAI-1) levels, as well as an increased white blood cell count, associate with T2D. Notably, the levels of these inflammatory markers can be reversed by improvements in lifestyle. Taken together, these findings strongly suggest that chronic inflammation is crucially involved in the development..
of cardiometabolic diseases, including atherosclerosis and T2D.

The obesity epidemic is now a global health burden. Obesity, particularly visceral obesity, is thought to be centrally involved in increasing the clinical risk of metabolic and cardiovascular diseases\(^{13}\). Although adipose tissue has long been considered a passive reservoir of lipids, it is now clear that this organ actively controls systemic energy homeostasis, not only by managing the storage and release of lipids, but also by acting as an endocrine organ that produces a variety of "adipokines"\(^{14, 15}\). For instance, leptin, which is pro-acting as an endocrine organ that produces a variety of adipokines, is now clear that this organ actively controls systemic energy homeostasis, not only by managing the storage and release of lipids, but also by acting as an endocrine organ that produces a variety of adipokines. For instance, leptin, which is produced by adipocytes, controls food intake and energy expenditure. Adipose tissue also produces adipokines with anti-inflammatory properties, such as adiponectin. However, obesity triggers the development of inflammation within visceral adipose tissue. Consequently, obese visceral adipose tissue secretes various proinflammatory cytokines, including IL-6 and TNF-\(\alpha\)\(^{10}\). The disruption of the balance between the production of these proinflammatory mediators and anti-inflammatory adipokines is thought to be an important contributor to adverse metabolic and cardiovascular effects. In addition, the release of free fatty acids (FFAs) is increased as a result of activated lipolysis.

The metabolic effects of adipose inflammation were first demonstrated by Hotamisligil \textit{et al.}\(^{17}\) who showed that an elevated level of the proinflammatory cytokine TNF-\(\alpha\) in obese adipose tissue induces insulin resistance. Since then, clinical and experimental evidence has firmly established the pivotal involvement of inflammation in the development of insulin resistance and, more recently, the role of inflammation in the onset of pancreatic \(\beta\) cell dysfunction. In 2007, it was reported that the number of macrophages increased in pancreatic islets in both obese rodent models and obese humans\(^{18}\). We have since demonstrated the pathological involvement of \(M1\) macrophages and islet inflammation in \(\beta\) cell dysfunction\(^{19}\).

Recent studies have also begun to elucidate the molecular links between adipose tissue inflammation and cardiometabolic disease. In this review, we focus on Toll-like receptor 4 (TLR4) signaling and FFAs as mediators linking chronic inflammation and cardiometabolic disease (e.g., T2D and atherosclerosis) in patients with obesity.

**Free Fatty Acid Species and Cardiometabolic Disease**

The intake of dietary fatty acids (FAs) is a strong predictor of metabolic syndrome, which is characterized by visceral obesity, hypertension, dyslipidemia and insulin resistance. Notably, it is the species of dietary FAs (quality), rather than the quantity of FAs, that is thought to be important for the development of metabolic syndrome and cardiovascular disease. Dietary FAs can be categorized into saturated FAs, monounsaturated FAs (MUFA)s and polyunsaturated FAs (PUFA)s. PUFA can be further subclassified into n-3 and n-6 PUFA\(\text{s}\) according to the position of the first double bond. Although the association between the type of dietary FA and the risk of metabolic syndrome is not completely consistent in the literature, most studies agree that a higher intake of a diet rich in saturated FAs correlates with higher risks of obesity, insulin resistance and cardiovascular disease. Among the saturated FFAs, palmitate, which has a carbon chain length of 16, is the major FFA circulating in both mice and humans. On the other hand, a diet rich in unsaturated FAs, including n-3 PUFA\(\text{s}\), which are enriched in fish, and n-9 MUFA\(\text{s}\), which are enriched in the Mediterranean diet, is inversely correlated with the risk of metabolic syndrome\(^{20, 21}\). Indeed, a prospective randomized controlled trial demonstrated that supplementation of n-3 PUFA reduces the incidence of acute coronary syndrome in patients with high cholesterol taking statins\(^{22}\). These clinical observations strongly suggest that each type of FA likely has its own specific effects on the incidence of cardiometabolic disease. Recent studies have focused on unraveling the molecular mechanisms underlying the differential effects of FAs.

**FFAs and TLR4 Signaling**

Many signaling pathways are known to be affected by FFAs. One pathway activated by saturated FFAs is mediated by TLR4, a key pattern recognition receptor contributing to innate immunity. TLRs recognize pathogen-associated molecular patterns (PAMPs) and in turn activate the innate immune response. TLR4 recognizes lipopolysaccharide (LPS), a component of the cell wall in Gram-negative bacteria and activates inflammatory signaling pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-\(\kappa\)B) pathway. Lipid A, the LPS component essential for the activation of TLR4, is composed of glucosamine and acyl chains (FA residues). The FA residues in lipid A are indispensable for TLR4 activation\(^{23}\). In lipid A derived from \textit{Escherichia coli}, all six of the FA chains are saturated FAs with a carbon chain length of 10 to 16. In contrast, lipid A derived from \textit{Rhodopseudomonas sphaeroides}, which lacks the ability to activate TLR4,
contains an unsaturated FA as one of its six FA residues. This suggests that saturated FA residues in LPS are necessary for the activation of TLR4 signaling. Consistent with this idea, saturated FAs with a carbon chain length of 12 (laurate) or 16 (palmitate) reportedly activate TLR4 signaling in RAW264.7 macrophages in vitro. The activation of TLR4 signaling by saturated FAs has also been reported in other cell types.

On the other hand, several reports have argued against the idea that TLRs are activated by saturated FFAs. For instance, Erridge et al. reported that the activation of TLRs by saturated FAs is due to contamination by LPS and lipopeptide of the FA-free bovine serum albumin (BSA) used to administer FFAs. In response to that study, Huang et al. showed that LPS-free palmitate is capable of activating TLR4 signaling in vitro. In addition, the results of many studies demonstrating that FFAs activate TLR4 signaling in a variety cell types and in vivo make it highly likely that FFAs affect TLR4 signaling both directly or indirectly. Our data show that palmitate binds to TLR4 in vitro, although it remains unclear whether FFAs directly activate TLR4 by binding to the receptor in the same manner as LPS. It is possible that additional molecules may mediate the effects of FFAs or that an additional signaling mechanism may indirectly lead to TLR4 activation. In this regard, a recent study proposed a model in which fetuin-A, a liver secretory protein, acts as an adaptor protein mediating the interaction between FFAs and TLR4. As mounting evidence indicates that FFA-induced TLR4 activation is crucially involved in the pathogenesis of cardiometabolic disease, it is important to further elucidate the precise molecular mechanism(s) linking FFAs and TLR4 signaling.

Additional TLR4 ligands involved in metabolic syndrome and atherosclerosis have also been identified. One is LPS itself. A prospective study demonstrated that the circulating LPS level correlates with the future risk of atherosclerosis of the carotid artery. One explanation for a higher circulating LPS level is chronic bacterial infection; another is that obesity increases the number of intestinal LPS-producing commensal bacteria as well as intestinal wall permeability, thus resulting in the enhanced entrance of LPS into the systemic circulation. Imajo et al. showed that, in diet-induced obese mice, Kupffer cells express higher levels of CD14, which enables the cells to respond to low-dose LPS, subsequently promoting nonalcoholic steatohepatitis. On the other hand, a germ-free environment reportedly provides no measurable protection from atherosclerosis in Apoe−/− mice.

Another possible TLR4 ligand is minimally modified LDL (mmLDL). Both macrophages and endothelial cells (ECs) are reportedly activated by mmLDL via TLR4. There are also reports that heat shock protein 60, S100A8, and advanced glycation end products (AGEs) of LDL activate TLR4 signaling. However, it remains unclear whether these non-LPS ligands directly activate TLR4. Further studies are therefore needed to clarify the actual modes of action of these potential endogenous ligands of TLR4.

The TLR4 signaling cascades in immune cells have been intensively analyzed, with several outstanding review articles. In brief, unlike other TLR types, which activate a single adaptor protein, such as myeloid differentiation primary response gene 88 (MyD88) or TIR-containing adaptor inducing interferon-β (TRIF), TLR4 activates both of these proteins. The MyD88 activated by TLR4 plays a dominant role in the activation of NF-κB and AP-1 during the early phase of TLR4 activation. TRIF also activates the transcription factor interferon regulatory factor 3 (IRF3) and participates in the activation of NF-κB and AP-1 during the late phase of TLR4 activation (Fig. 1).

**TLR4 Signaling Under Conditions of Insulin Resistance and Pancreatic β Cell Dysfunction**

Glucose intolerance is characterized by systemic insulin resistance, which subsequently impairs insulin secretion from pancreatic β cells. FFAs, the levels of which are elevated in patients with obesity due to increased release from inflamed obese visceral adipose tissue, have long been recognized to be a causative factor of obesity-induced insulin resistance. Shi et al. showed that FFAs activate TLR4 signaling in adipocytes, macrophages and likely skeletal muscle cells and are known to cause insulin resistance in mice. Adipocytes express functional TLR4 that responds to both FFAs and LPS and induces the expression of cytokine genes, such as Tnf and Il6. Notably, Tlr4−/− mice are protected from the expression of these proinflammatory cytokines in adipose tissue and the insulin resistance induced by lipid infusion, as well as being partially protected from high-fat diet (HFD)-induced systemic insulin resistance. Daniela et al. similarly showed that C3H/HeJ mice, which have a loss-of-function mutation in their Tlr4 gene, are protected against the development of HFD-induced insulin resistance. In C3H/HeJ mice, the HFD-induced activation of c-Jun N-terminal kinases (JNK) and IκB...
played by immune cells in metabolic tissue. Adipose tissue in particular contains a variety of immune cells, many of which are constitutively present but accumulate further under conditions of obesity. These immune cells appear to communicate with adipocytes and other cells within adipose tissue. For instance, inflammatory cytokines secreted by macrophages activate lipolysis in adipocytes by suppressing insulin signaling. FFAs released from the adipocytes then further activate macrophages via TLR4. Ogawa et al. therefore
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proposed that a vicious cycle governs the inflammatory activation of macrophages and adipocytes in obese adipose tissue. Similarly, macrophages and other immune cells likely to contribute to the development of insulin resistance in the liver and skeletal muscle. These studies suggest that saturated FFAs promote insulin resistance by activating inflammatory signaling, in part via TLR4 expressed on immune cells. In addition, several other signaling pathways also appear to promote lipid-induced insulin resistance. For instance, lipid infusion has been shown to increase the intramuscular levels of lipid metabolites, including fatty acyl-CoA and diacylglycerol (DAG). DAG is a potent activator of protein kinase C (PKC), and several PKC isoforms have been shown to interfere with insulin receptor signaling. FFAs also activate reactive oxygen species (ROS) and endoplasmic reticulum (ER) stress pathways, which may also contribute to the pathogenesis of insulin resistance.

In addition to stimulating insulin resistance, FFAs have also been shown to be responsible for pancreatic β cell dysfunction. The deleterious effects of FFAs on β cells are collectively termed “lipotoxicity,” with the direct activation of the ROS and ER stress pathways having been identified as the mechanism underlying β cell lipotoxicity. We recently found that palmitate-induced inflammation is critical for the development of pancreatic β cell dysfunction. Mechanistically, palmitate activates TLR4 on pancreatic β cells, and the resultant secretion of chemokines from the cells leads to the accumulation of M1-type classically activated macrophages within islets, thus initiating the onset of islet inflammation (Fig. 2). These results clearly demonstrate that inflammation is integral to lipotoxicity in vivo. M1 macrophages are also causatively involved in β cell dysfunction in obese db/db and KKAy T2D mouse models. Other reports have also shown that M1 macrophage accumulate within islets in obese mice. The involvement of TLR4 in the development of islet inflammation and β cell dysfunction is further supported by a recent analysis of mice fed an HFD. Taken together, these findings indicate that TLR4 signaling plays a pivotal role in the activation of inflammatory signals in metabolic tissues, which crucially contributes to the development of T2D. TLR4 on both non-immune cells (e.g., β cells) and immune cells (e.g., macrophages) appears to act as a sensor for saturated FAs, thereby triggering inflammatory processes in metabolic tissues. The pathological effects of saturated FAs (i.e., lipotoxicity) thus involve inflammation. That said, lipotoxicity also involves direct cytotoxic effects mediated by the ROS and ER stress pathways.

**Fig. 2.** Palmitate-TLR4 pathway leading to islet inflammation and β-cell dysfunction. Palmitate induces β cell dysfunction in vivo, at least in part, by activating inflammation within islets. β cells sense palmitate via the TLR4/MyD88 pathway and recruit inflammatory monocytes to islets by producing chemokines. The recruited monocytes differentiate into M1 macrophages that play a key role in the β cell dysfunction induced by palmitate. Modified from reference.
**TLR Signaling in Atherosclerosis**

TLR4 signaling is now known to contribute to the pathogenesis of cardiovascular disease. For instance, one prospective study showed an association between the TLR4 Asp299Gly polymorphism, which attenuates receptor signaling, and a smaller intima-media thickness in the common carotid artery. In addition, mice deficient in either Tlr4 or Myd88 are protected from atherogenesis against an Apoe-/- background. The arterial plaques observed in these mice have a smaller lipid content and show less macrophage infiltration and cyclooxygenase-2 (COX-2) immunoreactivity. These mice also exhibit lower serum levels of IL-12 and monocyte chemotactic protein-1 (MCP-1/CCL2), thus indicating the suppression of inflammation. The TLR2 expression is observed in ECs within atherosclerosis-prone regions with a disturbed blood flow, while hyperlipidemia has been reported to increase the endothelial TLR2 expression. Furthermore, bone marrow transplantation experiments have demonstrated that endothelial TLR2 is important for early intimal lipid and foam cell accumulation. It has also been reported that mmLDL-induced leukocyte adhesion is reduced in Myd88-/- ECs in vitro. These results indicate that TLR signaling in ECs contributes to atherogenesis. On the other hand, the promotion of atherogenesis by an exogenous TLR2 ligand, Pam3CSK4, is suppressed by Tlr2 deficiency in the bone marrow. In addition, while transplantation of Tlr2-/-Tlr4-/- bone marrow into low-density lipoprotein receptor knockout (Ldlr-/-) mice does not affect the lesion area, it suppresses the necrotic area, with a reduced rate of macrophage apoptosis. Moreover, bone marrow deficiencies in Trif and Tiam, two adaptor molecules for TLR4 and TLR3, reduce the atherosclerotic lesion size. Therefore, TLR signaling in bone marrow-derived cells, such as macrophages, plays a pivotal role in atherogenesis in the Ldlr-/- model.

While the studies outlined above all point to the proatherogenic effects of TLR2/4 signaling, one recent study also described the protective role played by TLR4 in Apoe-/- atherosclerosis, enhanced by the periodontal pathogen *Porphyromonas gingivalis*. In that model, systemic Tlr4 deficiency significantly exacerbates atherosclerotic lesions, increasing inflammatory cell infiltration, primarily by macrophages and Th17 cells. The authors proposed that Tlr4-/- dendritic cells (DCs) fail to secrete IL-12 and IL-10, which are important for polarization toward Th1 and regulatory T (Treg) cells, respectively, in response to *P. gingivalis* infection. The resulting Th17 skewing of the adaptive immune response may aggravate atherosclerosis. Similarly, Subramanian *et al.* reported that the conditional deletion of Myd88 from CD11c+ DCs in Ldlr-/- mice increases the size of atherosclerotic lesions. Mechanistically, while MyD88 signaling in CD11c+ DCs is required for the differentiation of both effector T cells and Treg cells, the net result in Ldlr-/- mice is the enhanced recruitment of monocytes to the plaque lesion due to the loss of the Treg-mediated suppression of MCP-1. These results indicate that the role of TLR4 signaling in atherogenesis is likely to be ligand-, timing-, and cell type-dependent. Another unanswered question is which actual ligands activate TLR4 in patients with atherogenesis. Although a number of ligands have been identified, as outlined in the previous section, it remains unclear whether and how these postulated non-LPS ligands directly activate TLR4. Nevertheless, TLR signaling appears to be an important regulator of atherogenesis.

**Vascular Lipotoxicity and TLR4 Signaling**

The results of multiple randomized controlled trials all support the idea that a diet rich in saturated FAs promotes atherogenesis. However, the mechanism by which FFAs promote atherogenesis is much less well understood than the mechanism of action of cholesterol. Previous in vitro studies have shown that palmitate impairs the insulin-dependent activation of endothelial nitric oxide synthase (eNOS) via the phosphorylation of IRS-1, as well as inducing the expression of IL-6 and intercellular adhesion molecule 1 (ICAM1). In addition, it has been reported that palmitate stimulates the expression of IL-8, CXCL3 and CCL20 in ECs in a manner dependent upon NF-κB signaling. In addition, it has been reported that palmitate increases the levels of ROS in cultured ECs and smooth muscle cells (SMCs), activates the ER stress pathway in ECs and alters the production of the extracellular matrix in cultured SMCs. In vivo, an HFD activates NF-κB signaling in the aorta of wild-type mice but not Tlr4-/- mice, while palmitate activates NF-κB signaling in aortic explants in a TLR4-dependent manner. These results suggest that palmitate promotes vascular disease by activating multiple inflammatory signaling pathways in ECs and SMCs, some of which are transduced by TLR4.

In order to directly test whether palmitate promotes vascular disease *in vivo*, we intraperitoneally injected mice with emulsified ethyl palmitate, which is rapidly hydrolyzed to palmitate. We found that...
intraperitoneal ethyl palmitate strongly aggravates neointima formation in a mouse carotid artery ligation model (Fig. 3A) and that the neointimal lesions consisted primarily of phenotypically modulated SMCs. Such aggravation of the neointima induced by intraperitoneal ethyl-palmitate is not observed in Myd88−/− mice, clearly indicating that MyD88 is indispensable for the vascular effect of palmitate noted in this model. Moreover, replacement of the bone marrow in Myd88−/− mice with wild-type bone marrow does not restore the adverse effects of palmitate on the neointima, indicating that the MyD88 expressed in vascular cells is essential for the response to palmitate. Mechanistically, palmitate activates TLR4 signaling in SMCs, which in turn activates ROS signaling via the serial activation of NF-κB and NOX1, a homolog of the catalytic subunit of NADPH oxidases, which constitute the major source of ROS in SMCs (Fig. 3B). The TLR4/NF-κB/NOX1/ROS pathway is largely responsible for the inflammatory phenotype in SMCs. Palmitate also promotes the dedifferentiation of SMCs, as mediated via an as yet unidentified signaling pathway. Although that study showed the essential involvement of TLR4 signaling in SMCs, it is very likely that TLR4 signaling in ECs and immune cells (e.g., macrophages) is also involved. In this regard, we previously found that palmitate induces the expression of paracrine factors in macrophages that promote SMC proliferation. This pathway promotes lesion formation, including Pdgfb, Mmp3 and Mmp9. Palmitate also appears to activate additional pathways, resulting in the dedifferentiation of SMCs, which is partly regulated by KLF5. Together, the palmitate-activated pathways promote the phenotypic modulation of SMCs within the injured arterial wall.

**Conclusion**

It is now widely appreciated that one of the unifying mechanisms underlying NCDs is chronic inflammation. In this review, we focused on saturated FFAs as a key mediator promoting inflammation in pancreatic islets, adipose tissue and the vasculature. The accumulation of excess lipids in metabolic tissues, including the liver and muscle, results in insulin resistance, cellular dysfunction and cell death, collectively known as lipotoxicity. Our discovery that palmitate exerts deleterious effects on both pancreatic islets and...
arteries by activating inflammatory processes via TLR4 signaling demonstrates that lipotoxicity involves inflammation and promotes vascular disease as well as metabolic dysfunction (Table 1). Mounting evidence indicates that TLR4 signaling plays a pivotal role in the pathological effects of lipids in the setting of cardiometabolic disease. However, the precise molecular mechanism leading to TLR4 activation is insufficiently understood. Further clarification of this mechanism is required in order to provide efficient therapeutic intervention affecting TLR4 signaling in patients with cardiometabolic disease.

In addition to FFAs, obese visceral adipose tissue is known to produce a variety of adipokines that also likely exert pathological effects on distant tissues. Future investigation of the pathogenic activities of adipokines and FFAs and their contribution to the progression of the chronic inflammation that accompanies expanding visceral adipose tissue is important. A noteworthy feature of the role of chronic inflammation in metabolic syndrome and atherosclerosis is that the immune responses are primarily driven by sterile endogenous signals. Further elucidation of the molecular mechanisms responsible for the activation of non-infectious inflammatory responses may lead to the development of methods for modulating the inflammatory processes involved in NCDs without inhibiting immune responses to classical non-host infections.

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### Conflicts of Interest

None.

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### Table 1. Palmitate-induced activation of TLR4 in multiple cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Downstream effectors</th>
<th>Major outcome</th>
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<tbody>
<tr>
<td>Endothelial cell</td>
<td>↑IL-6, ICAM1</td>
<td>Leukocyte adhesion</td>
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<tr>
<td></td>
<td>↓eNOS through inhibition of IRS-1</td>
<td>Endothelial dysfunction</td>
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<tr>
<td>Smooth muscle cell</td>
<td>↑PDGF-B, MMPs</td>
<td>SMC phenotypic modulation</td>
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<tr>
<td>Macrophage</td>
<td>↑Inflammatory cytokines</td>
<td>Inflammation</td>
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<tr>
<td>Pancreatic β cell</td>
<td>↑Chemokines</td>
<td>Recruitment of macrophages</td>
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<tr>
<td>Adipocyte</td>
<td>↑Inflammatory cytokines</td>
<td>Activation of macrophages and insulin resistance</td>
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</tbody>
</table>

Palmitate has been shown to activate TLR4 in multiple cell types. This table summarizes the known downstream targets of TLR4 signaling in each cell type and the pathological consequences.
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