1 Introduction

Non-alcoholic fatty liver had been believed to be a non-progressive benign disease. However, Ludwig et al. described some patients who exhibited progressive steatohepatitis without habitual alcohol drinking (Ludwig et al., 1980) and referred to this disease as non-alcoholic steatohepatitis (NASH). Later, NASH and non-progressive simple steatosis patients were included in the single disease entity of non-alcoholic fatty liver disease (NAFLD). Recently, non-progressive simple fatty liver has been renamed as non-alcoholic fatty liver (NAFL). The differential diagnosis of NAFL and NASH requires liver biopsy interpretation, and it is often difficult to clearly distinguish these two diseases. NASH is regarded as a more severe form of NAFLD and is broadly defined by the presence of steatosis with inflammation and progressive fibrosis (Matteoni et al., 1999; Brunt et al., 2009), ultimately leading to cirrhosis and hepatocellular carcinoma (HCC) (Yatsuji et al., 2009; Hatanaka et al., 2007; Fassio et al., 2004; Ono & Saibara, 2006). The mechanisms through which the subset of NAFLD patients develops NASH are poorly understood.

The development of NASH is generally thought of as a “two hit” process (Day & James, 1998). The first hit is the development of hepatic steatosis due to overeating, lack of exercise, or drug use. The second hit includes hepatic damage inducing cellular stresses such as oxidative stress, apoptosis, and gut-derived signals such as lipopolysaccharide (LPS). However, recently uncovered liver fat deposition and pro-inflammatory
mechanisms have revealed that inflammation could actually precede steatosis and might contribute to steatosis development. In experimental models, inflammatory macrophages could infiltrate before lipid deposition, and antioxidant treatment reversed inflammatory gene expression before the aberrant lipid metabolism related genes expression reversed (Shiri-Sverdlov et al., 2006). These results have encouraged us to define NASH pathogenesis as a “multiple parallel hits” process (Tilg & Moschen, 2010).

As one of the multiple hits, gut microbiota changes or disease susceptible genetic polymorphisms genotype could be included. A Western-style diet including high-fat and high-fructose characteristics can induce gut microbiota changes following inflammatory responses in the liver even without fat deposition. Patatin-like phospholipase 3 (PNPLA3) is a NASH susceptibility gene that is involved in hepatic fat metabolism. Although NAFL is usually non-progressive, patients harboring the risk allele (G-allele) of PNPLA3 are at increased risk for NASH progression (Romeo et al., 2008). Such dietary and genetic characteristics can precede the first hit of fat deposition. These components could be involved in the multiple parallel hits process. It is often difficult to distinguish the first and second hits. The multiple parallel hits theory has recently become widely accepted.

Many sources of cellular stress, including oxidative stress, apoptosis, and gut-derived LPS, trigger an inflammatory response and progressive liver damage (Csak et al., 2011). Oxidative stress is increased through the generation of reactive oxygen species (ROS), as well as by defects in redox defense mechanisms involving glutathione, catalase, or superoxide dismutase (Muriel, 2009). Mitochondria are the most important and abundant source of intracellular ROS. Therefore, mitochondrial dysfunction plays a central role in the pathological mechanisms of chronic inflammation and subsequent carcinogenesis in NASH. Although the mechanisms of mitochondrial dysfunction are not clearly understood, emerging data suggest that ROS, lipid peroxidation products, and tumor necrosis factor-α (TNF-α) are involved in the multiple hits, inducing the progression from simple steatosis to NASH (Takaki et al., 2014). ROS can educate adaptive inflammatory cells and induce directional migration of resident hepatic pro-fibrogenic cells, resulting in liver inflammation and fibrosis (Novo et al., 2011; Sutti et al., 2014).

Immune responses and inflammation are involved in metabolic diseases, such as diabetes mellitus, atherosclerosis, and NASH. Adipose tissue-derived cytokines promote metabolic disease progression. In obesity, excessive numbers of proinflammatory, M1-like macrophages accumulate in adipose tissue and the liver (Lanthier et al., 2010). Even in simple fatty liver, macrophage infiltration and the expression of the macrophage attractant chemokine monocyte chemotactic protein 1 (MCP1) are significantly increased (Gadd et al., 2013). Macrophages are an important mediator of inflammation and insulin resistance, which are the common phenomena of NAFLD. In advanced NASH, CD4 (+) and CD8 (+) T-cell infiltration increases, and the levels of inflammatory cytokines, such as IL-6 or IL-8, are also increased (Gadd et al., 2013).

Several studies have suggested that antioxidants such as vitamin E and 1-aminobenzotriazole confer benefits upon NAFLD patients, and the American Association for the Study of Liver Disease recommends the use of high-dose vitamin E for NASH (Chalasani et al., 2012). However, most clinical studies involving the treatment of
atherosclerotic diseases with dietary antioxidants have not generated clear results, partly because of the non-selective effects of these anti-oxidative drugs and difficulties associated with cytosolic distribution (Steinhubl, 2008). The clinical findings of antioxidant therapies have not always been favorable and are often associated with worsening pathology (Hackam, 2007). Thus, new treatment strategies are needed.

Here, we review the importance of immune reactions in the current understanding of NAFLD molecular pathogenesis and potential immune-related treatments to be considered in future therapeutic paradigms.

2 NASH Pathogenesis: General Characteristics

The pathogenesis of NASH is unclear. The multiple hits involved in NASH development include hepatic steatosis, gut-derived endotoxins, oxidative stress, or proinflammatory cytokines (Day & James, 1998) (Tilg & Moschen, 2010) (Tiniakos et al., 2010). The following factors are related to NASH progression.

2.1 PNPLA3 Genetic Background

PNPLA3 gene polymorphism is a susceptible genetic factor in NAFLD (Romeo et al., 2008). This genetic polymorphism differentiates between simple steatosis with or without minimal inflammation and fibrosis that progresses to NASH (Valenti et al., 2010; Kawaguchi et al., 2012). Patients with the NASH-sensitive single nucleotide polymorphism rs738409 G/G genotype might progress not only to simple steatosis but also to NASH, probably under the same types of metabolic stimulation. The function of PNPLA3 is not well known, since mice deficient in PNPLA3 develop neither fatty liver nor liver injury. However, the overexpression of sterol-regulated binding protein 1c (SREBP-1c) results in its binding to the transcription start site of the mouse PNPLA3 gene, while PNPLA3 knockdown can decrease the intracellular triglyceride content in primary hepatocytes (Qiao et al., 2011). Thus, PNPLA3 might function as a downstream target gene of SREBP-1c to mediate SREBP-1c stimulation of lipid accumulation. A meta-analysis revealed that this variant is associated with increased liver fat content when compared to weight-matched individuals not harboring the PNPLA2 polymorphism as well as increased risk of severe fibrosis, even in the presence of other etiologies of chronic liver diseases (Singal et al., 2014).

Not all patients with progressive NASH harbor the PNPLA3 risk allele; thus, differences in the characteristics of PNPLA3 risk allele–bearing and –nonbearing NAFLD patients have been demonstrated (Lallukka et al., 2013). PNPLA3-related NAFLD is not characterized by features typical of metabolic syndrome such as hyperinsulinemia, hypertriglyceridemia, and low HDL-cholesterol levels (Sookoian & Pirola, 2011). Obesity-related NAFLD patients exhibit the same distribution of the PNPLA3 genotype as non-obese patients, whereas inflammation-related genes are upregulated in adipose tissue.
2.2 Visceral Obesity and Adipokines

Obesity is a growing global epidemic among adults and children and is associated with many diseases such as hypertension, diabetes mellitus, hyperlipidemia, and NAFLD. Furthermore, obesity, hypertriglyceridemia, and hypertension are predictive risk factors for NAFLD (Tsuneto et al., 2010). Visceral fat accumulation in obesity correlates with various organ pathologies including cerebrovascular diseases, cancer, and NASH. Moreover, visceral fat accumulation is regarded as a significant risk factor for the development of NAFLD and NASH. A study from Japan found that the severity of hepatic steatosis, determined by ultrasound, was positively correlated with visceral fat accumulation and insulin resistance in both obese and non-obese individuals, suggesting that hepatic steatosis is influenced by visceral fat accumulation regardless of obesity (Eguchi et al., 2006).

Adipokines are multifunctional secreted factors that are primarily derived from adipose tissue. Adiponectin is the most abundant adipose tissue–specific adipokine. Mature adipocytes mainly produce adiponectin in white adipose tissue, and expression and secretion levels increase during adipocyte differentiation. Adiponectin levels are inversely correlated with visceral obesity, and insulin resistance and weight loss induce adiponectin synthesis. Proinflammatory adipokines such as TNF-a or IL-6 suppress adiponectin, which has anti-inflammatory, anti-atherogenic, and anti-diabetic properties (Polyzos et al., 2011). Adipose tissue is also the main producer of the adipokine leptin, and its levels directly correlate with body fat mass and adipocyte size (Carbone et al., 2012). Leptin production is mainly regulated by food intake, and hormones related to eating such as insulin increase leptin secretion and vice versa. Proinflammatory endotoxins, IL-1, and TNF-α increase the secretion of leptin, which has central and peripheral effects (Sarraf et al., 1997). Leptin acts on hypothalamic cells, inhibits anabolic pathways, activates catabolic pathways, inhibits appetite, and stimulates energy expenditure. Leptin-deficient (ob/ob) mice and leptin receptor–deficient (db/db) mice are severely obese and have increased pituitary and adrenal hormone production, hyperglycemia, elevated insulin, and decreased immune function (Cohen et al., 2001; Lindstrom, 2007).

Contrary to expectations, several groups have made the controversial observation that serum adiponectin levels are lower in NAFLD than in NASH, or are the same (Shimada et al., 2007; Younossi et al., 2008; Argentou et al., 2009; Lemoine et al., 2009). A meta-analysis of 27 studies of 698 controls and 1545 patients with NAFLD found that serum adiponectin levels were low in NAFLD and much lower in NASH (Polyzos et al., 2011). Since adiponectin and leptin exert antagonistic effects on liver fibrogenesis and inflammation, the ratio of adiponectin to leptin might be a better marker with which to distinguish NASH from NAFLD. Levels of adiponectin receptor II are decreased in human liver biopsy specimens and in mouse models of NASH (Kaser et al., 2005; Matsunami et al., 2011). However, since contradictory results have suggested that lower serum adiponectin levels induce high expression of hepatic adiponectin receptor II as a compensatory response, the function of these novel adipokines and receptors requires further investigation (Nannipieri et al., 2009; Ma et al., 2009).
2.3 Hepatic Steatosis and Oxidative Stress

Fatty liver is the basic feature of NAFLD and NASH. Lipid droplets are now considered as complicated organelles that exhibit many functions such as metabolic, inflammatory, and immunological responses. Lipid toxicity induces multiple effects such as oxidative stress, ER stress, and immune reactions (Takaki et al., 2014). Triglycerides are the main type of lipid stored in the liver of patients with NAFLD. The toxic lipids present in NASH and the non-toxic lipids in simple steatosis could differ (Yamaguchi et al., 2007). Diacylglycerol acyltransferase 2 (DGAT2) catalyzes the final step in hepatocyte triglyceride biosynthesis. Hepatic steatosis and the dietary triglyceride contents in a model of obese-simple fatty liver are reduced by DGAT2 antisense oligonucleotides in a manner that does not correlate with changes in body weight, adiposity, or insulin sensitivity (Yu et al., 2005). However, DGAT2 antisense oligonucleotide increased levels of hepatic free fatty acids, lipid oxidant stress, lobular necroinflammation, and fibrosis in mice fed a methionine choline-deficient (MCD) diet that generates inflammation and fibrosis with hepatic steatosis, whereas the hepatic triglyceride content decreased (Yamaguchi et al., 2007).

These results suggest that the pathogenesis and treatment of steatosis in simple fatty liver and in NASH are different. Human genetic variability analysis of lifestyle intervention has shown that the DGAT2 gene polymorphism is related to a decrease in liver fat, while changes in insulin resistance are not correlated (Kantartzis et al., 2009). Since insulin resistance is the key marker for NASH, DGAT2 gene polymorphism might only be associated with non-progressive fatty liver.

Excessive oxidative stress induced by mitochondrial, peroxisomal, and microsomal ROS in NASH results in apoptosis as well as nuclear and mitochondrial DNA damage. Limited antioxidant defenses contribute to the processes of both NASH and hepatocarcinogenesis (Kawai et al., 2012; Bugianesi, 2007). Physiologically low levels of ROS are involved in vital cellular processes, indicating that the balance of oxidative and antioxidative responses is important (Mittler et al., 2011). As mitochondria uptakes long chain fatty acid and provide to β-oxidation pathway and redox pathway finally produces detoxified water and ROS, its dysfunction leads to oxidative stress. Indeed, ultrastructural alterations, impaired ATP synthesis, and increased ROS production have been reported in liver mitochondria from NASH patients as well as in a rodent NASH model (Cortez-Pinto et al., 1999; Serviddio et al., 2008). Excess superoxide is generated within injured mitochondria through electron leakage and the resulting excess of superoxide would be converted to hydrogen peroxide (H$_2$O$_2$) by superoxide dismutase. Glutathione peroxidase or catalase can metabolize H$_2$O$_2$ to non-toxic H$_2$O; however, the Fenton and/or Haber-Weiss reactions generate the highly reactive and toxic hydroxyl radical.

Iron is the key mineral that induces oxidative stress produced via the Fenton reaction. Although its role in NASH is not fully understood, iron levels are elevated in NASH, which is an inducer of oxidative stress, and lowering iron levels has resulted in fair outcomes for patients with chronic liver diseases (Nelson et al., 2011). However, one-third of early stage NAFLD patients show iron deficiency correlated with the female sex, obesity, and type 2 diabetes (Siddique et al., 2013). We must wait for long-term,
follow-up studies to confirm whether iron-deficient obese patients progress to NASH, as well as the role of iron in NAFLD progression.

Autophagy is a catabolic process that degrades old proteins and cellular organelles such as mitochondria and endoplasmic reticulum (ER). Autophagy deficiency enhances ER stress and ROS production from abnormal mitochondria (Yang et al., 2010). Hepatic steatosis results in markedly decreased hepatic autophagy in chronic obesity mouse models via suppression of autophagy-related Atg7 expression or impairment of autophagosomal acidification and cathepsin expression (Singh et al., 2009; Inami et al., 2011). In human NAFLD liver, hepatic cathepsin expression was decreased and associated with the autophagic dysfunction–related protein P62 (Fukuo et al., 2014).

2.4 Insulin Resistance

Insulin resistance is a state of relative insulin insufficiency due to reduced tissue insulin responsiveness. Under normal physiological conditions, insulin secretion from pancreatic b cells is stimulated by postprandial increases in blood glucose, and insulin circulation generally normalizes blood glucose levels. Insulin stimulates glucose uptake by skeletal muscle and adipose tissue. Insulin also stimulates the liver to convert excess glucose into glycogen and triglyceride for storage. Insulin binds the insulin receptor and stimulates receptor autophosphorylation and internalization, which in turn recruits and activates insulin receptor substrate proteins 1 and 2 (IRS1/2). IRS1/2 can activate phosphatidylinositol 3-kinase, which converts PI bisphosphonate (PIP2) to PIP3. Cell proliferation transcription factor Akt binds PIP3 and is subsequently phosphorylated and activated. In muscle and adipose tissue, Akt stimulates the translocation of glucose transporters to the membrane to allow glucose uptake. In the liver, insulin binding promotes fatty acid synthesis through activation of SREBP 1. Akt kinase pathways have roles in cell growth, cell proliferation, fibrogenesis, and hepatocarcinogenesis (Larter et al., 2010). The gold standard methods to detect insulin resistance are the complexed clamp technique requiring the frequent sampling of intravenous glucose; however, simpler methods, such as the oral glucose tolerance test or homeostatic model assessment, are generally used.

Insulin resistance is concordant with NASH (Larter & Farrell, 2006). In the obese insulin-resistant model ob/ob mouse, liver insulin receptor knockout improved hepatic lipogenesis (Haas et al., 2012). This result indicates that insulin receptor signaling is required for hepatic steatosis. Insulin resistance could be induced by Kupffer cells, as depletion of Kupffer cells could attenuate systemic insulin resistance and improve liver autophagy in high-fat diet–fed mice (Zeng et al., 2015).

The NAFIC score is a NASH diagnostic screening tool developed for Japanese NASH patients; its criteria include high levels of ferritin, fasting insulin, and type IV collagen 7S (Sumida et al., 2011). As the fasting serum insulin level was significantly correlated with NASH prevalence, a modified NAFIC score with serum fasting insulin level stepwise refinement was more effective to diagnose NASH (Nakamura et al., 2013).

Insulin resistance is one of the most important factors that characterizes NASH and could be a treatment target.
2.5 Gut Microbiota Change and Toll-Like Receptor (TLR) Signaling in Liver Pathogenesis

Gut microbiota has been accepted as a key factor in several diseases. As healthy stool transplantation was proven to show surprising beneficial effects on *Clostridium difficile* enterocolitis, the clinical impact of gut microbiota on many diseases has been analyzed. NAFLD and hepatocellular carcinoma are obviously included in the relation. Endotoxin or LPS produced by gut microbiota could be delivered to the liver via the portal vein, which raises the question of why such toxic materials are capable of flowing into the portal vein through the intestinal barrier. Patients with biopsy-confirmed NAFLD have increased intestinal permeability with disrupted intercellular tight junctions in the intestine (Miele et al., 2009). These abnormalities are related to increased bacterial overgrowth in the small intestine. Murine NAFLD models of bacterial overgrowth develop compositional changes and increased intestinal permeability with a concurrent reduction in the expression of tight junction proteins (Brun et al., 2007). Plasma endotoxin levels are significantly higher in patients with NAFLD and in murine NASH models (Miele et al., 2009; Cani et al., 2008). A high-fat diet could increase LPS concentrations two- to three-fold (Cani et al., 2007). Proinflammatory inflammasomes induce inflammation in the liver of patients with NAFLD, but an inflammasome-deficient mouse model develops exacerbated hepatic steatosis and inflammation through the influx of TLR4 and TLR9 agonists into the portal vein (Henao-Mejia et al., 2012). The microbiota of these inflammasome-deficient mice differed from the microbiota of wild-type mice with NASH. Furthermore, co-housing inflammasome-deficient and wild-type mice resulted in intestinal inflammation and exacerbated hepatic steatosis in the wild-type mice (Henao-Mejia et al., 2012). This finding suggested that altered microbiota in inflammasome-deficient mice could be transferred to healthy mice, resulting in intestinal inflammation, increased permeability, and NAFLD.

The mechanisms by which commensal gut microbiota trigger hepatic steatohepatitis remain to be investigated. The gut microbiota release pathogen- or damage-associated molecular patterns (PAMPs or DAMPs), which are TLR ligands. TLR2, TLR4, and TLR9 have been intensively investigated and found to be involved in the pathogenesis of NASH (Takaki et al., 2014). TLR2 is a receptor for multiple glycolipids or lipoproteins in bacteria adhering to the cell surface of monocytes, myeloid dendritic cells or mast cells. TLR4 is an LPS receptor located on the surfaces of monocytes, myeloid dendritic cells, mast cells, β cells, and the intestinal epithelium. Toll-like receptor 9 is located on the ER or endosomes of plasmacytoid dendritic cells or β cells, and is regarded as a receptor for unmethylated CpG DNA particles that might be released from bacteria. These molecules have been analyzed in several NAFLD and NASH models. The results of TLR studies in different NASH models notably vary. For example, the choline-deficient amino acid-deficient diet model mouse develops steatosis with relatively mild hepatitis or fibrosis with obesity, whereas the methionine-choline-deficient (MCD) diet model mouse develops steatosis with severe hepatitis and fibrosis without obesity. In the TLR2 knockout mouse model, the choline-deficient amino acid-deficient diet induced mild NASH course improved, while the MCD diet induced severe NASH course
worsened (Miura et al., 2013; Rivera et al., 2010). As NAFLD includes heterogenous backgrounds and clinical courses, the pathogenesis should be variable. TLR4 and TLR9 agonists might flow into the portal veins of inflammasome-deficient MCD diet mouse models and thus exacerbate NASH (Henao-Mejia et al., 2012). Inflammasomes are multi-protein complexes composed of nucleotide-binding domain and leucine-rich repeat protein 3, apoptosis-associated speck-like protein containing CARD and procaspase 1, which are DAMP or PAMP sensors. Inflammasome activation leads to the processing and secretion of the proinflammatory cytokines IL1β and IL-18, whereas knockdown results in MCD NASH exacerbation (Henao-Mejia et al., 2012). These perplexing findings indicate that the intestinal DAMP or PAMP barrier function that is disrupted in inflammasome knockout mice overcomes the anti-inflammatory effect in the liver and TLR4 and TLR9 ligand outflow into the portal vein where they stimulate NASH progression.

The gut and oral periodontal status correlates with the progression of liver disease (Tamaki et al., 2011). Levels of the periodontopathic bacteria Porphyromonas gingivalis (P. gingivalis) are markedly higher in NASH patients than in NAFL patients and healthy subjects (Yoneda et al., 2012). In vivo infection of P. gingivalis in the high-fat diet-induced mouse NAFLD model resulted in fibrosis with proliferation of hepatic stellate cells (HSC) and collagen formation (Furusho et al., 2013). Treating periodontitis could improve transaminases in NAFLD and, in fact, several probiotics that control gut microbiota improve NAFLD (Yoneda et al., 2012) (Endo et al., 2013; Xu et al., 2012). Studies using models of hepatocarcinogenesis have found that a high-fat diet increases levels of deoxycholic acid, a gut bacterial metabolite that damages DNA and exacerbates hepatocarcinogenesis (Yoshimoto et al., 2013). Antibiotics could abrogate these effects. Gut microbiota affect not only NAFLD, but also obesity-related hepatocarcinogenesis.

3 NASH Pathogenesis: Multiple Hits and Correlation to Immune Responses

Since the significance of apparently similar fat droplets in simple fatty liver and NASH hepatocytes differs in DGAT2 knockdown experiments, analyzing the molecular pathogenesis of NASH at the cellular level is important. Hepatic inflammation and fibrosis, which are the characteristic features of NASH, involve immune reactions induced by cytokines, chemokines, adipokines, and inflammatory cell infiltrations (Figure 1).

Immune responses and inflammation are involved not only in NASH but also in most metabolic diseases, such as diabetes mellitus and atherosclerosis. Adipose tissue–derived cytokines promote metabolic disease progression. In obesity, excessive numbers of proinflammatory, M1-like macrophages accumulate in adipose tissue and the liver (Lanthier et al., 2010). Even in simple fatty liver, macrophage infiltration and macrophage attractant chemokine MCP-1 expression are significantly increased (Gadd et al., 2013). Macrophages are an important mediator of inflammation and insulin resistance, which are common phenomena in NAFLD. In advanced NASH, CD4 (+) and CD8 (+) T-cell infiltration increases, and the levels of inflammatory cytokines, such as IL-6 or IL-8,
3.1 Local Inflammatory Cell Infiltration and Cytokine Milieu

Even in NAFL, several inflammatory cytokines are elevated, evidenced by a report showing that IL-6 or IL-8 mRNA expression in liver biopsy specimens did not significantly differ between NAFL and NASH (Gadd et al., 2013). Inflammation occurs even in NAFL, but some factors exacerbate the inflammation and induce liver fibrosis as well. Fat-induced insulin resistance results in activation of serine kinases such as Jun-N-terminal kinase (JNK), inhibitor of nuclear factor kB (NF-kB) kinase (IKK), and novel isoforms of protein kinase C (Maher et al., 2008). JNK and IKK induce proinflammatory signaling. Excess fat can activate JNK and IKK in hepatocytes resulting in the increased expression of inflammatory cytokines and cell-adhesion molecules (Schattenberg et al., 2006). Hepatic lipid, especially the toxic oxidized lipid-induced oxidative stress product malonyldialdehyde, could be used as immunological antigens to activate proinflammatory cytokine expression in the MCD diet model (Sutti et al., 2014). The NASH characteristic features of insulin resistance and oxidative stress both affect the immune system to activate inflammation in the liver. The infiltrated cells have important correlations with cytokine and chemokine production, resulting in the advancement of steatosis, steatohepatitis, and steato-cirrhosis.

3.1.1 Macrophage and Kupffer Cells

Innate immune responses have great potential for amplification of inflammation in
NAFLD liver. CD68-positive macrophage infiltration and hepatic expression of macrophage chemotactic factor 1 (MCP1) are increased, even in NAFL, and have important roles in inflammatory cell recruitment and insulin resistance (Lanthier et al., 2010). MCP1 is a major chemokine inducing the recruitment of leukocytes into the liver during inflammation. These changes may be considered to be an early response by fatty liver. In the liver-infiltrated cells, the CD68-positive cells are comprised of inflammatory monocytes and liver resident macrophages, namely Kupffer cells. Kupffer cells are phagocytes of various cellular, viral, or bacterial components and are a source of hepatic pro-inflammatory and pro-fibrogenic cytokines. It is difficult to distinguish these cell types, although a recent report revealed that they could be distinguished by CD11b positivity expression. Kupffer cells might be the initiator of early inflammatory responses in NAFLD through TNF-α production (Tosello-Trampont et al., 2012).

Cholesterol phagocytosis by Kupffer cells can induce their activation along with TLR4 upregulation (Leroux et al., 2012). Aggregation of erythrocytes in inflammatory hepatic sinusoids can be seen in NASH patients. These erythrocytes often express phosphatidylserine on their surface that could be induced by oxidative stress (Otogawa et al., 2007). Such erythrocytes carry hemoglobin and iron that could be easily taken up by Kupffer cells following activation and oxidative stress induction.

Activated Kupffer cells can produce inflammatory monocyte chemoattractant chemokines such as MCP1 or interferon (IFN)-γ inducible protein (IP-10). IP-10 has been reported as a potential NAFLD biomarker that distinguishes NAFL from NASH (Zhang et al., 2014). Additionally, Zhang et al. revealed that IP-10 could induce several key inflammatory cytokines, such as TNF-α, IL-1β, and MCP-1, and the blockade of IP-10 was protective for steatohepatitis development in a mouse model of NASH. IP-10 might be an important biomarker and candidate treatment target. The chemokine- or cytokine-induced recruitment of monocytes accelerates inflammatory responses and activates HSCs to produce pro-fibrotic factors.

### 3.1.2 Natural Killer (NK) Cells and NKT Cells

NK and NKT cell infiltration is decreased in severe steatosis, and the same phenomenon is observed in a choline-deficient diet-induced mild inflammatory NASH mouse model (Kremer et al., 2010). However, in human advanced fibrotic NASH livers, more NKT cell infiltration occurs than in early fibrotic NASH livers, and NKT cell-deficient mice had less fibrosis than wild-type mice in severe fibrotic NASH model mice fed MCD (Syn et al., 2012). In mild NASH with severe obesity exhibiting KKAy type 2 diabetes mouse model exhibited decreased NKT cell expression and function, especially after high-fat diet supplementation (Yamagata et al., 2013). The function of NK or NKT cells could be different in the degree of obesity, insulin resistance, NAFL, NASH, and advanced NASH. As NAFL and NASH are different diseases, NKT cell function in NAFL and NASH progression is likely to be different.

IL-15 has been identified as a disease preventive cytokine in NAFL but a progression-related cytokine in NASH. IL-15 is an essential survival factor for natural killer (NK) cells, natural killer–like T (NKT) cells, and memory CD8+ T cells (Di Sabatino et al.,...
The role of NKT cells and related IL-15 in NAFLD is likely that they are preventive in the NAFL stage but disease progressive in fibrotic-stage advanced NASH.

### 3.1.3 Hepatic Stellate Cells (HSCs)

HSCs play significant roles in the progression of chronic liver inflammation and fibrosis (Rolo et al., 2012). Excess fatty acid accumulation in hepatocytes induces oxidative stress from mitochondria as well as peroxisomes or microsomes. These cytotoxic ROS and lipid peroxidation products are able to diffuse into the extracellular space, affecting Kupffer cells and HSC. Cellular oxidative stresses from hepatocytes and the direct uptake of free fatty acids or free cholesterol in Kupffer cells activates nuclear-factor kB, which induces synthesis of TNF-α and several proinflammatory cytokines such as IL-6 or IL-8 (Hui et al., 2004). Kupffer cells in patients with NASH produce TGF-β, resulting in HSCs acquiring a fibrogenic myofibroblast-like phenotype.

Exposure of primary HSC or HSC cell lines to H$_2$O$_2$ leads to increased gene expression of ER chaperone BIP binding transmembrane proteins such as inositol requiring enzyme 1 or activating transcription factor 4. ER stress in HSCs results in increased autophagy and HSC activation to fibrogenic status (Hernandez-Gea et al., 2013).

Levels of free cholesterol (but not of cholesterol ester) are increased in HSC in NAFLD, resulting in increased TLR4 protein levels and fibrogenic HSC (Tomita et al., 2013). Free cholesterol can accumulate in fibrogenic HSC, resulting in an increase in TLR4 by suppressing the endosomal-lysosomal degradation pathway of TLR4. The increased expression of TLR4 sensitizes cells to TGF-β–induced activation (Tomita et al., 2013).

As mentioned in section 2.3 of this article, autophagy is involved in steatosis-related ER stress and oxidative stress. In a mouse model of inflamed NASH liver, activated HSC has been shown to be in autophagy defect condition. However, cytoplasmic lipid droplets are maintained and remain quiescent in autophagy-defective HSCs, indicating that oxidative stress-induced ER stress and autophagy are key events in HSC activation (Hernandez-Gea et al., 2012).

### 3.1.4 T Cells

In advanced NASH, increases in CD4(+) and CD8(+) T-cell infiltration and inflammatory cytokines, such as IL-6 or IL-8, have been observed (Gadd et al., 2014). Adaptive immune response–linked CD8(+) T cells usually respond after innate immune cell reactions; however, obese adipose tissue could recruit CD8(+) T cells prior to macrophage accumulation (Nishimura et al., 2009). In an obesity-related NASH-hepatocarcinogenesis model, CD4(+) and CD8(+) T cells increased in the NASH condition with strong local activation marker CD44 and CD69 expression (Wolf et al., 2014). Moreover, significant increases in hepatic NKT cells and regulatory T cells were found in this model. Both NKT cells and CD8(+) T cells were necessary for hepatocarcinogenesis, while CD8(+) T cells did not mediate fatty acid uptake or HSC activation as efficiently as NKT cells. Depletion of CD8(+) T cells reversed liver damage but left cholesterol levels unchanged,
indicating that CD8(+) T cells are involved in liver inflammation rather than modulating lipid metabolism.

3.1.5 Cytokine Profile in NASH

Visceral obesity induces several cytokines including the inflammatory cytokine interleukin-17 (IL-17) (Fabbrini et al., 2013), which induces neutrophil chemokine expression via IL-17 receptor A, which is widely expressed in the liver. Controlling the IL-17–related pathway effectively treats NASH progression in mouse models (Harley et al., 2013). Elevated pre-therapy serum IL-17 levels in patients with HCC correlates with the risk of early recurrence after curative hepatectomy (Wu et al., 2012). Co-cultured HCC cell lines and T cells producing IL-17 in vitro augment the proliferation of HCC cells, suggesting the importance of IL-17 for NASH-related HCC pathogenesis.

Interferon (IFN)-γ–inducible protein (IP-10) is a pro-inflammatory cytokine that recruits inflammatory cells to damaged tissue and is associated with lipotoxicity. As mentioned in section 3.1.1. of this articles, IP-10 could be used as NAFL and NASH differential marker. In chronic hepatitis C, intrahepatic IP-10 levels have been correlated with necroinflammatory changes and fibrosis (Zeremski et al., 2008).

4 Treatment for NASH

Since NAFLD and NASH have emerged as lifestyle-associated diseases, lifestyle intervention is an important approach to their treatment. Healthy and Western-style diets differentiate the risk for NAFLD progression (Oddy et al., 2013). A one-year intensive lifestyle intervention, comprised of dietary modifications and physical activity, improved waist circumference, visceral abdominal fat, blood pressure, insulin resistance, and hepatic fat content in obese patients (Goodpaster et al., 2010). Western-style diets, especially those rich in trans-fatty acids, are powerful inducers of obesity and NAFLD and should be avoided (Neuschwander-Tetri et al., 2012). However, maintained compliance with therapeutic measures such as restrained food consumption is mentally challenging, and the opt-out rate is high (Soetens et al., 2008). Such patients require pharmacological therapies.

4.1 Anti-diabetic Therapy

Because the general characteristics of NASH comprise obesity and insulin resistance, anti-insulin resistance therapy has played a significant role. From this viewpoint, the ability of insulin sensitizing anti-diabetic drugs to treat NASH has been analyzed. Among these drugs, the PPAR-γ agonists pioglitazone and metformin have clinically improved NASH, although no histological improvements were shown in one-year studies (Aithal et al., 2008; Bugianesi et al., 2005).

Metformin increases intracellular levels of AMP after the activation of AMPK, which is a highly conserved heterodimeric serine-threonine kinase that serves as an en-
ERGY SENSOR IN EUKARYOTIC CELLS AND BRIDGES METABOLISM TO CARCINogensIS (Hardie et al., 1998). THE ACTIVATION OF AMPK SUPPRESSES CELL PROLIFERATION IN NON-MALIGNANT AND MALGignant cells via regulation of the cell cycle, apoptosis, autophagy, and the inhibition of fatty acid synthesis (Motoshima et al., 2006). Phospho (p)-AMPK IS DOWNREGULATED IN HCC tissues from patients, and low p-AMPK expression correlates with a poor prognosis, indicating the importance of AMPK signaling in HCC (Zheng et al., 2013). Adding metformin to hepatoma cell lines results in AMPK activation as well as dose- and time-dependent growth inhibition. Metformin might be a good candidate regarding NASH and NASH-related HCC prevention.

Glucagon like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are both incretins, a group of gastrointestinal hormones that cause increased insulin release from pancreatic β cells, and they represent good potential targets for NASH treatment. A GLP-1 receptor agonist analogue improved metabolic, biochemical, and histopathological indices of NASH in mice by restoring hepatic lipid oxidation (Svegliati-Baroni et al., 2011). Administration of a GLP-1 receptor agonist in type 2 diabetic patients with NAFLD caused a reduction in intrahepatic lipid content that correlated with diabetic control (Cuthbertson et al., 2012). Dipeptidylpeptidase-IV (DPP-IV) degrades GLP-1 and GIP, and thus the inhibition of DPP-IV extends the half-life of endogenous GLP-1 and GIP, resulting in diabetic control. The long-term administration of a DPP-IV inhibitor has reduced liver fat content in animals with diet-induced hepatic steatosis and insulin resistance (Kern et al., 2012).

A recently available anti-diabetic agent, sodium-glucose cotransporter 2 (SGLT2) inhibitor, provides a new treatment approach by lowering blood glucose levels via inhibition of glucose reabsorption from the proximal renal tubule (Kim & Babu, 2012). In type 2 diabetic patients, SGLT2 inhibitor administration induces significant body weight decrease and increased pancreatic β cell function, suggesting that it is a potential treatment for insulin resistance-related NASH (Rosenstock et al., 2012). One of the SGLT2 inhibitors, ipragliflozin, prevented hepatic triglyceride accumulation and large lipid droplet formation (Hayashizaki-Someya et al., 2015). Large clinical trials are needed to evaluate the effect of SGLT2 inhibitors on human NAFLD.

4.2 Anti-oxidant Therapy

The representative antioxidant vitamin E improved the non-alcoholic fatty liver disease activity scores (NAS) for clinical and histological activity within two years but increased insulin resistance and plasma triglyceride levels (Sanyal et al., 2010). However, the recovery of fibrosis progression was not proven (Hoofnagle et al., 2013). Controversy surrounds the value of ROS-scavenging agents because ROS have essential functions for life. Scavengers of ROS consistently exert effective chemical activities in vitro, but often not in vivo (Bast & Haenen, 2013). Scavenging of ROS is considered effective in preventing cancer development, while recent findings indicate that ROS also contribute to the progression of cancer (Watson, 2013). Stem cell–like cancer cells that express a CD44 variant have a powerful antioxidative phenotype that protects them from oxidative stress and prevents their apoptosis (Yae et al., 2012). The American Association for the
Study of Liver Disease recommends the daily administration of 800 IU of vitamin E, which is higher than that usually administered to treat NASH (Chalasani et al., 2012). This recommendation is based on a two-year randomized study of NASH treatment that resulted in improved ALT and histological activities (Sanyal et al., 2010). Further investigations of longer durations are required to determine the effects of vitamin E on NASH, including hepatocarcinogenesis.

L-carnitine is a precursor of carnitine-palmitoyltransferase 1, the rate-limiting enzyme for mitochondrial β-oxidation that affects mitochondrial function. Any deficiency in the mitochondrial carnitine-dependent transport system results in curtailed fatty acid oxidation. L-carnitine supplementation reduces TNF-α, liver function parameters, plasma glucose levels and histological scores (Malaguarnera et al., 2010).

Dietary intake of tomatoes has been reported to reduce the risk for human cancers (Ip & Wang, 2014). Tomatoes include vitamins A and C and phytochemicals such as carotenoids or flavonoids. Lycopene is the most abundant carotenoid found in tomato, tomato products, and other red fruits. Intake of lycopene inhibits NASH-promoted rat hepatic pre-neoplastic lesions (Ip et al., 2013). Fresh tomato contains 9-oxo-10,12-octadecadienoic acid (9-oxo-ODA), which acts as a PPARα agonist to improve NASH in mice. In addition, processed tomato products such as tomato juice, but not fresh tomato, contains 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), an isomer of 9-oxo-ODA that improves lipid and carbohydrate metabolism disorders in NAFLD model mice (Kim et al., 2012). Although clinical trials remain necessary, plasma carotenoid levels in NASH patients have been shown to be low, thus suggesting the possibility of a role for tomato intake in NASH prevention.

Molecular hydrogen has been shown to have powerful antioxidant effects with unique features (Ohsawa et al., 2007). In cultured cells, hydrogen scavenges hydroxyl radicals, but not superoxide, hydrogen peroxide (H₂O₂) or nitric oxide (NO), and prevents the decline in mitochondrial membrane potential and the subsequent decrease in cellular ATP synthesis, consistent with antioxidative effects. Kawai et al. reported that drinking hydrogen-rich water has favorable effects in NASH models (Kawai et al., 2012). Administration of hydrogen-rich water or the antioxidant pioglitazone reduced plasma transaminase levels, histological NAS, hepatic TNF-α, IL-6, fatty acid synthesis-related gene expression, and the oxidative stress biomarker 8-OHdG in the livers of MCD diet-induced NASH models.

As mentioned above, new antioxidants may be effective in controlling NAFLD progression. However, many investigations into the effects of antioxidants on diseases associated with oxidative stress have been disappointing, and the effects of newer antioxidants could prove similar. More basic and clinical experimentation into this novel potential treatment option is required.

4.3 Other Treatment Possibilities including Immune-targeted Therapy

Pentoxifylline is a methylxanthine derivative that increases red blood cell flexibility, reduces blood viscosity, and decreases platelet aggregation. In addition, pentoxifylline
suppresses TNF-α gene transcription and is a hydroxyl and peroxyl radical scavenger with anti-oxidative effects. A randomized controlled trial has proven that pentoxifylline decreases free-radical–mediated lipid oxidation and improves clinical and histological NASH (Zein et al., 2012; Zein et al., 2011).

TNF-α is one of the main cytokines involved in adipocyte-related inflammation including NASH. Powerful anti-TNF-α agents such as infliximab (a chimeric monoclonal antibody), adalimumab (a human monoclonal antibody), and etanercept (a fusion protein) have severe side effects, such as increased risk for tuberculosis, that render them unacceptable as therapies for NAFLD (Ford & Peyrin-Biroulet, 2013). The anti-oxidative agent pentoxifylline also has an anti-TNF-α function that is partially involved in its favorable effects on NASH.

Lipid-lowering drugs such as statins can also improve ALT and radiological steatosis in hyperlipidemic patients with NAFLD; however, histological improvements are not evident (Nelson et al., 2009). Ezetimibe is a Niemann-Pick C1-like protein inhibitor that can reduce the intestinal accumulation of free cholesterol. Ezetimibe showed histological NAS improvement in mice and in 10 patients with NASH, indicating the need for a larger randomized controlled trial (Deushi et al., 2007; Yoneda et al., 2010).

Ursodeoxycholic acid is also reportedly effective in some instances. Several randomized controlled trials have found improvements in ALT but not in liver histology, even at high doses (Leuschner et al., 2010). The combination of ursodeoxycholic acid and vitamin E showed improved ALT and histological NAS scores (Pietu et al., 2012).

A pro-inflammatory intestinal microbiome has been identified in mice and in patients with NASH. Probiotics, such as butyrate-producing agents, reduce hepatic triglyceride content and induce anti-oxidative enzymes that help to prevent the progression of NASH to hepatocellular carcinoma (Endo et al., 2013).

5 Conclusion

Inflammation is a representative finding in NASH pathogenesis. The immune system is an important regulator of the inflammatory process. While NAFL and NASH both present with liver steatosis, these diseases differ in their lipid deposition and infiltrating immune cell function. Thus, differentiating non-progressive NAFL and progressive NASH is a fundamentally important issue that remains to be resolved. Innate immune responses and adaptive immune responses are both involved in NASH-hepatocarcinogenesis mechanisms, while the important cell types differ in different clinical conditions. The treatment approach should be different in different clinical condition. Although vitamin E administration is the only effective treatment currently known, a patient follow-up of longer duration is necessary, as many studies have indicated that caution is required to avoid the potential life-threatening effects of long-term antioxidant therapy. Optimization of treatment protocols warrants further investigation, as does the search for novel therapeutic strategies.
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