Emerging Roles for Lipids in the Hepatic Innate Immune Response

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Abstract

The liver provides residence to one of the largest and most heterogeneous populations of innate immune cells within the body. Owing to its location downstream of the intestine and the constant interaction with gut derived factor, these immune cells protect both the liver and the periphery from a variety of invading pathogens while also contributing both positively and negatively to a number of chronic liver pathologies. Growing evidence indicates a critical role for lipid in the regulation of the hepatic innate immune response. Likewise, excess lipid consumption and accumulation within the liver represents a major clinical problem and nexus for lipid – immune cell interaction. The current review highlights our current understanding of the interactions of and mechanisms by which lipids may modulate the hepatic innate immunity.

INTRODUCTION

Non-alcoholic fatty liver disease represents a growing clinical problem worldwide [1,2]. Recent studies in a European population demonstrated NAFLD in 94% of patients with a BMI exceeding 30 kg/m² and perhaps more troubling in 25% of patients with a normal BMI (<25). Clear complications are associated with excess hepatic lipid accumulation from stimulation of marked inflammation to more severe tissue scarring and hepatocellular cancer development [3]. Given this strong connection between lipids and liver tissue pathologies and the importance of hepatic immune cell populations to both the promotion as well as potentially protection from these disease processes, a greater understanding of the impact of lipid on hepatic innate immune cell function is absolutely necessary to define new therapeutic approaches. The following section will highlight some new data defining the potential interactions among lipids and lipid derivatives and immune cell biology.

IMMUNOLOGY OF THE LIVER: AN OVERVIEW

Lying directly downstream of the intestine, the liver represents the first line of defense against the billions if not trillions of gut-derived bacterial pathogens [4]. To accomplish this role, the liver is equipped with a number of resident innate immune cell populations (Figure 1) including Kupffer cells, the hepatic macrophage, dendritic cells, natural killer (NK) cells, and natural killer T (NKT) cells [5,6]. Together, these resident innate immune cells operate to limit pathogen entry into the central circulation while also suppressing the inflammatory response to these routinely encountered pathogens. They also contribute to numerous liver pathologies from ischemic injury to viral infection and autoimmune disease [6]. Thus, they play important but potentially damaging roles in liver biology and pathobiology. The following sections will highlight each cell, their basic markers, and their general function in liver biology and pathobiology.

The Hepatic Innate Immune System

Also known as natural immunity, innate immunity is a response to common environmental antigens through conserved receptor complexes with similarities throughout vertebrates [7]. Being relatively non-specific, innate immune processes utilize a variety of pathways to destroy recognized antigens including complement, oxidant release, and/or phagocytosis. Kupffer cells, the resident liver macrophage, are the principle hepatic innate immune cell population and through expression of a number of receptors, most notably Toll like receptors and accessory molecules including CD14 and CD36, exert significant inflammatory responses to circulating pathogens including those derived from the gut [8-10]. In response to antigen such as endotoxin, KCs produce large quantities of tumor necrosis factor alpha (TNFα) and oxidants including NADPH oxidase-derived superoxide and endocytose bacteria carried through the portal circulation [9,11,12]. KCs also act as antigen presenting cells through processing and presentation via major histocompatability complex (MHC) class II molecules to hepatic
Figure 1 Diagram of the innate immune repertoire in the liver and their basic functions in liver biology. PV = portal vein; HA = hepatic artery; BD = bile duct; DC = dendritic cell; KC = Kupffer cell; NKT = natural killer T cell; Tc = T cell; NK = natural killer cell.

Lipid and the Hepatic Immune Response

Lipids represent an important regulator of metabolic processes both in the liver and in the periphery. Lipids or lipid derivatives such as arachidonic acid are also well appreciated for their ability to participate in the inflammatory response [37,38]. It is also becoming clear that these same processes can lead to the generation of anti-inflammatory mediators as well [39]. As noted earlier, excess lipid accumulation in the liver represents a major clinical problem where the innate immune response clearly functions to promote tissue damage [2]. Defining the mechanisms of and interactions between lipids and immune cell populations, particularly within the liver has thus gained significant research traction. To begin to understand these interactions, analysis of the impact of steatosis on the populations of hepatic innate immune cells present should be explored and is the subject of the following section.
Impact of fatty liver on resident innate immune cells

A number of studies have characterized the hepatic immune cell populations during the progression of fatty liver disease in a several models of injury. Accumulation of hepatic lipid promotes macrophage activation / accumulation and recruitment through induction of inflammatory chemokine signaling including CCR5 [40]. Genetic deletion of CCR5, or long-term depletion of macrophages reduces inflammatory liver damage in response to hepatic lipid accumulation but also reduces lipid accumulation itself through minimizing the inflammatory impact on hepatocyte metabolic function [41,42]. Indeed, TNFα production from KCs enhances hepatocyte sterol receptor element binding protein (SREBP) 1-c expression and subsequent lipid synthesis while it also reduces β-oxidation through inhibition of peroxisomal fatty acyl-CoA oxidase thus driving the hepatocellular lipid accumulation [43].

Recent evidence also indicates that other innate immune cells, particularly DCs, are increased in number within the steatotic liver. Henning et al. (Graffeo, Rehman, Fallon, Ochi) report a significant increase in hepatic DC number and maturation state exhibiting an activated phenotype with expression of TNFα, IL6, and MCP-1 (CCl2) as well as Tlr2, 4, 7, and 9 [44,45]. Intriguingly, loss of DCs promoted hepatic inflammation in NASH with increased KC accumulation and inflammatory mediator production [46]. These studies also demonstrated increased numbers of NK cells within the steatotic liver where they showed increased cytolytic activity in conjunction with up-regulated tumor necrosis factor related apoptosis-inducing ligand (TRAIL) receptor 2 [46].

In contrast to other hepatic innate immune cell populations, excess lipid accumulation is correlated with reductions in hepatic NKT cell populations [47]. Indeed, studies from our lab demonstrate a steatosis-dependent loss of NKT cells in a model of choline deficient diet induced steatosis. Further examination revealed a kupffer cell and IL12-dependent mechanism for their depletion. Depletion of macrophages with liposome encapsulated clodronate or genetic deletion of IL12 restored hepatic NKT cell numbers in the steatotic liver. Moreover, choline-deficient diet induced steatosis leads to a shifted, T helper 1 dominated (interferon gamma; IFNγ) inflammatory response which is nearly completely suppressed when NKT cells are restored [47,48]. The significance of NKT cell depletion in this model, aside from their potential as a regulatory cell population, remains to be determined though it is likely that NKT cell loss may predispose the liver to secondary pathologies including carcinoma development, a pathology associated with severe NAFLD.

As noted above, NKT cells respond to CD1d presented lipid derivatives opening the possibility that endogenous lipids may also contribute to their activation. For example, high levels of phosphatidylserine on the surface of apoptotic cells and its subsequent engulfment by APCs led to the rapid activation of NKT cells and their suppression of the immune response [49]. Excess lipid as seen with high dietary fat consumption may also alter NKT cell responses. Disruption in lipid processing in APCs may limit NKT cell activation [50]. Indeed, accumulation of lipids in β-galactosidase or lysosomal lipid transfer protein Niemann-Pick C deficient mice altered presentation of αGal by DCs to NKT cells in vitro [50]. Similar defects may also be present in the steatotic liver where excess lipid is likely accumulated in multiple cell populations including antigen presenting cells and may further negatively regulate their function or survival in the steatotic liver.

Lipids as regulators of hepatic innate immunity

It is clear from the above discussion that lipid accumulation within hepatocytes promotes an innate inflammatory response [51]. Activation of inflammatory cytokine production, up-regulation of key adhesion molecules, and recruitment of additional extrahepatic cells drive disease progression. A critical gap in our understanding of the mechanisms responsible for the initial inflammatory response exists. One plausible explanation is the induction of oxidant damage to hepatocytes which promotes inflammatory cell recruitment to repair tissue damage [2]. Growing evidence suggests that the process may be more direct, a scenario whereby the lipids themselves may regulate immune cell activation [52]. Specific transcription factors and signaling molecules are present in liver and immune cells which may act as sensors or mediators of lipid driven signals. These include members of the peroxisome proliferator activated receptor (PPAR) family, particularly PPARγ, retinoic acid receptors, and fatty acid binding proteins (FABPs) among others. Together, these factors provide a defined and potent mechanism by which lipids regulate immune cell function and could be critical for immune responses within the liver. The following sub-sections will highlight three of these regulators and their mechanisms of action and potential to alter hepatic immunity.

PPARγ

Perhaps one of the most well recognized lipid-associated transcription factors, PPARγ has shown significant influence on a variety of cell populations [53]. Activation by specific ligands (either endogenous or exogenous) promotes PPARγ dimerization with retinoid X receptor which initiates the recruitment of a wide variety of transcription co-activators including the steroid receptor co-activator 1, p300, and the PPARγ co-activator protein [54]. Binding of this complex to the peroxisome proliferator response element initiates transcription of an array of targets from cell cycle regulation to carbohydrate, lipid, and drug metabolism genes [55]. PPARγ has thus been strongly associated with lipid metabolism particularly in adipocytes and to a slightly lesser extent in hepatocytes. PPARγ also plays a prominent role in inflammatory responses [56,57]. Early investigation revealed the ability of endogenous or dietary PPARγ ligands to limit intestinal inflammation in two models of colitis in the mouse [58]. Additional studies highlighted the impact of PPARγ on macrophage responsiveness. Odegaard and colleagues demonstrated the interconnection of IL4 signaling and PPARγ induction on the M2 macrophage phenotype shift [59]. Macrophage specific loss of PPARγ limited IL4-induced arginase induction, a hallmark of M2 phenotype in murine macrophages. Similarly, mannose receptor up-regulation in response to IL13 was also abrogated in the absence of PPARγ again highlighting its importance in the induction of M2 macrophage responses [60]. In liver associated Kupffer cells, activation of retinoid X receptor or PPARγ with exogenous agonists limited LPS induced
PPARγ has also been shown to influence DC function. Unlike its effects on macrophages, ligand activation of PPARγ in DCs preferentially promotes CD1d expression and enhances antigen presentation to NKT cells thereby augmenting the immune response to specific antigens [64]. Consistent with these findings are those by Liu and others demonstrating the PPARγ-dependent induction of the activation marker CD40 and CD86 in response to oxidized low density lipoprotein [65]. Together, these studies demonstrate the functionality of PPARγ, a lipid sensitive transcription factor, on immune cell function. Future studies aimed at targeting or modulating activator or inhibitor production and delivery will likely provide new therapeutic options to treat acute and chronic liver pathologies.

### Fatty acid binding proteins

Fatty acid binding proteins (FABPs) are a family of abundantly expressed, 14-15kDa, intracellular cytosolic proteins that protect a cell's lipid balance [66]. FABPs are involved in the uptake and transport of hydrophobic ligands to specific cellular compartments based on the cellular needs of the cell. Structurally, FABPs consist of beta-barrels and an internal water-filled cavity where one ligand, such as a fatty acid, cholesterol, or retinoid is bound [67]. Fatty acids can be chaperoned by FABPs to the peroxisome for oxidation, the mitochondrion for β-oxidation, the endoplasmic reticulum for signaling and membrane synthesis, the cytosol to regulate a variety of enzyme activity, the nucleus for lipid-mediated transcriptional regulation, or to lipid droplets for storage [68]. The highly conserved, tissue specific, FABPs consist of at least 9 known isoforms, named for the tissue where each was discovered. The liver isoform, L-FABP (FABP1) is the most abundantly expressed of the family members, and accounts for as much as 5% of the cytoplasmic proteins in hepatocytes [69]. FABP2 and FABP3 isoforms are highly specific to the intestines and heart respectively. FABP4 was named due to its tissue distribution in the adipocyte and FABP5 in the epidermal. Other isoforms include ileal (FABP6), brain (FABP7), peripheral nervous system (FABP8), and testis (FABP9) [67]. Interestingly, despite the family's tissue specificity, FABP4 and FABP5 are the only isoforms co-expressed in the adipocyte as well as in the macrophage and thus potentially important for innate immune cell function [70]. Under normal conditions, FABP5 is more abundant in the macrophage than adipose tissue whereas FABP4 is more adipose specific. Disruption of FABP function allows excess lipids to accumulate in cells and can cause and may contribute to tissue damage including liver injury. The mechanisms underlying FABP signaling are poorly understood however they may control the lipid balance in inflammatory and metabolic cells like macrophages and adipocytes through lipid sensitive targets linked to inflammation signaling including as nuclear factor kappa B (NFκB), PPARγ, and LXR-α [71].

The integration of the inflammatory response and metabolic regulation is the focus of many metabolism disorders including type 2 diabetes, atherosclerosis, and fatty liver disease [72]. There is commonality between the metabolic regulators in adipocytes and the inflammatory response in macrophages, as both express cytokines, FABPs, nuclear hormone receptors, and many other factors [73]. Specifically, the co-expression of FABP-4 and FABP-5 in adipocytes and macrophages is of interest when studying metabolic diseases. Both FABP4 and FABP5 expression has been established by northern blot analysis in isolated peritoneal macrophages, western blot analysis in adipocyte and macrophage murine cells lines, and bone marrow derived macrophages [68,71]. As noted earlier, FABP4 expression is about 10,000 fold greater in adipocytes compared with macrophages [74]. Unlike the compensatory increase response of FABP4 seen in the deletion of FABP5, deletion of FABP4 does not cause this compensatory increased response of FABP5 [71]. Furthermore, Furuhashi showed that deletion of FABP4 in adipocytes resulted in reduced expression of inflammatory cytokines in macrophages, whereas the same deletion in macrophages led to enhanced insulin signaling and glucose uptake in adipocytes [68]. Likewise, A-FABP deficient macrophages exhibit a diminished production of inflammatory cytokines including TNFα, IL-6, IL1β, and CCl2 (MCP-1) [71]. Likewise, FABP4 potentiates lipopolysaccharide (LPS) induced macrophage activation augmenting a positive feedback loop involving c-Jun N terminal kinase (JNK) and activator protein 1 [75]. More specifically, the overlapping metabolic and inflammatory signaling and sensing pathways in adipocytes and macrophages are important to understand in the context of the hepatic immune response. Hoo et al. reported increased KC-associated FABP4 expression in obese mice as well as mice with LPS-induced acute liver injury [76]. Recently, BMS309403, an inhibitor of FABP4, has been shown to prevent the development of inflammation in adipocytes and macrophages [77]. Moreover, LPS induced FABP4 expression, alanine aminotransferase levels, TNFα, IL6, and CCL2 expression, and KC numbers were all significantly reduced when mice were pretreated with BMS309403 [77]. Taken together, these data demonstrate an influence of FABPs within immune cells and will aid in the understanding of their role in innate immunity and metabolism.

### Lipids and the Resolution of Inflammation

Synthesis of lipid derivatives with inflammatory properties is a critical component of acute inflammation [52,78]. Prostanoid (prostaglandins, thromboxanes) synthesis by cyclooxygenases, principally COX-2, promotes inflammatory responses through enhancement of immune cell recruitment (both directly as well as indirectly through endothelial cell adhesion molecule up-regulation) and their subsequent activation and production of inflammatory mediators promoting tissue damage. Recently, this pathway and enzyme complex has been shown to also produce key anti-inflammatory mediators which play a significant role in the resolution of acute inflammation [78]. Inhibition of COX-associated prostaglandin synthesis by aspirin limits the inflammatory response and is used routinely in the clinic. It is
also associated, through its inhibition of thromboxane synthesis, with reductions in platelet aggregation lending to its prophylactic use in patients with heart disease [79]. Intriguingly, acetylation of COX-2 by aspirin promotes the synthesis of key intermediaries of arachidonic acid, specifically 15R-hydroxyeicosatetraenoic acid (HETE) which is quickly transformed by 5 lipoxygenase (5-LOX) to 15-epi-lipoxin [79]. This and other lipoxins generated by 5-LOX limit inflammatory responses through inhibition of oxidant production, reduction in endothelial cell adhesion molecule expression, and limits inflammatory mediator production by macrophages, T cells, and neutrophils. This pathway is also induced normally by the inflammatory response whereby prostaglandins including E2 and D2 up-regulate 5-LOX thereby shifting, in a feed-forward manner, the production of anti-inflammatory mediators to limit tissue inflammation [79].

Similar to lipoxins, resolvins have also recently been described which show similar anti-inflammatory and pro-resolution properties important to normal tissue homeostasis [80,81]. Synthesized from eicosapentaenoic acid and docosahexaenoic acid, highly studied and abundant omega-3 fatty acids, through the actions of endothelial cell associated, aspirin-acylated COX-2 and subsequent conversion by 5-LOX, resolvin family members have shown great promise in limiting inflammatory reactions in a number of tissues and pathologies. For example, Arita et al demonstrated the ability of resolvin E1 to limit neutrophil infiltration, pro-inflammatory cytokine production, and weight loss in toxin-induced colitis in mice [82]. Similarly, in the kidney, resolvin D1 reduced tissue fibrosis and enhanced tissue function following acute ischemic insult in the murine kidney [83]. Thus, resolvins and lipoxins are potent inhibitors of the inflammatory process and provide new information into the protective effects of polyunsaturated fats, specifically omega-3 fatty acids.

The influence of these factors within the liver is less well characterized. Administration of resolvin E1 reduced macrophage recruitment and limited lipid accumulation in leptin deficient mice though the mechanism leading to this effect is unclear [84]. Schmoker and colleagues reported significant protection against D-galactosamine and TNFα induced liver injury in transgenic mice endogenously expressing omega-3 fatty acids [85]. These studies highlighted the ability of excess PUFAs to reduce inflammatory cytokine production within the liver, mediators likely derived from hepatic resident macrophages. Additional studies using these same transgenic animals revealed reduced tumorigenesis in the diethylnitrosamine liver tumor model [86]. Again, reduced TNFα and oxidant production were noted suggesting a connection to the innate immune response within the damaged liver. DHA supplementation and its conversion to 17S-hydroxy-DHA also proved effective at reducing carbon tetrachloride-induced tissue injury by limiting DNA damage in hepatocytes and subsequent necroinflammatory injury [87]. In addition, dietary treatment also reduced macrophage TNFα production likely through augmentation of PPARγ activation [84]. Given these and other studies, it is clear that these lipid derivatives play critical roles not only in inducing but also resolving inflammatory processes through inhibition of oxidant production, adhesion molecule expression, and immune cell function in vitro and in vivo and likely contribute significantly to the progression and resolution of tissue inflammation in the acute or chronically damaged liver.

### Cholesterol and the hepatic immune response

In addition to more traditional bioactive lipid compounds, growing evidence implicates cholesterol as a potential inflammatory mediator. Oxidation of low density lipoprotein is well appreciated as a mediator of macrophage activation and foam cell formation within the vasculature [88]. The impact of cholesterol on hepatic immunobiology is less clear. Cabellero and colleagues demonstrated a strong correlation between NASH and circulating free cholesterol suggesting an increase in cholesterol synthesis or availability [89]. and excess cholesterol has been shown to accelerate liver damage in mouse models of high fat diet induced fatty liver [90]. Intriguingly, these studies also demonstrated the ability of excess cholesterol in dying fatty hepatocytes to transform hepatic macrophages to foam-like cells with increased lipid accumulation and potential for secondary inflammatory responses, both in mice and in humans. Consistent with these findings, Nakashima and others demonstrated increased numbers of macrophages and their responsiveness to bacterial DNA motifs (e.g. CpG-ODN; Tlr9 agonist) in high fat and high cholesterol diet fed mice as measured by tumor necrosis factor alpha production ex vivo [91]. Further studies demonstrated that loss of CD36 or macrophage scavenger receptor provided protection against NASH likely through modifying cholesterol distribution of cholesterol storage in KCs from lysosome to cytoplasmic storage [92]. Thus, it is clear that cholesterol overload can alter hepatic macrophage function and promote inflammation and NASH like responses in mice and humans. It would be logical then that release or reductions in cholesterol storage may also provide protection in the context of macrophage function. To this end, Bie et al over-expressed cholesterol ester hydrolase specifically in macrophages and fed these mice high fat and high cholesterol diet [93]. Increased metabolism and release of cholesterol from macrophages in these transgenic mice reduced NASH progression and enhanced anti-inflammatory cytokine production by these cells further limiting tissue damage. Together, these studies highlight the impact of cholesterol on hepatic macrophage function and its ability to drive NASH induction and progression.

The impact of cholesterol on other innate immune cell populations is less well understood. DCs, similar to macrophages, show metabolic potential towards cholesterol and cholesterol derivatives [94]. Activation of Type I interferon receptors on DCs and macrophage promotes their expression of cholesterol 25-hydroxylase in a Stat1 dependent manner. Production of the oxysterol 25HC provides an important link between metabolism and innate immune cell function itself. Binding of 25HC to nuclear LXR limits dendritic cell CD86 up-regulation in response to antigen and suppresses IL12 production in favor of IL10 induction effectively squelching their ability to interact with and activate T cells [94].

### Nutrition and the Gut-Liver Axis: Potential avenues for secondary hepatic immune cell activation

Growing evidence supports a role for the gut in the modulation of hepatic immune cell responses [4]. It is clear from countless studies that gut-derived bacteria activate Kupffer cells to release key inflammatory cytokines including TNFα [12,95-97]. For...
example, sterilization of the gut limits macrophage activation and accumulation following chronic ethanol administration [8]. Likewise, depletion of gut bacteria with non-absorbable antibiotics reduced CCl4-induced tissue injury in conjunction with reduced macrophage accumulation and pro-fibrogenic cytokine production [19]. Interestingly, high fat diet feeding has been shown to promote intestinal inflammation leading to reduced barrier function and increased pathogen delivery to the liver. Ding and colleagues demonstrated significant interactions among bacteria and high fat diet to promote intestinal Nfkb activation and TNFa production as well as peripheral markers of insulin resistance and early metabolic syndrome [98]. Indeed, germ free mice fed a high fat diet showed significant reductions in inflammatory markers and their absence limited metabolic parameter disruption. Likewise, loss of Tlr4 was also shown to limit colonic inflammation following high fat diet feeding further demonstrating the dynamics of lipid and gut bacteria in the promotion of intestinal inflammation [99]. Intriguingly, excess fat was also shown to alter gut microbiota populations and promote increased plasma endotoxin levels suggesting damaged to the gut barrier itself [99]. Supporting this notion, Serino and others demonstrated reduced occludin and junctional adhesion molecule A expression in jejunal and ileal segments [100]. Together, these studies and numerous others demonstrate 1) the ability of excess dietary lipid to promote intestinal inflammation, decrease barrier integrity, and promote pathogen delivery to the liver where they may activate awaiting innate immune cells to promote liver various liver pathologies and 2) highlight the multiple levels by which dietary lipid may regulate liver specific responses.

CONCLUSION

It is clear from the current review that lipids play a critical role in the regulation of the hepatic innate immune response. Clear mechanisms exist for their potential to both promote as well as resolve the inflammatory response in a number of liver pathologies including non-alcoholic fatty liver disease. Key questions remain including the endogenous source(s) of many of these inflammatory or protective mediators and the potential for diet to enhance or regulate their function and availability. Given the growing problem of obesity and associated hepatosteatosis, understanding the dynamics of and mechanisms governing lipid – immune cell interactions, particularly the innate immune response, is critical to developing pharmacological and potentially dietary therapies to treat or prevent numerous chronic liver pathologies.

REFERENCES

1. TIlg H, Hotamisligil GS. Nonalcoholic fatty liver disease: Cytokine-adipokine interplay and regulation of insulin resistance. Gastroenterology. 2006; 131: 934-945.
YS. Kuppfer cells trigger nonalcoholic steatohepatitis development
Tosello-Trampont AC, Landes SG, Nguyen V, Novobrantseva TI, Hahn
Dependent suppression of peroxisome proliferator-activated receptor
of macrophages promotes nonalcoholic steatohepatitis through CCR2.
Serhan CN, Yacoubian S, Yang R. Anti-inflammatory and proresolving
Fattahi MJ, Mirshafiey A. Prostaglandins and rheumatoid arthritis.
Induction of vascular adhesion molecule-1 in rheumatoid arthritis
Wu CY, Chi PL, Hsieh HL, Luo SF, Yang CM. TLR4-dependent
cutting edge: activation of NK T cells in IL-12-mediated rejection of tumors. Science.
1997; 278: 1623-1626.
Farrell GC, Monsalve FA, Pyarasani RD, Delgado-Lopez F, Moore-Carrasco R.
Ahmadian M, Sih JM, Hah N, Liddle C, Atkins AR, Downes M, et
Serhan CN. Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? Am J Pathol. 2010; 177: 1576-1591.


