REVIEW

Gut and Liver, Vol. 6, No. 2, April 2012, pp. 149-171

NASH is an Inflammatory Disorder: Pathogenic, Prognostic and Therapeutic Implications

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While non-alcoholic fatty liver disease (NAFLD) is highly prevalent (15% to 45%) in modern societies, only 10% to 25% of cases develop hepatic fibrosis leading to cirrhosis, end-stage liver disease or hepatocellular carcinoma. Apart from pre-existing fibrosis, the strongest predictor of fibrotic progression in NAFLD is steatohepatitis or non-alcoholic steatohepatitis (NASH). The critical features other than steatosis are hepatocellular degeneration (ballooning, Mallory hyaline) and mixed inflammatory cell infiltration. While much is understood about the relationship of steatosis to metabolic factors (over-nutrition, insulin resistance, hyperglycemia, metabolic syndrome, hypoadiponectinemia), less is known about inflammatory recruitment, despite its importance for the perpetuation of liver injury and fibrogenesis. In this review, we present evidence that liver inflammation has prognostic significance in NAFLD. We then consider the origins and components of liver inflammation in NASH. Hepatocytes injured by toxic lipid molecules (lipotoxicity) play a central role in the recruitment of innate immunity involving Toll-like receptors (TLRs), Kupffer cells (KCs), lymphocytes and neutrophils and possibly inflammasome. The key pro-inflammatory signaling pathways in NASH are nuclear factor-kappa B (NF-κB) and c-Jun *N*-terminal kinase (JNK). The downstream effectors include adhesion molecules, chemokines, cytokines and the activation of cell death pathways leading to apoptosis. The upstream activators of NF-κB and JNK are more contentious and may depend on the experimental model used. TLRs are strong contenders. It remains possible that inflammation in NASH originates outside the liver and in the gut *microbiota* that prime KC/TLR responses, inflamed adipose tissue and circulating inflammatory cells. We briefly review these mechanistic considerations and project their implications for the effective treatment of NASH. **(Gut Liver 2012;6:149-171)**

Key Words: Non-alcoholic fatty liver disease; Hepatic fibrosis; Non-alcoholic steatohepatitis

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the commonest form of liver disease in all regions of the world with modern industrialised economies, including Korea and many other Asian countries.[1-6] Patients usually present without symptoms or clinical features are non-specific. Instead, liver abnormalities are found incidentally by hepatic imaging, particularly ultrasonography, and/or there are raised liver enzymes (alanine aminotransferase [ALT] and gamma-glutamyltranspeptidase).[7-9] The diagnosis of NAFLD requires exclusion of other disorders, particularly viral hepatitis, significant alcohol intake, and exposure to potentially hepatotoxic medications. By agreements such as the Asia-Pacific Guidelines on NAFLD,[6] the term NAFLD is now retained for cases of fatty liver associated with metabolic complications of over-nutrition, usually with central obesity and overweight.

We and others have stressed that NAFLD is closely allied to pre-diabetes and metabolic syndrome.[3,10,11] As recently reviewed,[3] the evidence for this includes the strong risk factors for NAFLD posed by obesity, insulin resistance, glucose intolerance and one or more components of metabolic syndrome, and the corresponding strong risk for onset of type 2 diabetes and cardiovascular disease/events conferred by a fatty liver.[12-16] Community based studies from Korea, Japan and other areas in North Asia have been highly informative for understanding that NAFLD is not so much a "Western disease" as the inevitable result of changes in prosperity and lifestyle that have increased the prevalence of overweight/obesity, insulin resistance, type 2 diabetes and cardiovascular risk factors (clustered as metabolic syndrome).[3,4] Thus the community prevalence of NAFLD in

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Received on January 4, 2012. Accepted on January 18, 2012.
pISSN 1976-2283 eISSN 2005-1212 http://dx.doi.org/10.5009/gnl.2012.6.2.149

this region increased from less than 10% in the 1980s, through 10% to 20% in the 1990s, to current rates of 15% to 30% or higher.[4,17]

The known ethnic differences in metabolic complications of over-nutrition, such as insulin resistance, diabetes, metabolic syndrome and hypoadiponectinemia, are also consistent with the proposition that, like them, NAFLD is a genetic disorder.[3,18,19] Thus, an encompassing concept for NAFLD pathogenesis is that it represents the outcome of genetically determined interactions between a changing environment and a susceptible host. In this case, the environmental factors include too much energy intake, particularly in the form of cheap, highly processed simple carbohydrates and saturated fats, and reduced levels of physical fitness resulting from sedentary lifestyles.[20,21] Of particular interest to the present review, one prevalent genetic polymorphism predisposing to steatosis in overweight persons of European or Hispanic ancestry, *PNPLA3*, does not operate by increasing the risks of diabetes or metabolic syndrome.[18,22-25] Instead, it correlates with serum ALT levels,[26] reflecting liver injury or inflammation, and with more severely fibrotic liver disease in both NAFLD/non-alcoholic steatohepatitis (NASH) and alcoholic cirrhosis.[27,28] This point emphasises that not all cases of NAFLD have the same implications for liver disease.

NAFLD embraces a pathological spectrum of liver disease, from cases of steatosis with virtually no evidence of hepatocellular injury or liver inflammation, often referred to as simple steatosis or "not NASH," through steatohepatitis (NASH), to cases with cirrhosis.[29-31] The latter are often complicated by portal hypertension and hepatic decompensation, and occasionally present with hepatocellular carcinoma (HCC).[32] At this late stage, steatosis and liver inflammation may both have resolved; they are cases of "cryptogenic cirrhosis." As discussed next, natural history and clinical outcome studies based on community and liver clinic cohorts indicate a nearly 2-fold increase in standardized mortality rates among persons with NAFLD.[33-37] Further, while cardiovascular disease and common cancers remain the two most common causes of death, liver-related mortality ranks the third most common, as compared to 13th in the general community.[36] A key question emerges: what aspects of liver pathology, and what disease mechanisms, account for progression of NAFLD to cirrhosis and its fatal complications?

WHICH ASPECTS OF NAFLD PATHOLOGY HAVE PROGNOSTIC AND MANAGEMENT IMPLICATIONS

1. Fibrotic severity

The observation that histologic characteristics are useful in predicting the outcome of patients with NAFLD is best exemplified for patients at either end of the pathological spectrum. At one end, individuals with only hepatic steatosis (simple steatosis) infrequently show signs of any histologic progression, and are not at significant long-term risk of liver-related death.[33,34,38] By contrast, those with advanced hepatic fibrosis (bridging fibrosis [F3] and/or cirrhosis [F4]) are likely, in time, to experience liver-related complications (ascites, variceal bleeding, and/or HCC).[35-37] While cardiovascular disease and cancer head the list of causes of death, 7- to 10-year liver-related mortality (12% to 25%) ranks third overall.[2,36,37] In fact, the outcome of patients with advanced NAFLD (Child-Pugh B and C) is similar to that of individuals with hepatitis C virus-related cirrhosis.[35,37]

In reaching these general conclusions, certain assumptions are implied. First, the necessity for histologic appraisal is problematic because liver biopsies are performed less often outside research studies and clinical trials due to patient and clinician perceptions that the result will not influence management, and the concerns about biopsy-related complications. While non-invasive assessment of hepatic necroinflammatory activity and hepatic fibrosis (serum biomarkers, transient elastography) is increasingly advocated,[39-44] it is most reliable at either end of the clinical spectrum of severity (mild, severe), when histology is most predictable. It remains suboptimal in the substantial number of patients in patients with mild-moderate hepatic fibrosis (F1, F2), among whom liver disease may progress.[34]

Second, in patients with only hepatic steatosis there can be changes in host characteristics over time, such as increasing body weight or worsening insulin resistance and/or development of diabetes, and baseline steatosis and necroinflammatory severity have not been correlated with such progression of metabolic disease.[12,34] These considerations not withstanding, most gastroenterologists and hepatologist would generally reassure patients with isolated hepatic steatosis about their liver prognosis, but recommend primary care follow-up of cardiovascular risk factors and lifestyle interventions to address these. Conversely patients with advanced hepatic fibrosis should enter a more rigorous liver follow-up protocol.

2. Presence of NASH (versus "not NASH")

Current uncertainty about how "progressive' this condition really is at least partly stems from the use of differing operational definitions for NASH.[45,46] Thus, NASH has been variously defined to include cases with hepatic steatosis and lobular inflammation (regardless of hepatic fibrosis),[29] hepatic steatosis with lobular inflammation and ballooning of hepatocytes with or without fibrosis,[33,47,48] or as separate scoring systems for "activity" (the NAFLD activity score, which assigns numerical scores to steatosis, lobular inflammation and ballooning and fibrosis (the latter usually F0-F4).[30] The Brunt system[29] was developed by correlating histologic changes with serum aminotransferases (AT) as a measure of hepatic necroinflammatory activity, and not with clinical outcome, whereas the scoring system proposal by Kleiner *et al*.[30] was never intended for diagnosis but was to be used as a tool for assessing serial liver biopsies in clinical trials. The premise has been that small changes could be identified more clearly and reliably by assigning numerical values than by

descriptive remarks.[45]

A head-to-head comparison of these different histologic classification systems has recently been reported,[47] and an editorial based on additional data from Korea reached similar conclusions.[46] Both authors recommended the following. First, for routine clinical use (i.e., for diagnosis), an indication that there is or is not steatohepatitis is probably sufficient, with an intermediate category of "borderline" steatohepatitis where there is some uncertainity. Second, among the various components of steatohepatitis, ballooning degeneration of hepatocytes is broadly favoured for defining NASH.[45-47] In one study, ballooning degeneration was found to correlate with liver-related mortality, but only by univariate analysis.[47]

In summary, the combination of hepatic fat and lobular inflammation is now regarded as insufficient for a diagnosis of NASH. However, other features such as the presence of "more than mild" portal inflammation,[29,33] or the presence of panacinar steatosis (as compared to isolated zone 3 steatosis),[48] have also been associated with advanced hepatic fibrosis. The latter is the best histologic predictor of liver-related mortality irrespective of the degree of steatohepatitis.[49] As expected from the earlier discussion, classification systems incorporating hepatic fibrosis in the definition of NASH correlate well with liver-related mortality,[33,47] while systems that do not are not predictive of future outcome.[29,30] It needs to be stated, however, that the latter systems do include staging for hepatic fibrosis, but do not require its presence for the definition of NASH.

3. Extent of necroinflammatory activity

Having established that fibrotic NASH is all that matters, is there any value in assessing the degree of necroinflammatory activity? It would be if it could be determined that the grade of inflammation is a predictor of future hepatic fibrosis (in the case of liver outcomes) or metabolic syndrome-related disorders (in the case of overall mortality). Some evidence supports this view,[50] although negative studies have also been reported.[12] A systematic review showed clearly that age and inflammation on the initial biopsy (hazard ratio, 2.5) were the main indepen-

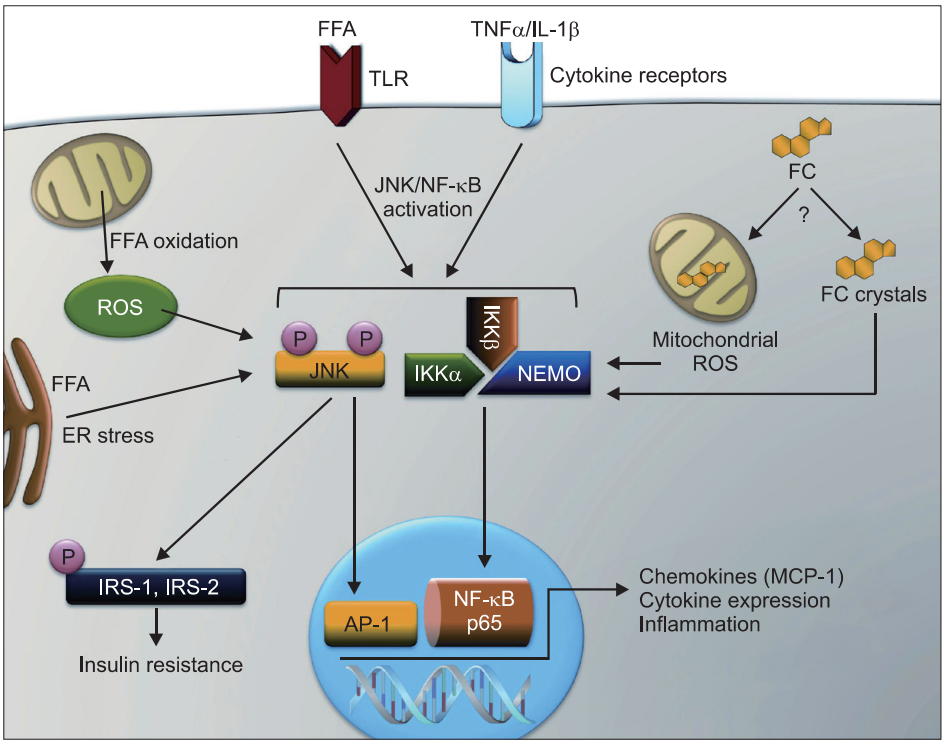

Fig. 1. Excess lipid accumulation activates inflammatory pathways and induces insulin resistance. Extracellular free fatty acids (FFA) activate toll-like receptors (TLR), causing downstream activation of c-Jun N-terminal kinase (JNK) and IκB kinase (IKK) complex (composed of IKKα, IKKβ and NF-κB essential modulator [NEMO]). IKK heterotrimeric holocomplex catalyzes downstream activation of nuclear factor-kappa B (NF-κB), allowing p65 (also known as RELA), a proinflammatory transcription factor, to enter the nucleus where it induces transcriptional expression of multiple proinflammatory chemokines (e.g., macrophage chemotactic protein 1 [MCP-1]), cytokines, and adhesion molecules (e.g., vascular cell adhesion molecule-1). Once activated, JNK activates c-Jun which is involved with hepatocellular cell death, and via formation of heterodimeric c-Jun:c-Fos forms the pro-inflammatory transcription factor, activator protein 1 (AP-1). In addition to TLR activation, some intracellular lipid molecules (Table 2) may result in JNK/NF-κB activation by formation of reactive oxygen species (ROS); ROS may arise from excessive β-oxidation of FFA, uncoupling of oxidative phosphorylation and mitochondrial damage caused by free cholesterol (FC) accumulation and crystallization. Alternatively, some intracellular lipids may induce endoplasmic reticulum (ER) stress, leading to JNK/NF-κB p65 activation (see Fig. 3 for more details). JNK activation can also phosphorylate insulin receptor substrates (IRS)-1 and -2, which by blocking insulin receptor signal transduction leads to insulin resistance.
TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β.

dent risk factors for fibrosis progression.[51] These findings are not surprising because clinicians are familiar with the need to 'damp down' hepatic inflammation in chronic viral hepatitis B or C and autoimmune hepatitis in order to achieve a favourable clinical outcome by preventing or reversing progression of hepatic fibrosis.

4. Liver histology and cardiovascular outcomes

After establishing that NASH is the hepatic component of the metabolic (insulin resistance) syndrome,[2,3,52,53] it was to be anticipated that morbidity and mortality from cardiovascular disease would be highlighted in long-term studies of NAFLD. Surrogate markers of atherosclerosis (e.g., carotid intima-media thickness) are present even in adolescents with NAFLD, and clinical endpoints such as deaths from myocardial infarction/need for coronary revascularisation have been documented in several natural history studies of NAFLD.[36,37] The concept that fatty liver may also drive the inflammatory cascade of atherosclerosis is now gaining acceptance. There is some evidence that individuals with NASH have a worse atherogenic profile,[54] and are more likely to have overt cardiovascular disease than patients with hepatic steatosis alone.[12] In summary, based on present somewhat limited evidence, it can be concluded that ongoing hepatic necroinflammatory activity in patients with NAFLD increases the risk of future cardiovascular disease, and confers a higher risk of unfavourable liver-related outcomes by promoting development of hepatic fibrosis.

WHAT ARE THE ORIGINS OF LIVER INFLAMMATION IN NASH?

Inflammation is a critical response to tissue damage or infection in which secreted mediators such as cytokines, chemokines and eicosanoids coordinate cellular defences and tissue repair. Since this is generally a whole body response, it is possible that inflammation affecting or infiltrating the liver in NASH may originate outside the liver. One site of interest is the adipose, particularly visceral adipose which is expanded in NAFLD.[3,55,56] Visceral adipose is inherently pro-inflammatory,[57-59] but inflammation also occurs in stressed, de-differentiated subcutaneous adipose tissue in obesity. Important consequences include the release of macrophage chemokines and cytokines, notably macrophage chemotactic protein 1 (MCP-1) and tumor necrosis factor-α (TNF-α). A recent time course study showed that in mice fed a high fat (HF), cholesterol-enriched diet, macrophage and cytokine transcripts were up-regulated in adipose at 6-16 weeks, before their appearance in liver from 16 to 26 weeks.[60] Key inflammatory signals, including interleukin (IL)-1β, IL-1 receptor antagonist, TNF-α and CD11b+ and CD11c+ macrophages, were particularly associated with liver inflammation. Lanthier *et al*.[61] have likewise shown that macrophage inflammation of adipose is responsible for the early stages of both hepatic and peripheral insulin resistance in HF-fed mice, but deletion of adipose macrophages cannot reverse the later phase once liver inflammation is established.

In other research, a consistent increase in serum MCP-1 has been noted to be part of the systemic and adipose inflammatory state in metabolic syndrome.[62-67] Adipocyte-derived MCP-1 (also known as CCL-2) stimulates recruitment of chemokine (C-C motif) receptor 2 (CCR-2)-expressing macrophages into adipose. MCP-1 and its cognate receptor, CCR2, are potentially important molecules in NASH since, like NF-κB and c-Jun *N*-terminal kinase (JNK), they unite the inflammatory response with insulin resistance,[68,69] as reviewed by Maher *et al*.[70] and depicted in Fig. 1. MCP-1 also stimulates lipogenesis in the liver.[71] In this way, adipose inflammation can exacerbate steatosis and connect to innate inflammatory responses within the liver.

Inflammation and de-differentiation of adipose also alters release of the key insulin-sensitizing and anti-inflammatory adipokine, adiponectin. Adiponectin blocks elaboration and release of TNF-α.[72,73] Serum adiponectin levels fall in metabolic syndrome and type 2 diabetes, while low serum adiponectin levels in NAFLD are inversely related to steatosis severity, and in some studies to the presence of NASH.[72-75] Key signalling pathways that explain some of the connections between hepatic inflammation and insulin resistance include the IκB kinases (IKK)/nuclear factor-kappaB (NF-κB) and JNK, as discussed later and reviewed.[70]

In addition to macrophages recruited to inflamed adipose, circulating lymphocytes and macrophages also contribute to systemic inflammation in metabolic syndrome. For instance, raised serum cholesterol levels are associated with increased secretory function of circulating lymphocytes.[76] Conversely, treatment with simvastatin and/or ezetimibe reduced plasma levels of highly-sensitivity C-reactive protein and intercellular adhesion molecule 1. Statin or combination treatment also significantly reduced lymphocyte release of TNF-α, interferon-gamma (IFN-γ) and IL-2, an anti-inflammatory effect that was most marked for patients with insulin resistance.[76]

Another tissue compartment that could contribute to liver inflammation in NASH is the gastrointestinal tract, more specifically, the gut *microbiota*. There is evidence of altered gut flora in obesity,[77] and of increased mucosal permeability in NASH.[77-80] Further, in some animal models sterilisation of gut contents or their modification by probiotic administration to suppress endotoxin production altered liver inflammation or liver injury,[81] albeit the models do not conform to what we now categorize as NASH. The topic of intestinal-liver interactions in obesity and fatty liver disease has been reviewed elsewhere,[70,80,82,83] and will be mentioned later in respect to activation of innate immunity in the liver.

Notwithstanding the potential relevance of adipose inflammation,[84] circulating chemokines, cytokines and inflammatory cells, and the gut *microbiota* to NASH pathogenesis, the per-

spective we will take in this review is that one may not need to look much further than at the liver itself to understand the origins of inflammation in NASH.

LIVER CELL TYPES AND INFLAMMATION IN NASH

The liver is comprised of several cell types, each of which could potentially activate or be influenced by hepatic inflammation. Hepatocytes comprise 60% to 80% of all liver cells and conduct the metabolic, biosynthetic, detoxification and biliary secretory functions of the liver. In fatty liver, hepatocytes stain positive for triacylglycerides (TG), and in NASH the defining pathological element is hepatocellular injury, evident as ballooning, Mallory bodies and apoptosis. Among other liver cell types, Kupffer cells (KCs), the liver's resident macrophage population, natural killer (NK) cells, NK T cells, T cells, sinusoidal endothelial cells (SECs) and hepatic stellate cells (HSCs) can each play pro-inflammatory roles.[85,86]

Several possible mechanisms activate pro-inflammatory pathways in livers with NASH, leading to release of chemokines, cytokines and other pro-inflammatory molecules, as summarised in Table 1. Chemokine release is particularly responsible for recruitment of infiltrating monocyte-derived macrophages, and neutrophils, which together with lymphocytes comprise the mixed cell type inflammatory infiltrate in NASH. Oxidative stress and necrosis can provoke a neutrophil inflammatory response.[87] In general, pro-inflammatory signalling in NASH is mediated by activation of innate immune mechanisms. These may be primed by gut-derived endotoxin, but there is increasing evidence that this is in response to lipotoxicity and/or molecules released by stressed hepatocytes (discussed below).

HEPATOCYTE STRESSES

1. Lipotoxicity

The appearance of simple steatosis in the majority of cases

Table 1. Some Key Pro-Inflammatory Molecules in Non-Alcoholic Steatohepatitis (NASH)

Molecule	Category	Activated by	Actions	Evidence for involvement in NASH*
IKK	Protein kinase (signalling molecule)	ROS, ER stress, cytokine/growth factor receptors, TLRs (Fig. 1)	Phosphorylates IκB, leading to NF-κB activation; can cause insulin resistance	Consistent activation of NF-κB in human NASH and experimental models; blockade modifies experimental steatohepatitis
NF-κB	Transcription factor (signalling molecule)	IKK, Myd88, ER stress (Figs 1, 3 and 5)	Up-regulates multiple pro-inflammatory molecules	Consistent activation in human NASH and all experimental models; blockade modifies experimental steatohepatitis (multiple studies)
JNK	Protein kinase (signalling molecule)	ROS, cytokine/growth factor receptors, TLRs (Figs 1 and 5); saturated fatty acids, FC, lysophosphatidyl choline	Mitochondrial cell death pathway; via AP-1 (c-jun:c-fos) multiple pro-inflammatory molecules; causes insulin resistance	Consistent activation in human NASH and all experimental models; blockade modifes MCD steatohepatitis; lowering hepatic FC abolishes hepatocyte JNK activation and liver inflammation/apoptosis in *foz/foz* mice
MCP-1	Chemokine	NF-κB; may arise from adipose (visceral) and liver	Recruits CD11b macrophages; lipogenesis (insulin resistance)	Circulating levels rise in multiple models. One of several factors that may connect metabolic responses (lipogenesis, insulin resistance) to inflammatory recruitment in NASH
CCR-2	Chemokine receptor (for MCP-1)	NF-κB	Part of macrophage recruitment	Tissue expression increased in several models
MIP-1	Chemokine	NF-κB	Neutrophil (PMN) recruitment	Increased in experimental models
TNF-α	Cytokine	NF-κB, AP-1	Cytolytic (but not to NF-κB-expressing normal hepatocytes); activates neutrophils; indirectly pro-fibrotic; causes insulin resistance (via IKK and JNK); opposes adiponectin secretion by adipose	Circulating levels increase in obesity but are similar with simple steatosis and NASH; experimental evidence conflicting (see text): no change in fatty liver phenotype in absence of TNF-α or its type 1 receptor (3 studies), but 2 others (MCD model) found less inflammation or fibrosis

Table 1. Continued

Molecule	Category	Activated by	Actions	Evidence for involvement in NASH*
IL-1β	Cytokine	NF-κB, AP-1; inflammasome-mediated activation of caspase 1 (cleaves pro-interleukin 1)	Similar to TNF-α	Increased in some models; pathogenic involvement less clear
IL-18, IL-33	Cytokines	Often coupled to IL-1β reflecting inflammasome activation		As above
IL-6	Cytokines	NF-κB, AP-1	Stat3 activation; further chemokine and cytokine release	Specific roles less clear in NASH vs not-NASH NAFLD
IFN-γ	Cytokine	TLRs via IRFs (Fig. 5)	Lymphocyte recruitment	
ROS	Cause oxidative stress	Mitochondrial uncoupling; lipid peroxidation (CYPs2E1 and 4A); peroxisomes; PMNs and KCs/macrophages	NF-κB activation via IKK; JNK activation; HMGB1/TLR signalling; other effects on chemokines	Protective role of antioxidant vitamin E in some (not all) clinical trials and in MCD model; protective efficacy of anti-oxidant heme oxygenase-1 experimentally
COX-2	Eicosanoid synthetic enzyme	NF-κB, AP-1; possibly cytokines	Synthesis of pro-inflammatory eicosanoids	Pathogenic role in MCD model
ICAM, VCAM	Adhesion molecules	NF-κB, AP-1; possibly cytokines	Promote inflammatory recruitment to liver	Up-regulated in several models; pathogenic roles unclear

IKK, IκB kinase; ROS, reactive oxygen species; Myd88, myeloid differentiation primary response gene 88; ER, endoplasmic reticulum; TLRs, toll-like receptors; NF-κB, nuclear factor-kappa B; JNK, c-Jun N-terminal kinase; FC, free cholesterol; AP-1, activator protein 1; MCD, methionine and choline deficiency; MCP-1, macrophage chemotactic protein 1; CCR-2, chemokine (C-C motif) receptor 2; MIP-1, macrophage inflammatory protein 1; PMN, polymorphonuclear neutrophils; TNF-α, tumor necrosis factor-α; NAFLD, non-alcoholic fatty liver disease; IFN-γ, interferon-gamma; IRFs, interferon-regulatory factors; CYPs2E1, cytochromes P450 2E1; KCs, Kupffer cells; HMGB1, high mobility gel box 1; COX-2, cyclooxygenase 2; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.
*for further details and references, see text.

indicates that fatty livers are not necessarily pro-inflammatory. However, it now seems likely that the steatotic hepatocytes in NASH contain excess lipid molecules other than TG, and there is mounting evidence that such non-TG lipid molecules are implicated in the pathogenesis of NASH by the process of lipotoxicity.[3,88-92] Conversely, formation of TG may actually be a cytoprotective mechanism in liver.[89,90] Candidate lipotoxic molecules in NASH have been reviewed;[90,92,93] they are summarized in Table 2.

Lipidomic analyses of human fatty livers have identified free cholesterol (FC) but not free fatty acids (FFA), diacylglycerides (DAG) or ceramide among the potential lipotoxic molecules that accumulate selectively in NASH but not in "not NASH' NAFLD livers.[84,91,93-95] Lysophosphatidylcholine has also been implicated in a small study.[95] Another consistent feature is depletion of very long chain polyunsaturated fatty acids (PUFA); the potential relevance could be impaired production of hepatoprotective eicosanoids. Consistent with this proposal, the plasma lipidomic signature of NASH indicates over-production of proinflammatory (15-hydroxyeicosatetraenoic acid) rather than anti-inflammatory products of lipooxygenase.[96]

Some potential lipotoxic lipid species implicated in NASH have been explored experimentally, particularly saturated FFA and FC, but also (mostly in dietary studies) PUFA,[97,98] sucrose,[99] and fructose.[100] Such studies demonstrate the unequivocal potential of such lipid molecules to kill cells of hepatocyte lineage, by directly or indirectly activating JNK and the mitochondrial/lysosomal cell death pathway,[101] and also to stimulate pro-inflammatory signalling via NF-κB and JNK/activator protein 1 (AP-1), as discussed later. In general, saturated long chain fatty acids (such as palmitic and stearic acids) are more toxic than mono-unsaturated FFA.[102-105] There are also data that the effects of palmitic acid may be exerted via formation of lysophosphatidylcholine,[95,106] or via reactive oxygen species (ROS),[107] or endoplasmic reticulum (ER) stress.[105] To date, however, most such studies have been in immortalised cell lines (typically human HCC cells) whose biology differs from well-differentiated hepatocytes and the intact liver (Table 2).

Alternatively, investigators have used animal models whose pathology may resemble NASH but the pathogenesis does not involve obesity, insulin resistance and hypoadiponectinemia.[108,109] For example, Marí *et al*.[110] have elegantly demon-

Table 2. Lipids Implicated (or Not) in Lipotoxicity to the Liver and Hepatocytes

Lipid type*	Accumulation discriminates NASH from "not NASH" liver pathology	Comments: evidence of liver lipotoxicity
TG	No (clinical samples, experimental models)	Does not cause tissue injury or inflammation/fibrosis; TG formation may be protective; role in hepatic insulin resistance controversial
DAG	No (fewer data)	Potential pro-inflammatory pathway (via protein kinase C activation); favoured role in mediating insulin resistance
FFA (long chain), saturated	No (clinical samples, lipidomic readouts of experimental models)	Palmitic acid activates JNK and causes lipoapoptosis in HCC cells and primary hepatocytes, possibly via formation of lysophosphatidyl-choline or ROS; in some animal models, saturated (or trans) fat in diet worsens insulin resistance and liver pathology; blockade of TG formation causes FFA accumulation and worse inflammation/fibrosis
FC	Yes (2 human studies; several metabolic syndrome models in mice, rats and opossum)	Yes; activates JNK, at least in macrophages; depletes mitochondrial GSH rendering hepatocytes susceptible to TNF-α or Fas-mediated cell death
Total cholesterol (mostly CE)	Less clear-cut differences	Formation of CEs may play similar role as TG formation, countering lipotoxic effects of FC and FFA (but this has not been demonstrated experimentally)
Ceramide	No (several studies)	Favoured role in some neurotoxicities, but no evidence for role in liver lipotoxicity
Lysophosphatidyl choline	Unclear (one small study with little information on disease phenotype)	Causes lipoapoptosis to primary hepatocytes/HCC cell lines (and see palmitic acid)
Other: e.g., mono-acylglycerides, long chain FA CoA esters	No (few informative data)	Potential implication as mediating insulin resistance

NASH, non-alcoholic steatohepatitis; TG, triglyceride; DAG, di-acylglycerides; FFA, free fatty acids; JNK, c-Jun N-terminal kinase; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; TG, triacylglycerides; FC, free cholesterol; GSH, glutathione; TNF-α, tumor necrosis factor-α; CE, cholesterol ester; FA, fatty acyl.
*for further comments and references, please refer to the text.

strated how FC accumulates in livers of animals fed a high (2%) cholesterol, choline-deficient diet or high cholesterol/cholate-supplemented diet, sensitizing hepatocytes prepared from such livers to apoptosis via the mitochondrial cell death pathway. In this work, cholesterol-loaded hepatocytes were also exquisitely sensitive to TNF-α-mediated cytolysis, despite unchanged NF-κB expression, which usually confers hepatoprotection to hepatocytes, unless they are depleted of reduced glutathione (GSH).[111] Cholesterol loading appears to deplete mitochondrial GSH, rendering cells susceptible to apoptosis via cytokine death receptor signalling (Fas or TNF-R). Such a phenomenon has also been demonstrated for Fas-mediated apoptosis of FC-loaded macrophages.[112,113]

The most compelling evidence that hepatocytes may be the source of liver inflammation in NASH comes from studies in obese rodents with insulin resistance that leads to hyperinsulinemia and diabetes. We have used mice with a spontaneous mutation of the murine homology of the Alström gene (*Alms1*[termed *foz/foz*]),[108,114,115] while others have used wildtype (WT) C57B6 mice or rats.[100,116-118] *Foz/foz* mice exhibit hyperphagia with early onset obesity and insulin resistance, the phenotype of Alström syndrome. Feeding them a high carbohydrate, HF diet with 0.2% cholesterol accelerates onset of diabetes with marked hypoadiponectinemia.[114] The resultant liver pathology shows NASH with fibrosis,[108,114,115] whereas chow-fed *foz/foz* NOD.B10 mice and WT NOD.B10 mice fed the same diet develop only steatosis. Feeding WT C57B6 mice a similar HF diet, and particularly diets with higher cholesterol content (1% or 2%, often supplemented with cholic acid) also leads to unequivocal NASH; the onset is generally later, varying between 6 and 15 months in different reports.[119-121] A similar approach, typically with cholesterol-enriched HF diet, can also produce NASH in some lines of rats[116,117] and in a line of opossums (ABCB4) that are genetically predisposed to hypercholesterolemia.[122] Finally, a HF diet rich in trans fats combined with high-fructose corn syrup equivalent and inactivity (the American Lifestyle-Induced Obesity Syndrome) also caused obesity-related steatosis with moderate necroinflammatory change, albeit in this and most other animal models (the *foz/foz* mouse is an exception), hepatocellular ballooning, a cardinal feature of human NASH is inconspicuous and there was no fibrosis.[100]

In HF-fed *foz/foz* mice, onset of NASH is associated with

more than 200-fold increase in liver cholesterol esters (CE), and ~8-fold increase in FC.[115] Removal of cholesterol from the HF diet reduces hepatic CE and FC content and ameliorates the severity of liver injury and steatohepatitis.[115] Likewise, pharmacological treatments that lowered hepatic cholesterol dampened necroinflammatory severity in this NASH model.[123] Conversely, increasing dietary cholesterol (to 2% in *foz/foz* mice,[115] or 1% in other studies with C57B6 mice[108-121]) worsens inflammation and liver injury in experimental NASH. It is plausible that FC or other cholesterol fractions (7-ketocholesterol and other oxysterols are candidates) could activate KCs and recruited macrophages directly, analogous to processes implicated in atheroma,[112,113,124] and demonstrated in low density lipoprotein receptor knockout and apoE knock-in mice.[125,126] However, immunofluorescence studies in *foz/foz* mice (unpublished data) and human livers[94] show that hepatocytes are the cell type most conspicuously laden with FC in NASH. The subcellular compartments involved are the plasma membrane, ER and mitochondria.[94,115] A noteworthy feature of our studies has been the location of macrophages and neutrophils around heavily lipid-laden and swollen hepatocytes, some of which are ballooned (Fig. 2). Cellular processes could lead hepatocytes to incite inflammatory recruitment in NASH are discussed next.

2. Cytokines and oxidative stress

An earlier concept of NASH pathogenesis envisaged a "two hit" process, in which the abnormal metabolic milieu causing steatosis comprised the "first hit," and the vulnerability of a fatty liver to a separate injurious process ("second hit") resulted in cell death and inflammation.[127] Fifteen years ago, the injurious processes of interest were oxidative stress and cytokines, particularly those stimulated by endotoxin (lipopolysaccharides), such as TNF-α.[128,129] While both oxidative stress and cytokines are clearly evident in livers with NASH,[130-133] the weight of evidence is that TNF-α is a consequence rather than cause of liver inflammation in NASH.[2,72,134] Further, serum TNF-α levels increase in obese people, most likely originating from macrophages in the inflamed adipose;[84] importantly, values in NAFLD patients do not discriminate NASH from "not NASH."[74] It is also salient that some experimental forms of steatohepatitis, including a forced over-nutrition model,[135] can occur in the absence of TNF-α or its NF-κB signalling type 1 receptor.[136,137]

Oxidative stress is a key pro-inflammatory pathway in acute liver injury, such as ischemia-reperfusion injury[87] and in some types of steatohepatitis,[134] including alcohol-related liver disease, methionine deficiency,[138] and methionine and choline deficient (MCD).[137,139-142] Older studies employing immunohistochemistry demonstrated evidence of oxidized proteins, lipids and DNA in NASH livers,[143-145] but this could be a consequence of inflammation rather than its cause. A potential distraction has been identification of multiple sources of pro-oxidants in NASH, such as mitochondria (from uncoupling of oxidative phosphorylation to release reactive oxygen species), from ER (induction of cytochromes P450 [CYP] 2E1 and 4A),[146,147] peroxisomes[89] and inflammatory cells (NADPH oxidase).[134,148,149] Hepatoprotection from anti-oxidants and anti-oxidant pathways (such as heme oxygenase) has been demonstrated in MCD steatohepatitis,[150,151] and vitamin E may have some efficacy against necro-inflammatory change in NASH,[152] but there is less evidence for operation of oxidative stress in murine models that link metabolic syndrome to NASH. We agree with the interim conclusion reached by several experts,[89,91] that oxidative stress and/or cytokines are not likely to be the initiators of liver inflammation in NASH, although roles in insulin resistance, perpetuation of necroinflammatory change, fibrogenesis and progression towards cirrhosis and hepatocarcinogenesis remain likely.

3. ER stress

Accumulation of unfolded proteins within the ER is often observed in cells like hepatocytes that have high rates of protein synthesis. The cellular responses, collectively known as the unfolded protein response (UPR), involve provision of chaperones,

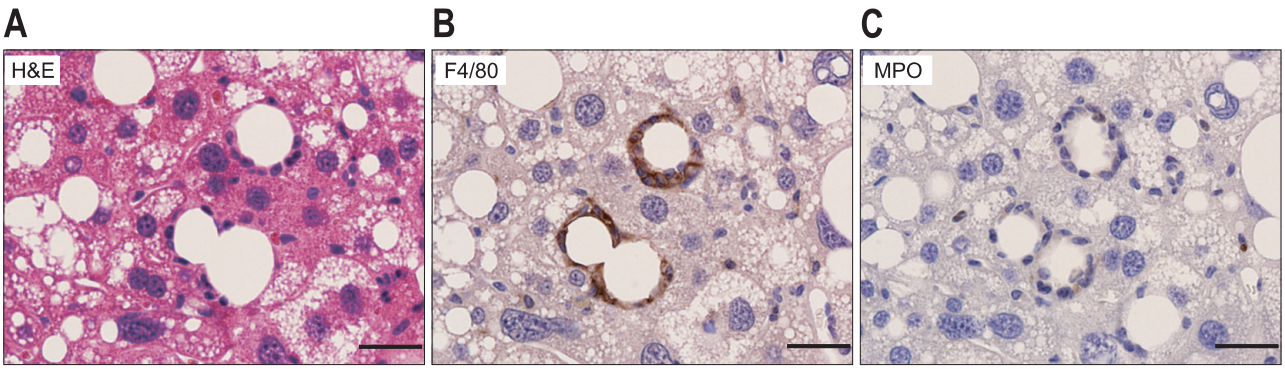

Fig. 2. Inflammatory cell recruitment and localization around lipid-laden hepatocytes in HF-fed *foz/foz* mice with non-alcoholic steatohepatitis (NASH). (A) H&E-stained liver section from HF-fed (0.2% cholesterol) *foz/foz* mouse with NASH, showing several enlarged hepatocytes with macrosteatotic vacuoles, and at least one ballooned hepatocyte (bottom right). (B) Macrophages (F4/80 positive), and (C) neutrophils (myeloperoxidase positive) accumulate around hepatocytes showing macrosteatotic vacuoles. These livers contain large amounts of free cholesterol.[115] Scale bars represent 20 μm.

such as 78 kDa glucose-regulated protein (GRP78), for protein refolding and transport out of the ER, and suppression of further protein synthesis.[153-155] Failure to mount an adequate UPR triggers a set of intracellular molecular "switches" that comprise the ER stress response. The three key pathways are depicted in Fig. 3. Through these pathways, ER stress activates NF-κB, JNK and C/EBP, with downstream effects on inflammatory recruitment, phosphorylation of insulin receptor signalling intermediates (to worsen insulin resistance), lipogenesis, and oxidative stress. These processes can ultimately lead to dismantling of the cell by apoptosis, particularly involving C/EBP-homologous protein, which transcriptionally suppresses anti-apoptotic Bcl-2 and induces pro-apoptotic Bim (Fig. 3).

Relationships between hepatic ER stress, lipogenesis, insulin resistance and hepatic steatosis in obesity and metabolic syndrome have been the subject of intense scrutiny,[155,156] and ER stress has been proposed as a mechanism in diverse experimental forms of liver injury (alcohol-related, drug-induced).[154-157] In obese humans, UPR (typically GRP78 expression) and ER stress markers have been noted in the adipose, liver and pancreatic beta cells.[158] To date, however, the evidence for operation of hepatic ER stress in human NAFLD/NASH is limited and inconsistent; some pathways seem to be activated, others are not,[159] and there have not been informative correlations between pathways and disease phenotype. Likewise, the evidence for operation of ER stress in animal models is conflicting.[155,160-163] In particular, there is little evidence that ER stress is a pro-inflammatory pathway in models that exhibit both the metabolic determinants of NAFLD and steatohepatitis pathology, such as HF-fed *foz/foz* mice (van Rooyen, unpublished data).

Impaired activity of sarcoplasmic-ER calcium ATPase-2b (SERCA), the ER calcium sequestering pathway, appears to be a key mediator of cellular responses to ER stress.[164] Such inhibition could deplete ER calcium stores, causing cytoplasmic ionic calcium concentrations to rise, increasing its movement into mitochondria with implications for mitochondrial injury, but this has not yet been demonstrated. Enrichment of the ER membrane with cholesterol also inhibits SERCA activity in parallel

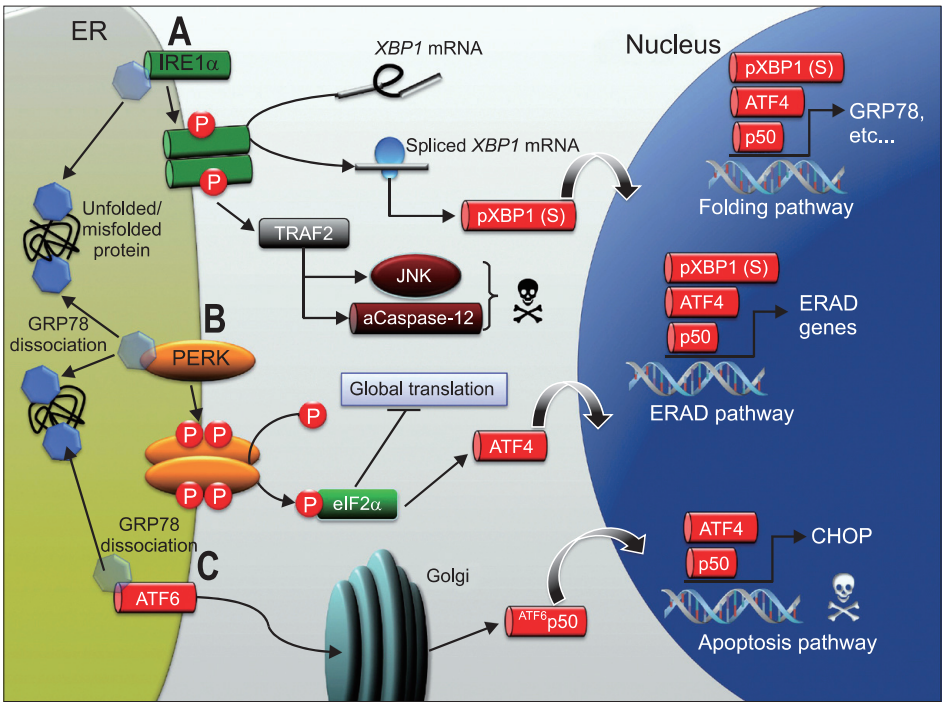

Fig. 3. Mammalian unfolded protein response (UPR) pathways. The UPR is triggered by several events, including protein unfolding/misfolding, hypoxia, low adenosine triphosphate levels, ER calcium depletion, and protein/sterol over-expression, causing dissociation of 78 kDa glucose-regulated protein (GRP78) from the three UPR sensors, (A) inositol-requiring enzyme 1α (IRE1α), (B) protein kinase RNA-like endoplasmic reticulum kinase (PERK), and (C) activating transcription factor-6 (ATF6). Activated IRE1α undergoes dimerization and autophosphorylation to generate endogenous RNase activity; in turn, this is responsible for splice truncation of X-box binding protein 1 (XBP1S) mRNA. Additionally, IRE1α may also activate the extrinsic apoptosis pathway, in which tumor necrosis factor (TNF) receptor-associated factor 2 (TRAF2)-dependent downstream activation of c-Jun N-terminal kinase (JNK) and caspase-12 takes place. Once activated, PERK undergoes homodimerisation and autophosphorylation to activate eukaryotic translation initiation factor 2 (eIF2α). In turn, this induces ATF4 expression. Separately, dissociation of GRP78, allows ATF6 processing by the Golgi complex, where proteases S1P and S2P cleave an active 50 kDa (p50) ATF6 domain that is free to translocate to the nucleus. Xbp1s, ATF4 and ATF6, as well as other unlisted factors, are responsible for three dominant cell responses to UPR. The folding pathway induces increased expression of molecular chaperones, including GRP78, assisting in compensatory ER protein folding. Alternatively, the cell may respond by increasing ER-associated protein degradation (ERAD) pathway, whereby gene products target and degrade unfolded proteins in the ER. Prolonged UPR results in the activation of the intrinsic apoptosis pathway; this ATF6 and ATF4-dependent process induces C/EBP-homologous protein (CHOP) expression. In turn, CHOP inhibits B-cell lymphoma 2 and induces apoptosis.

with increased membrane order parameter.[155] This has potential implications for NASH because ER is one site of increased cholesterol deposits (van Rooyen, unpublished data). Ultimately, the mechanistic relevance of ER stress as a disease pathway must come from *in vivo* studies of chemical chaperones that block its operation.[165] One such chaperone is tauroursodeoxycholic acid, an agent that appears to have little if any therapeutic efficacy against NASH.[166,167]

4. Mitochondria, autophagy and the regulation of inflammation

Ultrastructural studies have consistently shown intramitochondrial crystals in NASH, the identity of which has not been resolved,[168,169] and the association with decreased hepatic adenosine triphosphate (ATP) levels is also consistent with mitochondrial uncoupling or injury.[170,171] Mitochondria are a major source of ROS. Physiologically, about 2% of oxidative phosphorylation is uncoupled, but during hibernation, obesity and in several experimental models of NAFLD expression of uncoupling proteins (UCP), particularly UCP2, increases.[172,173] Damage to mitochondrial DNA and proteins, saturated FFAs and excessive ionic calcium could further uncouple oxidative phosphorylation, thereby generating oxidative stress. As mentioned earlier, FC impairs GSH uptake into mitochondria with similar deleterious effects.[110,174] In addition, permeabilization of the inner mitochondrial membrane by opening of the mitochondrial permeability transition pore is a key pathway to initiation of cell death by apoptosis or necrosis.[175]

A critical cellular response to mitochondrial injury or starvation (energy depletion) is autophagy (termed mitophagy when confined to mitochondria).[176-179] During mitophagy, damaged mitochondria are eliminated in a controlled process of lysosomal membrane and macromolecular turnover. This counters cellular degeneration and prevents unnecessary cell loss or, in the face of insurmountable damage, prepares residual cellular remnants (apoptotic bodies) for macrophage-mediated clearance in the more organised cell death pathway of apoptosis.[176] By augmenting apoptosis, autophagy tends to dampen inflammation, whereas necrotic cell death can promote it.[87,178-180] Mitochondria play a central role in inflammatory pathways, such as NF-κB

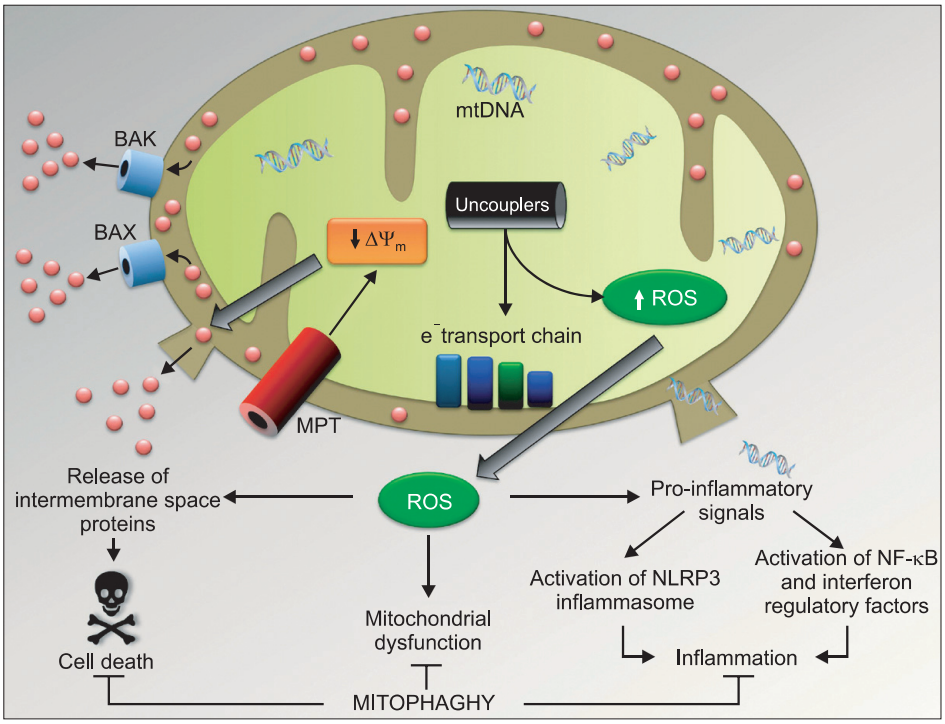

Fig. 4. Mitophagy inhibits pathways of mitochondrial dysfunction and associated cell death and inflammation. Mitophagy restitutes physiological cell functioning by inhibiting mitochondrial-related cell death and/or injury arising either from the generation of reactive oxygen-species (ROS) or pro-inflammatory signals, or as a result of mitochondrial membrane permeability transition (MPT). During activation of the intrinsic apoptosis pathway, BH3-only protein members, including BAK and BAX, effect mitochondrial membrane permeabilisation (MOMP) and release of intermembrane space proteins, including cytochrome *c* which induces a downstream caspase cascade activation that leads to apoptosis. Alternatively, necrotic cell death may be initiated by cyclophilin D-dependent initiation of MPT pore. Once opened, MPT destroys the mitochondrial transmembrane potential (ΔΨm), thereby abrogating oxidative phosphorylation and exacerbating ROS generation. Excessive ROS formation can activate the NACHT, LRR and PYD domains-containing protein 3 (NALP3) inflammasome. Uncoupling of oxidative phosphorylation can also trigger MPT, during which mtDNA may undergo cytoplasmic translocation, leading to nuclear factor-kappa B (NF-κB) and interferon regulatory factor-dependent inflammatory pathway activation. Importantly, excess intra-mitochondrial ROS is able to mutate mitochondrial DNA (mtDNA), leading to premature aging and mitochondrial inefficiency post-replication (this in turn exacerbates ROS generation through impaired oxidative phosphorylation).

and interferon-responsive factors (IRF), as depicted in Fig. 4, as well in the induction of inflammasomes (discussed below). There is also an interaction between impairment of autophagy and induction of ER stress.[181] The recent interest in whether abrogation of autophagy contributes to inflammatory recruitment in NASH has been reviewed.[179]

5. The inflammasome

The inflammasome is a larger multimeric structure that regulates caspase 1 activation.[182,183] The NLRP3 (nucleotide-binding domain, leucine-rich repeat containing) inflammasome (also known as cryopyrin or NALP-3) is expressed by myeloid cells and is up-regulated by pathogen-associated molecular patterns (PAMPs). It requires a caspase recruitment domain, and can recruit pro-caspase 1 in the presence of the adapter protein ASC (apoptosis-associated speck-like CRD-domain containing protein). Once all the components of the NALP3 inflammasome are assembled in the cytosol, caspase 1 is released and can promote the cleavage and therefore maturation of pro-inflammatory cytokines (pro-IL-1β, pro-IL-18, and IL33) to promote and sustain inflammation. NLRP3 inflammasome can be activated by several endogenous and exogenous agonists, as reviewed elsewhere.[182,183] Salient to NASH, palmitic acid (but not oleic acid) induces activation of the NLRP3-ASC inflammasome to activate caspase 1 and cause IL-1β and IL-18 production.[183] This pathway involves mitochondrial production of ROS (Fig. 4). Other agonists that could be relevant include uric acid crystals, which can precipitate in the extracellular space of dying cells, and extracellular DNA, possibly including mitochondrial DNA.

The inflammasome is activated in experimental alcohol-induced liver injury,[83] and in mice fed the MCD diet, but not in HF diet-induced simple steatosis.[184] Csak *et al*.[184] exposed hepatocyte cultures to palmitic acid, and showed that this sensitised liver cells to release IL-1β following the further addition of lipopolysaccharide. In addition, palmitic acid provoked hepatocytes to release undefined "danger signals," which then activated the inflammasome in liver lymphocytes and macrophages to augment release of IL-1β and TNF-α. Other work has confirmed that, under certain circumstances, hepatocytes can themselves secrete chemokines and cytokines.[185] Thus, activation of the inflammasome is one of several models by which hepatocytes could play a central role in inflammatory recruitment in NASH, but as indicated next, there are other potential pathways.

6. Ballooned hepatocytes and inflammatory recruitment; is the p53/senescence pathway involved?

Early studies identified ballooning as one of few histological features associated with risk of cirrhosis development in NAFLD.[33] While not always confirmed by subsequent studies, in which presence of fibrosis and histology as "definite NASH" tend to over-ride ballooning in multivariate analyses,[12,47] a link between ballooning and portal fibrosis has been emphasized by Richardson *et al*.[186] These authors also found a strong link between ballooning and lobular inflammation in NASH, which is consistent with the proposal that ballooning attracts inflammatory cells, as indicated by their co-localisation in experimental studies (Fig. 2), and their implication in secretion of Hedgehog ligands.[187-189] This family of fibrogenic transcription factors also plays a pro-inflammatory as well as pro-fibrotic role. Thus ballooned hepatocytes have been shown to be a focus for both HSC activation and hepatic precursor cell recruitment, both of which are under cytokine regulatory control.[189]

Ballooned hepatocytes often contain Mallory's hyaline (also known as Mallory-Denk bodies), which are derived from ubiquitin-modified intermediate (cytokeratin [CK]) filaments; ubiquitin staining can be used to identify ballooned cells more clearly.[190-193] This destruction of intermediate filaments might indicate that cytoskeletal disruption leads to ballooning, but ultrastructural studies are limited. There is also evidence that foamy, lipid micro-droplets confer the glazed appearance of ballooned hepatocytes rather than hydropic change.[190,192,193] Apoptosis is increased in livers with steatohepatitis,[101,104,132,193] while circulating peptides liberated by caspase 3 cleavage of CK18, an hepatocyte-specific CK, serves as a biomarker for NASH versus "not NASH."[194,195] The original term for apoptosis was "shrinkage necrosis"; therefore, the presence of ballooning seems more likely to reflect imminent cell necrosis rather than apoptosis. If so, the disintegration products could be pro-inflammatory, and it is well recognized that necrosis, an unregulated form of cell death, activates macrophages, neutrophils (e.g., by high mobility gel box 1 [HMGB1]) and other pro-inflammatory pathways,[87,180,196] including the inflammasome discussed earlier.

An alternative possibility is that ballooned hepatocytes are a reflection of cellular senescence in the liver. In epithelial cells, stressors such as oxidative stress and DNA damage can lead to replicative senescence.[196] In humans, this is particularly associated with shortened telomere length such that cell division is no longer possible.[197] Most interest in senescence as a disease mechanism has been for neurodegenerative disorders and cancer;[196,197] it does not appear to have been much studied in NASH. However, cirrhosis is associated with loss of telomere length,[198] and p53, the guardian of senescence,[196,199] is up-regulated in several types of fatty liver disease.[200] Senescence arrests cell division by inducing cell cycle inhibitors (p21, $p16^{INK4A}$, Rb) and has a characteristic molecular expression profile closely linked to regulation of an inflammatory response in neighbouring tissues.[197] The pro-inflammatory molecules involved include cytokines (IL-1, IL-6, IL-8), chemokines (IL-8, MCP-1, GRO α/β/γ) and chemokine receptors (CXCR2), most of which are consistently found to be up-regulated in experimental NASH (Table 1).[72,86,132,137,201] Further research is required to establish whether hepatocyte senescence is inherent to inflammatory recruitment in the transition of steatosis to NASH.

PRO-INFLAMMATORY SIGNALS

A common outcome of the above subcellular stress processes is the activation of intracellular pathways that signal pro-inflammatory responses. These signalling pathways include ionic calcium, protein kinase and transcription factor activation, and the most consistent are activation of NF-κB and JNK. These pathways will now be considered separately, but it should be noted that they are usually activated in tandem and often co-regulate the same gene products.

1. Activation of NF-κB

NF-κB is a transcription factor comprised of five peptides that form homodimeric or heterodimeric complexes; p65 and p50 are highly expressed in liver. NF-κB p65:p50 heterodimers regulate the transcription of several hundred pro-inflammatory molecules (p50:p50 tends to be inhibitory), including cytokines, chemokines, adhesion molecules, nitric oxide and cyclooxygenase 2.[141] In resting (G_0) hepatocytes, NF-κB is sequestered in the cytosol bound to inhibitory (IκB) proteins. Their phosphorylation, mediated by IKK, and subsequent ubiquitination targets the NF-κB -IκB complex to the 26S proteasome for degradation. This liberates NF-κB in a form that can be transported into the nucleus. Detection of p65 in nuclear extracts, or binding to cognate oligonucleotides in gel shift assays serve as indicators of NF-κB activation, together with increased levels of transcripts for "NF-κB-responsive genes." IKK is activated directly by oxidative stress and other cellular stressors (such as ER stress), or via liganding of NF-κB-signaling receptors.

NF-κB activation is uniformly found in human NASH[202] and in all animal models in which it has been studied. Using MCD fed mice, we employed TNF-α and TNF-R1 knockout animals, and *in vivo* transfection of WT mice with non-degradable mutant-IκB to show that NF-κB activation is essential for hepatic inflammatory recruitment in steatohepatitis;[137] further, such NF-κB activation occurs independently of TNF-α. Other work using the MCD dietary model has produced conflicting findings; curcumin, which blocks oxidative stress-mediated NF-κB activation provided protection,[203] but TNF-α anti-serum reduced liver injury in rats administered the MCD diet,[204] while Tomita *et al*.[205] found that TNF-R knockout mice had protection against liver fibrosis in their MCD experiments.

Fractionation of livers from HF-fed *foz/foz* mice (Larter, unpublished data) and MCD-fed animals[137] shows that NF-κB activation is most prominent in non-parenchymal cells (KCs, SECs, HSCs), but it is also evident in hepatocytes.[137,206] The emerging concepts of metabolic stress mentioned earlier provide some indication that pro-inflammatory pathways in NASH could eminate from stressed hepatocytes via activation of NF-κB. Alternatively, TNF-α, IL-1β and other cytokines released from NF-κB-activated KCs could activate NF-κB in neighbouring hepatocytes.

Myeloid differentiation primary response gene 88 (Myd88) null mice are refractory to dietary steatohepatitis caused by a choline deficient and defined amino acid (CDAA) diet.[207] Using bone marrow chimeric (WT/Myd88$^{-/-}$) mice, Miura and colleagues showed that the KC compartment was essential for inflammatory recruitment in this model.[207] Further, the upstream stimulus to Myd88/NF-κB activation appeared to be Toll-like receptor 9 (TLR9),[207] located in endosomes/lysosomes and most responsive to unmethylated CpG-containing DNA. The implication of TLRs and their role in the innate immune response and activation of NF-κB in NASH is discussed later.

2. JNK

Like NF-κB, the JNKs (1 and 2) can be activated directly by oxidative stress and by lipotoxic molecules (FFA, FC), or as the result of ligand binding to growth factor and TNF superfamily death-signalling receptors (Fas, TNF-R1, TNF-related apoptosis-inducing ligand death receptors) or TLRs.[208] JNK activates the mitochondrial apoptosis pathway and forms the c-jun:c-fos heterodimer, AP-1; AP-1 is pro-inflammatory, typically inducing similar genes as NF-κB.

JNK appears always to be activated in lipotoxicity and in both experimental and human forms of NASH.[159,209-213] In seminal work, Schattenberg *et al*.[209] showed that activation of JNK1 (but not JNK2) was essential for inflammatory recruitment in MCD-induced steatohepatitis; others have confirmed this.[210-212] Saturated fatty acids activate JNK in primary hepatocytes and tumour cells of hepatocyte lineage;[95,101,106,214] and this was a critical pathway to cell death by the mitochondrial apoptosis pathway.[101,214]

In the *foz/foz* diabetes/metabolic syndrome model, we have noted that both JNK1 and JNK2 are activated with NASH, but not in genotype or dietary controls with simple steatosis.[212] Further, dietary or pharmacological measures that lowered hepatic FC virtually abrogated JNK activation in association with mitigation of liver injury (ALT elevation), hepatocyte apoptosis and macrophage accumulation.[123] These observations are consistent with the proposal that JNK activation is a key injury and inflammatory pathway in metabolic syndrome-related NASH.

INNATE IMMUNITY IN NAFLD: TLRS, KCS AND LYMPHOCYTES

There is little doubt that innate immunity is involved in the inflammatory response in NASH, and this topic has been reviewed elsewhere.[70,82,83,85,208] Only the most salient aspects will be mentioned here.

1. Why could innate immunity be relevant to inflammation in NASH?

As mentioned, necrotic cell death elicits an inflammatory response. This concept was refined in 1994 when Matzinger[215] proposed the "danger hypothesis" as a way in which the in-

nate immune system can respond to key molecules released by damaged cells, thereby eliminating them. The mechanism by which stressed or dead cells trigger inflammation and adaptive immune responses involves damage-associated molecular patterns (DAMPS),[180,216-218] also termed alarmins. Intracellular pro-inflammatory DAMPS include high-mobility group gel box 1 (HMGB1),[218] heat shock proteins, fibrinogen and fibrinonectin, and mitochondrial products such as formyl peptides and mitochondrial DNA.[217] Although they differ from PAMPs, some DAMPS can be recognised by similar receptors, particularly TLRs (e.g., TLR4 responds to both HMGB1 and lipopolysaccharide).[217,219]

2. TLRs and NASH

Eight TLRs are expressed in mammalian liver (TLRs 1, 2, 4, 6-10), with varying levels of expression on KCs, hepatocytes, SECs and HSCs.[85] Most are expressed on the cell surface, but TLRs 1, 3 and 9 are intracellular (endosomal/lysosomal) proteins. TLRs recognise molecular patterns present on a broad range of pathogens and altered or specialised host molecules. Upon ligand binding and with recruitment of certain co-factors (e.g., myeloid differentiation factor 2 [MD2]), they signal via overlapping protein casettes to trigger inflammatory and antiviral responses, as well as maturation of dendritic cells to activate adaptive immunity.[216] Individual TLRs interact with different combinations of adapter proteins (e.g., MD2) and activate transcription factors such as NF-κB, AP-1 (via JNK) and interferon-responsive factors (IRF). As shown in Fig. 5, MyD88 is shared by almost all TLRs and recruits members of the IL-1 receptor-associated kinase family. In fact, the intracellular domain of

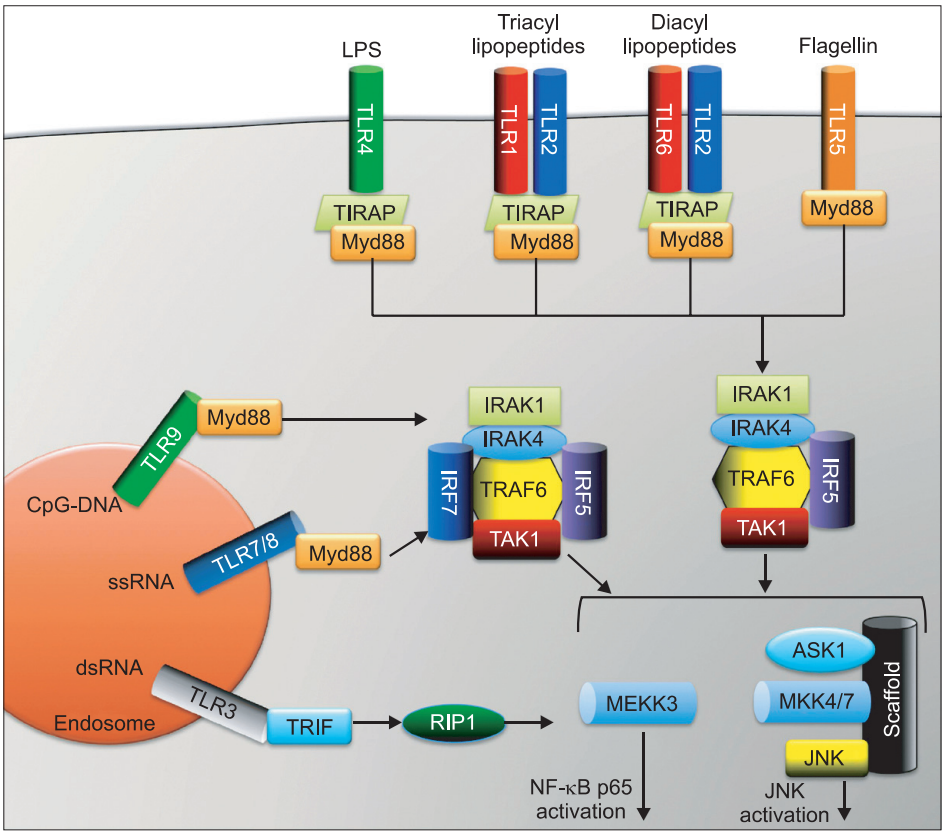

Fig. 5. Toll-like receptor (TLR) signalling involves JNK and NF-κB p65 activation. Toll-like receptors (TLR) constitute a family of receptors involved in pro-inflammatory signalling in the innate immune system, responsible for the recognition of pathogen-associated molecular patterns (PAMPs) and exogenous stimuli, such as pathogens, or endogenous agonists, such as sterile tissue damage; the later are termed danger-associated molecular patterns (DAMPs). Of the 9 known TLR receptors, four (TLR-3, -7, -8, and -9) are expressed on the endosomal membrane and are responsible for viral particle surveillance, including detection of deoxy-cytidylate-phosphate-deoxy-guanylate DNA (CpG-DNA), and single- and double-stranded RNA. The remaining TLRs are expressed on the plasma membrane and are responsible for the detection of extracellular microbial pathogens. Relevant PAMPs include: LPS, diacyl- and triacyl lipopeptides, and flagellin, as well as several DAMPs, including HMGB1. Activated TLR3, as well as TLR4, signal through adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF), which in turn recruits RIP1 to activate the IKK complex, thereby activating nuclear factor-kappa B (NF-κB). The other TLRs signal through toll/interleukin-1 receptor domain containing adaptor protein (TIRAP) and myeloid differentiation factor 88 (Myd88). Activated Myd88 induces the recruitment of IL-1R-associated kinase (IRAK) 4, as well as IRAK1, which bind TRAF-6 and transforming growth factor-β activated kinase (TAK)-1. IRF5 and IRF7 are then recruited to the post-Myd88 protein complex. Interferon-regulatory factor 7 (IRF7) recruitment is dependent upon on TLR7 and TLR9 signalling. The IRAK1/4/TRAF6/TAK1/IRF5/7 complex is responsible for downstream Myd88-dependent activation of c-Jun N-terminal kinase (JNK) and NF-κB. TRAF, tumor necrosis factor (TNF) receptor-associated factor; MEKK, MAP kinase kinase kinase; MKK, mitogen-activated protein kinase kinase; ASK, apoptosis signal-regulating kinase.

plasma membrane expressed TLRs exhibits IL-1 receptor motifs, and their intracellular signalling shares several intracellular adapter molecules with IL-1 (Fig. 5).

When released from necrotic cells, HMGB1 stimulates KCs and monocytes to produce pro-inflammatory mediators by acting as an endogenous ligand for TLR4, although it might do that by forming highly inflammatory complexes with other molecules (ssDNA, endotoxin, IL-1β, nucleosomes).[218] TLR4 is involved in acute liver injury, such as hepatic ischemia-reperfusion injury,[220-222] in alcoholic liver injury (when priming by gut-derived endotoxin is pivotal).[82] and is also up-regulated in MCD steatohepatitis[219,223] and fructose-induced hepatic steatosis (not NASH).[224,225] Saturated FFA can also bind to TLR4.[70,90,226-228] TLR4 and MD2, its co-receptor for endotoxin, are expressed on KCs, hepatocytes and HSCs. Deletion of either TLR4 or MD2 dampens (but does not abolish) necroinflammatory activity of MCD steatohepatitis, with the most impressive effects being on NADPH oxidase expression and activation of inflammatory cells.[229]

Other research has identified activation of TLRs2 and 9 in various experimental models of NAFLD or NASH.[225] As mentioned earlier, TLR9 is located within the cell and is most responsive to unmethylated CpG-containing DNA, but it also binds HMGB1. TLR9-deficient mice are protected from steatohepatitis in the CDAA model.[207] TLR2 (but not TLR4) expression by hepatocytes can be induced by lipopolysaccharide, TNF-α and IL-1β via NF-κB activation, while signalling cross-talk between TLR4 and TLR9 amplifies the inflammatory response of macrophages,[230] indicating other potential loops for perpetuation of inflammation in NASH. TLR5 is not expressed in the liver, but it has recently been reported that TLR5 knockouts have altered gut flora and develop obesity and metabolic syndrome, including insulin resistance and steatosis.[231] Any relevance to NASH has yet to be established, although a fascinating finding was that transfer of the altered gut flora from $TLR5^{-/-}$ mice to healthy animals resulted in a similar disease phenotype, including (non-inflamed) fatty liver.[231]

3. Kupffer cells

KCs are specialised tissue macrophages in the liver. They not only contribute to insulin resistance in fatty liver disease,[61] but unite the inflammatory responses in many liver diseases.[232] KCs are particularly sensitive to gut-derived endotoxin, acting through CD14, TLRs 2 and 4 and adapter proteins such as MD2 to activate NF-κB via MyD88.[70,229] Other intracellular signalling molecules lead to induction of IFN-γ, which is important for lymphocyte recruitment.[86,208] In chimeric mice with KCs derived from $MyD88^{-/-}$ bone marrow donors,[207] there was amelioration of the inflammation and fibrosis induced in the CDAA model of steatohepatitis compared with WT mice, demonstrating a key role for KC activation in this model. To the authors' minds, the data are more convincing than those in a recent study (using an irradiated, 2% cholesterol HF diet that is hepatotoxic) in which the authors proposed that TLR4 on hepatocytes, not KCs, responds to HMGB1 by NF-κB activation.[206] Other earlier work showed that engulfment of cellular fragments denoted as apoptotic bodies from UV-treated murine hepatocytes (which would also causes oxidative stress) activated KCs to generate FasL and TNF-α.[233] Ablation of KCs (e.g., by gadolinium or clodronate liposomes) reduces severity of liver injury and inflammation in alcohol-related liver injury in rodents, as does measures to change the gut flora in favour of non-endotoxin producing organisms. In a HF-fed mouse model, KC ablation ameliorated severity of steatosis by releasing hepatocytes from IL-1β and NF-κB-dependent suppression of peroxisome proliferator-activated receptor (PPAR)-α activity, thereby allowing PPAR-γ to exert its effects on fatty acid oxidation.[234]

4. Lymphocytes

Several types of lymphocytes are present in the normal liver, including NK cells, NK T cells, and T cells.[86] Hepatic NK cells can be regulated by KC-derived cytokines (IL-1, IL-18), and in turn generate IFN-γ which participates directly in cell killing and in modulation of T cell responses. Lymphocytes accumulate in NASH livers, but which subpopulations predominate and their pathogenic roles in injury and inflammation have not yet been fully characterized.

5. Neutrophils

The presence of neutrophils (polymorphonuclear neutrophils, [PMNs]) among the liver inflammatory infiltrate of alcoholic steatohepatitis has long been recognized. Neutrophils are also present in NASH,[29,31] where their possible pathogenic significance remains obscure. In the *foz/foz* metabolic syndrome model of NASH, the reduction of hepatic cholesterol stores which ameliorates liver injury, apoptosis and macrophage recruitment does not appear to alter accumulation of myeloperoxidase positive cells (PMNs) (van Rooyen, unpublished data). On the other hand, reduction of hepatic triglyceride stores and lipogenesis either by a dietary reversion strategy or with Wy-14,643 (a potent PPAR-α) agonist, has more impressive effects on neutrophils than on macrophages.[212] Dietary reversion (from HF to chow) suppressed UCP2 expression and increased hepatic ATP levels, which would favour operation of apoptosis (and this was observed) rather than ROS-mediated necrosis (Larter and Farrell, unpublished data). Combined use of M30 and full-length CK8/18 in patients with NASH indicates that both apoptosis and necrosis occur in humans with the inflammatory form of NAFLD.[195] It is therefore possible that neutrophil accumulation is associated with necrosis in NASH, and it may be regulated by different pathways than those important for macrophage and lymphocyte recruitment and activation.[87] These important and rather neglected issues require further study.

FUTURE DIRECTIONS, CLINICAL AND THERAPEUTIC IMPLICATIONS

The two hits concept of NASH pathogenesis served to dissect injury and pro-inflammatory pathways from the metabolic causes of steatosis. Insights gained since then indicate that the lipid molecules that accumulate, together with TG, in some NAFLD livers can themselves participate directly in pathogenesis of the necroinflammatory element of NASH. The fact that steatosis (which biochemically is TG accumulation) does not inevitably predispose to NASH is better understood by recent studies showing that TG formation is protective against injury and inflammation, not predisposing to such inflammation.[235,236] On the other hand, FC, certain FFA, DAG and some phospholipids can directly injury liver cells and mediate subcellular stresses (mitochondrial, ER, oxidative) that incite hepatocellular injury, cell death and inflammatory recruitment in NASH. Thus, the essential difference between the two extremes of liver pathology (NASH versus not-NASH) is not attributable to the amount of fat (TG) in the liver, but rather the type of lipid molecules that accumulate.

Research in NASH pathogenesis has reached the exciting stage where investigation of potential lipotoxic molecules is being refined. Arguably the most critical future direction, however, is to perform more definitive lipidomic studies in human liver so as to clearly identify which lipid species are unambiguously implicated, and the genetic and environmental reasons for their accumulation. Such measurements must also establish correlations between candidate lipotoxic mediators, pro-inflammatory (and pro-fibrotic) pathways and liver pathology. *In lieu* of such human data, researchers (and journal editors) might better focus their attention on models where development of NAFLD across the pathological spectrum that includes NASH is clearly related at least to over-nutrition and insulin resistance, and ideally to obesity, type 2 diabetes and hypoadiponectinemia, the metabolic determinants of human NASH.[2,3] Other models have taught us what can occur in steatohepatitis pathogenesis, but hepatologists are most interested in what does occur in NASH. Therefore, nutritional depletion models like the MCD dietary model developed in the author's laboratory in 1996, choline deficiency, the CDDAA and similar deprivations, 2% cholesterol (equivalent to 20 kg of cholesterol a day for humans!) plus cholate diets, or genetic knockout and knock-in models of disordered adipokine (leptin, adiponectin) or lipid and cholesterol handling should no longer, in our view, receive the high level of current attention simply because of the "cute" reductionist science used, when clinically more relevant (and equally convenient) alternatives have been characterized metabolically and pathologically.[108,109,135]

Establishing whether the pro-inflammatory pathways in NASH eminate from hepatocytes subjected mitochondrial injury, impaired autophagy, the inflammasome, or from processes like ER stress, oxidative stress/necrosis, senescence and p53 expression is pertinent to the design of novel, mechanism-based therapies for NASH. Current approaches, vitamin E, PPAR-γ ("glitazone") agonists, ursodeoxycholic acid, lipase inhibitors, generally effect some reduction in steatosis severity, but effects on inflammation are less consistent (ezetimibe may be an exception), and there are few reliable reports of fibrosis reduction. Despite interest in the gut-liver axis in obesity, type 2 diabetes and NAFLD, there are few data on clinical improvement with use of probiotics, other measures to alter the intestinal *microbiota* or use of incretin mimetics. Only bariatric surgery, or other effective means of weight loss coupled to increased physical activity that improve insulin sensitivity seem to combat both steatosis and inflammation in NASH. We therefore need to learn whether this is because of a primary effect on improving insulin sensitivity and reducing hyperinsulinemia, with possible secondary changes to turnover and storage of hepatotoxic lipid species,[237] such as we recently clarified for disordered hepatic cholesterol homeostasis.[115] If so, the findings could direct a radically different therapeutic approach, perhaps even finding a cure for NASH without what seems presently to be essential- a change in lifestyle and a decrease in body weight.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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