Nonalcoholic Fatty Liver Disease

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Key Points

- NAFLD is primary hepatic steatosis with inflammation and ballooning hepatocyte injury with or without fibrosis.
- Insulin resistance and chronic low-grade inflammation lead to the development of NAFLD in a genetically predisposed individual.
- Hepatic steatosis is the initiating event, whereas ER stress, oxidative stress, and mitochondrial injury propagate the liver injury.
- Chronic inflammation in NAFLD starts within the adipose tissue.
- It persists as free fatty acids and intestinal microbiome activate TLRs and inflammasome.
- Inflammation worsens insulin resistance and NAFLD.

Defining Nonalcoholic Fatty Liver Disease: Phenotypes of the Disease

Nonalcoholic fatty liver disease (NAFLD) is defined by fat deposition in the liver in the absence of secondary causes for steatosis. The disease spectrum of NAFLD varies from simple steatosis, through steatosis with inflammation with or without hepatocyte injury, to cirrhosis at the other end of the spectrum [1]. Nonalcoholic steatohepatitis (NASH) is a part of NAFLD spectrum and is characterized by the presence of hepatic fat deposition, inflammation, and most importantly hepatocyte damage in the form of characteristic ballooning injury. Current AASLD consensus guidelines require the presence of liver injury in the form of ballooning to distinguish NASH from other disorders of the NAFLD disease spectrum. On the other hand, the term nonalcoholic fatty liver (NAFL) is classically used to describe steatosis in the absence of ballooning [2–4]. The histological criterion for diagnosing NAFLD is fat infiltration in more than 5 % of the hepatocytes. The accumulation of fat usually starts in zone 3 that is the peri-sinusoidal region. Although hepatic steatosis or inflammation in itself does not define NASH, both have been associated with liver-related mortality. Steatosis has been linked to increased cardiovascular mortality [4]. Some studies have determined that inflammation that extends beyond the portal tracks has been correlated with advanced fibrosis, while others have not found this relation. Similarly, evidence suggests that pan-acinar steatosis is predictive of fibrosis [5, 6]. Age and degree of inflammation on biopsy performed at diagnosis have been correlated to progression of fibrosis in a systematic review of several clinical trials. Of the several histological systems proposed for NAFLD diagnosis, those incorporating fibrosis are predictive of long-term mortality. Fibrosis is the only histological feature that is individually related to prognosis [5–8]. On the other hand, clinical presence of obesity and type 2 diabetes has been associated with disease progression [1, 9]. Outcomes of advanced NAFLD (Child-Pugh B and C) have prognosis comparable to those with similar stage of hepatitis C-related liver disease [10, 11].

The pathology in NAFLD arises from the complex interaction of environmental factors such as sedentary lifestyle and excess energy intake in a genetically susceptible host. NAFLD is associated with metabolic syndrome and insulin resistance. This has been well established by several animal and human studies. The role of the immune system in NASH, in terms of its relationship to prognosis, has been observed from several animal studies and human data. However, the role of inflammation in NAFLD etiopathogenesis in terms of the origin, initiation and propagation of inflammation, the involved tissues, cell types, and inflammatory mediators is only beginning to be understood. In this chapter we summarize the current evidence with respect to activation of the innate immune system in NAFLD and its implications on preventing the progression of disease and therapeutic options.

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Diagnosis and Epidemiology

NAFLD is now the most common cause of liver disease in the world. The worldwide prevalence of NAFLD varies from 15 to 45 %. Ultrasound-based studies have reported the prevalence of NASH from 17 to 46 % [12]. On the other hand, histologically confirmed NASH in potential organ donors has ranged from 20 to 51 % [13]. A higher prevalence has been reported in developed countries where its prevalence corresponds to the increasing prevalence of metabolic syndrome. Although NAFLD has been historically known as a disease of the developed world, accumulating evidence supports increasing incidence in several countries of the Asia-Pacific region [12, 14]. The difference in prevalence noted by different studies depends upon the diagnostic tool used by that particular study and the population under consideration [15, 16]. Diagnosing the disease continues to remain a challenge given the limitation of liver enzymes and ultrasound to appropriately identify patients. Several new diagnostic tools have been developed including noninvasive assessment of liver fat by magnetic resonance imaging and spectroscopy and transient elastography, clinical scoring systems, and plasma CK-18 levels [17, 18]. While promising, these diagnostic options need further validation by large-scale studies and are currently reserved as research tools. The gold standard for diagnosis is still liver biopsy, which is rarely performed except in specialized centers. Given these limitations, current AASLD guidelines recommend against a routine screening of patients for NAFLD [2].

Overall mortality in NAFLD patients is two times that of the general population [19]. Morbidity and mortality from hepatic dysfunction in NAFLD vary with the histological severity of the disease [6]. While simple steatosis, the most common pathology seen in NAFLD, is not known to be related to increased disease-related mortality, it is frequently associated with metabolic syndrome and complications thereof. At the same time, steatosis puts patients at an increased risk of morbidity and mortality from other chronic liver diseases. Depending on the length of observation, studies have noted that a third to a half of all patients with simple steatosis eventually progress to NASH. Cirrhosis occurs in up to 15 % of all patients of NAFLD, and about a fifth of the patients with NASH-related cirrhosis develop hepatocellular cancer. This is significant as the third most common cause of mortality in people with NAFLD is liver related compared to 13th in the general population. In fact most cases of cryptogenic cirrhosis are now attributed to NAFLD [4, 10, 20]. This increase mortality is particularly significant given the widespread prevalence and increasing incidence of the disease as determined by population studies. This represents a pressing need for the scientific community to accurately determine the factors that determine disease progression and poor outcome and device preventive and therapeutic measures.

Pathogenesis of NAFLD Disease Initiation: Hepatic Steatosis

Hepatic fat infiltration is central to the pathogenesis of NAFLD. The factors that lead to initiation of hepatic steatosis and those that cause the disease to progress are interrelated and work in concert. The initial two-hit hypothesis proposed to explain NAFLD pathogenesis has now been largely rejected due to inability of currently available data to pinpoint precise triggers for disease initiation vs. progression. Insulin resistance and a state of low-grade chronic inflammation contribute to the pathogenesis of NAFLD, but the precise sequence of events which leads to disease progression to more severe phenotypes in not entirely understood (Fig. 23.1).

Composition of Intrahepatic Fat in NAFLD

Triglycerides (TG) represent the predominant type of fat that is deposited in the liver in NAFLD (Table 23.1). Lipidomic studies in humans have revealed that NAFLD is associated with an increase in diacylglycerol (DAG), triacylglycerol (TAG), and free cholesterol and an increase in omega-6 unsaturated fatty acids with a relative decrease in omega-3 unsaturated fatty acids [21]. In vivo evidence from animal models shows that mice genetically engineered to selectively overexpress diacylglycerol acyltransferase (DGAT) 2, the enzyme that catalyzes the final step in TG formation, had hepatic steatosis with increased amounts of TG compared to controls; however, the animals did not develop insulin resistance [22]. In another in vivo study, feeding a methionine and choline-deficient (MCD) diet to mice that are genetically prone to obesity results in the animals developing the entire spectrum of NASH but with a decrease in hepatic triglyceride content over time. More interestingly, blocking DGAT2 expression produced an expected reduction in hepatic TG content accompanied by an increase in hepatic free fatty acid (FFA) content which was associated with worsening of hepatic inflammation, lipid peroxidation, oxidative stress, hepatocyte injury, and fibrosis [22–24]. Thus, TG accumulation may in fact represent a protective mechanism against FFA-induced lipotoxicity. FFAs are the building blocks for hepatic steatosis [25].

Among FFAs, saturated long-chain fatty acids (such as palmitic and stearic acids) have been shown to be toxic, whereas monounsaturated FFAs are likely to be protective in NASH [26–28]. Cells cultured in the presence of unsaturated

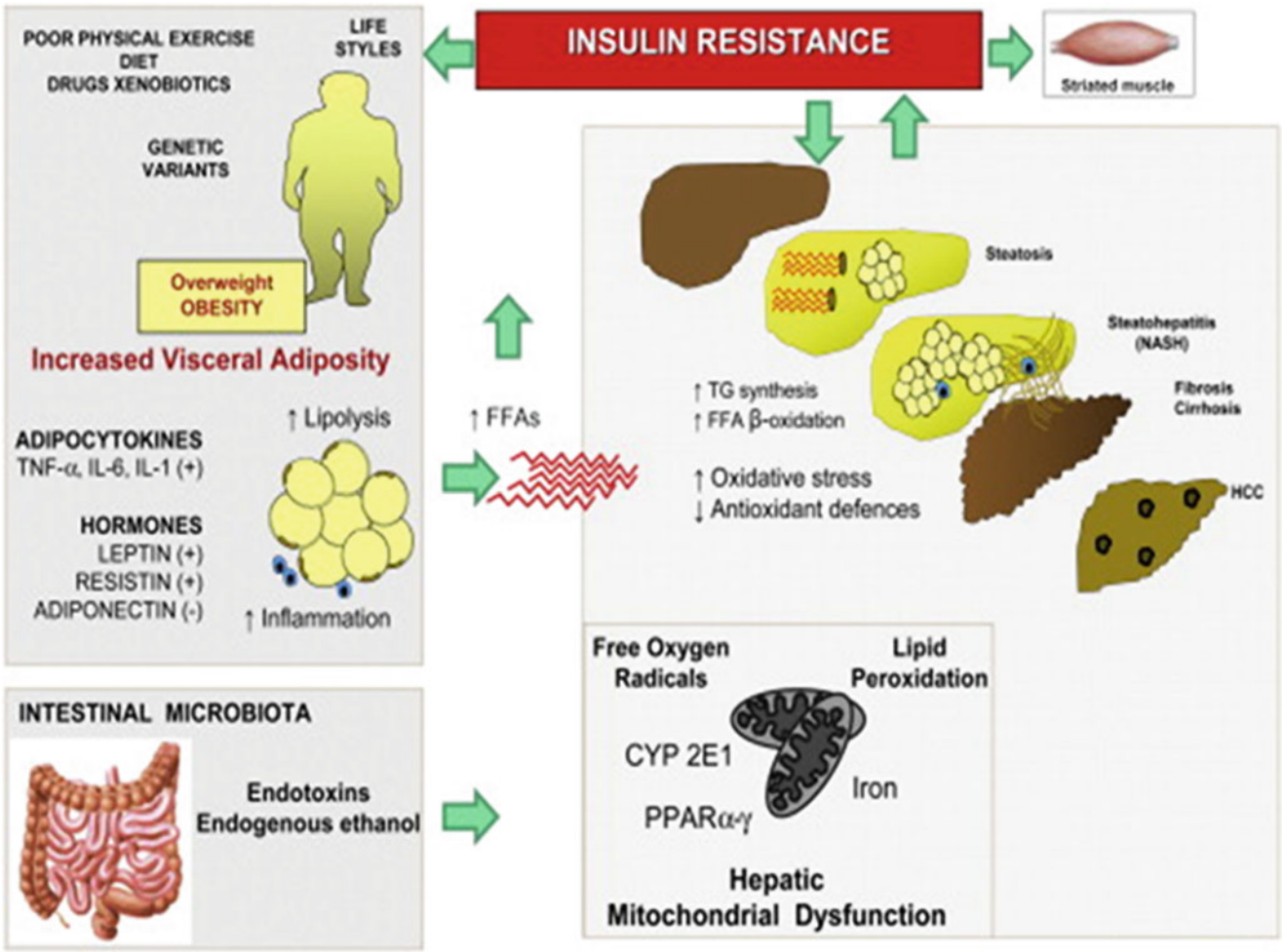

Fig. 23.1 Overview of NAFLD Pathogenesis: The complex interaction involving increased visceral adiposity, altered adipocytokines, adipose tissue inflammation, increased lipolysis and flux of FFAs to the liver, the intestinal microflora, increased hepatic-free oxygen radicals and lipid peroxidation lead to the pathology seen in NAFLD. *TG* triglycerides, *FFA* free fatty acids, *TNF* tumor necrosis factor, *PPARs* peroxisome proliferator-activated receptors, *HCC* hepatocellular carcinoma. *Symbols*: increased (↑); decreased (↓); increased/positive effect (+), decreased/inhibitory effect (−). Adapted from Krawczyk et al. 10.1016/j.bbr.2011.03.031, 2010

Table 23.1 Definition of obesity and its classification based on body mass index

BMI (kg/m^2)	Classification
• <18.5	Underweight
• 18.5–24.9	Normal weight
• 25.0–29.9	Overweight
• 30.0–34.9	Class I obesity
• 35.0–39.9	Class II obesity
• ≥40.0	Class III obesity

Obesity is defined as a BMI more than or equal to 30 kg/m^2
Note: class III obesity is also referred to as severe or morbid obesity

FFA had no change in viability but accumulated significant amounts of TG. On the other hand, saturated fatty acid (SFA) treatment resulted in an increase in apoptotic death without an increase in the amount of intracellular TG accumulation. In addition, FFAs exert hepatotoxicity via several mechanisms which includes formation of lysophosphatidylcholine, reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, c-Jun N-terminal kinase (JNK) activation, and mitochondrial and lysosomal cell death pathway and stimulates pro-inflammatory signals via direct interaction with Toll-like receptors (TLRs) and interferes with insulin signaling [26–28].

Evidence also suggests that FC is pathogenic in NAFLD. It stimulates macrophage JNK activation and depletes mitochondrial-reduced glutathione rendering hepatocytes susceptible to TNF-α or Fas-mediated apoptosis [24, 25].

Although intrahepatic FFAs are not increased in NAFLD as discussed above, serum FFAs, particularly the SFA, palmitate, are increased significantly in human studies of NAFLD [29–31]. FFAs that lead to steatosis are derived from the combined effect of diet, adipose tissue lipolysis, and de novo fatty acid synthesis. In NAFLD, 15 % of liver fat derives from dietary FFA, but de novo lipogenesis increases

from 5 % in healthy subjects to 26 % in NAFLD. However, the largest source of hepatic FFAs (60–80 %) is influx of FFA from adipose tissue as a result of adipose tissue lipolysis.

In summary, there is mounting evidence to suggest that non-TG lipid molecules, especially FFA and free cholesterol (FC), play a key role in the pathogenesis of NASH by leading to lipotoxicity. Fat infiltration in the liver does not necessarily correspond to inflammation. The quality of the fat deposits and not just the quantity is what seems to determine the pathogenesis of NAFLD.

Obesity, Metabolic Syndrome, Insulin Resistance (IR), and Their Relationship to NAFLD

Obesity is defined as a body mass index (BMI) of more than or equal to 30 (Table 23.2). Several clinical studies have demonstrated the association between obesity and NAFLD [32, 33], and as described above, an improvement in the disease is noted with diet- and/or exercise-induced weight loss [34–36]. Not unlike other diseases that fall into spectrum of metabolic syndrome, NASH correlates better with visceral obesity when compared to BMI [37–39]. See Table 23.2 for definition of metabolic syndrome. Our lab has previously published that both visceral fat and dorsocervical lipohypertrophy are associated with severity of disease in NAFLD [40].

IR is the central physiological mechanism of metabolic syndrome, including NAFLD. IR is characterized by an inability of tissues to respond to insulin despite a relative abundance of insulin [41]. The result that IR has depends upon the organ under consideration and the function of insulin in that organ. Peripheral IR results in poor glucose uptake and utilization by skeletal muscle and decreased suppression of lipolysis in adipose tissue leading to hyperglycemia and an increased FFA delivery to the liver [41, 42]. In the liver, IR results in hyperglycemia by impairment of glycogenesis and an increase in gluconeogenesis and glycogenolysis [41, 43]. In addition, the effect of insulin on several intracellular transcription factors involved in lipid homeostasis is altered. Hepatic IR leads to an increase in the activity of the liver X receptor (LXR), carbohydrate-responsive element-binding protein (ChREBP), and sterol-responsive element-binding protein 1c (SREBP-1c), thus increasing hepatic lipogenesis [44, 45]. Nuclear receptors, LXR and retinoid X receptor, work in concert to activate ChREBP and SREBP-1c, which in turn transcriptionally regulate as fatty acid synthase (FAS) and acetyl-CoA carboxylase, the key enzymes needed for de novo fatty acid synthesis in the liver [44, 45]. Another nuclear receptor, peroxisome proliferator-activated receptor-γ (PPAR-γ), leads to steatosis [46–48] but increases insulin sensitivity and suppresses inflammation by increasing serum adiponectin levels [49–51]. In addition, IR-led hyperinsulinemia induces oxidative stress, causes upregulation of connective growth factor and stimulates hepatic stellate cells (HSC) to proliferate, and secretes extracellular matrix [42, 52].

Table 23.2 Key cytokines in NAFLD

TNF-α	Pro-inflammatory, proapoptotic, promotes insulin resistance, activates neutrophils, and opposes adiponectin secretion by adipose
	Circulating TNF-α levels are significantly higher in NAFLD compared to obese controls, and its hepatic expression correlates with the severity of fibrosis. While some studies have noted that TNF levels predict disease severity in NAFLD, several studies have not found a difference in TNF levels across the disease spectrum of NAFLD
IL-6	Chronic elevation is proapoptotic, pro-fibrotic, worsens liver injury and insulin resistance. It activates STAT-3 leading to further inflammatory cytokine secretion. In human studies, serum IL-6 levels increase in patients with NAFLD, and its hepatic levels correlate with degree of steatosis, hepatocyte injury, and fibrosis
IL-4	Leptin-deficient and diet-induced obesity mice have decreased numbers of IL-4-producing NKT cells which correlates with severity of liver disease. Replacement of IL-4-producing NKT cells results in improvement in hepatic steatosis in these mice
MCP-1	Secreted by adipocytes and binds to its macrophage receptor CCR-2. In animal studies, deficiency of either MCP-1 or CCR-2 results in protection from macrophage infiltration into adipose tissue, diet-induced hepatic steatosis, and insulin resistance. Hepatic levels may correlate with NAFLD severity
CCR-2	See above
IL-1β	Activated by caspase-1 as part of inflammasome as well as NF and AP-1 and leads to neutrophil recruitment and insulin resistance via the IKK and JNK pathway
IL-18	As a part of inflammasome, same as above
MIP-1	Secreted by adipose tissue and recruits neutrophils; increased in animal models of NAFLD
Visfatin	Predominantly expressed in visceral adipose tissue and its levels decrease in serum and visceral adipose tissue in NAFLD, and its levels negatively correlate to the degree of hepatic steatosis

Role of Diet in NAFLD

Several animal models have been developed to study the role of dietary factors in NAFLD. Several types of diet have been used to generate these animal models. More commonly used steatosis-inducing diets include MCD diet, high-fat diet with varying amounts of cholesterol, and diets containing high amounts of fructose. Feeding a high-carbohydrate, HF diet with 0.2 % cholesterol to animals that are genetically prone to develop diabetes and hypo-adiponectinemia

leads to classical NASH with fibrosis [53–55], whereas chow-fed animals develop only steatosis. WT C57B6 mice also develop NASH but with diets containing higher percentage of cholesterol 1 or 2 %. In these mice the degree of liver injury is more pronounced with increasing percentage of dietary cholesterol [55–57]. Finally, an HF diet rich in trans saturated fats combined with high-fructose corn syrup equivalent also caused obesity-related steatosis with moderate necroinflammatory change; however, this failed to reproduce ballooning and fibrosis. Conversely, elimination of cholesterol from the HF diet or treatment with drugs that lower hepatic cholesterol results in decreased severity of steatohepatitis [55, 58].

Human studies evaluating dietary intake in NAFLD have shown that patients typically consume a diet with excess amount of cholesterol and saturated fat but lower in polyunsaturated fats, vitamins C and E, and fiber. This disproportionally high consumption of saturated fats by NAFLD patients has been confirmed by other reports [59]. Compared to patients with simple steatosis, subjects with NASH consume more carbohydrates but a lower amount of proteins and zinc [57]. Consumption of a fast food-based high-calorie diet is associated with increase in ALT and hepatic steatosis even in healthy subjects [60]. Several studies have noted an improvement in liver enzymes with diet- and exercise-induced weight loss in NAFLD patients [34–36].

Genetic Predisposition to NAFLD

Obesity, IR, and sedentary lifestyle are all risk factors for NAFLD that are quite widespread in the general population. In spite of this, only a small fraction of people develop steatosis and an even small percentage progress to NASH. This observation indicates that certain individuals are probably genetic predisposed to develop the disease. However, given the complexity of the disease, a Mendelian pattern of inheritance is unlikely, and both familial- and population-based studies can be helpful in understanding the inheritance pattern of NAFLD.

Familial clustering of NASH has been noted although a specific pattern of inheritance has not been identified. In a familial study, 20 % of patients were identified as having first-degree relatives with NASH [61]. In another report, hepatic steatosis was seen in 17 % of siblings and 37 % of parents of overweight children without NAFLD compared to 59 and 78 % in siblings and parents, respectively, of children with NAFLD [62].

The most important mutation identified that predisposes an individual to NAFLD is in the gene encoding patatin-like phospholipase domain-containing (PNPLA) 3 gene. This gene is regulated by insulin and increased with obesity in animals. It is also expressed predominantly in the adipose tissue and liver making it an interesting candidate gene. The single-nucleotide polymorphism (SNP) rs738409[G] of PNPLA3 encoding I148M (rs738409[G]) correlates with degree of steatosis and inflammation in NAFLD [63]. Other SNPs have been identified in PNPLA3 that predict heritability and ethnic differences in NAFLD.

Population-based studies have identified several other candidate genes in NAFLD. The SNP rs1801278 in insulin receptor substrate 1 (IRS1) that affects insulin receptor activity, predisposes to liver damage and decreases hepatic insulin signaling in patients with NAFLD [64]. Similarly, SNPs in adiponectin gene 45GT and 276GT and the SNP rs2241766 of adiponectin C1Q and collagen domain containing (ADIPOQ) are associated with NAFLD [65, 66]. Polymorphisms in apolipoprotein C3 (APOC3) and apolipoprotein E genes have been shown to increase risk for development of fatty liver disease, insulin resistance, and plasma triglyceride levels [67, 68]. Similarly, genetic polymorphisms of genes encoding Kruppel-like factor 6, microsomal triglyceride transfer protein, and manganese superoxide dismutase (MnSOD) have been associated with NAFLD. Kruppel-like factor 6 (wild type) predicts fibrotic severity of NASH while T/T genotype of MnSOD was noted to be more frequent in NASH patients compared to controls. This is plausible as MnSOD deficiency results in an accumulation of superoxide anion resulting in increased oxidative stress [69]. Several candidate genes involved in lipid metabolism, inflammation, oxidative stress, and insulin sensitivity have been identified to potentially play a role in inheritance and progression of metabolic syndrome and NAFLD and have recently been extensively reviewed [70].

Role of Oxidative Stress

Excess FFAs that accumulate as a result of the processes that are described above, in an insulin-resistant state, are further metabolized by physiologic β-oxidation in mitochondria. Mitochondria have structural and functional defects in NAFLD. Uncoupling of oxidation and phosphorylation leads to generation of ROS [31]. Peroxisomal oxidation of very long-chain fatty acids and the ER induction of cytochromes P450 [CYP] 2E1 and 4A also contribute to the ROS load in NAFLD [71–74]. These ROS are central to the pathogenesis of NAFLD. They drive cell injury by interfering with mitochondrial electron transport chain, damage mitochondrial DNA, block ATP generation, and cause peroxidation of cellular lipids leading to membrane defects. Several studies in human NASH livers have shown the presence of lipid peroxidation products [74]. Polyunsaturaed fatty acids (PUFA) are especially important in the context.

The aldehyde products generated as a result of PUFA peroxidation not only retain prooxidant properties but have a longer half-life and by diffusing to surrounding tissues stimulate stellate cell proliferation leading to fibrosis and neutrophil phagocytosis [75].

However, animal models of high-fat diet-induced obesity have failed to demonstrate a clear contribution of oxidative stress in liver injury in NAFLD. In a major clinical trial, PIVENS, treatment with antioxidant vitamin E treatment in NAFLD resulted in improved disease severity in patients without cirrhosis or diabetes mellitus. In children vitamin E improved NASH but was not associated with sustained improvement in liver enzymes [76]. Thus, oxidative stress may contribute to liver injury in NAFLD but is not the sole mechanism involved.

Endoplasmic Reticulum (ER) Stress Response

The unfolded protein response (UPR) is the physiological pathway triggered by the ER to eliminate excess or mis-/unfolded proteins within the cell. It can also be triggered by ER calcium depletion and cellular energy depletion, both of which are seen in NAFLD. Mis-/unfolded proteins, sequester glucose-regulated protein 70 kDa (GRP78) from the three UPR sensors, inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6). The three UPR sensors undergo activation by phosphorylation and dimerization (or cleavage in case of ATF-6). IRE1α and PERK in turn activate X-box-binding protein 1 (XBP1S) and ATF-4, respectively, which together with cleaved ATF-6 comprise the effector molecules for the UPR response. These molecules lead to protein folding via increased transcription of GRP78 and stimulate the endoplasmic-reticulum-associated protein degradation (ERAD) pathway by which mis-/unfolded proteins are eliminated [77, 78].

However, in case of excess protein synthesis, the adaptive UPR response fails resulting in the accumulation of mis-/unfolded proteins within the ER. This precipitates ER stress by which the ER sets off signals that lead to cell senescence and death by apoptosis, but the process may increase inflammation. IRE1α can activate the extrinsic apoptosis pathway via JNK and caspase-12 activation. ATF6 and ATF4 can induce C/EBP-homologous protein (CHOP) expression which inhibits B-cell lymphoma 2 and induces proapoptotic Bim, thus leading apoptosis via the intrinsic apoptotic pathway. Although in obesity, markers of ER stress are increased in liver along with other tissues [79, 80], in NAFLD the current evidence that ER stress plays a major role in the pathogenesis of NAFLD is inconsistent [81–83] (Fig. 23.2).

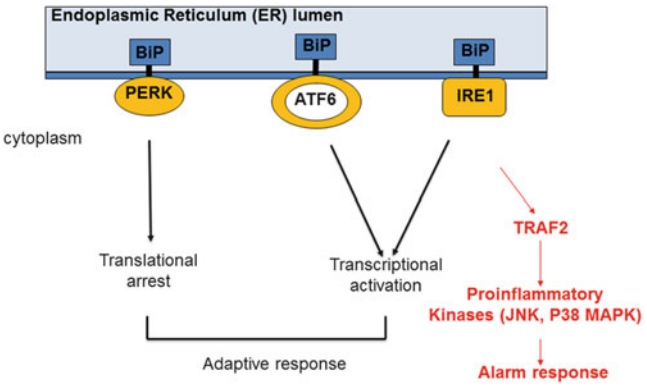

Fig. 23.2 The unfolded protein response (UPR): The three UPR sensors, inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF6). Once activated the UPR sensors, lead to arrest of further protein synthesis and folding response. If the ER response fails, it leads to ER stress via TRAF2 activation

Mitochondrial Injury

Mitochondria in NAFLD have structural and functional defects including defects in its DNA, ATP depletion, and uncoupling of oxidative phosphorylation by overexpression of uncoupling proteins such as UCP-2 [84, 85]. Mitochondria respond to injury or energy depletion by mitophagy, a form organelle restricted autophagy. This process avoids excessive inflammation. However, ROS accumulation within hepatocytes can induce mitochondrial membrane permeability termed mitochondrial permeability transition (MPT). The MPT pore leads to mitochondrial death by intrinsic apoptotic pathway, but at the same time the MPT pore propagates further ROS formation and necrosis [86]. Loss of mitochondrial membrane integrity leads to a loss of the transmembrane potential required for sustaining electron transport chain. The failure to link oxidation to phosphorylation results in ROS generation. ROS have many biological effects described earlier in the paper including activation of NF-κB and inflammasome leading to inflammation and insulin resistance. MPT pore induces necrosis, and necrosis by itself can drive further inflammation.

Role of Innate Immunity in the Pathogenesis of NAFLD

The innate immune system is the first line of defense against foreign substances entering a host organism. It is essentially composed of epithelial barriers, certain proteins, and phagocytic cells that are capable of delivering a rapid defense against potential threat to the organism. Unlike the adaptive

pathway that is initially slow to recognize but remembers a potential pathogen, innate immune responses are nonspecific and rapid. Innate immune responses are initiated when the body recognizes molecular patterns on the invading substance as foreign. Many of these molecules are recognized by TLR proteins, which are highly conserved across from plants to vertebrates and expressed by several cells mediating innate immunity. Exposure to these triggers then leads to activation of phagocytic and antigen-presenting cells including macrophages, natural killer (NK) T cells, and dendritic cells. The phagocytic cells once activated release a slew of chemicals including enzymes, antimicrobial peptides, and ROS that leads to kill the invading microorganisms and/or metabolism of the foreign material. In vertebrates, microbial surface molecules also activate complement system. Ultimately, these mediators of innate immunity signal an inflammatory response and trigger activation of adaptive immunity. Several components of innate immune system are activated in NAFLD as described below.

Activators of Innate Immunity in NAFLD

Adipokines and Cytokines

Over the past decade we have learned that adipose tissue is not just a depot for storage of fat but rather a dynamic organ that secretes several cytokines, termed adipokines. Accumulation of visceral adiposity leads to worsening of metabolic syndrome leading to a low-grade chronic inflammatory state. Increased deposits of visceral fat by imaging studies have been correlated with adverse NAFLD outcomes [38–40]. Both visceral and subcutaneous adipose tissues have a variable propensity to induce insulin resistance; visceral fat is inherently more inflammatory than subcutaneous fat. This is attributed in part to a difference in the maturity of adipocytes at the two sites [87]. In the physiological state the predominant adipokine secreted by adipose tissue is adiponectin, which functions to sensitize the peripheral tissues to insulin. On the other hand, in obesity the levels of adiponectin decline, whereas there is an increase in several inflammatory cytokines such as leptin, resistin, interleukin (IL)-6, IL-1B, tumor necrosis factor (TNF)-α, and monocyte chemoattractant protein (MCP)-1. The net result of adipose dysfunction is propagation of systemic inflammation and peripheral insulin resistance via NF-κB and JNK activation [88]. Below we summarize the role of some of the key adipokines in NAFLD.

Adiponectin

Leptin-deficient obese mice, genetically engineered to produce high levels of adiponectin, have a greater overall amount of adipose tissue but interestingly a lower number of macrophages in adipose tissue and lower levels of systemic IL-6 and TNF-α [89]. Overexpression of adiponectin in obese mice results in a greater proportion of alternatively activated M2 macrophages in their adipose tissue. These studies suggest that adiponectin promotes a decrease in macrophage infiltration of adipose tissue and favors their M2 differentiation [90]. In human studies, adiponectin levels correlate inversely to degree of steatosis, necroinflammation and fibrosis in NAFLD, BMI, percentage of body fat, fasting insulin concentration, and plasma triglyceride levels. Similarly deficiency has been noted of the hepatic receptor for adiponectin, AdipoR2. AdipoR2 expression is lower NASH liver compared to controls and correlates inversely with the severity of steatosis and fibrosis in NASH [91, 92]. Thus the evidence strongly suggests that a relative deficiency of adiponectin contributes to progressive inflammation and overall disease in NAFLD making it an attractive therapeutic option.

Leptin

Leptin is anorexigenic and promotes expenditure of energy. However in obesity and NAFLD serum, leptin levels are increased. Experimental evidence suggests that leptin may promote immune cell activation and phagocytosis and can stimulate hepatic fibrosis by stellate cell activation. Treatment with leptin in human studies is associated with improvement in steatosis and hepatocyte injury suggesting that elevated leptin levels in NAFLD may in fact be representative of a state of resistance to the hormone, not unlike an insulin-resistant state [88, 92–94]. Additional cytokines are summarized in Table 23.3.

Intestinal Microbiome

The total number of bacteria in our gut is nine times that of the number of cells in our body and 15,000–35,000 species of bacteria reside in human gut [95, 96]. It is intuitive hence that these bacteria have a significant influence on our health and disease states and are collectively referred to as the intestinal microbiome.

The intestinal microbiome affects the nutritional state of the host [95, 96]. Chow-fed conventionally reared mice have a 40 % higher body fat than gnotobiotic mice in spite of consumption of fewer calories. Transplanting bacteria from obese mice to lean mice, without a change in diet, resulted in the latter rapidly gaining weight. In addition to influencing the nutritional state of the host, the gut microbiome presents a large amount of endotoxin load to the liver via the portal circulation [97, 98]. In NAFLD the size of the microbiome is increased, and its composition is distinct from controls. Also NAFLD is associated with increased intestinal permeability from defects in tight junctions. The net result is endotoxinemia which via activating TLR signaling in the liver contributes to the development of NAFLD [99, 100].

Table 23.3 Lipids implicated in the pathology of NAFLD

Triglycerides	Largest type of fat that is deposited in the liver in NAFLD. Represents an adaptive or protective change. Does not cause tissue injury or inflammation/fibrosis. May play a role in promoting insulin resistance
Diacylglycerol	Leads to insulin resistance via protein kinase C activation
Free fatty acid	Long-chain, saturated FFA, i.e., palmitic acid leads to in vitro ROS generation, pro-inflammatory (activates JNK), and causes lipoapoptosis in hepatocytes. Promote TLR activation in Kupffer cells
	In animal models, FFA leads to blockade of TG synthesis and worsening of steatohepatitis
	Diet worsens insulin resistance and liver pathology
Lysophosphatidylcholine	Apoptosis of hepatocytes
Ceramide	Increased in NAFLD in lipidomic studies
Polyunsaturated fatty acids	Protective in NAFLD, especially omega-3 unsaturated fatty acids. Anti-inflammatory, inhibit hepatic stellate cells and Kupffer cell activation
Free cholesterol	Pro-inflammatory (activates JNK), promotes ROS formation, depletes mitochondrial GSH rendering hepatocytes susceptible to TNF-α or Fas-mediated apoptosis

Recently a lot of attention has been brought to an additional mechanism by which the microbiome may propensate NAFLD. Endogenous ethanol and acetaldehyde are produced by gut microflora and have been observed in obese subjects, patients with intestinal blind loops, and in those with small intestinal bacterial overgrowth [101, 102]. These can enter the liver by the portal system and initiate hepatic steatosis by several well-studied mechanisms of liver injury [103].

Probiotics have been used in animal and human studies of NAFLD with reports of improvement in overall disease [104, 105], but further studies are warranted before they can be adapted in clinical practice.

Cellular Elements of Innate Immunity Involved in NAFLD Pathogenesis

Role of Adipose Tissue Macrophages: Adipose Tissue-Liver Signaling

Adipose tissue is inherently pro-inflammatory in obesity. However the questions that still remain unanswered are whether adipose tissue inflammation leads to NASH, and if so how? An interesting animal study has helped shed light on this question. In mice fed a high-fat cholesterol-rich diet for 26 weeks, inflammatory signals were detected from adipose tissue between 6 and 16 weeks before their appearance in the liver at 16–26 weeks, indicating that macrophages in adipose tissue are activated in the adipose tissue before a similar process occurs in the liver [106]. However other studies have confirmed that adipose tissue inflammation, once started continues throughout the pathological spectrum of NASH and once hepatic inflammation is established despite deletion of Kupffer cells, inflammation in NASH fails to resolve. It is interesting that deletion of Kupffer cells before onset of hepatic inflammation prevents the onset of NAFLD despite high-fat diet-induced obesity, systemic inflammation, and insulin [107]. These data indicate that inflammation may originate in adipose tissue, but once established it is further driven by both the adipose tissue and hepatic macrophages [108].

Altered balance between pro- and anti-inflammatory adipokines leads to activation of resident macrophages in the adipose tissue and additional recruitment of macrophages from the circulation. For the latter process, monocyte chemoattractant protein-1 (MCP-1) and TNF-α are particularly important. MCP-1 binds to C-C chemokine receptor-2 (CCR2) receptors on macrophages and triggers their activation [109]. Adipose tissue histology in obesity shows clustering of macrophages in the pathognomic "crown-like" clusters surrounding necrotic adipose tissue cells and these are believed to propagate the cycle of inflammation. Tissue macrophages have been functionally classified into M1/M2 macrophages homologous to Th1/Th2 phenotype to T cells. While resident macrophages in healthy adipose tissue mostly express the M2 phenotype, in obesity and diabetes mellitus, they are typically pro-inflammatory or of the M1 phenotype [110, 111].

Role of Kupffer Cells

Similar to adipose macrophages, Kupffer cells have also been proposed to play a significant role in pathogenesis of NAFLD [112, 113]. In MCD diet-induced NAFLD in mice, liposome-encapsulated dichloromethylene bisphosphonate (clodronate) eliminates macrophages and prevents development of steatohepatitis [112]. In metabolic syndrome, an increased number of monocytes have been identified in circulation [114]. Also, the overall number of macrophages has been shown to increase in the liver in NAFLD patients and this correlates with the severity of disease [115]. Interestingly while simple steatosis has a more diffuse distribution of Kupffer cells, in NASH the increased numbers of Kupffer cells are mostly present in the perivenular region [115]. However it is unclear whether the increased macrophages in the liver in NAFLD are derived from blood monocytes or represent an expansion of resident hepatic Kupffer cells as currently reliable markers to distinguish between the two do

not exist. Interestingly, although the number of Kupffer cells is increased in NAFLD, imaging studies utilizing superparamagnetic iron oxide (SPIO)-magnetic resonance imaging which relies on uptake of labeled iron for detection of macrophages demonstrated decreased uptake suggesting impaired phagocytic function of Kupffer cells in NAFLD [116].

Subcellular Pathways of the Innate Immune Pathway in NAFLD

TLR Signaling and Its Role in NAFLD

TLRs are a group of extra- and intracellular receptors that are capable of recognizing nonprotein microbial sequences and damaged or altered host molecules. Of the 13 types of TLRs known to exist in mammals, so far 8 have been identified in human liver and are expressed by several cells within the liver including hepatocytes, Kupffer cells, and HSC [117, 118]. The ligand sequences that bind to and activate TLRs are called pathogen-associated molecular patterns (PAMPs) or disease-associated molecular patterns (DAMPs) depending upon whether they are nonself or originate within the host organism. TLRs recognize PAMPs from a wide variety of pathogens including protein and nonprotein molecules of bacterial, viral, and fungal origins [119, 120]. Most important of these is lipopolysaccharide, a component of the cell wall of Gram-negative bacteria which results in activation of TLR4 [119]. Downstream targets of TLR4 activation depend on the adaptor molecules recruited in the activation process [121–123]. TLR4 activation leads to activation of nuclear factor (NF)-κB and AP-1 by engaging myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein or MyD88 adaptor-like (TIRAP/Mal). TLR4 also signals via TIR domain-containing adaptor inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) leading to activation interferon regulatory factor 3 (IRF3) and thus transcription of interferon-β [117, 118]. Binding of these ligands to TLRs triggers a signaling cascade that results in activation of transcription factors involved in inflammatory pathways such as NF-κB, AP-1, and interferon-responsive factors (IRF). SFAs have been shown to activate TLR4 signaling in macrophages through both Myd88-dependent and TRIF-dependent pathways. By contrast, polyunsaturated fatty acids inhibit these pathways [124, 125]. TLR4-mediated cellular events escalate liver injury in several forms of hepatic steatosis [117]. LPS levels are elevated in several animal models of NAFLD including the high-fat (HF) diet, fructose-rich diet, MCD diet, and choline-deficient amino acid-defined (CDAA) diet, and treating with antibiotics or TLR4 mutation protects the animals from hepatic steatosis [112, 126].

TLR9 may also play a significant role in NAFLD. It recognizes DNA containing an unmethylated-CpG motif on DNA that is characteristic of bacterial DNA. A recent murine study reported that bacterial DNA is detectable in the blood in NASH, even without cirrhosis, and that bacterial DNA binding to TLR9 contributes to the development of steatohepatitis. WT mice on a CDAA-defined diet developed severe steatohepatitis with insulin resistance. In contrast, TLR9-deficient mice had less steatohepatitis even though bacterial DNA was present in the blood [127, 128] (Fig. 23.3).

Probiotics can improve NAFLD in animals and humans and one proposed mechanism is via suppressing TLR activation [104, 105]. While SFAs promote TLR signaling, polyunsaturated fatty acids improve steatohepatitis by inhibiting TLR signaling [129] (Fig. 23.3).

Role on Inflammasome in NAFLD

The nucleotide-binding domain, leucine-rich repeat-containing (NLRP3) inflammasome, also known as cryopyrin or NALP-3, is a multimeric structure and is expressed by myeloid cells that regulates inflammation [130]. Once the complex which requires pro-caspase and adaptor protein recruitment is assembled in the cytosol, caspase-1 is released. Caspase-1 then promotes the cleavage of pro-inflammatory cytokines, namely, pro-IL-1β, pro-IL-18, and IL33, to their respective active forms.

The inflammasome is activated by several stimuli including PAMPs and DAMPs. SFAs, such as palmitate, are well-recognized DAMPs, which, via mitochondrial ROS formation, activate NLRP3 inflammasome to release IL-1β and IL-18. In addition, palmitate-conditioned hepatocytes activate the inflammasome in liver lymphocytes and macrophages to augment release of IL-1β and TNF-α [130–132]. In vivo studies reveal that inflammasome is activated in mice with MCD diet-induced fatty liver, but not in HF diet-induced simple steatosis [132]. A recent study shed more light on this interesting topic as the authors showed that mice lacking inflammasomes NLRP6 and NLRP3 and IL-18 develop progressive NAFLD and metabolic syndrome. Moreover, cohousing inflammasome-deficient mice with wild-type mice led to worsening of hepatic steatosis and obesity [133].

Innate Immunity and Insulin Resistance

We have previously explained that NAFLD is a disorder characterized by insulin resistance [30, 31, 134]. The insulin receptor is a transmembrane tetrameric complex, which upon binding to insulin signals autophosphorylation of tyrosine residues and sets off a signaling cascade including phosphorylation of the Janus-activated kinases (JAK) which leads to phosphorylation and activation of insulin receptor substrates (IRS)-1 and IRS-2 that mediate various intracellular functions

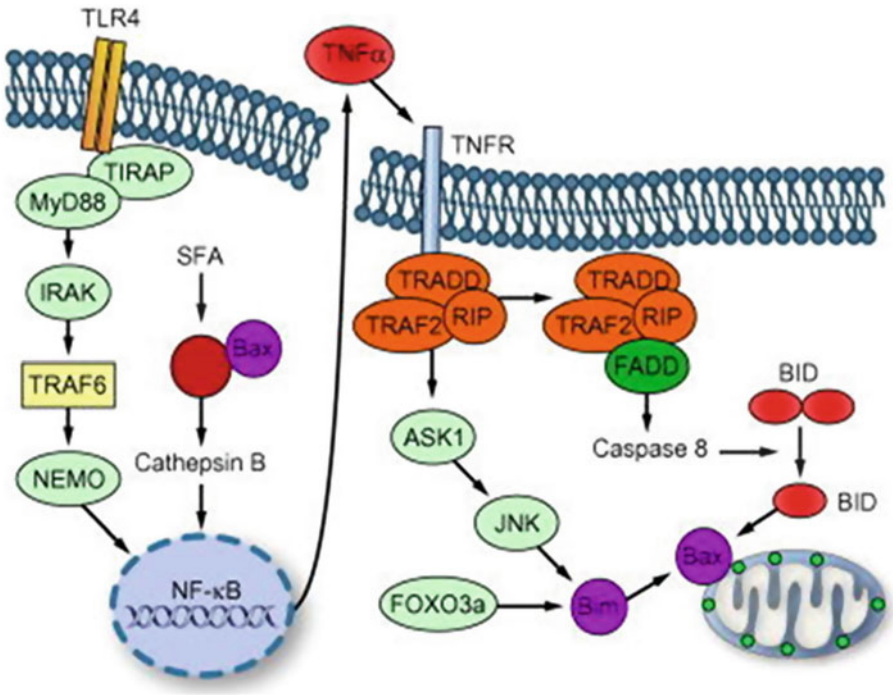

Fig. 23.3 TLR4 activation recruits several downstream adaptor molecules ultimately leading to NFκB activation and TNFα production. TNFα binds to its transmembrane receptors and causes downstream activation of proapoptotic pathways. *SFA* saturated fatty acid, *Bim* Bcl-2 protein family member, *ASK1* apoptosis signal-regulating kinase, *1Bax* B-cell lymphoma 2-associated X protein, *TIRAP* Toll/IL-1 receptor domain containing adaptor protein, *MyD88* myeloid differentiation factor 88, *IRAK* interleukin 1 receptor-associated kinase, *TRAF2/6* TNF receptor-associated factor 2/6, *NEMO* NFκB essential modulator, *TRADD* TNF receptor-associated death domain protein, *RIP* receptor interacting protein, *FADD* Fas-associated protein with death domain, *BID* proapoptotic BCL-2 interacting domain, *FoxO3a* forkhead box-containing protein, class O member 3a, *TNFα* tumor necrosis factor α, *NFκB* nuclear factor κ B. Adapted from Fuchs and Sanyal, J Hepatolology, 2011

of insulin. Serine–threonine kinases via phosphorylation and activation of IRS-1 and IRS-2 can lead to direct activation of the pathway and interfere with normal insulin signaling, thus leading to insulin resistance [135–137]. Fatty acids can activate IRS-1 and IRS-2 causing insulin resistance. Several other factors that exist in NAFLD lead to activation of these kinases including hyperinsulinemia pro-inflammatory cytokines, oxidative stress, and TLR activation. The three serine kinases that have been linked to insulin resistance are JNK, inhibitor of nuclear factor κB (NF-κB) kinase (IKK), and certain isoforms of protein kinase C (PKC) [138–140]. Among these, JNK and IKK are known to stimulate inflammatory pathways through their activation of activator protein-1 (AP-1) and NF-κB, respectively. JNK and IKK promote the expression of lipogenic genes, cytokines, and cell-adhesion molecules and mediate SFA-induced apoptosis of hepatocytes [137, 141].

Another group of molecules in this context is the suppressors of cytokine signaling (SOCS). By competing for insulin-binding sites, SOCS can directly lead to IRS-1 and IRS-2 activation and thus IR [142–144]. Hence, signaling molecules of the innate immune system mediate propagation of insulin resistance in NAFLD (Fig. 23.4).

Innate Immune Mechanisms Promote Hepatic Fibrosis in NAFLD

LPS is elevated in the systemic and portal circulation in patients with cirrhosis [145]. Reduction of gut microflora by nonabsorbable broad-spectrum antibiotics results in a decrease in serum LPS levels and inhibits experimental liver fibrosis. TLR signaling has been implicated in stimulating HSC and inducing hepatic fibrosis in several models of chronic liver injury [146]. TLR4 signaling promotes activation of quiescent HSC via an MyD88-dependent pathway leading to increased chemokine production and leads to KC chemotaxis. Mice mutant in TLR co-receptors had lesser degree of hepatic fibrosis despite a similar level of plasma LPS [147]. Another proposed mechanism for hepatic fibrosis via TLR signaling is via the adaptor molecule MAP3K tumor progression locus-2 (Tpl2). TLR4 and TLR9 activation leads to downstream activation of Tpl2 that ultimately leads to ERK signaling and increased expression of fibrogenic genes in HSC in vitro. Tpl2 knockout mice on an MCD diet have a significant reduction in fibrosis compared with wild-type controls [148].

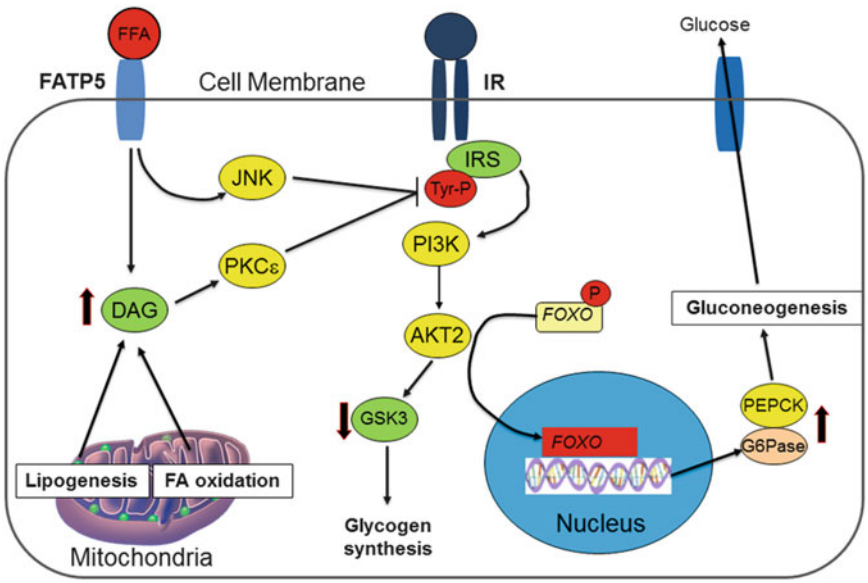

Fig. 23.4 Mechanism for lipid induced insulin resistance. Free fatty acids (FFA) and Diacylglycerol (DAG) increase from diet, lipogenesis, and β-oxidation of fatty acids. Both can lead to activation of insulin receptor substrates (IRS-1 and -2) via protein kinase-ε (PK-ε) and Janus kinase (JKN)-mediated pathways. The net result is worsening of insulin resistance due to decreased glycogen synthesis, increased FOXO-1 phosphorylation and nuclear translocation resulting in increased gluconeogenesis

Conclusion

Our insight into the pathophysiology of NAFLD has expanded tremendously over the past decade. We now understand that hepatic pathology in NAFLD evolves in a genetically susceptible individual exposed to an environment of nutrient excess and sedentary lifestyle. NAFLD is not just a liver exclusive disease, rather a hepatic manifestation of a systemic disease state characterized by insulin resistance and chronic low-grade inflammation. Innate immune responses, once initiated, undergo further amplification via interrelated pathways of the innate and adaptive immune systems. IKK and JNK activated by several intracellular pathways described above or via TLR signaling converge to stimulate hepatocytes, Kupffer cells, and possibly several other resident liver cells to produce cytokines and chemokines which can then further compound the process of inflammation, insulin resistance, and hepatocellular cell damage.

While our knowledge continues to increase on the topic, several questions still remain unanswered. We have yet to generate practical tools for making the diagnosis of NAFLD easier and have just started developing effective therapies that may help arrest the disease progression and repair damage. And although we do know that in NAFLD, there exists a dysregulation of immune system, we have still not determined which comes first, immune activation or insulin resistance, and whether this originates in the adipose tissue or gut microbiome. Nevertheless, ways to regulate the immune imbalance that occurs in NAFLD will hold the key to ultimately treating one of the root causes of the disease. The rapidly increasing worldwide prevalence of NAFLD only makes these questions all the more intriguing and the challenge more formidable at the same time.

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