Pathogenesis of Nonalcoholic Steatohepatitis: An Overview

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Nonalcoholic fatty liver disease (NAFLD) is a heterogeneous group of liver diseases characterized by the accumulation of fat in the liver. The heterogeneity of NAFLD is reflected in a clinical and histologic spectrum where some patients develop isolated steatosis of the liver, termed nonalcoholic fatty liver, whereas others develop hepatocyte injury, ballooning, inflammation, and consequent fibrosis, termed nonalcoholic steatohepatitis (NASH). Systemic insulin resistance is a major driver of hepatic steatosis in NAFLD. Lipotoxicity of accumulated lipids along with activation of the innate immune system are major drivers of NASH. Lipid-induced sublethal and lethal stress culminates in the activation of inflammatory processes, such as the release of proinflammatory extracellular vesicles and cell death. Innate and adaptive immune mechanisms involving macrophages, dendritic cells, and lymphocytes are central drivers of inflammation that recognize damage- and pathogen-associated molecular patterns and contribute to the progression of the inflammatory cascade. While the activation of the innate immune system and the recruitment of proinflammatory monocytes into the liver in NASH are well known, the exact signals that lead to this remain less well defined. Further, the contribution of other immune cell types, such as neutrophils and B cells, is an area of intense research. Many host factors, such as the microbiome and gut–liver axis, modify individual susceptibility to NASH. In this review, we discuss lipotoxicity, inflammation, and the contribution of interorgan crosstalk in NASH pathogenesis. (*Hepatology Communications* 2020;4:478-492).

Nonalcoholic fatty liver disease (NAFLD), the most common chronic liver disease in the United States, is a heterogeneous disorder.[1] Based on histology, pathogenesis, and natural history, the NAFLD disease spectrum is characterized by excess fat deposition in the liver that is unassociated with injury or inflammation (isolated steatosis or nonalcoholic fatty liver [NAFL]) on one end and hepatocyte ballooning, liver injury, inflammation, and varying degrees of fibrosis (nonalcoholic steatohepatitis [NASH]), ultimately leading to cirrhosis and the associated risks of end-stage liver disease and hepatocellular carcinoma (HCC), on the other end.[2] Fibrosis has been reported in some subjects with NAFL, although NAFL is generally considered nonprogressive. Isolated steatosis is characterized by predominantly macrovesicular lipid accumulation in 5% or more hepatocytes, typically beginning around central veins. Hepatocellular ballooning, Mallory-Denk bodies, and inflammation are observed additionally in NASH. Chronic inflammation is associated with fibrosis, which initially is pericellular and can progress to bridging fibrosis and cirrhosis. Thus, the two components of histologic assessment are disease activity (scored on steatosis, ballooning, and lobular inflammation) and fibrosis stage.[3] Subject to the caveat that there is significant collinearity between the NAFLD activity score (NAS) and fibrosis, fibrosis is

the only histologic factor associated with mortality.[4] Modern multiomics approaches confirm the relevance of histologic observations by demonstrating a correlation between genetic predictors of progression and histologic assessment of the NAS.[5] Here, we discuss the key molecular and cellular mechanisms that form the underpinnings of the observed histologic changes and global transcriptomics changes in NAFLD.

Steatosis and Lipotoxicity

The pathogenesis of NAFLD is multifactorial, and several systemic alterations have been implicated.[2] The primary insult of lipid excess is followed by variable contributions from pathogenic drivers, such as lipotoxicity and immune system activation, and modifiers, such as genetic susceptibilities, alcohol, and dysbiosis. However, there is considerable heterogeneity in NAFLD progression and NASH development, and only a subset of NAFLD develops NASH. Potential explanations for this variability include differences in etiopathogenic drivers,[2] dynamic multiphasic progression,[5] or that they represent distinct diseases. Alcohol is a well-recognized disease modifier. Recognizing the arbitrary cutoffs that define the level of intake, even modest levels of alcohol consumption have effects on NASH progression, including a worse histology and a risk for fibrosis progression.[6] Biologic sex modulates NAFLD pathobiology both in experimental models and humans,[7,8] with women being relatively protected from disease.

HEPATIC STEATOSIS

One key concept is the presence of a perturbed systemic energy balance state, characterized by substrate surplus, predominantly carbohydrates and fatty acids.[9,10] The major sources of nonesterified fatty acid (NEFA) delivery to the liver are increased release from adipocytes (accounting for approximately 60%), conversion from carbohydrates within the liver (*de novo* lipogenesis, 26%), and excess dietary intake (14%)[9] (Fig. 1). Insulin resistance (IR) and NAFLD are crucially linked[2,11]; IR leads to reduced glucose uptake in adipocytes and muscles, and hepatocytes can secrete dipeptidyl peptidase 4, which promotes adipose tissue inflammation and IR.[12]

At the adipocyte level, metabolic dysregulation due to impaired insulin postreceptor signaling leads to excess lipolysis of triglycerides (TGs) and NEFA release into the circulation. Albumin-bound NEFAs are delivered to the liver. Hepatocyte NEFA uptake is mediated by fatty acid transport proteins, cluster of differentiation 36 (CD36), caveolins, and to a lesser extent passive diffusion.[13] Additionally, *de novo* lipogenesis (DNL) from glucose and fructose occurs in the hepatocytes and is increased in subjects with NAFLD.[9,14] Unlike glucose, entry of fructose metabolites into the DNL pathway is not regulated by glycolysis.[15] Fructose also induces the carbohydrate response element binding protein independent of insulin and promotes hepatic steatosis. The predominant fate of NEFAs in the liver is to either undergo mitochondrial beta-oxidation or be esterified to form TGs. Partitioning of NEFAs into

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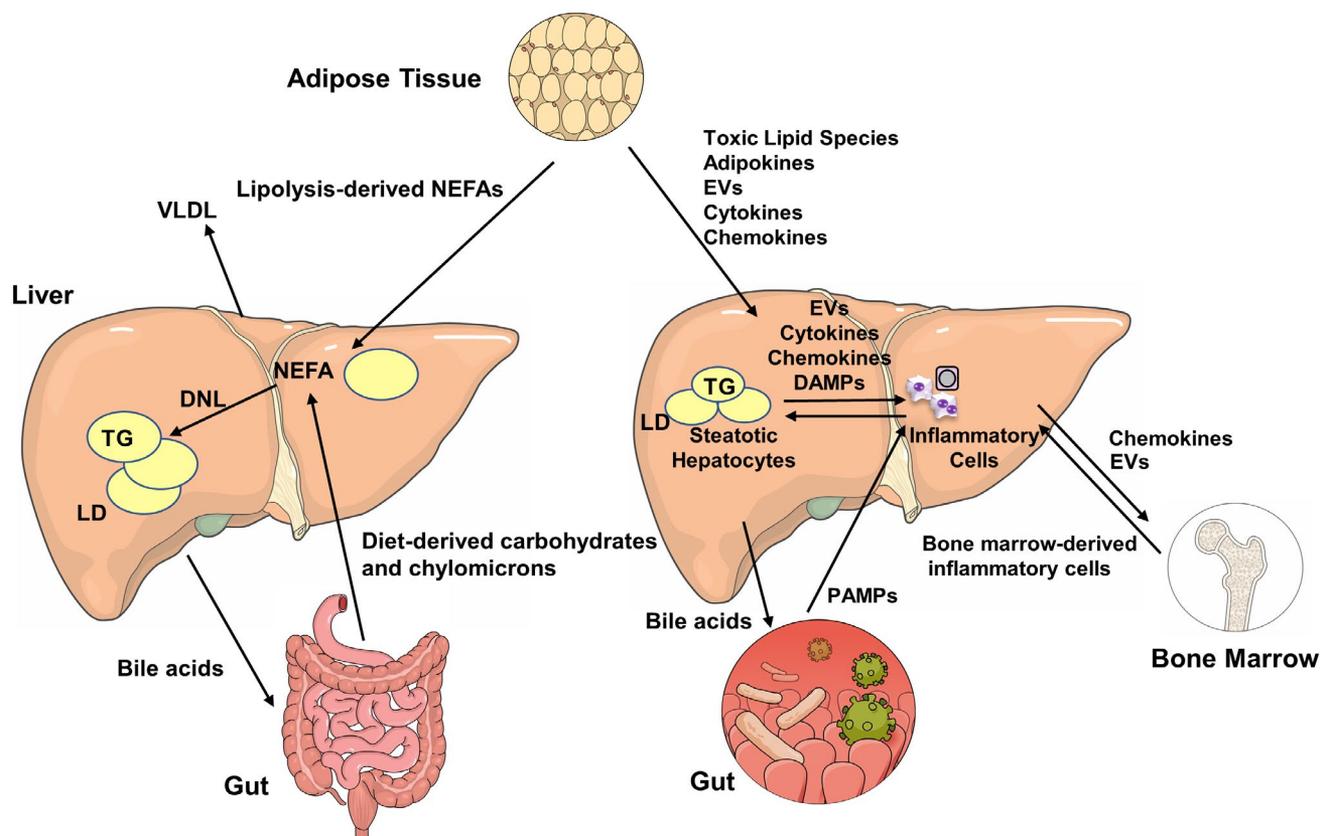E-mail: Malhi.harmeet@mayo.edu
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FIG. 1. Metabolic interorgan crosstalk in NAFLD. This illustration depicts interorgan crosstalk in NAFL on the left and NASH on the right. Hepatic NEFAs are predominantly derived from three sources: lipolysis in adipose tissue, dietary lipid absorption, and DNL from carbohydrates in the liver. These NEFAs are stored in the liver as TG-rich lipid droplets leading to hepatic steatosis or may be exported out of the liver as very low-density lipoprotein to adipose tissue. Bile acids from the liver are key regulators of the gut–liver axis. Several mediators orchestrate the inflammatory milieu in the liver that results in NASH and fibrosis. Lipotoxic lipid species lead to hepatic stress and subsequent release of EVs, cytokines, chemokines, and DAMPs from liver cells. This results in recruitment of immune cells from the bone marrow. Bile acids from the liver, PAMPs from the gut, and adipokines from adipose tissues also influence various steps in this process. Abbreviations: LD, lipid droplet; VLDL, very low-density lipoprotein.

other lipid classes, such as phospholipids and ceramides, is also increased by enhanced NEFA influx into the liver.[16,17] Formation of TGs, a relatively inert storage form, appears to be an adaptive mechanism to protect the liver from toxic lipids. TGs can be exported as very low-density lipoprotein particles or stored as lipid droplets. Lipolysis of these droplets releases NEFA back into the hepatic pool, and the regulation of this step is important in the pathogenesis of NASH.

The most strongly associated genetic variant with NASH is a single-nucleotide polymorphism (I148M) in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) gene,[18] which encodes a lipid droplet protein and is involved at this lipolytic step.

I148M variant PNPLA3 is degradation resistant, accumulates on lipid droplets, and is sufficient to induce steatosis.[19] Hydroxysteroid 17β-dehydrogenase 13 (HSD17B13), another lipid droplet protein, is up-regulated in NASH.[20] Variants of HSD17B13 are associated with increased steatosis but decreased inflammation and lower alanine aminotransferase in NAFLD. Thus, genetic studies point to an important role for lipid droplet proteins in regulating features of NAFLD; this is an area that needs further mechanistic studies to understand how steatosis can be protective against liver injury yet lipid droplet proteins may be deleterious.

Apart from quantity, the type of NEFAs that accumulate in NAFLD are also altered, with significantly

more saturated fatty acids than monounsaturated and polyunsaturated fatty acids (MUFAs and PUFAs, respectively). The 16-carbon palmitate and 18-carbon stearate are major saturated fatty acids that accumulate and are associated with disease progression.[21] Other implicated lipotoxic species include diacylglycerols, ceramides, lysophophatidyl choline (LPC), and free cholesterol.[21,22] Given the key role in the pathobiology of NAFLD and NASH, a strategy to decrease substrate delivery to the liver or to promote disposal of NEFAs from the liver represents an attractive therapeutic target (Table 1). This could be achieved, for example, by increasing fatty acid oxidation (peroxisome proliferator-activated receptor [PPAR]α/δ agonists; fibroblast growth factor [FGF]21 agonists; thyromimetics), inhibition of DNL (acetyl-coenzyme A [CoA] carboxylase inhibitor), increasing fatty acid desaturation (stearoyl-CoA desaturase inhibitor), or improving IR (PPARγ and glucagon-like peptide 1 [GLP-1] agonists). However, worsening dyslipidemia has been observed with several pharmacologic agents that directly or indirectly target NEFA flux, lending some caution to this approach. Altered lipid flux may also impact accumulation of toxic lipids, which is discussed more in the next section.

LIPOTOXIC HEPATOCELLULAR STRESS

In addition to the histologic or imaging-based recognition of fat accumulation in the liver, there has been a rapid increase in the understanding of the deleterious role of lipotoxicity in NAFLD since the first description of lipotoxicity by Roger Unger in 1994 and the earlier recognition that many lipid species are bioactive. Concomitantly, there has been an expansion of hepatic and plasma lipidomics in NAFLD.[23-25] Lipidomics analyses have typically been stochastic rather than paired and kinetic over the natural history of NAFLD. This has limited their interpretation to correlations; although *in vitro* observations and mouse models have elucidated the signaling pathways triggered by toxic lipids.

Lipotoxic Lipid Classes

Mechanistic studies in isolated cells and animal models have elucidated the role of several lipid classes in hepatocellular toxicity, liver injury, and inflammation. Among these, saturated NEFAs, predominantly palmitate, the glycerophospholipid LPC, free cholesterol, sphingolipids (including ceramides), and sphingosine 1-phosphate (S1P) are well studied, although other classes of lipid, and their biosynthetic pathways are also deranged in NASH.[22] While not the focus of this review, it is interesting to briefly note an increase in the ratio of n-6 to n-3 PUFAs and their inflammation-regulating derivatives in NASH, especially an increase in linoleic acid and its oxidized products[26] as well as an increase in oxysterols, which may have proinflammatory roles through activation of innate immune cells.[27] Toxic lipids accumulate and provoke injury in hepatocytes as well as in nonparenchymal liver cells. Several extrahepatic factors, such as intestinal dysbiosis and adipokines, modulate lipotoxic exposure to the liver and subsequent injury and inflammation, with variable contributions across individuals and different stages of disease pathology.[2] At a molecular level, lipotoxicity leads to endoplasmic reticulum (ER) stress, lysosomal dysfunction, inflammasome activation, cell death, and activation of inflammatory responses due to lethal and sublethal hepatocellular injury.[28]

TABLE 1. THERAPEUTIC STRATEGIES TARGETING LIPOTOXICITY IN NAFLD

Target	Mechanism	Example Drug
FXR	Agonist: improve insulin sensitivity; anti-inflammatory and antifibrotic	Obeticholic acid
Acetyl-CoA carboxylase 1/2	Antagonist: decrease DNL	PF-05221304
FGF19/21	Agonist: decrease bile acid synthesis; anti-inflammatory and antifibrotic	NGM282
PPARα/γ/δ	Agonist: increase fatty acid oxidation; improve IR; anti-inflammatory	Elafibrinor
Steroyl CoA-desaturase 1	Antagonist: decrease DNL	Aramchol
Thyroid hormone receptor β	Agonist: decrease circulating lipids	MGL-3196
Niacin-R	Agonist: decrease lipolysis in adipose tissue; decrease TG synthesis and increase fatty acid oxidation	Niacin
Sirutin-1	Agonist: decrease DNL; increase fatty acid oxidation; anti-inflammatory	Resveratrol
Ketohexokinase	Antagonist: decrease DNL	PF-06835919

Molecular Mechanisms of NEFA-Induced Lipotoxic Cell Death

Palmitate is elevated in plasma and accumulates in its esterified form in the liver in NASH, as demonstrated by a preponderance of palmitate-containing TGs in mouse models and humans with NASH.[29] The molecular pathways that mediate the toxicity of palmitate have been elegantly elucidated in cultured hepatocytes.[21,22] Palmitate can activate both the intrinsic- and extrinsic-mediated (death receptor) apoptotic machinery in hepatocytes.[21] The intracellular balance of proapoptotic versus antiapoptotic proteins is shifted in palmitate-treated hepatocytes toward apoptosis (Fig. 2). This includes the activation of intracellular stress-activated kinase c-jun N-terminal kinase (JNK),[30] up-regulation of the proapoptotic proteins Bim and p53 up-regulated modulator of apoptosis (PUMA),[31,32] degradation of the antiapoptotic proteins B-cell lymphoma-extra large (Bcl-XL) and myeloid cell leukemia 1 (Mcl-1),[33] and inhibitor of apoptosis proteins.[34] Many of these perturbations occur downstream of the stress kinase JNK and organelle dysfunction, such as lysosomal permeabilization and ER stress, leading to transcriptional up-regulation of proapoptotic
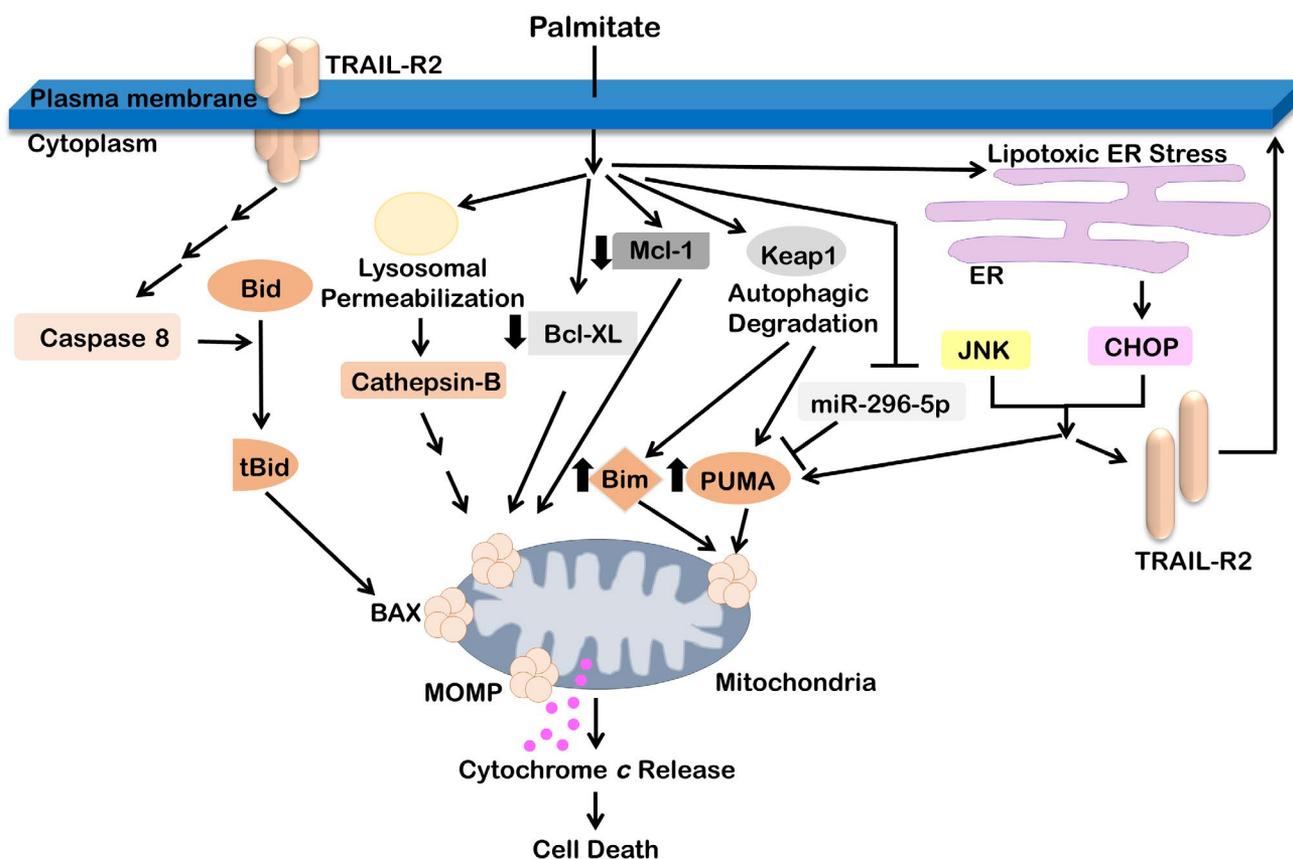
FIG. 2. Molecular pathways of palmitate-induced lipotoxicity in hepatocytes. Palmitate activates the extrinsic death receptor-mediated pathway of apoptosis and also activates the intrinsic pathway of apoptosis. Lysosomal permeabilization leads to the release of the protease cathepsin B. Lipotoxic ER stress leads to up-regulation of the proapoptotic transcription factor CHOP. The stress-induced kinase JNK and CHOP induce the death receptor TRAIL-R2 and the proapoptotic Bcl-2 family proteins PUMA and Bim. PUMA and Bim are also up-regulated by palmitate-induced autophagic degradation of Keap1. Palmitate decreases the expression of antiapoptotic proteins Mcl-1 and Bcl-XL. TRAIL-R2 can undergo ligand-independent oligomerization, cleavage-induced activation of caspase 8, Bid cleavage to tBid, and activation of Bax. Ologomeric Bax results in mitochondrial outer membrane permeabilization, release of cytochrome *c*, activation of effector caspases, and apoptosis. Abbreviations: BAX, B-cell lymphoma 2-like protein 4; Bcl-XL, B-cell lymphoma-extra large; Bim, B-cell lymphoma 2-like protein 11; Keap1, Kelch-like ECH-associated protein 1; Mcl-1, induced myeloid leukemia cell differentiation protein; MOMP, major outer membrane protein; tBid, truncated p15 BID.

proteins. Posttranscriptional up-regulation of PUMA has been described by palmitate-induced repression of microRNA-296-5p.[35] Palmitate also sensitizes cells to tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL)-induced cell death by transcriptional up-regulation of TRAIL receptor 2 (TRAIL-R2) expression, which can lead to ligand-independent activation of the extrinsic apoptotic pathway.[36] Both the extrinsic and intrinsic pathways converge on Bax-induced mitochondrial permeabilization, release of cytochrome c, and activation of effector caspases. Palmitate toxicity may in part be mediated by LPC, which accumulated intracellularly in palmitate-treated hepatocytes and activated the intracellular proapoptotic pathways previously defined for palmitate, including JNK, C/EBP homologous protein (CHOP), and PUMA.[37]

Protective Lipid Classes

In addition to toxic classes of lipids, two broad categories of protective lipids are associated with obesity-associated NAFLD.[22] The MUFAs palmitoleate and oleate can reduce the toxicity of palmitate in cultured hepatocytes, although they promote TG formation, suggesting that the sequestration of palmitate into neutral TG is cytoprotective.[22,38] In a mouse model, adipose tissue-derived palmitoleate suppressed hepatic steatosis and improved muscle IR, suggesting a different mechanism for its protective effect.[39] The second class of lipids of interest is a set of PUFA-derived specialized proresolving mediators (SPMs), which, through receptor-mediated effects on immune cells, limit inflammation. These consist of n-3 PUFA-derived lipoxins, resolvins, maresins, and protectins.[22] Exogenous resolvin D1 administration promoted the resolution of inflammation in a mouse dietary withdrawal model of NASH resolution, suggesting a therapeutic role for SPMs in NASH.[40]

Organelle Stress

Subcellular organelle stress, including lysosomal permeabilization and ER stress, occurs in palmitate-treated hepatocytes and eventually contributes to cell death by up-regulation of proapoptotic signaling (Fig. 2). Lysosomal permeabilization was mediated by translocation of Bax to the lysosomal membrane.[41]

Palmitate-induced ER stress occurs due to an increase in saturated acyl chains and is referred to as lipotoxic or lipid bilayer ER stress.[42] Indeed, palmitate-induced apoptosis was partially dependent on the ER stress-induced proapoptotic transcription factor CHOP, which in turn transcriptionally up-regulated TRAIL-R2 and PUMA expression.[43] A recent study has linked ER stress to inflammatory responses elicited by steatotic hepatocytes through the release of proinflammatory extracellular vesicles (EVs).[44] ER stress has been implicated in obesity-associated IR and also in inflammation in various models.[45] The contribution of ER stress to inflammation in NASH needs further exploration.

Relevance of Cell Death in NASH Models

A comprehensive multiomics study identified hepatocyte apoptosis as a key early signaling event in high-fat diet-induced NASH in mice.[46] In keeping with this observation, mice deficient in apoptosis are protected from NASH.[47] TRAIL receptor knockout mice are resistant to high-fat diet-induced obesity and diminished macrophage inflammatory responses, pointing toward a broader role for death receptor-induced inflammatory signaling in obesity-associated inflammation. Besides apoptosis, other forms of hepatocyte cell death have been described in NASH models. Pyroptosis, an inflammatory form of cell death that occurs due to the activation of inflammasomes and caspase 1 leading to cleavage-induced activation of gasdermin D, is implicated in animal models of NASH and in liver biopsies of patients with NASH[48]; however, there are no clear links between palmitate and pyroptosis in hepatocytes. Free cholesterol, on the other hand, is known to activate the inflammasome.[49] Free cholesterol accumulation in hepatocytes and cholesterol crystal formation in hepatocyte lipid droplets in subjects with NASH and not isolated steatosis is associated with aggregation of Kupffer cells (KCs) and fibrosis, suggesting that perhaps inflammasome activation may play a role in NASH.[50,51] Similarly, ferroptosis, a form of cell death dependent on iron and oxygenated phosphatidyl ethanolamine (PE), is also reported in NASH.[52] Oxygenated lipids, such as PE, may be a mediator or a correlate of lipotoxicity due to other lipids in these models. Further, although necroptosis in hepatocytes

with intact caspase signaling remains controversial, the receptor-interacting protein kinases are implicated in NASH.[53] This may be due to their roles in inflammatory signaling and cell death secondary to immune cell activation.

Mechanistically diverse types of hepatocellular death are observed in NASH in both animal models and human specimens. In these models, hepatocellular death is linked to inflammation, and these two processes may form a feed-forward loop such that cell death triggers inflammatory signaling and immune cells secrete mediators, such as TRAIL or Fas, which can initiate apoptotic signaling. Although the question of primacy in these processes has not been answered, experimental data show that interruption of this loop mitigates NASH.[54] Furthermore, as discussed above, rather than apoptosis alone, several other forms of cell death may play a role in activation of the immune response. Death receptor activation also leads to the release of chemokines, including interleukin (IL)-6, IL-8, C-X-C motif chemokine ligand 1 (CXCL1), and C-C motif chemokine ligand 2 (CCL2), which can promote macrophage chemotaxis toward dying cells.[55]

Mechanisms of Inflammation

INFLAMMATION AND IMMUNE DYSREGULATION

Activation of Immune Receptors

Inflammasomes are intracellular pattern recognition receptors (PRRs) that trigger the maturation of proinflammatory cytokines, such as IL-1β or IL-18.[56] While the expression of the Nod-like receptor protein 3 (NLRP3) inflammasome components is very low in healthy hepatocytes, during NASH, the expression of NLRP3 components is increased in animal models[57] and humans.[58] Selective pharmacologic inhibition[57] or genetic deletion[58] of the NLRP3 inflammasome or its components results in improved NASH pathology, including hepatocyte inflammation and fibrosis. The NLRP3 inflammasome is predominantly expressed in injured hepatocytes, KCs, and liver sinusoidal endothelial cells.[59] It is also present in hepatic stellate cells

where it is required for the development of fibrosis, at least in experimental mouse models.[60] The NLRP3 inflammasome can be activated by multiple ligands, including pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs). In NASH, potential triggers of its activation include lipopolysaccharide (LPS) and danger signals released from hepatocytes undergoing palmitate-induced apoptosis, mitochondrial DNA released following fatty acid stimulation,[61] cholesterol, and microvesicles released from fat-laden cells undergoing lipotoxicity.[62-64]

Toll-like receptors (TLRs) are transmembrane PRRs that sense invading pathogen or endogenous damage signals.[65] Several studies have shown that TLR4 expression, especially in macrophages, is increased in patients with NASH[66] and mouse models of disease.[67] Translocation of TLR4 agonists, such as LPS and bacterial components from the gut, activate TLR signaling and has been shown to drive the progression of NASH.[68] The expression of TLR9, a TLR that mainly recognizes bacterial DNA, increases in multiple mouse models of NASH, and its genetic deficiency leads to improved steatosis, inflammation, and liver fibrosis, suggesting that TLR9 promotes the progression of NASH.[69]

IMMUNE CELL-MEDIATED INFLAMMATION

Crosstalk between immune cells in metabolic tissues dictates the overall inflammatory tone and systemic metabolic homeostasis. Recent evidence indicates that immunologic imbalances in the liver support the maintenance and progression of inflammation in NAFLD. In general, NASH is characterized by a robust recruitment of immune cells into the liver where they become activated and have the capacity to release molecules that cause inflammation (Fig. 3; Table 2). While a dysregulated immune response can lead to disease, the inflammatory response early during liver injury may also be important for healing and tissue repair.[70] Although innate immune mechanisms are considered a major contributing factor to the inflammatory process in NASH, recent evidence indicates that adaptive immunity has an important role in the progression of this disease.[71] Here, we highlight the major immune cell types involved in the progression of NASH.
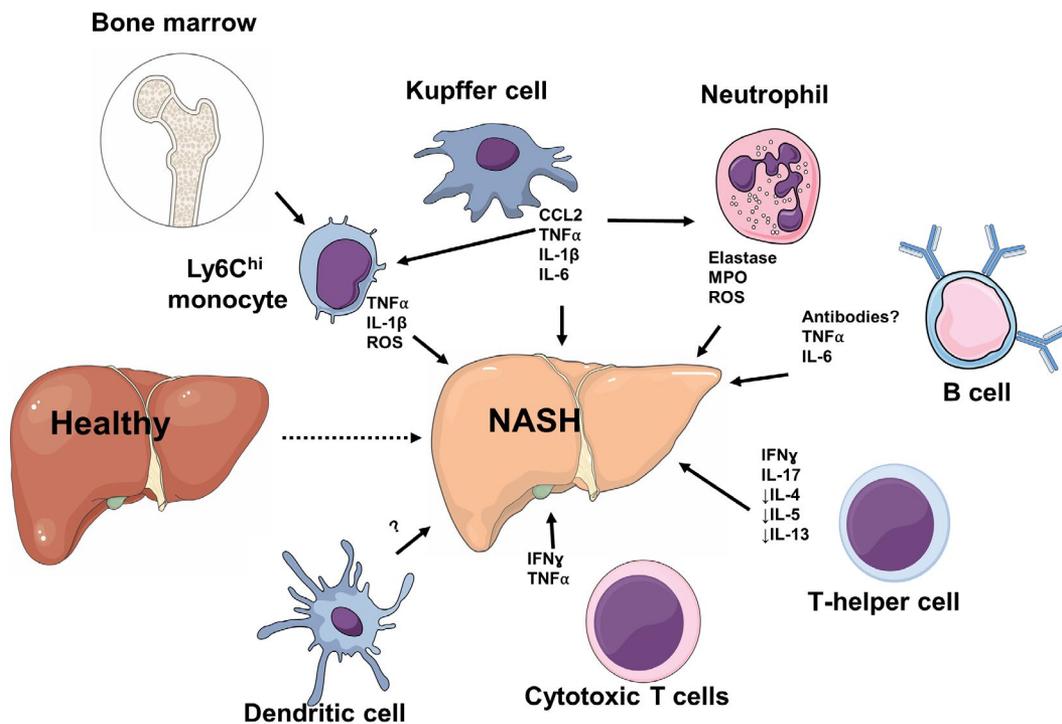
FIG. 3. Immune dysregulation in the pathogenesis of NASH. Activation of liver-resident KCs results in the release of CCL2 and other proinflammatory cytokines, such as TNFα, IL-1, and IL-6, leading to the recruitment of bone marrow-derived Ly6C^{hi} monocytes and neutrophils that further contribute to the inflammatory response. Activated neutrophils promote NASH by releasing elastase, MPO, and ROS. B cells can accumulate during NASH and produce TNFα and IL-6. NASH is characterized by excessive Th1- and Th17-derived IFNγ and IL-17, respectively, and a deficiency in Th2-derived IL-4, IL-5, and IL-13. Cytotoxic CD8+ T cells are supported by type I IFN responses and lead to the production of IFNγ and TNFα. The role of DCs and their subsets in NASH is unclear as animal studies show contradictory results and it has not been investigated in humans. Abbreviation: ROS, reactive oxygen species.

TABLE 2. IMMUNE CELLS INVOLVED IN HEPATIC INFLAMMATION DURING NASH

Cell Type	Activator/Recruiting Factor	Role in NASH
Kupffer cells	NLRP3 inflammasome, TLRs, IFN genes	Promote NASH in early phase. Produce increased TNFα and CCL2
Bone marrow-derived monocytes	CCL2/CCR2 axis	Promote inflammation and fibrosis in NASH
Dendritic cells		Unclear. Increased intracellular lipid and proinflammatory phenotype
Neutrophils		Promote NASH through antimicrobial secretion and NETs
Th1 and Th17 cells		Promote NASH through increased production of IFNγ and IL-17
Th2 cells		Decreased production of the anti-inflammatory cytokines IL-4, IL-5, and IL-13
Cytotoxic T cells	Type I IFNs	Promote NASH and HCC. Produce increased amounts of IFNγ and TNFα
B cells		Unclear, promote fibrosis in mouse models of hepatic injury

Macrophages

Liver macrophages are a heterogeneous population consisting of yolk sac-derived tissue-resident macrophages or KCs and bone marrow monocyte-derived macrophages. In the healthy liver, KCs exist within the hepatic sinusoids where they scavenge bacteria and microbial products from the intestine while mature monocytes show a patrolling behavior. In mouse models of NASH, KCs contribute to the early

phase of disease through increased production of TNFα and CCL2.[72] Indeed, depletion of KCs attenuates liver steatosis and hepatic IR in rats fed high-fat or high-sucrose diets.[73] Determining the factors that trigger the activation of innate immune cells, such as KCs, and transition from isolated steatosis to NASH is one of the major goals in the field. KCs express the highest levels of inflammasome components among liver cell types, and NLRP3 activation in KCs promotes IL-1β secretion fueling the progression of NASH.[61] Activation of the NLRP3 inflammasome in KCs can be caused by mitochondria DNA release in response to NEFA[61] or through the stimulator of interferon (IFN) genes, which induces inflammation through nuclear factor kappa B.[74]

The recruitment of bone marrow-derived monocytes has been shown to be a critical event in the progression of NASH. KC-derived factors facilitate a substantial infiltration of these monocytes into the liver, where together with KCs they contribute to the triggering and progression of disease through their inflammatory functions.[75] In mouse models of NASH, inhibition of CCL2 or CCL2 receptor (CCR2) leads to reduced recruitment of monocyte-derived macrophages, improved IR, hepatic inflammation, and fibrosis.[76] Although it has been proposed that infiltrating monocytes differentiate into liver macrophages, single-cell RNA sequencing of myeloid cells in mice fed a Western diet has shown that NAFLD also induces functional changes in their bone marrow precursors that remain stable throughout their migration into the liver,[77] suggesting a role for liver–bone marrow crosstalk in maintaining hepatic inflammation in NASH.

Dendritic Cells

In the steady state, liver dendritic cells (DCs) function as antigen-presenting cells (APCs) that internalize blood-derived antigens and transport them to regional lymph nodes. Liver DCs, however, are poor APCs with less capacity to activate T cells compared with DCs from other tissues.[78] While the role of DCs in human NASH is unclear, animal studies show contradictory results as depletion of hepatic DCs has resulted in both ameliorated or aggravated liver fibrosis and inflammation. These conflicting results are likely due to the heterogenicity of hepatic DCs and the low specificity of the methods used to

experimentally manipulate DCs. Hepatic DCs can be divided into three subtypes with diverse functional capacity: classical type 1, classical type 2, and plasmacytoid. Despite this heterogeneity, most studies of liver DCs have focused on total CD11c⁺ or major histocompatibility complex II⁺ DCs. Regardless of their subclass, hepatic DCs that have increased intracellular lipid content show a proinflammatory phenotype in both mice and humans.

Neutrophils

The infiltration of neutrophils into the liver contributes to the progression of NASH through the secretion of cytokines and active molecules. Western diet-fed mice develop increased expression of neutrophil elastase, and its genetic depletion results in decreased liver steatosis and inflammation.[79] Similarly, myeloperoxidase (MPO) deficiency ameliorates liver inflammation and fibrosis in mice fed a high-fat diet, suggesting a pathogenic role of neutrophils in NASH.[80] Neutrophils can release extracellular traps (NETs) composed of nucleic acids and antimicrobials to entrap pathogens and limit infection. Increased production and reduced clearance of NETs has been linked to chronic sterile inflammation in autoimmune and inflammatory diseases. Markers of NETs are elevated in the serum of patients with NASH, and blockade of NET formation in mice protects mice from inflammation and NASH-driven HCC.[81] Another mechanism by which neutrophils can aggravate NASH is through human neutrophil peptides that induce cytokine and chemokine release during inflammation. Transgenic expression of human neutrophil peptide 1 in mice fed a NASH diet aggravated hepatic fibrosis through induction of hepatic stellate cell proliferation.[82] Although the majority of studies suggest that neutrophils are pathogenic in the progression of NASH, they play a critical role in the reparative phase of sterile liver injury as they remove dead vasculature and create new channels for vascular regrowth. Whether neutrophils have divergent roles in NASH by promoting early inflammation but favoring later resolution, including fibrosis, remains to be investigated.

T-Helper Cells

T-helper (Th) cells are key players of adaptive immune responses as they assist B cells, macrophages, and

cytotoxic T cells in eliminating pathogens and infected cells. After immune activation, Th cells can differentiate into Th1, Th2, and Th17 effector cells, depending on the cytokines in their environment. In general, NASH is characterized by excessive Th1-derived cytokines, such as IFNγ, and a deficiency in Th2-derived cytokines, including IL-4, IL-5, and IL-13.[83] IL-17-producing Th17 cells accumulate in the liver of mice and humans with NASH and have been shown to aggravate inflammation and fibrosis through effects on macrophages and hepatic stellate cells, respectively.

Cytotoxic T Cells

In mice and humans, cytotoxic CD8+ T cells accumulate in the liver during NAFLD, and their pharmacologic[84] or genetic ablation[85] results in decreased steatosis, IR, inflammation, and hepatic stellate cell activation. Activation of these cytotoxic CD8+ T cells is supported by type I IFN responses and leads to the production of the proinflammatory cytokines IFNγ and TNFα.[85] Cytotoxic CD8+ T cells have also been shown to promote NASH development and subsequent transition to HCC in a process that requires crosstalk with natural killer T cells.

B cells

B cells have recently emerged as critical regulators of inflammation in adipose tissue during obesity.[86] However, their role in the development of NASH is not well understood. In general, B cells can be divided into B1 and B2 subsets with differing immunophenotypes, functions, and cytokine secretion profiles. B2 cells accumulate in peripheral tissues, such as adipose tissue, where they contribute to IR through activation of T cells and secretion of proinflammatory cytokines. In contrast, adipose tissue B1 cells have protective roles against obesity-related IR. In mice with NAFLD, B cells accumulate in the liver where they express increased amounts of TNFα and IL-6 and have a higher capacity to activate T cells.[87] B cells have also been shown to promote hepatic fibrosis in a mouse model of acute liver injury. In the same model, specific B-cell deletion of the adaptor protein myeloid differentiation primary response 88 (MYD88) results in reduced infiltration of monocytes and DCs, suggesting that B cells are among the first responders during hepatic injury.[88] Despite these findings, the exact mechanisms by which B cells become activated in the liver and promote NASH are unclear.

Intercellular and Interorgan Crosstalk

Intercellular and interorgan crosstalk are necessary for the development of liver injury and inflammation in NASH. Both soluble mediators and circulating EVs have been implicated in this crosstalk. Here, we review the adipose–liver axis, gut–liver axis, and the role of EVs in inflammatory crosstalk in NASH (Fig. 1).

ADIPOSE–LIVER AXIS

Adipose tissue metabolic dysfunction is closely related to liver inflammation and fibrosis in humans and is a central driver of NASH development.[89] During obesity, failure of the adipose tissue to expand and store excess energy leads to increased lipolysis and subsequent secretion of NEFAs. Diminished adipose tissue expandability and not obesity itself is believed to be the key factor linking positive energy balance and metabolic disease.[90] Increased influx of adipose tissue-derived NEFAs is a substantial source of substrate for the formation and storage of TGs in the liver, resulting in steatosis.[91] Not only is the adipose tissue the major source of NEFA but it is also an endocrine organ secreting adipokines with systemic regulatory effects. Leptin and adiponectin produced by visceral adipocytes influence NAFLD and other components of the metabolic syndrome through regulation of food intake, body fat composition, insulin sensitivity, and inflammation.[91] In addition, excessive production of proinflammatory cytokines by visceral adipose tissue macrophages is considered to be critical in obesity-associated adipose tissue inflammation.[92] Activated adipose tissue macrophages secrete cytokines and chemokines, including TNFα, IL-1β, IL-6, and CCL2, which cause local IR resulting in dysregulated lipid metabolism but which can also reach the circulation leading to systemic IR.[93] Immune activation in adipose tissue likely precedes liver inflammation as mice with NASH have increased expression of macrophage and inflammatory genes in adipose tissue before the liver.[89]

GUT−LIVER AXIS

There is a bidirectional relationship between the gut, which represents an important port of first entry for external environmental influences, and the liver, which is the first line of receipt and processing of these factors. The gut contributes to both homeostasis in health as well as the pathogenesis of liver disease through several intermediaries that interact with one another, including the microbiota, bile acids, luminal products, immune mediators, and gut hormones. While causal links between the microbiota and NAFLD have not been fully elucidated, disruption in intestinal permeability[94] and bacterial-derived ligands (e.g., LPS) and metabolites (e.g., secondary bile acids, short chain fatty acids)[95] are putative mediators of this association. Microbial-derived PAMPs are capable of inciting an immune reaction and inflammation in the liver. Recently, the presence of a strain of bacterium (*Klebsiella pneumoniae*) that produces high levels of endogenous alcohol was associated with NAFLD in a human cohort.[96] Bile acids are synthesized and secreted by hepatocytes and are involved in the absorption of dietary lipids. They are transported back to the liver by enterohepatic circulation and act on the nuclear farnesoid X receptor (FXR), which is also expressed on hepatocytes, to influence glucose[97] and lipid metabolism.[98] Further, release of FGF19 after ileal FXR activation is a feedback mechanism that reduces bile acid synthesis and also hepatic steatosis and IR.[99] Bile acids, through their antimicrobial effects, also modulate the relationship between gut microbiota and chronic liver disease[100] and improve glucose metabolism by activation of G-protein coupled bile acid receptor (GPBAR1) in enterocytes.[101] Therefore, targeting these mechanisms, for example, with an FXR agonist, is an attractive strategy in therapy for NAFLD.[101,102] Gut-derived hormones, such as GLP-1, play a crucial role in controlling nutrient intake, absorption, and metabolism and are attractive targets for metabolic disease in general as well as in the liver.[2]

EXTRACELLULAR VESICLES

Recent studies have examined the role of EVs as a vector for cell-to-cell communication in NASH.[103] Circulating EVs are elevated in human NASH samples as well as mouse models of NASH.[44] Numerous bioactive cargoes have been defined for these EVs as well as the recipient cell responses activated by hepatocyte-derived EVs.[103] In NASH models, circulating EVs also differ in their cell of origin. EVs from adipocytes, macrophages, neutrophils, and platelets are reported to be elevated.[104] Mechanistic studies have demonstrated that both microvesicles and exosomes are released by lipotoxic hepatocytes using distinct cell signaling pathways. LPC-stimulated EV release was dependent on mixed lineage kinase 3. EVs released from palmitate- or LPC-treated hepatocytes required activation of the death receptor 5 signaling axis leading to caspase-mediated cleavage and activation of the rho-associated coiled-coil-containing protein kinase 1 (ROCK1).[103] In additional experiments, palmitate-stimulated small EV release was dependent on the *de novo* synthesis of ceramide and preserved in the presence of caspase inhibitors, suggesting that lipotoxic EVs are heterogeneous in origin.[44]

Similar to the mechanism of their origin, EVs in NASH contain multiple bioactive cargoes. CXCL10 and S1P on EVs are implicated in macrophage chemotaxis, whereas TRAIL activated a proinflammatory response in macrophages.[103] Vanin-1-enriched EVs were targeted to endothelial cells where they activated angiogenic pathways.[103] Mitochondrial DNA-enriched microparticles were also elevated in mouse models of NASH and found to activate myeloid cell TLR9-dependent inflammation in the liver.[103] Systemic injections of circulating EVs derived from high-fat-fed mice led to an increase in myeloid cells in circulation and hepatic accumulation of monocyte-derived macrophages, suggesting that lipotoxic EVs can educate monocytes in the circulation to home into the liver.[103] Thus, EVs may play a role in the local microenvironment of the liver, in circulation, and in interorgan crosstalk.

Conclusions

In the multifactorial pathogenesis of NASH, both hepatocyte lipotoxicity and immune-mediated inflammation play key roles. Activation of hepatic stellate cells occurs as a consequence of signaling from stressed or apoptotic hepatocytes and macrophages, although other immune cells may play a role as well (Fig. 4). This triadic lesion forms the cornerstone of progressive NASH. However, recognizing the heterogeneity of NAFLD,
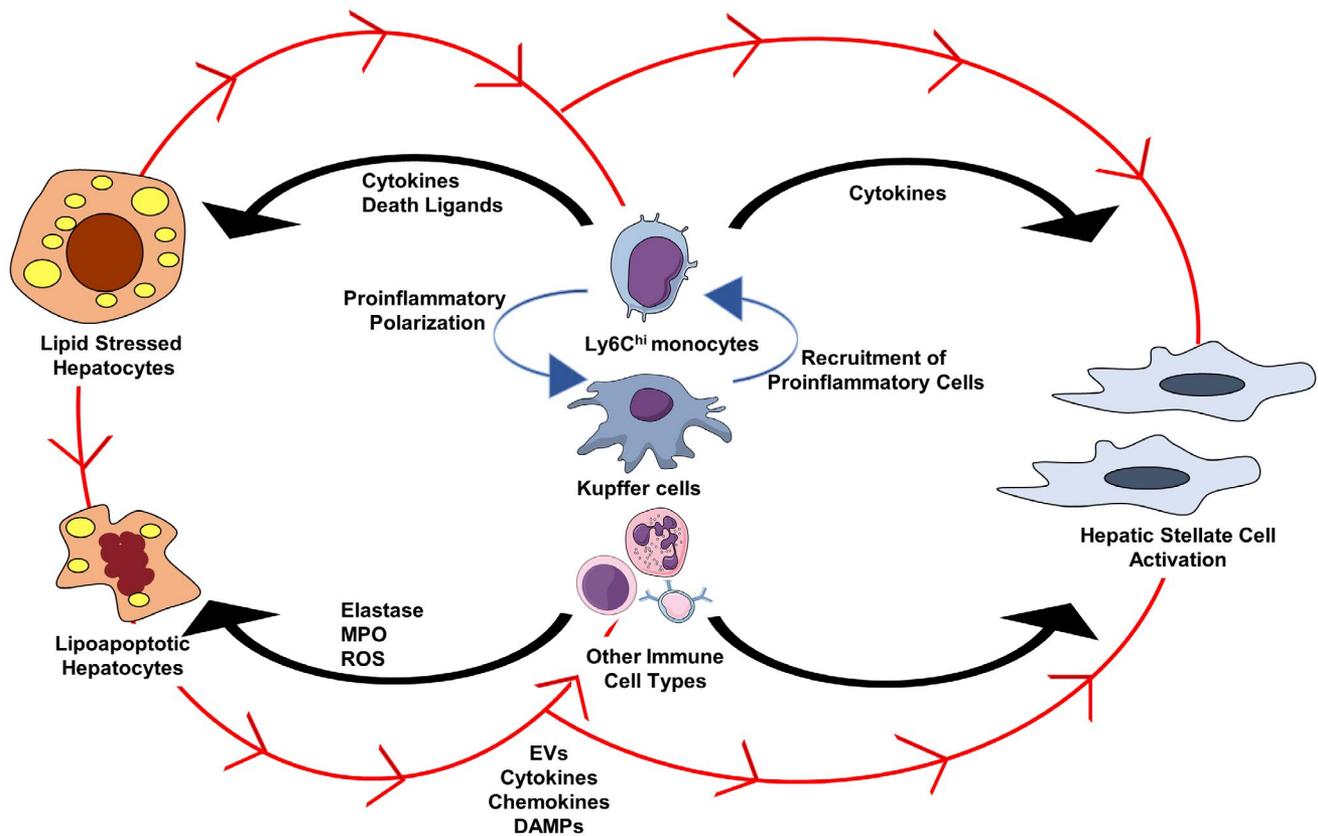
FIG. 4. Triadic lesion in the pathogenesis of NASH. Hepatocyte injury, macrophage-mediated inflammation, and hepatic stellate cell activation comprise the key mechanistic abnormalities in NASH. Soluble and EV signals from hepatocytes lead to proinflammatory activation of macrophages, can recruit proinflammatory monocytes into the liver, and also lead to hepatic stellate cell activation (depicted in red arrows). Other immune cells, such as neutrophils and B cells, may also respond to hepatocyte-derived signals. Activated macrophages release cytokines and chemokines that can promote hepatocyte apoptosis, attract other immune cells into the liver, and also influence the activation of hepatic stellate cells. Abbreviation: ROS, reactive oxygen species.

it is very likely that our classification of NAFL and NASH will evolve as our understanding of the complexity of NASH pathogenesis grows. For example, future definitions may stratify the type of NASH based on key features in the pathogenesis continuum, such as ER stress-predominant NASH versus apoptosis-predominant NASH versus macrophage-predominant NASH or B-cell-predominant NASH. Furthermore, each of these types of NASH may have variable activation of hepatic stellate cells. Our nascent understanding of this heterogeneity would also suggest that combination therapy, although logical, may uncover potentially redundant pathways in NASH pathogenesis. Regardless, mechanistic studies that answer fundamental questions in NASH pathogenesis are still needed. These include exploration of mechanistic questions that address early events in liver injury in NASH, such as the

consequences of sublethal hepatocyte injury. Another key area is the definition of the triggers, kinetics, and magnitude of immune-cell activation responses along the NAFLD spectrum and to define the individual and synergistic contribution of each type of immune cell.

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