Kupffer cells in fatty liver disease : Does size really matter?

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Kupffer cells in fatty liver disease

Does size really matter?

Veerle Bieghs

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Contents

Chapter 1	Introduction	7
Chapter 2	Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of non-alcoholic steatohepatitis	23
Chapter 3	The LDL receptor knock-out mouse is a physiological model particularly vulnerable for studying inflammation in non-alcoholic steatohepatitis	47
Chapter 4	Role of Scavenger Receptor A and CD36 in diet-induced non-alcoholic steatohepatitis in hyperlipidemic mice	63
Chapter 5	CD36 and SR-A contribute similarly and independently to non-alcoholic steatohepatitis in hyperlipidemic mice	85
Chapter 6	Myeloperoxidase promotes progression of non-alcoholic steatohepatitis in mice	101
Chapter 7	CYP27: a novel player in diet-induced non-alcoholic steatohepatitis	121
Chapter 8	Pneumococci: a new treatment for non-alcoholic steatohepatitis?	139
Chapter 9	General discussion	159
	Summary	177
	Samenvatting	181
	Dankwoord	185
	Curriculum Vitae	191
	List of abbreviations	199

Chapter 1

General introduction

The metabolic syndrome

Obesity is the main causal factor in the development of the metabolic syndrome (MetS). This growing multiple risk factor syndrome is characterized by a constellation of metabolic risk factors for the development of atherosclerotic cardiovascular disease and diabetes. Clustering of these disorders has been independently suggested by several scientists as early as the 1920's, and different definitions and names have been given, including syndrome X, insulin resistance syndrome, Reaven's syndrome and CHAOS¹.

There are six cardiovascular risk factors that accompany the MetS: abdominal obesity, atherogenic dyslipidemia [characterized by elevated apolipoprotein B, elevated triglycerides, small low density lipoprotein (LDL) particles, and low high-density lipoprotein (HDL) cholesterol levels], elevated blood pressure, glucose intolerance, a prothrombotic state and a pro-inflammatory state. The likelihood of an individual developing MetS is enhanced by underlying risk factors, most notably, obesity, insulin resistance (IR), lack of physical activity, advancing age and hormonal factors^{2,3}. Whereas the MetS is present in only about 5% of people with normal body weight, 22% of those who are overweight and 60% of those who are considered obese have the disorder. A family history of IR, hypertension and early heart disease greatly increases the chance of an individual developing the MetS⁴. The clinical consequences of the MetS are coronary heart disease and stroke, type 2 diabetes and its complications, cholesterol gallstones, some forms of cancer, and fatty liver disease. As the liver is a crucial organ in lipid and glucose homeostasis, it likely plays an essential role in the MetS. This thesis will focus on diet-induced liver pathology.

Figure 1.1 The multiple risk factors and consequences of the metabolic syndrome.

The liver as a central organ in the metabolic syndrome

Liver function in health and disease

The liver is one of the most essential organs for life. Its main functions are glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production and detoxification. The liver also manufactures and secretes bile to aid in the digestion and uptake of lipids from the intestine. In addition, enzymes in the liver modify the biochemistry of many substances, such as carbohydrates, proteins, lipids, and steroids. In all of these processes, the interaction between liver cells and circulating blood is the central principle around which the microanatomy of the liver is organised⁵.

Macroscopically, the liver is divided into four lobes, namely, the left lobe, right lobe, quadrate lobe and caudate lobe. Microscopically, the liver consists of several cell types, such as hepatocytes, stellate cells and Kupffer cells (KCs) (Figure 1.2). Hepatocytes are the so-called “liver cells”, and constitute up to 70% of the total liver mass. Hepatocytes enable the liver to carry synthesis, storage and secretion. Hepatocytes are separated by sinusoids, which comprise the vascular channels of the liver. Stellate, or Ito cells are pericytes located in the perisinusoidal space (also known as the space of Disse), which is a space between the sinusoids and the hepatocytes. Stellate cells represent 5 to 8% of the total liver mass, and are the main cellular source of extracellular matrix proteins in the liver^{6,7}. KCs were first described by Karl Wilhelm von Kupffer in 1876, and are specialized tissue macrophages present in the sinusoids⁸. They represent 10-15% of the total liver cell population, and comprise 80-90% of all tissue macrophages in the body. KCs are responsible for the rapid repression and clearance of exogenous particulates and immunoreactive materials that are perceived by the body to be foreign and harmful⁹. Like other macrophages, KCs also sense endogenous molecular signals that may result from the perturbed homeostasis of the host. KCs rapidly recognize potential danger and undergo activation. Activated KCs are able to: (1) initiate interactions with hepatocytes and other liver cells through the release of active mediators, including cytokines, chemokines, eicosanoids, proteolytic enzymes, reactive oxygen species (ROS), and nitric oxide; (2) recruit and retain neutrophils and natural killer (NK) T lymphocytes, NK cells and blood monocyte-derived macrophages; (3) engulf, ingest and eliminate solid particles, including microorganisms, apoptotic cells and cellular debris; and (4) process and present antigens to attract cytotoxic and regulatory T cells, thereby contributing to adaptive immunity⁹⁻¹⁴. All of these functions need to be rigorously controlled to avoid escalation of the inflammatory response.

Figure 1.2 Schematic structure of the liver sinusoidal endothelium. Liver sinusoids are lined by liver sinusoidal endothelial cells (LSEC), which separate the sinusoid lumen from hepatocytes. Kupffer cells patrol the sinusoids and bind to LSEC and, occasionally, hepatocytes, through the gap between two adjacent LSEC. Stellate cells are located in the space of Disse. (Figure taken from Bertolino et al.¹⁵).

The migration of leukocytes into tissue is a key element of innate and adaptive immunity. Chemokines greatly influence the inflammatory infiltrate in chronic liver disease, by differentially recruiting leukocytes into the liver. Chemokines involved in the recruitment of immune cells to the liver, are either secreted by the infiltrating cells or by resident cells, such as hepatocytes or Kupffer cells, within the liver. However, increased recruitment is in itself insufficient to generate chronic inflammation, because powerful mechanisms have evolved to ensure that, in most cases, acute inflammation is resolved and tissue integrity is restored after the original insult is dealt with¹⁶. Resolution occurs when clearance of infiltrating cells by apoptosis, or exit through the lymphatic system (or both), outweighs the influx of cells from the circulatory system. In chronic inflammation, these resolution and healing mechanisms fail, resulting in ongoing lymphocyte recruitment, tissue damage and fibrosis. Thus, lymphocyte recruitment from the circulatory system is a critical step in the development of inflammation. The complex local interactions between different infiltrating and resident cell types depend upon local cytokine secretion to maintain the survival and activation of intra-hepatic effector cells, as well as the secretion of chemokines, that determine the recruitment and retention of specific subsets of leukocytes that result in chronic inflammation¹⁷.

NAFLD as the hepatic manifestation of the metabolic syndrome

The MetS was found to be a strong predictor of the development of non-alcoholic fatty liver disease (NAFLD). NAFLD is characterised by the accumulation of fat in the liver (steatosis) in the presence of <10g of daily alcohol intake. Alcoholic and non-alcohol induced fatty liver diseases are indistinguishable morphologically, and accurate diagnosis requires clinicopathological correlations, rooted in a thorough clinical history that requires truthful subjective reporting on the level of daily alcohol consumption. The core characteristic features of the MetS were found to be associated with NAFLD, including: obesity, diabetes, IR and hyperlipidemia¹⁸. In fact, clear correlations between the MetS and NAFLD were found not only with obesity, but also with type 2 diabetes mellitus and hyperlipidemia¹⁹. It is estimated that 75% of type 2 diabetes patients have some form of fatty liver disease^{20,21}. Since NAFLD affects only 2.6 % of normal weight children, but 22.5²² to 52.8%²³ of obese children, there is a clear link between obesity and NAFLD in children. However, several reports claim that steatosis is independent from obesity^{24,25}. In contrast, these reports state that steatosis is affected by fat distribution. In this regard, waist circumference correlates closely with steatosis development, indicating that abdominal fat is the most harmful¹⁸.

The incidence of dyslipidemia is also high in NAFLD patients. Dyslipidemia includes hypertriglyceridemia, hypercholesterolemia and either an abnormal increase in low-density lipoproteins or a decrease in high-density lipoprotein cholesterol. It has been postulated that all of these abnormalities in lipid metabolism may be contributing factors in non-alcoholic steatohepatitis (NASH) development²⁶ (discussed in more detail below). In addition, genetic hyperlipidemic disorders such as familial hypercholesterolemia (FH) are characterized by elevated plasma LDL levels, and are associated with NAFLD²⁷. Combined, these observations suggest a close relationship between hyperlipidemia and NAFLD. Although NAFLD has not been included as a component of the MetS, the increased onset of NAFLD is being viewed by experts as an early event in the development of IR, and therefore as an indicator or predictor of future MetS. Environmental factors, and in particular dietary composition, undoubtedly play a role in facilitating the imbalance between energy intake and consumption that underlies the present pandemic of “overnutrition”. For example, in atherosclerosis, there is strong evidence to support the hypothesis that dietary factors, such as diets high in saturated fats and cholesterol and low in polyunsaturated fats, are correlated with both an increased risk for developing this disease and poor prognosis²⁸. With NAFLD and NASH, however, the picture is less clear. While it is known that caloric excess is associated with obesity, this risk factor alone does not distinguish between patients with NASH, simple steatosis or even normal liver histology²⁹.

Non-alcoholic steatohepatitis

NAFLD can further progress to a more dangerous condition known as NASH. NASH is characterized by steatosis, in combination with hepatic inflammation. Although steatosis alone is generally considered to be a reversible, benign condition for the liver itself, the presence of inflammation in a fatty liver is a key feature of NASH, and represents an irreversible step with a worse prognosis. The inflamed liver preludes further disease progression, and allows more advanced stages of the disease, such as fibrosis, cirrhosis and eventually liver failure or hepatocellular carcinoma (requiring liver transplantation) to develop^{30,31}. Thus, the progression from steatosis to hepatic inflammation represents a key step in the development of NASH.

Epidemiology

In Western populations, estimates of NAFLD prevalence vary between 20 and 30%, rising to as high as 90% in morbidly obese individuals. Although NAFLD occurs in all ethnic groups, it appears to have a lower prevalence in African-Americans compared with Hispanic and European Americans. This difference remains even after controlling for obesity and IR, and may be related to ethnic differences in lipid homeostasis^{32,33}. NASH is less common, affecting only 2-3% of the general population and up to 37% of the morbidly obese. The increasing prevalence of NAFLD in children and young adults is of particular concern. Studies have reported a 3% prevalence of NAFLD in the general pediatric population, rising to as high as 53% in obese children. In addition, NAFLD is strongly associated with type 2 diabetes, with steatosis present in 70% of type 2 diabetes patients. Therefore, NAFLD is now recognized to be the hepatic manifestation of the MetS^{22,23,32,34-36}.

Histology

The major histological hallmarks of NASH include micro- and macrovesicular steatosis, infiltration of inflammatory cells and hepatocyte ballooning, with or without the presence of collagen strand depositions (fibrosis). Ballooning of the hepatocytes is a form of liver cell injury that may result from intracellular fluid accumulation and other toxic cell damage, and is characterized by swelling of the hepatocytes, resulting in a clear to pale cytoplasm. Collagen deposition can progress from periportal expansion to septal fibrosis to complete cirrhotic remodelling of the liver³⁷⁻³⁹.

Pathogenesis

The progression from simple steatosis to steatohepatitis is a key concern in the NASH field (Figure 1.3). Currently, it is not known why some individuals with NAFLD develop an aggressive form of the disease, while others do not⁴⁰. Although this likely results from the interplay of different determinants, including genetic and environmental

factors, the sequence of events is not known. The so-called “two-hit hypothesis” postulates a sequential evolution from bland steatosis to NASH, where IR represents the first hit, which induces steatosis and renders the liver more susceptible to injury. Following IR, a second hit (drugs, ischemia, endotoxin-induced damage, etc.) would promote progression from steatosis to NASH. The damage to fat-laden hepatocytes results mainly from the production of ROS from FA metabolism via oxidation, which causes cell death, and triggers hepatic inflammation and fibrogenesis due to activation of hepatic stellate cells and other fibrogenic cell types. This theory has been reviewed extensively and consideration has been given to the possibility that some subjects may be directly committed to NASH development from the beginning of the disease⁴¹. Similarly, several reports call into question the notion that steatosis is a prerequisite for the development of inflammation during NASH progression. Mari *et al.* demonstrate that free cholesterol (FC) accumulation, but not triglycerides (TG) and free fatty acids (FFA), plays a key role as a first hit, because FC sensitizes hepatocytes to TNF- and Fas-induced apoptosis and NASH⁴². The benign effect of TG accumulation was confirmed by Yamaguchi *et al.*, who demonstrated that TG themselves are not hepatotoxic, and in fact protect the hepatocytes from lipotoxicity by buffering the accumulation of FFA⁴³. Furthermore, Matsuzawa *et al.* demonstrated that cholesterol can induce oxidative damage to the liver, and that a high fat diet further aggravates oxidative stress and NASH, possibly by inducing IR and down-regulating antioxidant genes⁴⁴. In agreement with these observations, our group has previously shown that hyperlipidemic mouse models have already developed NASH after 2 days of high fat cholesterol (HFC) feeding. In this case, inflammation developed alongside, rather than following steatosis⁴⁵.

Figure 1.3 Histology of a normal liver, NAFLD and NASH resp.

Diagnosis

In the majority of cases, NAFLD and NASH patients are asymptomatic; although sometimes, patients complain about fatigue, malaise and upper right quadrant discomfort⁴⁶. Despite the fact that elevated liver enzymes, such as aminotransferase (ALT), in the blood usually suggest liver damage, a fraction of patients with NAFLD or

NASH were found to have normal liver enzyme levels⁴⁷. However, most blood tests lack the specificity and sensitivity to distinguish NASH from simple NAFLD. The lack of clear diagnostics for NASH represents a key clinical problem, because patients with NASH need close monitoring and frequent follow-up care.

In clinical practice, liver biopsies are currently the primary method used for the detection of liver inflammation. However, there are several important limitations to liver biopsies, including patient stress and discomfort, risk of bleeding and long hospitalizations. In fact, it has been estimated that complications occur in about 0.5% of liver biopsy procedures⁴⁸. Furthermore, NASH lesions are known to be unevenly distributed throughout the liver parenchyma⁴⁹. Considering the minute portion of the liver sampled (0.002%), histological analysis of biopsy tissue remains prone to insensitivity and staging inaccuracies⁵⁰. These limitations prevent the use of biopsies as a clinical screening procedure for liver inflammation. In light of this, several imaging modalities have been advocated as non-invasive diagnostic tests for simple liver steatosis, including ultrasonography, computer tomography, magnetic resonance imaging, and, more recently, magnetic resonance spectroscopy^{32,51,52}. Unfortunately, these imaging techniques have insufficient sensitivity and specificity to distinguish NASH from the benign non-inflammatory fatty liver disease.

Therapy

Simple lifestyle modifications, such as body weight management and appropriate nutritional counseling (with or without regular physical exercise and cognitive-behaviour programs), are the mainstays of therapy for patients with the MetS. Body weight reduction leads to a loss of the adipose tissue, which further leads to improvements in peripheral and hepatic insulin sensitivity, and prevention of hepatic injury. Although specific diet and exercise guidelines for MetS patients have not been published, there are several types of diets which have been proposed for the treatment of obesity and MetS by different medical and commercial sources⁵³⁻⁶¹. In addition to lifestyle changes, current therapies utilized include insulin sensitizers (e.g. metformin and the thiazolidinediones), weight loss drugs (e.g. orlistat and sibutramine), and possibly bariatric surgery for morbidly obese patients. However, liver transplantation remains the only curative treatment option for end-stage cirrhosis. Given the fact that hypertriglyceridemia and low HDL levels are defining elements of the MetS, lipid lowering agents are also possible candidates for NASH treatment. For example, Statins and fibrates, or a combination of the two, are considered to be good treatments for NASH. However, the use of lipid lowering drugs is controversial because of their potential hepatotoxicity in patients with underlying hepatic disease^{62,63}. Therapies currently under evaluation for the treatment of NASH include anti-oxidants, such as vitamins C, E^{64,65} and betaine⁶⁶, iron depletion⁶⁷, ursodeoxycholic acid^{68,69} and pentoxifylline^{70,71}. Because none of these treatments have yet to show any convincing

evidence of benefit, new therapy options should be considered. To gain new insight into the mechanisms by which hepatic inflammation is triggered, animal models for NASH are necessary. Studies in animal models have the potential to provide crucial insights into NASH pathogenesis and may lead to the development of novel therapeutic options for the treatment of this disease.

Hyperlipidemic mice as a physiological model for NASH

The best characterized and most widely used animal models for NASH are leptin (*ob/ob*) deficient mice, or mice fed a diet deficient in methionine and choline (MCD). However, *ob/ob* mice do not spontaneously develop liver inflammation, but require a second hit, such as the administration of lipopolysaccharide (LPS), to activate inflammatory signaling pathways. Furthermore, mutation of the leptin gene is not common in human obese NAFLD patients. In contrast, MCD fed mice display all the hallmarks of NASH, from steatosis to inflammation and fibrosis development. However, MCD fed mice tend to lose weight and display lowered plasma TG levels, and are therefore very different from human NASH patients, who are in general obese and/or hyperlipidemic⁷². MetS and steatosis development are associated with high fat feeding and hyperlipidemia⁷³. Therefore, there is a need for NASH animal models that incorporate these features. Feeding hyperlipidemic mice a diet resembling the atherogenic diet frequently consumed in industrialized countries, i.e. enriched in carbohydrates, lipids and cholesterol, has been shown to induce severe steatosis and hepatic inflammation^{74,75}. These mouse models are thus particularly interesting for the study of NASH pathogenesis.

Hyperlipidemic mouse models are generated based on key genes involved in the transport and uptake of dietary lipids in the body. Because lipids are water insoluble, they are packaged into lipoproteins during transport throughout the body in the circulatory system. Lipoproteins generally consist of a hydrophobic core, composed of cholesterol esters and TGs. This hydrophobic core is surrounded by a monolayer of phospholipids, unesterified cholesterol, and apolipoproteins (Apo). These Apos solubilize and stabilize the lipid content and, in addition, have specialized functions in lipoprotein metabolism. One of the mouse models used in this thesis, *APOE2^{ki}*, bears a human mutation in the gene encoding for ApoE. In this “humanized” mouse model, the human *APOE2* allele is replaced by the murine ApoE gene. Thus, these mice express human *APOE* under the control of the endogenous promoter sequences in a tissue specific manner and at physiological levels. *APOE2* has a markedly reduced affinity for low density lipoprotein (LDL) receptors, leading to a plasma lipoprotein profile resembling human type III hyperlipoproteinemia (HLP)⁷⁷. Additionally, more than 90% of all type III HLP subjects are homozygous carriers of *APOE2*. A similar mouse model for the study of NASH is the *Ldlr*^{-/-} mouse. By eliminating the functional gene for the LDL receptor (*Ldlr*), Ishibashi *et al.* generated a mouse model that

displayed mildly elevated cholesterol levels on a high fat, high cholesterol diet. The largest amount of cholesterol was present in the IDL/LDL fraction. This profile is comparable to the human plasma lipoprotein profile, in which cholesterol is mainly confined to the LDL fraction⁷⁸. Both hyperlipidemic mice have a human-like lipoprotein profile and may serve as physiological mouse models to study the progression of NASH.

Figure 1.3 Major functions of ApoE and Ldlr. Triglycerides (TGs), cholesterol and other lipids are packaged into chylomicrons (CMs) after digestion and absorption in the intestine. CMs contain the apolipoproteins ApoB, ApoC and ApoE. The CMs enter the circulation, where their TGs are hydrolyzed by LpL, which can be modulated by ApoE. The resulting CM remnants (CMRs) are rapidly taken up by the liver through binding of ApoE to the Ldlr and the LRP. ApoE also stimulates hepatic VLDL production and secretion. Circulating VLDL particles, which contain both ApoE and ApoB, also undergo lipolysis, turning them into VLDL remnants or IDL. IDLs are internalised by the liver through binding to the Ldlr and LRP, suggesting that this process is also dependent on ApoE. Further lipolysis of remaining IDLs results in LDL formation. These lipoproteins can be taken up by the Liver through binding to the ApoB particles present in the LDLs. ApoE can also modulate cholesterol efflux out of the peripheral tissues to small circulating HDL. (image retrieved from Wouters *et al.*⁷⁶)

Thesis aim and outline

Currently, it is not known why some individuals with NAFLD develop NASH, while others do not. Therefore, in this thesis, we used hyperlipidemic mouse models to study the pathogenesis of NASH, since we aim to establish the mechanisms by which hepatic inflammation is triggered.

Chapter 2 describes the role of dietary cholesterol during the early inflammatory processes of NASH. The causal role of dietary cholesterol was investigated in different hyperlipidemic mice models of NASH, e.g. *Ldlr*^{-/-} and *APOE2ki* mice. **Chapter 3** illustrates the long-term effects of HFC feeding on the later consequences of NASH in hyperlipidemic mouse models (*APOE2ki* and *Ldlr*^{-/-} mice). This chapter demonstrates the differences between the two models after prolonged high fat feeding. In **Chapter 4**, we investigated the role of scavenger receptors (CD36 and SR-A) in diet induced NASH. To evaluate the involvement of scavenger receptor mediated uptake of modified lipoproteins by KCs, *Ldlr*^{-/-} mice were transplanted with bone marrow from *Cd36*^{-/-}/*Msr1*^{-/-} mice and fed an HFC diet. Furthermore, Chapter 4 also describes the effect of deletion of both scavenger receptors on hepatic inflammation and the development of 'foamy' Kupffer cells. **Chapter 5** explores the individual contributions of both CD36 and SR-A in diet-induced NASH. This chapter also describes the association between lysosomal cholesterol accumulation inside KCs and hepatic inflammation. In **Chapter 6**, the role of myeloperoxidase (MPO), an oxidant-generating neutrophil enzyme which can lead to the oxidation of cholesterol, was examined in the progression of hepatic and visceral adipose tissue inflammation. For this purpose, *Ldlr*^{-/-} mice were transplanted with *Mpo*^{-/-} bone marrow. **Chapter 7** describes the function of CYP27, an enzyme which produces 27-hydroxycholesterol, in diet-induced NASH. It has been shown using *in vitro* studies that 27-hydroxycholesterol can reduce lysosomal cholesterol accumulation. Therefore, we investigated the role of CYP27 on lysosomal cholesterol accumulation and hepatic inflammation by transplanting *Ldlr*^{-/-} mice with *Cyp27*^{-/-} bone marrow. **Chapter 8** describes the effect of an immune response against oxidized cholesterol during NASH. *Ldlr*^{-/-} mice were immunized with heat-inactivated pneumococci, as these have the same epitope as naturally occurring antibodies against oxidized LDL. This approach was tested as a possible therapeutic option for the prevention of NASH. Finally, **Chapter 9** will discuss the major findings of the thesis, place them in the context of the current state of the field, and address the clinical implications of the results.

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Chapter 2

Dietary cholesterol, rather than liver steatosis, leads
to hepatic inflammation in hyperlipidemic mouse
models of non-alcoholic steatohepatitis

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Abstract

Introduction

Non-alcoholic steatohepatitis (NASH) involves liver lipid accumulation (steatosis) combined with hepatic inflammation. The transition towards hepatic inflammation represents a key step in the pathogenesis, as it will set the stage for further liver damage, culminating in hepatic fibrosis, cirrhosis and liver cancer. The actual risk factors that drive hepatic inflammation during the progression to NASH remain largely unknown.

Aim

The role of steatosis and dietary cholesterol in the etiology of diet-induced NASH was investigated by using hyperlipidemic mouse models fed a western diet.

Materials and methods

Livers of male and female hyperlipidemic (low density lipoprotein deficient [*Ldlr*^{-/-}] and apolipoprotein E2 knock-in [*APOE2ki*]) mouse models were compared with livers of normolipidemic wild-type (WT) C57Bl/6 mice after short term feeding with a high fat diet with high cholesterol (HFC) and without cholesterol (HFNC).

Results

Whereas WT mice displayed only steatosis after a short term on HFC diet, female *Ldlr*^{-/-} and *APOE2ki* mice showed steatosis with severe inflammation characterized by infiltration of macrophages and increased nuclear factor kappa beta (NF-κB) signaling. Remarkably, male *Ldlr*^{-/-} and *APOE2ki* mice developed severe hepatic inflammation in the absence of steatosis after 7 days on HFC diet compared to WT animals. HFC diet induced bloated 'foamy' Kupffer cells in male and female *Ldlr*^{-/-} as well as in *APOE2ki* mice. Hepatic inflammation was found to be linked to increased plasma very low density lipoprotein (VLDL) cholesterol levels. Omitting cholesterol from the HFC diet lowered plasma VLDL cholesterol and prevented the development of inflammation and hepatic foam cells.

Conclusion

These findings indicate that dietary cholesterol, possibly in the form of modified plasma lipoproteins, is an important risk factor for the progression to hepatic inflammation in diet-induced NASH.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition ranging from benign lipid accumulation in the liver (steatosis) to steatosis combined with inflammation. The latter is referred to as non-alcoholic steatohepatitis (NASH). NAFLD may be considered the hepatic event in the metabolic syndrome and is, therefore, linked with all common metabolic syndrome risk factors like obesity, insulin resistance, hypertension and dyslipidemia¹. The prevalence of NAFLD in the general population is increasing, but only a small proportion will develop NASH. Estimates in USA are that only 2-3% of all adults have NASH, compared to an estimation of 20% of Americans with NALFD².

Steatosis alone is considered a relatively benign and reversible condition. The transition towards NASH represents a key step in pathogenesis, as it will set the stage for further damage to the liver, including fibrosis, cirrhosis and liver cancer. The actual risk factors that drive hepatic inflammation during the progression to NASH remain largely unknown. Therefore, knowledge about events that induce hepatic inflammation is of great importance for the diagnosis and treatment of NASH.

Currently, NASH is thought to develop via the “two-hit model”³. According to this hypothesis, hepatic steatosis is the critical first hit and prerequisite for further liver injury to develop. A second hit can be represented by oxidative stress⁴. However, recent reports doubt steatosis being a prerequisite for development of inflammation during NASH progression⁵⁻⁷. In line with these observations, we have previously shown that in APOE2 knock-in (*APOE2ki*) mice, a mouse model with a human-like lipoprotein profile, hepatic steatosis and inflammation (i.e. steatohepatitis) develop very rapidly when fed a western type diet with moderate amounts of fats. Already after 2 days of feeding a western diet enriched with triglycerides (TGs) and cholesterol, female *APOE2ki* mice show a marked liver inflammation⁸. Inflammation thus developed alongside rather than subsequent to steatosis. The *APOE2ki* mouse carries the defective human APOE2 isoform that replaces the endogenous mouse apoe gene. ApoE is highly expressed in macrophages and has been shown to influence several inflammatory processes⁹. Therefore, to exclude defects in ApoE as the cause of such early inflammation in the liver, we first investigated diet induced NASH development in another hyperlipidemic mouse model that does not have a defect in ApoE, i.e. the *Ldlr*^{-/-} mouse¹⁰. C57Bl/6 wild-type (WT) mice, with the genetic background of both mouse models, were used as control. Second, pilot experiments revealed a difference between female and male *Ldlr*^{-/-} and *APOE2ki* mice as the male mice did not develop steatosis after short term high fat feeding.

Consequently, we investigated male hyperlipidemic *APOE2ki* and *Ldlr*^{-/-} mice to unravel whether steatosis is necessary for hepatic inflammation to develop. Third, based on the results from these diet intervention studies, a correlation was found between plasma total cholesterol (TC) and hepatic inflammation. Therefore, the role of plasma TC was further investigated.

Materials and methods

Mice and diet

Mice were housed under standard conditions given free access to food and water. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University. Several experiments were performed. In the first experiment, 12-week old female C57Bl/6 and *Ldlr*^{-/-} mice were fed a high fat diet containing 21% milk butter and 0.2% cholesterol (HFC), for 2, 4, 7 and 21 days. A single group was kept on a standard chow diet until the age of 12 weeks and served as control group. In the second experiment, male C57Bl/6, *Ldlr*^{-/-}, and *APOE2ki* mice were put either on HFC for 7 days or were kept on chow diet. In the third experiment, male and female C57Bl/6, *Ldlr*^{-/-}, and *APOE2ki* mice were fed HFC or the high fat diet without the added cholesterol (HFnC) for 7 days. The HFnC diet contained only the residual cholesterol derived from the butter component, i.e. 0.05%. Collection of blood, sacrificing of the mice, and tissue isolation was done as described previously⁸.

Lipid analysis

Approximately 50 mg of frozen liver tissue was homogenized as described previously⁸. Both plasma and liver lipid levels were measured with enzymatic color tests (1489232, cholesterol CHOD-PAP, Roche, Basel, Switzerland; TR0100, TG GPOtrinder, Sigma Aldrich, St. Louis, MO, USA; 999-75406, NEFAC, ACS-ACOD, Wako Chemicals, Neuss, Germany) as described before⁸.

RNA isolation and first strand cDNA synthesis

Total RNA was isolated from approximately 25 mg of mouse liver tissues as described previously⁸. All applications were done according to manufacturer's protocols. Total RNA (500 ng) from each individual mouse was converted into first strand cDNA with iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Quantitative PCR

The changes in gene expression of inflammatory markers were determined by quantitative PCR on a Bio-Rad MyIQ with the IQ5 v2 software (Bio-Rad, Hercules, CA, USA) by using IQ SYBR Green Supermix with fluorescein (170-5006CUST, Bio-Rad, Hercules, CA, USA) and 10 ng of cDNA. For each gene a standard curve was generated with a serial dilution of a liver cDNA pool. To standardize for the amount of cDNA, Cyclophilin A (*Ppia*) was used as the reference gene. Primer sets for the selected genes were developed with Primer Express version 1.5 (Applied Biosystems, Foster City, CA, USA) using default settings.

Primer sequences:

MCP1-forward, 5'-GCTGGAGAGCTACAAGAGGATCA-3';

MCP1-reverse, 5'-ACAGACCTCTCTCTTGAGCTTGGT-3';

CD68-forward, 5'-TGACCTGCTCTCTAAGGCTACA-3';

CD68-reverse, 5'-TCACGGTTGCAAGAGAAACATG -3';

TNF α -forward, 5'-CATCTTCTCAAAATTCGAGTGACAA-3';

TNF α -reverse, 5'-TGGGAGTAGACAAGGTACAACCC-3';

Ppia-forward, 5'-TTCCTCTTTCACAGAATTATTCCA-3';

Ppia-reverse, 5'-CCGCCAGTGCCATTATGG-3'.

Data from qPCR were analyzed according to the relative standard curve method.

Taqman® Low Density Arrays

To perform gene-expression analysis on a medium scale, Taqman® Low Density Arrays (TLDA) 96a (PN 4342259) were used. Each TLDA plate contained 4 x 96 annotated and validated individual TaqMan® Gene Expression Assays (forward primer, reversed primer and Taqman® Probe) (supplementary Table 2.1). Per individual assay 2 ng cDNA of a single liver was loaded together with the TaqMan® Universal PCR Master Mix (PN 4324018). Of each group, mRNA from 5 individual mice was used. TLDA plates were run on an ABI Prism 7900HT Sequence Detection System with a TaqMan® Low Density Array Upgrade. Data were analyzed by using RQ Manager 1.2 software. Technically failed assays were omitted from analysis. Materials, equipment and software necessary to perform TLDA gene expression studies were obtained from Applied Biosystems, Foster City, CA, USA. All data were normalized to Ppia expression. Significant differences were determined with student t-tests; p-values <0.05 were considered significant.

Liver histology

Four μ m paraffin embedded liver sections were stained with Haematoxylin/Eosin (HE) and Periodic acid Schiff (PAS)-diastase. Frozen liver sections (7 μ m) were fixed in acetone and stained with CD68 (FA11) or Mac1 (M1/70). Pictures were taken with a Nikon® digital camera DMX1200 and ACT-1 v2.63 software (Nikon® Corporation, Tokyo, Japan).

Table 2.1 Liver Gene expression

A			
Gene	Group	WT HFC vs. chow	<i>Ldlr</i> ^{-/-} HFC vs. chow
<i>Cd36</i>	Lipid Uptake	1.4	1.0
<i>Fabp1</i>	Lipid Uptake	1.2	0.9
<i>Lipc</i>	Lipid Uptake	1.1	0.9
<i>Lpl</i>	Lipid Uptake	1.3	3.7
<i>Lrp1</i>	Lipid Uptake	1.5	1.1
<i>Slc27a1</i>	Lipid Uptake	1.1	0.9
<i>Acaa1a</i>	Lipid Oxidation and efflux	1.3	1.0
<i>Acox1</i>	Lipid Oxidation and efflux	1.2	0.8
<i>Cpt1a</i>	Lipid Oxidation and efflux	1.5	0.8
<i>Crot</i>	Lipid Oxidation and efflux	1.5	0.9
<i>Ech1</i>	Lipid Oxidation and efflux	0.9	0.8
<i>Hadha</i>	Lipid Oxidation and efflux	1.1	1.0
<i>Mttp</i>	Lipid Oxidation and efflux	1.0	0.8
<i>Ppara</i>	Lipid Oxidation and efflux	1.4	0.9
<i>Ppard</i>	Lipid Oxidation and efflux	1.2	1.2
<i>Pparg</i>	Lipid Oxidation and efflux	1.1	1.1
<i>Fasn</i>	Lipid Met. Synthesis	0.5	0.4
<i>LXRa</i>	Lipid Met. Synthesis	1.6	1.1
<i>FXR</i>	Lipid Met. Synthesis	1.1	0.7
Gene	Group	WT HFC vs. chow	<i>Ldlr</i> ^{-/-} HFC vs. chow
<i>Scd1</i>	Lipid Met. Synthesis	1.2	0.6
<i>Srebf1</i>	Lipid Met. Synthesis	1.2	1.3
<i>Abca1</i>	Cholesterol efflux	1.5	1.7
<i>Cyp7a1</i>	Cholesterol efflux	2.0	1.6
<i>Scarb1</i>	Cholesterol efflux	1.8	1.4
<i>Adfp</i>	Intracellular lipid distribution	1.3	0.8
<i>Cav1</i>	Intracellular lipid distribution	1.2	0.9
<i>Cav2</i>	Intracellular lipid distribution	1.2	0.9
<i>M6prbp1</i>	Intracellular lipid distribution	1.4	1.0
<i>Npc1</i>	Intracellular lipid distribution	1.0	0.6
<i>Idi1</i>	Cholesterol metabolism	0.2	0.1
<i>Insig2</i>	Cholesterol metabolism	1.5	0.7
<i>Cyp8b1</i>	Other	1.3	0.3
<i>Cd19</i>	Cell Markers	0.8	1.4
<i>Cd4</i>	Cell Markers	2.0	1.3
<i>Cd68</i>	Cell Markers	1.7	3.1 (a)
B			
Gene	Group	WT HFC vs. chow	<i>Ldlr</i> ^{-/-} HFC vs. chow
<i>Cd8a</i>	Cell Markers	1.4	2.0
<i>Fcgr1</i>	Cell Markers	2.2	2.4 (a)
<i>Itgam/Mac1</i>	Cell Markers	1.5	5.7 (a)
<i>Mpo</i>	Cell Markers	3.0	7.9
<i>Icam1</i>	Cell Markers	1.9	3.0
<i>Vcam1</i>	Cell Markers	1.0	3.0
<i>Vegfa</i>	Cell Markers	1.1	1.0
<i>Ccl2/mcp1</i>	Chemotaxis	3.0	9.5 (b)
<i>Ccl3/mip1</i>	Chemotaxis	3.4	11.2 (b)
<i>Ccr2</i>	Chemotaxis	1.8	1.9 (b)

B (continued)			
Gene	Group	WT HFC vs. chow	<i>Ldlr</i> ^{-/-} HFC vs. chow
<i>Ptgs2/Cox2</i>	Chemotaxis	1.2	<u>25.4</u> (b)
<i>Cat</i>	Oxidative stress	1.1	0.8
<i>Gsta2</i>	Oxidative stress	<u>0.5</u>	<u>0.2</u>
<i>Hmox1</i>	Oxidative stress	1.8	<u>2.8</u>
<i>Ikbkb</i>	Oxidative stress	1.0	1.3
<i>Por</i>	Oxidative stress	1.3	0.8
<i>Bcl2</i>	Apoptosis	1.2	<u>2.0</u>
<i>Fasl</i>	Apoptosis	2.0	<u>2.2</u>
<i>Il10</i>	Anti-inflammatory	2.5	1.7
<i>Il10ra</i>	Anti-inflammatory	1.5	<u>2.9</u>
<i>Socs1</i>	Anti-inflammatory	<u>2.6</u>	<u>2.3</u>
<i>Socs3</i>	Anti-inflammatory	1.3	<u>1.9</u>
<i>Cd14</i>	General Inflammation	1.8	<u>4.5</u>
<i>Cd40</i>	General Inflammation	2.1	<u>2.7</u>
<i>Cd80</i>	General Inflammation	2.7	<u>2.7</u>
<i>Cd86</i>	General Inflammation	2.2	<u>3.0</u>
<i>CsfF</i>	General Inflamm.	0.6	0.9
<i>Cxcl10</i>	General Inflammation	3.0	<u>3.5</u>
<i>Ifng</i>	General Inflammation	1.1	1.7
<i>Il18</i>	General Inflammation	1.0	0.9
<i>Il18r1</i>	General Inflammation	1.9	<u>4.0</u>
<i>Il1b</i>	General Inflammation	1.4	<u>6.3</u>
<i>Il1r1</i>	General Inflammation	0.8	<u>2.0</u>
<i>Il1r2</i>	General Inflammation	1.1	5.4
<i>Il6</i>	General Inflammation	1.6	6.0
<i>Il6ra</i>	General Inflammation	0.6	0.7
<i>Nfkbia</i>	General Inflammation	1.2	1.4
<i>Saa1</i>	General Inflammation	<u>2.3</u>	<u>19.9</u>
<i>Stat1</i>	General Inflammation	1.8	<u>1.8</u>
<i>Stat3</i>	General Inflammation	1.2	1.4
<i>Tlr2</i>	General Inflammation	<u>2.3</u>	<u>4.9</u>
<i>Tlr4</i>	General Inflammation	1.5	<u>2.4</u>
<i>Tnf</i>	General Inflammation	3.0	<u>10.0</u>
<i>Tnfrsf1a</i>	General Inflammation	1.3	1.0
<i>Tnfrsf1b</i>	General Inflammation	1.3	<u>1.4</u>

Table shows gene abbreviation and classification according to function. Expression is shown as fold change compared to levels of animals on standard chow diet for WT and *Ldlr*^{-/-} female mice after 7d of HFC. Significant changes (student t-tests), compared to chow are shown bold and underlined. A. Genes involved in lipid metabolism and B. Genes involved in inflammation. Regulated genes used as cell markers are marked by (a); regulated genes involved in chemotaxis are marked by (b).

Electron microscopy

Livers were freshly isolated from the mice, perfused and fixed overnight with 2.5% glutaraldehyde (Ted Pella, Redding, CA, USA). Tissue fragments were washed and post-fixed in 1% osmium tetroxide. Tissues were subsequently dehydrated through 100% ethanol, cleared with propylene oxide, and embedded in epoxy resin. Sections of 1 µm were stained with toluidine blue to identify the presence of foamy Kupffer cells. Next,

sections of 70-90 nm were cut on an ultra-microtome, mounted on Formvarcoated (1595E, Merck) 75 mesh copper grids and counterstained with uranyl acetate and lead citrate before analysis on a Philips CM100 transmission electron microscope.

Statistical analysis

Data were analyzed using Graphpad Prism 4.0. Groups were compared using 2-tailed non-paired t-tests or ANOVA with a Dunnett post test, based on the statistical relevance. Data are expressed as means $\pm$ SEM and considered significant at $p < 0.05$.

Results

Plasma lipid levels in female hyperlipidemic mouse models

Female *Ldlr*^{-/-}, *APOE2ki* and WT mice were fed an HFC diet for up to 3 weeks and changes in plasma lipids were monitored (experiment 1). Results on female *APOE2ki* mice were published earlier⁸ and are included in Figure 2.1 (black bars) for clarity. WT mice only displayed minor changes in their lipid profile. Compared to control chow levels, female *Ldlr*^{-/-} mice showed increased plasma TGs (Figure 2.1A) and free fatty acids (FFA) (Figure 2.1B) levels and diet-induced hypercholesterolemia (Figure 2.1C) upon HFC feeding. Historical data of female *APOE2ki* mice (black bars) shows that these animals have elevated basal plasma TG levels that tended to decrease within time (Figure 2.1A), while FFA remained at basal levels (Figure 2.1B) and TC levels increased markedly throughout the dietary period (Figure 2.1C)⁸.

Only female *Ldlr*^{-/-} and *APOE2ki* mice develop liver inflammation

APOE2ki and *Ldlr*^{-/-} mice showed equal accumulation of lipid droplets in their livers after 7 days of HFC which was comparable with livers of control WT animals (Figure 2.2A). Figure 2.2B shows that all mouse models had similar increases of liver TGs after short periods of HFC feeding. Liver TC showed a similar response (Figure 2.2C). HFC feeding thus induced a degree of hepatic steatosis in *Ldlr*^{-/-} and *APOE2ki* mice similar to what was observed in WT animals. These observations were confirmed by Oil red O (ORO) staining (data not shown). As HE staining revealed inflammatory clusters in the livers (Figure 2.2A), antibodies against the macrophage marker Mac1 were used to identify inflammatory cells. The number of Mac1 positive cells was counted to determine the level of liver inflammation. Interestingly, WT livers were completely free of inflammation upon HFC feeding, while *Ldlr*^{-/-} mice displayed inflammatory cell clusters similar to those observed previously in *APOE2ki* mice⁸ (Figure 2.2D), albeit that inflammation was less severe in *Ldlr*^{-/-} mice. Gene expression analysis of several inflammatory genes was performed in livers of the mouse models outlined at 2, 4, 7 and 21 days after HFC feeding. These genes were monocyte

chemoattractant protein 1 (*Mcp1*) (Figure 2.2E), *Cd68* (Figure 2.2F), a macrophage marker, and tumor necrosis factor (*Tnf*) (Figure 2.2G), a cytokine. Expression of these genes in *Ldlr*^{-/-} and *APOE2ki* mice was strongly up-regulated after HFC feeding. In contrast, control WT mice showed only a moderate increase in liver gene expression, apparently insufficient to drive an overt inflammatory response as determined by liver histology. Hence, in HFC-induced fatty liver, female *APOE2ki* and *Ldlr*^{-/-}, but not WT mice, are sensitive to developing inflammation, indicating that diet-induced steatosis does not necessarily lead to the immediate development of an inflammatory response in the liver.

Figure 2.1 Plasma lipid levels in female mice. Figure shows plasma lipids of three mouse models at several time points after HFC treatment: 2, 4, 7 and 21 days (d) compared to chow-fed control mice. (A) Plasma triglycerides (TGs), (B) plasma free fatty acids (FFA) and (C) plasma total cholesterol (TC). Bars represent time points (2, 4, 7 and 21 days) and are grouped per genotype: WT mice (grey bars), *Ldlr*^{-/-} mice (white bars) and *APOE2ki* mice (black bars). Statistical analysis was done with one way ANOVA with Dunnet's post test; * indicates significant difference from levels on chow diet.

Liver gene expression profiling reveals an inflammatory profile in *Ldlr*^{-/-} but not in WT female mice

To investigate the hepatic response to HFC feeding in more detail, custom TLDA assays were designed to compare the expression of 96 genes involved either in lipid transport and metabolism, or in inflammation. The expression of *Mcp1*, *Cd68* and *Tnf*, determined by QPCR, was similar between *Ldlr*^{-/-} and *APOE2ki* mice (Figure 2.2G).

Figure 2.2 NASH parameters in female mice. (A) Representative pictures (200x) of HE stained liver sections were taken of female mice after 7 days high fat, high cholesterol (HFC) diet. Black arrows indicate inflammatory cell clusters. (B) Liver triglycerides (TGs) and (C) total cholesterol (TC) levels were quantified biochemically. (D) Liver sections were stained for Mac1 (CD11b) and counted. Gene expression analysis with quantitative RT-PCR for three known inflammatory marker genes: (E) *Mcp1*, (F) *Cd68* and (G) *Tnf*. Data was set relative to wild-type (WT) animals on chow diet. Bars represent time points (2, 4, 7 and 21 days) and are grouped per genotype: WT mice (grey bars), *Ldlr*^{-/-} mice (white bars) and *APOE2ki* mice (black bars). Statistical analysis was done with one way ANOVA with Dunnet's post test; * indicates significant difference from levels on chow diet.

Additionally, previous microarray analysis has already shown a profound inflammatory response after feeding female *APOE2ki* mice HFC diet⁸. The response of *Ldlr*^{-/-} mice to 7 days of HFC feeding was compared to WT mice. Table 2.1 shows changes in expression levels for these genes and indicates the pathway to which each gene belongs to. Genes involved in lipid metabolism showed only few changes after 7 days of HFC diet in either mouse model (Table 2.1A). Inflammatory gene expression, including several known targets of NF- κ B, was markedly up-regulated in female *Ldlr*^{-/-} mice, but not in female WT mice (Table 2.1B). The inflammatory response consisted mainly of the increased expression of macrophage specific genes (Table 2.1B), like *Cd68*, Fc Gamma receptor 1 and *Mac1*, but not other immune cell specific genes, like *Cd19* (B-cells), *Cd4* (TH-cells), *Cd8a* (TC-cells), and myeloperoxidase (*Mpo*) (neutrophils). These data suggest that inflammation is mainly related to macrophage accumulation and activation. Also *Icam1* and *Vcam1*, involved in inflammatory cell migration and invasion, were up-regulated. Additionally, genes involved in chemotaxis (Table 2.1B), such as *Ccl2* (*Mcp1*), *Ccl3* and *Ptgs2* were strongly upregulated, indicating an important role of these gene products in the development of hepatic inflammation.

Steatosis is not necessary for the development of HFC diet induced hepatic inflammation

Male *Ldlr*^{-/-} and *APOE2ki* mice were put either on chow diet or HFC diet for 7 days (experiment 2). Male WT animals were used as control. Both male *Ldlr*^{-/-} and *APOE2ki* mice showed increased diet-induced plasma TGs (Figure 2.3A) and TC (Figure 2.3B) compared to control mice, although *APOE2ki* mice were less responsive than *Ldlr*^{-/-} mice. The changes in TGs were similar to those observed in female mice for the *Ldlr*^{-/-} animals, while *APOE2ki* mice differed in their TG response as female *APOE2ki* mice had elevated starting levels of TGs that did not change after dietary intervention (Figure 2.1A). While in the female animals the *APOE2ki* mice were more responsive with respect to plasma TC (Figure 2.1C), male *APOE2ki* mice had a smaller increase than *Ldlr*^{-/-} mice. Biochemical assessment of liver lipids showed no increase in liver TG levels in male hyperlipidemic mice. Male WT mice did show an increase in hepatic TGs, however, the levels after 7 days of HFC did not exceed the ones displayed in the *Ldlr*^{-/-} and *APOE2ki* males (Figure 2.3C). On the other hand, liver TC levels did rise significantly after HFC in all models (Figure 2.3D). Likewise, ORO staining did not reveal overt steatosis (data not shown). Lipid accumulation is generally considered an initial and causal factor in the progression from steatosis to NASH¹¹. Surprisingly, despite the lack of steatosis in male mice, there was a severe inflammatory response, reflected by a 3- to 5-fold increase in *Mac1* positive cells (Figure 2.3E) and increased gene expression of *Mcp1* (Figure 2.3F), *Cd68* (Figure 2.3G) and *Tnf* (Figure 2.3H) compared to controls. These responses were more pronounced in *APOE2ki* than in *Ldlr*^{-/-} male mice. Overall, this indicates that liver lipid accumulation is not a prerequisite for hepatic inflammation to develop in these mouse models.

Figure 2.3 Plasma and liver lipid levels in male mice. (A) Plasma TGs and (B) TC were determined of male mice. Data from animals fed chow diet or 7 days of HFC. (C) Liver triglyceride and (D) cholesterol levels were quantified biochemically. (E) Liver sections were stained with antibodies against Mac1 (CD11b) and counted. Gene expression analysis with quantitative RT-PCR for three known inflammatory genes: (F) *Mcp1*, (G) *Cd68* and (H) *Tnf*. Data was set relative to WT animals on chow diet. Bars are grouped per genotype: WT mice (grey bars), *Ldlr*^{-/-} mice (white bars) and *APOE2ki* mice (black bars). Statistical analysis was done with student t-tests; * indicates significant difference from levels on chow diet.

Figure 2.4 Hepatic foam cells. (A) Representative pictures (400x) of female *APOE2ki* liver sections stained against CD68 on control chow diet and after 7 days of HFC. (B) Representative pictures (400x) of toluidine stained liver sections from *APOE2ki* female mice on a chow diet and after 7 days of HFC. Black arrows indicate foamy Kupffer cells. (C) Representative electron microscopy pictures (1550-fold magnification) of female *APOE2ki* mice fed either chow or HFC diet. Cholesterol crystals are indicated with black arrows.

Foam cells and modified lipoproteins in hyperlipidemic mice

Previously, we found that female *APOE2ki* mice displayed an increase in the size of CD68 positive cells after dietary intervention⁸. Immunostaining against CD68 now

indicated a comparable increase in size rather than number of CD68 positive cells, i.e. Kupffer cells and macrophages, in livers of male and female *Ldlr*^{-/-} and *APOE2ki* mice, but not of WT mice (Figure 2.4A) (experiments 1 and 2). Additionally, toluidine blue staining clearly illustrated that these cells with foamy appearance are located in the sinusoidal space of the liver, suggesting that these cells are KCs (Figure 2.4B). Further detail was provided with electron microscopy (Figure 2.4C). EM pictures showed clear differences between livers of the animals. KCs appeared to have more cytoplasm and filled a larger fraction of the sinus, indicating that these cells are swollen compared to chow fed animals. In HFC fed animals, cytoplasm of KCs contained lipid droplets and filled lysosomes. Moreover, these cells contained cholesterol crystals, which is indicative for the uptake of cholesterol by these cells.

Figure 2.5 Treatment with HFNC diet lowers VLDL cholesterol levels. Figure shows lipoprotein fractions after 7 days HFC and 7 days HFNC in (A) female *Ldlr*^{-/-}, (B) female *APOE2ki*, (C) male *Ldlr*^{-/-} and (D) male *APOE2ki* mice

Omitting dietary cholesterol reduces plasma VLDL TC and protects against developing hepatic inflammation

A consistent finding was that plasma TC was increased in the mouse models that developed hepatic inflammation. Accordingly, we hypothesized that plasma TC is an important determinant of hepatic inflammation. To test this hypothesis, mice (both male and female) were put on the high fat diet with or without added cholesterol (HFC and HFNC; high fat no cholesterol) (experiment 3). Omitting dietary cholesterol

induced lower levels of VLDL-TC than HFC diet in both sexes of the hyperlipidemic mice (Figure 2.5) and lowered total plasma TC by approximately 50% in *APOE2ki* and *Ldlr*^{-/-} animals (data not shown). Female WT mice also displayed lowering of TC levels, while this was not observed in male WT mice (data not shown).

Omission of dietary cholesterol did result in lower TGs and TC levels in WT controls, while it did not diminish liver TG content in *APOE2ki* and *Ldlr*^{-/-} mice (Figure 2.6A, 2.6B). In male mice, omitting cholesterol even tended to enhance TG content in the livers (Figure 2.6C). The level of liver TC remained low in female *APOE2ki* mice and in male *Ldlr*^{-/-} mice (Figure 2.6B, 2.6D).

Figure 2.6 Omitting cholesterol from the high fat diet prevents inflammation development without affecting steatosis. Biochemical measurements of liver lipids are shown for TGs in (A) female and (C) male mice and for TC content in (B) female and (D) male mice after 7 days of treatment with HFC or HFNC. Counting of Mac1 positive cells is shown in liver sections of (E) female and (F) male mice. Data are shown from WT (grey bars), *Ldlr*^{-/-} mice (white bars) and *APOE2ki* mice (black bars). Statistical analysis was done with student t-tests; * indicates significant difference from levels on HFC diet.

Figure 2.7 HFNC diet does not induce hepatic foam cells. Representative pictures (400x) of HE-stained liver sections from *Ldlr*^{-/-} and *APOE2ki* male and female mice after 7 days HFC and HFNC. Black arrows indicate foamy Kupffer cells. Inserts show foamy Kupffer cells when present on higher magnification.

Strikingly, Mac1 staining of liver sections showed that macrophage infiltration was limited when *Ldlr*^{-/-} and *APOE2ki* mice of both sexes were put on the HFNC diet (Figure 2.6E, 2.6F). Control mice did not show hepatic inflammation in any of the conditions. HE staining revealed that upon feeding HFNC diet, in male and female *Ldlr*^{-/-} and *APOE2ki* mice no swollen, foamy KCs were detected (Figure 2.7). Additional evidence was found with PAS-diastrase staining. Figure 2.8 shows the lack of swollen KCs upon feeding the mice HFNC compared to mice fed HFC diet. Accordingly, expression levels of *Mcp1*, *Cd68* and *Tnf* were down-regulated in female mice fed HFNC compared to HFC and similar trends were observed in male mice (Figure 2.9). Thus, lower levels of diet-induced VLDL-TC are associated with less hepatic inflammation in *Ldlr*^{-/-} and *APOE2ki* mice.

Figure 2.8 PAS-diastase staining. Representative pictures of liver sections stained with PAS-diastase. The displayed pictures are from male *APOE2ki* mice that received either HFC diet or HFNC diet. Black arrows indicate Kupffer cells.

Figure 2.9 Gene expression in mice fed HFNC diet. Gene expression analysis in female and male mice with quantitative RT-PCR for two known inflammatory genes: (A+B) *Mcp1*, (C+D) *Cd68*, and (E+F) *Tnf*. Bars are grouped per genotype: WT mice (grey bars), *Ldlr*^{-/-} mice (white bars) and *APOE2ki* mice (black bars). Data was set relative to WT animals on HFC diet. Statistical analysis was done with student t-tests; * indicates significant difference from levels on chow diet.

Discussion

This study clearly dissociates steatosis and inflammation in the livers of hyperlipidemic mice. HFC feeding induced a very early inflammatory response in the livers of *Ldlr*^{-/-} and *APOE2ki* female mice. Contrary, female WT mice developed comparable steatosis but no inflammation. Additionally, we found that in male mice, inflammation increased rapidly in the absence of steatosis. Together, these results suggest that liver inflammation can arise independently of steatosis upon high fat feeding. Subsequent experiments showed that omitting cholesterol from the HFC diet prevented VLDL-TG accumulation and hepatic inflammation, while parameters of steatosis remained largely unaffected. The presence of bloated foamy KCs only in HFC fed hyperlipidemic mice suggests that scavenging of modified lipoproteins by KCs may initiate this early inflammation.

Hyperlipidemic mice are sensitive to develop early diet induced NASH

Until now, the best characterized and most known models for NASH are mice deficient for leptin (*ob/ob*) or mice fed a diet deficient in methionine and choline (MCD)¹¹. However, *ob/ob* mice do not spontaneously develop liver inflammation but require a second hit, like the administration of lipopolysaccharide (LPS) to activate inflammatory signaling. Furthermore, mutation of the leptin gene is not common in human obese NAFLD patients. MCD fed mice display all hallmarks of NASH, from steatosis to inflammation and fibrosis development. However, MCD fed animals tend to lose weight and display lowered plasma TG levels¹¹ and are therefore very different from human NASH patients, who are mostly obese and/or hyperlipidemic. Dyslipidemia is commonly associated with NAFLD. It was postulated that abnormalities in lipid metabolism such as the increase of serum TGs, TC and LDL-TG levels and decrease of HDL-TG may be contributing factors of NASH development¹². Consequently, hyperlipidemic mice have been shown previously to develop diet-induced NASH, not only in *APOE2ki* mice⁸, but also in *Ldlr*^{-/-} and ApoE deficient (*apoE*^{-/-}) mice¹³. Unlike human subjects, WT mice carry most of their lipids in highdensity lipoproteins. In contrast, *Ldlr*^{-/-} mice and mice with various defects in the ApoE protein have a human-like lipoprotein profile and may serve as physiological mouse models to study the early progression of NASH.

Medium-scale gene expression analysis showed that, despite the presence of steatosis, not many lipid genes were regulated in either *Ldlr*^{-/-} or in WT control mice. This is probably due to the early time point of 7 days of high fat feeding, which might be too early to evoke a large transcriptional response of these genes. In line with this, we have found that HFC-induced expression of genes involved in lipid metabolism increases gradually with time⁸. Alternatively, the physiological response on lipids may

also be due to secondary feedback mechanisms regulated on the protein level rather than the gene level, resulting in the lack of transcriptional regulation.

In contrast to the lipid genes, a large set of inflammatory genes was regulated in the HFC fed *Ldlr*^{-/-} mouse, but not in WT mice, confirming our histological data. The data show that the inflammatory response consisted mainly of genes involved in chemotaxis and infiltration of macrophages. Furthermore, many genes that were up-regulated are known targets of the transcription factor *Nf-κb*, suggesting an important role for this transcription factor in response to HFC feeding and NASH development, as was previously postulated in HFC fed *APOE2ki* mice⁸ and in MCD fed mice with C57Bl/6 background¹⁴. Moreover, in NASH patients, *Nf-κb* expression was found to be up-regulated and correlated with hepatic inflammation and fibrosis¹⁵. The other regulated genes are involved in several inflammatory signaling pathways, such as *Il18*, *Il1*, *Il6*, *Tnf* and toll-like receptor signaling.

Steatosis is dissociated from the development of hepatic inflammation

In contrast to the two-hit model, where hepatic steatosis is generally considered as the first hit in the transition towards inflammation¹, male mice had an inflamed liver even without steatosis. The lack of steatosis development in male mice compared to female mice was somewhat surprising, as estrogen is normally known to be protective against NASH. It has been shown that estrogen replacement in deficient mice lowers steatosis development¹⁶. We postulate that the lack of steatosis in male mice may be explained by the fact that in the livers of male mice, PPAR isoforms are up to 100-fold more active than in livers of female mice¹⁷. Therefore, it is conceivable that in male mice, activation of PPAR can compensate for the increased lipid load. In support of this, we showed previously that feeding female *APOE2ki* mice HFC for short periods did not activate PPARα significantly⁸. Another gender-specific difference was found in *APOE2ki* mice, as female *APOE2ki* mice displayed no change in plasma TG levels, while male *APOE2ki* mice did show increases upon HFC feeding. A possible explanation for this observation may be that estrogens can increase activities of hepatic lipase and lipoprotein lipase¹⁸, which could consequently lead to increased hydrolysis of TGs in the plasma. Clearly, steatosis is not mandatory for progression towards hepatic inflammation in the mouse models used. In fact, others have postulated TG accumulation in the liver may even serve as a protective mechanism against inflammation development, by acting as a reservoir for harmful FFA's⁶.

Plasma cholesterol may mediate hepatic inflammation

We found a correlation between plasma TC and the development of hepatic inflammation, rather than with steatosis. This observation led us to hypothesize plasma TC as an important cause for the development of inflammation in these animals. Feeding the animals HFNC diet kept plasma VLDL-TC low and prevented the

development of inflammation in all hyperlipidemic mouse models, without diminishing liver TGs. Liver TGs even increased in male *APOE2ki* mice without any apparent explanation, an observation that further strengthens the dissociation of steatosis from hepatic inflammation. Recent publications also point to the importance of dietary cholesterol in liver inflammation and NASH in both rodents^{5,19} and rabbits²⁰. Moreover, in human subjects, it was found that high cholesterol feeding increased C- reactive protein and Serum Amyloid A levels²¹.

Recently, Mari *et al.* reported that high cholesterol feeding may serve as the first hit that sensitizes rat livers to develop hepatic inflammation after exposure to a second hit, like TNF or fatty acid synthase (FAS)⁵. In contrast, in the current study, we observed that high cholesterol feeding alone is sufficient to cause a very early inflammatory response. Several differences between the studies could explain this dissimilarity. First, Mari *et al.* used rats, which are known to be more resistant to developing hyperlipidemia than mice²². Our current study utilizes mouse models with genetic modifications specifically involved in plasma lipoprotein clearance and lipid metabolism. Consequently, it is possible that other mechanisms may be of greater importance in the rat model that was used in their study. Second, the manner of inducing steatosis was different. In our study, a mild high fat diet was used while Mari *et al.* used choline deficiency to evoke steatosis in the rats. Moreover, the diet of Mari *et al.* contained high levels of cholesterol (2%) supplemented with sodium cholate but no elevated TG content. In these concentrations, both cholesterol and cholate have been shown to induce an inflammatory response in livers of C57Bl/6 mice²³. The diet used in our study contained elevated TG levels to evoke steatosis and had cholesterol levels of only 0.2%, which is much closer to the average daily cholesterol intake in humans. Therefore, the diet used in our study appears to be more relevant in terms of induction of NASH in human patients.

Interestingly, in line with the present observations, male *apoE*^{-/-} mice were shown to develop liver inflammation when fed cholesterol levels of 0.25% and higher²⁴. This again points to an important role for dietary cholesterol in hepatic inflammation. However, this inflammatory response was investigated after several weeks of high fat diet and in the presence of steatosis. Our data suggests a very early impact of dietary cholesterol, possibly in the form of plasma VLDL-TC, on hepatic inflammation regardless of steatosis development.

Kupffer cells may initiate the early hepatic inflammation by scavenging modified lipoproteins

The mice used in our study are commonly used in atherosclerosis research as they have atherogenic lipoprotein profiles, because of increased modified remnant lipoproteins (oxLDL)²⁵. OxLDL binds to the scavenger receptors CD36 and SRA, which

are also present on KCs, and can trigger an inflammatory response^{26,27}. In livers of the mice with hepatic inflammation, we found bloated, foamy cells, which resemble lipid laden KCs, as has been described previously^{13,28}. Injection of modified LDL has been shown to result in an immediate uptake preferentially by non-parenchymal cells, such as KCs^{29,30}. Moreover, it has been shown that modified LDL injection in mice activates the hepatic NF- κ B pathway and subsequent inflammation³¹. Therefore, it is possible that in our hyperlipidemic mouse models, circulating levels of modified lipoproteins are scavenged by hepatic KCs, thereby triggering an inflammatory response. In WT mice on the other hand, cholesterol-rich lipoproteins are rapidly cleared from the blood by hepatocytes via the Ldlr, before they can be modified and taken up by KCs. Taken together, it is possible that oxLDL may be a causal factor for the development of diet-induced hepatic inflammation. Recent papers have shown a correlation between postprandial LDL conjugated dienes and hepatic necroinflammation and fibrosis development in human subjects³². LDL levels, possibly in modified form, were also found to be increased in NASH patients¹², confirming the clinical relevance of our findings.

In conclusion, this study demonstrates that dietary cholesterol, possibly in the form of modified plasma lipoproteins, rather than liver steatosis, may be a risk factor for progression of NASH development. Currently, most therapies for NASH patients involve weight loss and most diagnostic tests of NASH severity depend solely on the degree of steatosis. Further studies may unravel the exact contribution of cholesterol to the risk of developing NASH and may provide evidence for alternative strategies for new therapies and markers for diagnostic tests.

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Chapter 3

The LDL receptor knock-out mouse is a physiological
model particularly vulnerable for studying
inflammation in non-alcoholic steatohepatitis

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Submitted

Abstract

Background & aims

Non-alcoholic steatohepatitis (NASH) involves steatosis combined with inflammation, which can progress into fibrosis and cirrhosis. Exploring the molecular mechanisms of NASH is highly dependent on the availability of animal models. Currently, the most commonly used animal models for NASH imitate particularly late stages of human disease. Thus, there is a need for an animal model that can be used for investigating the factors that potentiate the inflammatory response within NASH. We have previously shown that 7-day high-fat- high-cholesterol (HFC) feeding induces steatosis and inflammation in both *APOE2ki* and *Ldlr*^{-/-} mice. However, it is not known whether the early inflammatory response observed in these mice will sustain over time and lead to liver damage. We hypothesized that the inflammatory response in both models is sufficient to induce liver damage over time.

Methods

APOE2ki and *Ldlr*^{-/-} mice were fed a chow or HFC diet for 3 months. C57Bl/6 mice were used as control.

Results

Surprisingly, hepatic inflammation was abolished in *APOE2ki* mice, while it was sustained in *Ldlr*^{-/-} mice. In addition, hepatic fibrosis developed only in *Ldlr*^{-/-} mice. Finally, bone-marrow-derived-macrophages of *Ldlr*^{-/-} mice showed an increased inflammatory response after oxidized LDL (oxLDL) loading compared to *APOE2ki* mice.

Conclusion

Ldlr^{-/-} mice, but not *APOE2ki* mice, developed sustained hepatic inflammation and liver damage upon long term HFC feeding due to increased sensitivity for oxLDL uptake. Therefore, the *Ldlr*^{-/-} mice are a promising physiological model particularly vulnerable for investigating hepatic inflammation in non-alcoholic steatohepatitis

Introduction

Non-alcoholic fatty liver disease covers a disease spectrum ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma¹. Whereas steatosis might not adversely affect outcome, inflammation determines the long-term prognosis of this disease²⁻⁵. It is still not known why some patients progress towards inflammation, while others do not.

Exploring the molecular basis of the hepatic alterations associated with the metabolic syndrome is highly dependent on the availability of animal models which mimic the human condition from the physiological and metabolic points of view^{6,7}. To date, the most comely used animal models for NASH imitate particularly late stages of human disease. Thus, there is a need for animal models that can be used for investigating the factors that potentiate the inflammatory response within NASH.

NASH is a component of the metabolic syndrome and therefore it is frequently associated with hyperlipidemia and atherosclerosis⁸. One of the commonly used models for atherosclerosis studies is the low density lipoprotein (LDL) receptor knock-out (*Ldlr*^{-/-}) mouse. The LDL receptor plays a major role in the clearance of apoB and apoE-containing lipoproteins⁹. Another mouse model for atherosclerosis is the apolipoprotein E2 knock-in (*APOE2ki*) mouse. In *APOE2ki* mice, the murine *apoe* gene is replaced by the human *APOE2* allele. The APOE2 protein has a markedly reduced affinity for the LDL receptor, leading to a plasma lipoprotein profile resembling human type III hyperlipoproteinaemia (HLP)¹⁰. Previously, we used these 'humanized' *APOE2ki* mice and *Ldlr*^{-/-} mice to study NASH. Both hyperlipidemic mice developed early hepatic inflammation and steatosis when fed a high-fat-high-cholesterol (HFC) diet, whereas C57Bl/6 mice only developed steatosis¹¹. Unlike the lipoprotein profile in wild-type (WT) profile, in which most cholesterol is present in the HDL fraction, the profile of the *Ldlr*^{-/-} mouse and *APOE2ki* is more comparable with the human plasma lipoprotein profile, in which cholesterol is mainly confined to the LDL fraction¹². Thus, when fed a HFC diet, both *APOE2ki* and *Ldlr*^{-/-} mice have the potential to be used as animal models for investigating the factors that potentiate the inflammatory response within NASH. However, data from the literature regarding the effect of inflammation on liver damage in hyperlipidemic mice on NASH progression are partial and inconclusive.

Based on the pronounced inflammatory response observed upon short-term HFC feeding, we hypothesized that the inflammatory response in both models will sustain over time and will be sufficient to induce fibrosis and liver damage. To test this hypothesis, male *APOE2ki* and *Ldlr*^{-/-} mice were fed a HFC diet for 3 months and normolipidemic C57Bl/6 (WT) mice were used as the control group. Surprisingly, although plasma and liver lipid levels were still elevated in both hyperlipidemic models after 3 months of HFC feeding, the inflammatory response was only sustained in the

Ldlr^{-/-} mice. In addition, hepatic fibrosis only developed in *Ldlr*^{-/-} mice. By analysing the effect of oxidized LDL (oxLDL) loading on macrophages from the different models, we showed that these differences are most likely attributable to an increased sensitivity for oxLDL-induced inflammation in *Ldlr*^{-/-} mice compared to *APOE2ki* mice. All together, the *Ldlr*^{-/-} mice are a promising physiological model particularly vulnerable for investigating hepatic inflammation in the context of NASH.

Materials and methods

Mice and diet

The mice were housed under standard conditions and given free access to food and water. The experiments were performed according to Dutch laws and were approved by the Committee for Animal Welfare of Maastricht University. Twelve-week-old male C57Bl/6, *APOE2ki* and *Ldlr*^{-/-} mice, with the same genetic background, were fed a high-fat-high-cholesterol diet containing 21% milk butter and 0.2% cholesterol (HFC) for 3 months (n=8). The control groups for each genotype were kept on a standard chow diet for the same period. The collection of blood, sacrificing of the mice, and tissue isolation were performed as described previously¹¹.

Lipid analysis

Liver and plasma lipid analysis were performed as described previously¹¹.

Liver histology

Frozen liver sections (7 µm) were stained for infiltrated macrophages (macrophage marker, Mac-1), CD68 Kupffer cells (CD68 marker, FA11), T cells (T-cell marker, KT3), fibroblasts (fibroblast marker, ERTR7) and neutrophils (neutrophil marker, NIMP) as described previously¹³. Paraffin-embedded liver sections (4 µm) were stained with Sirius Red as described previously¹³.

Gene expression analysis

Total RNA isolation from mouse liver tissues, cDNA synthesis and Q-PCR analysis were performed as described previously¹¹.

Measuring auto-antibody titers against modified LDL

Specific antibody titers against modified LDL in plasma were determined as described elsewhere^{14,15}. Plasma was diluted and antibody binding measured by chemiluminescent enzyme linked immunosorbent assay (ELISA).

In vitro murine macrophage culture

Bone marrow cells were isolated from the femurs and tibiae of C57Bl/6, *APOE2ki* and *Ldlr*^{-/-} mice. The cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, the Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, the Netherlands), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) (all GIBCO Invitrogen, Breda, the Netherlands) (R10) supplemented with 15% L929-conditioned medium (LCM) for 8-9 days to generate bone marrow-derived macrophages (BMM), as described previously¹⁶. The cells were treated for 24 h with 25 µg/ml of oxidized LDL (Intracel, Frederick, USA). Uptake of oxLDL particles that were Dil-labelled was assessed by flow cytometry after residual oxLDL was washed away.

Statistical analysis

The data was statistically analysed by performing two-tailed non-paired *t*-tests using GraphPad Prism, version 4.03 for Windows. Data were expressed as the mean ±SEM and considered significant at *p*<0.05. *, ** and *** indicate *p*<0.05, 0.01 and 0.001, respectively.

Results

Hepatic inflammation is only sustained in *Ldlr*^{-/-} mice after 3 months of the HFC diet compared to 7 days of the HFC diet

In order to determine whether hepatic inflammation was sustained in both *Ldlr*^{-/-} and *APOE2ki* mice after 3 months (long-term) of HFC feeding, the numbers of infiltrated macrophages, neutrophils and T cells and the gene expression levels of *Tnf*, *Mcp-1* and *Cd68* in these mice were compared with control mice fed on chow and with mice on the 7-day (short-term) HFC diet (Figure 3.1). Normolipidemic C57Bl/6 mice were used as the control group. To follow the changes in hepatic inflammation within time, we included the data of the 7-day HFC diet (dotted bars) in the first figure, which originates from Wouters *et al.*³². These data indicated that both *Ldlr*^{-/-} and *APOE2ki* mice had increased inflammation after 7 days of HFC feeding compared to mice on the chow diet and normolipidemic C57Bl/6 mice, as reflected by elevated numbers of infiltrated macrophages, neutrophils and T cells. In the control mice, only a minor increase in the number of infiltrated cells was observed after 7 days of HFC feeding (Figure 3.1A). Gene expression analysis for *Tnf*, *Mcp-1* and *Cd68* confirmed these findings. Comparing 7 days and 3 months of HFC feeding demonstrated that the inflammatory response was only sustained in *Ldlr*^{-/-} mice. In contrast, the number of infiltrated macrophages, neutrophils and T cells was significantly reduced in *APOE2ki* mice after 3 months of HFC feeding (Figure 3.1C). Moreover, the inflammatory cells were more clustered in *Ldlr*^{-/-} mice compared to *APOE2ki* and C57Bl/6 mice

(Figure 3.1B). In addition, the observed differences in hepatic inflammation between the hyperlipidemic models and between short- and long-term HFC diets were confirmed by gene expression analysis for *Tnf*, *Mcp-1* and *Cd68*, where the inflammatory response was sustained in the *Ldlr*^{-/-} mice and abolished in the *APOE2ki* mice after 3 months of the HFC diet compared to 7 days (Figure 3.1C).

Figure 3.1 Parameters of hepatic inflammation. (A) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP) and T cells (CD3) and counted as cells/mm². (B) Representative pictures of Mac-1 stained liver sections of control C57Bl/6, APOE2ki and Ldlr^{-/-} mice, respectively (×200 magnification). (C) Hepatic gene expression of *Tnf*, *Mcp-1* and *Cd68*. Pointed bars indicate the earlier time point of 7 days. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively.

Hepatic steatosis is present in all models after 3 months of the HFC diet

In order to investigate if the inflammatory response was associated with steatosis after 3 months of the HFC diet, liver lipid levels and Oil red O staining were performed on the livers of the three different mouse models. The liver cholesterol and triglyceride levels were increased in all C57Bl/6, APOE2ki and Ldlr^{-/-} mice after 3 months of HFC feeding compared to mice on the chow diet. Furthermore, the levels of cholesterol and

triglycerides were significantly higher in *Ldlr*^{-/-} mice compared to C57Bl/6 and *APOE2ki* mice after 3 months of the HFC diet (Figure 3.2A+B). These findings were confirmed by Oil red O staining (Figure 3.2C-E).

Only *Ldlr*^{-/-} mice have increased fibrosis after 3 months of the HFC diet

One of the features of advanced stages of NASH is hepatic fibrosis. To investigate whether or not the 3 mouse models differed in the extent of fibrosis, Sirius Red (data not shown) and fibroblast (ERTR7) staining (Figure 3.3A+B) were performed. Both stainings demonstrated that *Ldlr*^{-/-} mice have increased hepatic fibrosis after 3 months on the HFC diet compared to C57Bl/6 and *APOE2ki* mice. Likewise, the collagen content and fibroblast presence were greater around the portal areas. The histological findings were confirmed by the gene expression analysis of *Tgf-β*, *Mmp-9* and *Col1a1* (Figure 3.3C).

Figure 3.2 Oil red O staining on frozen liver sections. (A-C) Representative pictures (×100 magnification) of the Oil red O staining after the chow diet and 3 months of the HFC diet in C57Bl/6, *APOE2ki* and *Ldlr*^{-/-} mice.

High plasma cholesterol levels and foamy Kupffer cells in all hyperlipidemic models after 3 months of the HFC diet

Plasma cholesterol levels were significantly increased after 3 months of HFC feeding in all three models. No differences were observed between *APOE2ki* and *Ldlr*^{-/-} mice (Figure 3.4A). Moreover, the size of foamy KCs did not differ between the hyperlipidemic models. The KCs of these models were swollen compared to chow and C57Bl/6 mice (Figure 3.4B).

A

B

C

Figure 3.3 Parameters of hepatic fibrosis. (A-B) Representative pictures (magnification $\times 400$ (A), magnification $\times 100$ (B)) of ERTR7-(fibroblast marker) positive sections after 3 months on the HFC diet in C57Bl/6, APOE2ki and Ldlr^{-/-} mice, respectively. (C) Gene expression analysis of *Tgf-β*, *Col1a1* and *Mmp-9*. Data were set relative to the C57Bl/6 mice on the chow diet. *Significantly different from chow group. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively

Figure 3.4 Plasma cholesterol levels and foamy Kupffer cells. (A) Plasma total cholesterol levels after chow and 3 months of the HFC diet in the three different models. (B) Representative pictures (magnification $\times 200$) after 3 months of the HFC diet for C57Bl/6, *APOE2ki* and *Ldlr*^{-/-} mice, respectively. *Significantly different from chow group. * and *** indicate $p < 0.05$ and 0.001 , respectively.

Ldlr^{-/-} mice have increased hepatic expression levels of lipid-related genes

To further investigate the effect of HFC feeding on hepatic cholesterol metabolism, gene expression levels of the lipid-related genes were analysed. As expected, the hepatic expression of *Cd36*, *Sr-a*, *Lpl*, *Abcg1*, *Abca1* and *Ppar γ* were all elevated in mice on the HFC diet. In addition, *Ldlr*^{-/-} mice on the HFC diet showed higher expression levels of these genes (except for *Abca1*) than *APOE2ki* mice (Figure 3.5).

Figure 3.5 Cholesterol-related hepatic gene expression. Gene expression analysis of *Cd36*, *Sr-a*, *Lpl*, *Abcg1*, *Abca1* and *Pparγ*. Data were set relative to the C57Bl/6 mice on the chow diet. *Significantly different from chow group. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively.

Bone marrow-derived macrophages of *Ldlr*^{-/-} mice are more inflammatory after oxidized LDL loading compared to *APOE2ki* macrophages

We have previously shown that the level of naturally occurring antibodies against oxLDL in the plasma correlates with hepatic inflammation⁴. To investigate whether the differences in hepatic inflammation between *Ldlr*^{-/-} and *APOE2ki* correlate with the plasma levels of these antibodies, measurements of IgM and IgG antibodies against oxLDL were performed. These levels were comparable between the three different mouse models, suggesting a similar generation of oxidation-specific epitopes (data not shown). Moreover, bone marrow-derived macrophages (BMM) of these mice were loaded with oxLDL, which leads to foam cell formation and inflammation *in vitro*. Gene expression analysis of the inflammatory gene *Tnf*, *Il6*, *Mcp-1* and *Cd68* after oxLDL loading demonstrated that *APOE2ki* BMM have a lower expression compared to C57Bl/6 and *Ldlr*^{-/-} BMM. Additionally, the two main scavenger receptors responsible for the uptake of modified lipids, *Cd36* and *Sr-a*, showed increased expression in *Ldlr*^{-/-} compared to *APOE2ki* (Figure 3.6A). The percentage of positive BMM and the positivity of BMM were increased upon oxLDL loading compared to basal levels, but were not different between the groups (Figure 3.6B-C). In conclusion, although the uptake of oxLDL was the same between the different models, BMM of *APOE2ki* mice were less inflammatory compared to C57Bl/6 and *Ldlr*^{-/-} mice.

Figure 3.6 Loading of bone marrow-derived macrophages with oxLDL. (A) Bone marrow-derived macrophages (BMM) of C57Bl/6, APOE2ki and Ldlr^{-/-} mice were loaded with oxidized low-density lipoprotein (oxLDL) and gene expression of tumor necrosis factor (*Tnf*), interleukin 6 (*Il6*), monocyte chemoattractant protein 1 (*Mcp-1*), *Cd68*, *Cd36* and scavenger receptor A (*Sr-a*) was analysed. Data were set relative to the basal levels of C57Bl/6 bone marrow. (B) Percentage of positive BMM at basal levels and after oxLDL loading. (C) Positivity of BMM at basal levels and after oxLDL loading. * indicate p<0.05.

Discussion

The present study illustrates that the Ldlr^{-/-} mice are a promising long term NASH model, while in contrast to our expectations, APOE2ki mice are not. The HFC diet led to sustained hepatic inflammation and fibrosis in Ldlr^{-/-} mice, but not in APOE2ki mice. This difference was most likely a consequence of the increased sensitivity for oxLDL-induced inflammation in Ldlr^{-/-} mice compared to APOE2ki mice. These novel observations indicate that hyperlipidemia and steatosis are not sufficient for maintaining the inflammatory response in the liver. In addition, our data demonstrate that the Ldlr^{-/-} mouse model can be used as an excellent physiological model particularly vulnerable for investigating hepatic inflammation in the context of NASH.

Lack of a suitable animal model that faithfully recapitulates the pathophysiology of human NASH is a major obstacle in delineating mechanisms responsible for the

progression of steatosis to NASH. The best characterized and most widely used genetic model for NASH is the leptin-deficient mouse (*ob/ob*). The *ob/ob* mice develop hepatic steatosis but not hepatic inflammation or fibrosis, possibly due to the loss of normal leptin signalling¹⁷. Therefore, these *ob/ob* mice need a pro-injurious stimulus, such as an endotoxin (LPS)¹⁸. The most well-known nutritional model for NASH is a diet deficient in methionine and choline (MCD). These mouse models display all of the hallmarks of NASH, from steatosis to inflammation and fibrosis development¹⁹. However, mice fed an MCD-deficient diet tend to lose weight and display lowered plasma TG levels and are therefore very different from NASH in human metabolic syndrome or diabetes patients who are mostly obese and/or hyperlipidemic⁶. Therefore, the currently available genetic and nutritional models are especially useful to investigate late stages of NASH but have some limitations restricting their use to serve as a physiological model to study the development of hepatic inflammation in the context of NASH^{6,8}.

Based on the analogy between the mechanisms of NASH and atherosclerosis, an emerging trend in NASH research is to utilize the mouse models traditionally targeted for studies of atherosclerosis, including *APOE2ki* and *Ldlr*^{-/-} mice. The *APOE2ki* mouse, a humanized mouse model of hyperlipidemia, has outstanding potential as it is highly responsive to dietary factors and pharmacological interventions²⁰. As these mice are not commonly used, the effect of feeding a long-term HFC diet on the liver of *APOE2ki* mice had so far not been investigated. In contrast, several studies have been performed to determine the consequences of a long-term HFC diet on *Ldlr*^{-/-} mice. The *Ldlr*^{-/-} mice are mildly hypercholesterolemic due to the absence of LDL receptors, which prolongs the plasma half-life of VLDL and LDL²¹. These mice were utilized by Kong *et al.*, who revealed that 5 months of HFC feeding in male *Ldlr*^{-/-} mice induced macrovesicular steatosis, but not inflammatory cell infiltration²². In contrast, Yoshimatsu *et al.* demonstrated that 3 months of HFC feeding of female *Ldlr*^{-/-} mice resulted in steatosis, infiltration of neutrophils into the liver and increased serum aminotransferases (ALT) levels. However, the diet was highly enriched with cholesterol (1.25%) and cholic acid (0.5%), and the latter is a primary bile acid that is known to cause hepatic toxicity. In addition, the effect on hepatic fibrosis was not investigated²³. Finally, Gupte *et al.* demonstrated that middle-aged (12-month-old) male *Ldlr*^{-/-} mice fed an HFC diet for 3 months developed steatosis, inflammation, fibrosis, oxidative stress and elevated liver injury markers²⁴. However, the authors concluded that the advanced age of these mice exacerbated the HFC-induced fibrosis²⁵. Thus, the current data regarding the effect of a long-term HFC diet on NASH progression in hyperlipidemic mice is partial and inconclusive. By using young mice with a physiological diet, we proved for the first time that the *Ldlr*^{-/-} mice are a promising model for investigating hepatic inflammation in NASH and can be a valuable tool for conducting interventional studies; these findings should eventually lead to a better

understanding of human NASH and the development of an efficient therapy for this condition.

Studies in *apoE*^{-/-} mice show that ApoE influences several inflammatory processes due to the fact that it is produced by a wide variety of cell types, including macrophages²⁶⁻²⁹. However, our data suggest that the APOE2 isoform is not directly involved in inflammation as the inflammatory response was not sustained in the *APOE2ki* mice. Unlike *APOE2ki* mice, *apoE*^{-/-} mice have been extensively used as models for atherosclerosis. It was shown that the mean cross-sectional area of the plaque in the *APOE2ki* mice is approximately half that seen in age-matched *apoE*^{-/-} mice with a similar genetic background and the plaques of the *APOE2ki* mice are less mature³⁰⁻³². Moreover, it was also demonstrated that apoE has allele-specific effects in protecting cells from oxidative cell death, with E2 the most effective one³³. In addition, the circulation time of atherogenic particles is also reduced in *APOE2ki* mice compared to *apoE*^{-/-} mice, which can result in an decreased inflammatory response in the body^{34,35}. Altogether, these evidences suggest that *APOE2ki* mice are less inflammatory compared to the *apoE*^{-/-} mice.

Where *Ldlr*^{-/-} mice require an atherogenic diet to develop atherosclerosis, *APOE2ki* mice spontaneously develop the full spectrum of atherosclerotic lesions, even on a regular chow diet. Moreover, an atherogenic diet, high in fat and cholesterol, further exacerbates the development of atherosclerosis and xanthomas in these *APOE2ki* mice. However, it was shown that the lesions in these *APOE2ki* mice mainly consisted of foam cells and had relatively fewer fibrous caps, cholesterol clefts and necrotic cores¹⁰. Thus, in line with our observations, it is likely that the foamy macrophages from the atherosclerotic plaques of *APOE2ki* mice are less inflammatory compared to those in *Ldlr*^{-/-} mice. Currently, *APOE2ki* mice are still accepted as an established model for atherosclerosis. Altogether, based on our data and data from the literature, this paradigm should be re-evaluated.

Although hepatic inflammation was completely abolished in *APOE2ki* mice after 3 months of HFC feeding, plasma and liver lipid levels were still elevated. Previously, we reported that elevated plasma cholesterol levels can trigger hepatic inflammation¹¹. In addition, omitting cholesterol from the diet even resulted in a dramatic inhibition of hepatic inflammation. In the present study, however, neither plasma cholesterol, nor anti-oxLDL antibodies were correlated with hepatic inflammation. These observations suggest that the differences in inflammation are not related to systemic difference in lipids and oxidation, but rather to differences in the activity of intracellular inflammatory pathways. In line with this hypothesis, KCs from both hyperlipidemic models still had a foamy appearance after 3 months of HFC diet. Moreover, BMM of *Ldlr*^{-/-} mice showed increased expression of *Il-6* and *Cd36* compared to macrophages from *APOE2ki* and C57Bl/6 mice after oxidized LDL loading.

Relevantly, IL-6 was shown to dictate the transition from acute to chronic inflammation by changing the nature of leukocyte infiltration (from polymorphonuclear neutrophils to monocyte/ macrophages). In addition, IL-6 exerts stimulatory effects on T- and B-cells, thus favoring chronic inflammatory responses³⁶. Thus, these data suggest that, unlike macrophages from *APOE2ki* and C57Bl/6 mice, macrophages of *Ldlr*^{-/-} mice produce higher levels of IL-6 and therefore are more prone to develop chronic inflammation. In addition, gene expression of *Cd36*, the main scavenger receptor responsible for the uptake of oxidized cholesterol and involved in inflammatory signal transduction, was also higher in BMM of *Ldlr*^{-/-} mice after oxLDL loading compared to *APOE2ki* and C57Bl/6 mice. Interestingly, oxLDL up-regulates CD36 expression via the peroxisome proliferator-activated receptor (PPAR) γ , and this may initiate a feed forward loop of CD36 expression that amplifies the inflammatory response³⁷. In support of this view, the expression levels of several target genes for *Ppar γ* (*Abcg1*, *Lpl*, *Cd36*) were also reduced in the livers of *APOE2ki* mice compared to *Ldlr*^{-/-} mice. Thus, it is possible that this loop mechanism does not occur in *APOE2ki* mice and therefore these mice are less sensitive to the inflammatory cascade.

So far, there is no suitable physiological model for studying the hepatic inflammation in a metabolic context that faithfully recapitulates the pathophysiology of human NASH. In this study, the hepatic inflammatory response induced by prolonged HFC feeding in *APOE2ki* and *Ldlr*^{-/-} mice was investigated. We demonstrated that *Ldlr*^{-/-} mice have increased sensitivity for oxLDL-induced inflammation and fibrosis compared to *APOE2ki* mice. Therefore, the *Ldlr*^{-/-} mouse model is particularly useful for understanding the relationships between lipid metabolism, inflammatory recruitment and fibrogenesis in the context of NASH. This model may therefore be an excellent platform for the assessment of therapeutic strategies for hepatic inflammation.

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Chapter 4

Role of Scavenger Receptor A and CD36 in diet-induced non-alcoholic steatohepatitis in hyperlipidemic mice

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Abstract

Background & aims

Non-alcoholic steatohepatitis (NASH) is a disorder which consists of steatosis and hepatic inflammation. It is not known why only some people with steatosis develop NASH. Recently we identified dietary cholesterol as a factor that directly leads to hepatic inflammation and hepatic foam cell formation. We propose a mechanism where Kupffer cells (KCs) take up modified cholesterol-rich lipoproteins via scavenger receptors (SRs). KCs thereby accumulate cholesterol, become activated, and may then trigger an inflammatory reaction. Scavenging of modified lipoproteins mainly depends on CD36 and SR-A (MSR1).

Methods

To evaluate the involvement of SR-mediated uptake of modified lipoproteins by KCs in the development of diet-induced NASH, female low density lipoprotein (LDL) receptor-deficient (*Ldlr*^{-/-}) mice were lethally irradiated and transplanted with bone marrow from *Msr1*^{+/+}/*Cd36*^{+/+} or *Msr1*^{-/-}/*Cd36*^{-/-} mice and fed a Western diet.

Results

Macrophage and neutrophil infiltration revealed that hepatic inflammation was substantially reduced by approximately 30% in *Msr1*^{-/-}/*Cd36*^{-/-} transplanted (-tp) mice compared to control mice. Consistent with this, the expression levels of well-known inflammatory mediators were reduced. Apoptosis and fibrosis were less pronounced in *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice, in addition to the protective phenotype of natural antibodies against oxidized LDL in the plasma. Surprisingly, the effect on hepatic inflammation was independent of foam cell formation.

Conclusions

Targeted inactivation of scavenger receptor pathways reduces the hepatic inflammation and tissue destruction associated with NASH, independent of hepatic foam cell formation.

Introduction

Non-alcoholic steatohepatitis (NASH) is considered to be the hepatic event of the metabolic syndrome. It is characterized by hepatic lipid accumulation (steatosis) combined with inflammation. Estimates of the general USA population indicate that approximately 2-3% of all adults have NASH and this prevalence is expected to rise rapidly due to the increasing prevalence of obesity¹. The current view on the pathogenesis of diet-induced liver inflammation is that hepatic steatosis is a critical prerequisite for the development of inflammation. While steatosis itself is generally considered benign and reversible, the presence of inflammation can lead to further progression of NASH, resulting in liver fibrosis, cirrhosis and eventually, liver failure and hepatocellular carcinoma². Thus, the progression towards hepatic inflammation represents a key step in NASH development. It is not yet known why only a small percentage of people with steatosis develop NASH. Although several processes have been identified that participate in the development of hepatic inflammation³⁻⁷, the actual trigger for the inflammatory response remains uncertain.

We and other researchers have recently shown that dietary cholesterol is an important risk factor for the development of hepatic inflammation⁸⁻¹⁰. Interestingly, hyperlipidemic mice fed a high-fat, high-cholesterol (HFC) diet develop early hepatic inflammation, which is associated with bloated Kupffer cells (KC), resembling foam cells in atherosclerosis. These KCs with a foamy appearance have also been identified in other studies in the sinusoidal space of the liver^{11,12}. Strikingly, omitting cholesterol from the diet in these mice resulted in a dramatic inhibition of hepatic inflammation and foam cell formation⁸. An attractive hypothesis is that, due to the decreased lipoprotein uptake by hepatocytes in hyperlipidemic mice, lipoproteins have a longer residence time in the plasma and are therefore more prone to be modified and subsequently scavenged by KCs. This process ultimately results in KC activation, which may be responsible for triggering the hepatic inflammation and KC cholesterol accumulation.

Like typical macrophages, KCs express scavenger receptors (SRs)¹³ and are thus capable of taking up modified lipoproteins. *In vitro*, foam cell formation has been shown to depend mainly on two different SRs: SR-A (macrophage scavenger receptor 1 (MSR1)) and CD36¹⁴. MSR1 was found to account for the majority (80%) of macrophage uptake of acetylated LDL (acLDL), but has a lower affinity for oxLDL^{14,15}. CD36 binds moderately oxLDL, rather than acLDL¹⁶, and was found necessary for oxLDL-induced JNK activation¹⁷. Despite numerous studies addressing the role of these two SRs in atherosclerosis, their roles remain uncertain. While clear effects of MSR1 and CD36 on cholesterol uptake and foam cell formation have been found *in vitro*¹⁴, the results *in vivo* are less consistent. Findings in mouse models of atherosclerosis lacking one or both of these SRs have ranged from reduced foam cell formation and atherosclerosis

to no effect, or to greater foam cell formation and increased atherosclerosis^{16,18,19}. Whereas the reasons for these discrepancies are unclear, there is a growing appreciation of the numerous functions that scavenger receptors can play in addition to lipoprotein uptake, including their roles in inflammatory signal transduction and tissue homeostasis.

To investigate the contribution of *Msr1* and *Cd36* expression by macrophages on hepatic modified lipoprotein uptake and inflammation, we performed a bone marrow transplantation (BMT) of donor bone marrow from mice with targeted deletions for *Msr1* and *Cd36* into lethally irradiated *Ldlr*^{-/-} recipient mice. We hypothesized that *Msr1*^{-/-}/*Cd36*^{-/-}-transplanted (-tp) mice upon high fat diet would develop reduced levels of hepatic inflammation compared to mice with normal hematopoietic cells due to a decreased uptake of modified lipoproteins by KCs. Surprisingly, while deletion of *Msr1* and *Cd36* in hematopoietic cells failed to block cholesterol accumulation in KCs, these transplanted mice showed reduced markers for NASH, including less inflammation, apoptosis and fibrosis after high fat feeding. These data suggest that SR expression by KCs in the liver increases the risk for NASH in the presence of high levels of plasma-modified lipoproteins.

Materials and methods

Mice, diet and bone marrow transplantation

Mice were housed under standard conditions and given free access to food and water. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University. One week before transplantation, ten-week-old female *Ldlr*^{-/-} mice were put into filter-top cages and received acidified water supplemented with neomycin (100 mg/l; N1142, Sigma-Aldrich, St. Louis, MO, USA) and polymyxin B sulphate (60,000 U/l; 21850-029, Life Technologies Cooperation, Carlsbad, Ca, USA). One day before transplantation, mice were subjected to a full body irradiation with a lethal dose of 10 Gy. The bone marrow of five female wild-type mice and five female *Msr1*^{-/-}/*Cd36*^{-/-} littermates was collected and pooled. Each irradiated *Ldlr*^{-/-} mouse received 10⁷ bone marrow cells by injection into the tail vein. After a recovery period of 9 weeks, the mice were given a HFC diet for 7 days (n=8 in *Msr1*^{+/+}/*Cd36*^{+/+} and n=8 in *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice) and 3 months (n=7 in *Msr1*^{+/+}/*Cd36*^{+/+}-tp group, n=8 in *Msr1*^{-/-}/*Cd36*^{-/-}-tp group), containing 21% butter and 0.2% cholesterol (diet 1635, Scientific Animal Food and Engineering, Villemoisson-sur-Orge, France). Chow fed mice that were sacrificed after 9 weeks recovery were used as control group (n=7 in both *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice). Blood from the tail vein was collected before the dietary period, after 7 days on the HFC diet in the short-term group, and after 3 months in the long-term group on the day of sacrifice

after a 4-hour fast period. The mice were then sacrificed by cervical dislocation. Tissues were isolated and snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/PBS.

Plasma lipid analysis

Both plasma and liver lipid levels were measured with enzymatic color tests (cholesterol CHOD-PAP, 1489232, Roche, Basel, Switzerland, TG GPOtrinder, TR0100, Sigma Aldrich, St. Louis, MO, USA; NEFAC, ACS-ACOD, 999-75406, Wako Chemicals, Neuss, Germany) according to the manufacturer's protocols on a Benchmark 550 Micro-plate Reader (170-6750XTU, Bio-Rad, Hercules, CA, USA).

Liver lipid analysis

Approximately 50 mg of frozen liver tissue was homogenized for 30 s at 5000 rpm in a closed tube with 1.0 mm glass beads and 1.0 ml SET buffer (Sucrose 250 mM, EDTA 2 mM and Tris 10 mM). Complete cell destruction was done by two freeze-thaw cycles and three times passing through a 27-gauge syringe needle and a final freeze-thaw cycle. Protein content was measured with the BCA method (23225, Pierce, Rockford, IL, USA). Cholesterol, TG and FFA were measured as described above. Protocols were followed according to the manufacturers' instructions.

Liver histology

Frozen liver sections (7 μm) were fixed in acetone and subsequently blocked for endogenous peroxidase by incubation with 0.25% of 30% H_2O_2 for 5 minutes. Primary antibodies used were against infiltrated macrophages and neutrophils (rat-anti-mouse Mac-1 (M1/70)) and T-cells (rat-anti-mouse CD3) (both generous gifts from Prof. Kraal, Free University, Amsterdam, The Netherlands), neutrophils (rat-anti-mouse Ly6-C, clone NIMP-R14) (generous gift from Prof. Heeringa, Groningen, The Netherlands), KCs (rat-anti-mouse CD68, clone FA11) (generous gift from Prof. Gordon, Oxford, UK) and apoptosis (rabbit-anti-mouse cleaved caspase 3) (9661Lg, Cell Signalling, Danvers, MA, USA). 3-Amino-9-ethylcarbazole (AEC) (A85SK-4200.S1, Bio-connect, Huissen, the Netherlands) was applied as color substrate and haematoxylin (4085.9002, Klinipath, Duiven, The Netherlands) for nuclear counterstaining. Sections were enclosed with Faramount aqueous mounting medium (S302580, DAKO, Glostrup, Denmark). For the lipid stainings, the neutral lipid marker oil red o (ORO, O0625, Sigma-Aldrich, Saint Louis, MO, USA) and the fluorescent free cholesterol marker Filipin (F9765, Sigma-Aldrich, Saint Louis, MO, USA) were used. The filipin staining was quantified by scoring all filipin positive KCs between 0 and 3, where score 0 indicates not positive and score 3 indicates extremely positive for cholesterol content inside the KCs. Paraffin embedded liver sections (4 μm) were stained with Hematoxylin-Eosin (HE, Haematoxilin, 4085.9002, Klinipath, Duiven, The Netherlands and Eosin, E4382, Sigma-

Aldrich, Saint Louis, MO, USA), Sirius Red (Direct Red 80 , 43665, Sigma-Aldrich, Saint Louis, MO, USA) and rabbit-anti-mouse myeloperoxidase (MPO, A0398, DAKO, Glostrup, Denmark). Pictures were taken with a Nikon digital camera DMX1200 and ACT-1 v2.63 software (Nikon Instruments Europe, Amstelveen, The Netherlands). Immune cells were counted in six microscopical views (magnification 200x) and were noted as cells/mm².

RNA isolation and quantitative PCR

Total RNA was isolated from approximately 25 mg of mouse liver tissues as described previously¹. All applications were performed according to the manufacturers' protocols. Total RNA (500 ng) from each individual mouse was converted into first strand cDNA with the iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The changes in gene expression of inflammatory markers were determined by quantitative PCR on a SDS 7900HT by using Power Sybr Green mastermix (4329001 and 4368708, both Applied Biosystems, Foster City, CA, USA) and 10 ng of cDNA. For each gene, a standard curve was generated with a serial dilution of a liver cDNA pool. To standardize for the amount of cDNA, Cyclophilin A (*Ppia*) was used as the reference gene. Primer sets for the selected genes were developed with Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA) using default settings. Primer sequences are given in Table 4.1. Data from qPCR was analyzed according to the relative standard curve method.

Table 4.1 Primer sequences for quantitative PCR.

Gene	Primer forward	Primer reverse
<i>Tnf</i>	CATCTTCTCAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
<i>Il6</i>	GCTACCAAACGGATATAATCAGGAAA	CTTGTTATCTTTAAGTTGTTCTTCATGTACTC
<i>Tlr-4</i>	TATCCAGGTGTGAAATTGAAACAATT	GGGTTTCCTGTCAAGTATCAAGTTTG
<i>Tlr-2</i>	AATTGCATCACCGGTCAGAAA	GTTTGCTGAAGAGGACTGTTATGG
<i>Ppar-α</i>	TTCCTGTTTGTTGGTGCTAT	TGCAACTTCTCAATGTAGCCTATGTT
<i>Ppar-γ</i>	TCGCTGATGCACTGCCTATG	GAGAGGTCCACAGAGCTGATT
<i>Cd68</i>	TGACCTGCTCTCTAAGGCTACA	TCACGGTTGCAAGAGAAACATG
<i>Abca1</i>	CCCAGAGCAAAAAGCGACTC	GGTCATCATCACTTTGGTCCTTG
<i>Abcg1</i>	TCGGACGCTGTGCGTTTT	CCCACAAATGTCGCAACCT
<i>Col1a1</i>	AACCTGCCCGCACATG	CAGACGGCTGAGTAGGGAACA
<i>Mmp-13</i>	ACAAAGATTATCCCCGCTCATA	CACAATGCGATTACTCCGATACTG
<i>Timp-1</i>	CGCCTAAGGAACGGAAATTTG	GATAGATAAACAGGGAAACACTGT
<i>Tgf-β</i>	AGCGCTCACTGCTCTTGGA	GTCGCCCCGACGTTTG
<i>Cd36</i>	GCCAAGCTATTGCGACATGA	AAAAGAATCTCAATGTCCGAGACTTT
<i>Sr-a</i>	CATACAGAAACACTGCATGTCAGAGT	TTCTGCTGATACTTTGTACACACGTT
<i>Lox1</i>	TCCCGTTCTGGATTGGAT	TTGCCTGATGAATATAGCTGTAAGAAA
<i>Sr-b1</i>	TTCTTCACTACGCGCAGTATGTG	CACTCCAAAACAAAAGCATTTCCTC
<i>Sr-cl</i>	TAGACGGGTACCTGTTGATTACA	ATTGATTTCACTACACTGGAAGTCAT
<i>Sr-ec</i>	GACGACTCCTTCTTCTGATCCT	TGGGCCATAGGGACCATCT
<i>Psox</i>	TGCAGTCCAAAAGCGTGTGT	GTGGTGAAAACCTCTCCCATGAC
<i>Ppia</i>	TTCCTCCTTTCACAGAATTATCCA	CCGCCAGTGCCATTATGG

Chimerism determination by quantitative PCR

For the determination of the chimerism in the transplanted mice we have made use of the knowledge that donor bone marrow has a *Ldlr*^{WT} origin whereas recipient bone marrow a *Ldlr*^{-/-} origin. Genomic DNA was isolated using the NucleoSpin Blood QuickPure DNA isolation kit (740569, Macherey Nagel, Düren, Germany). A standard curve was generated by mixing DNA from *Ldlr*^{-/-} and *Ldlr*^{WT} bone marrow cells at different ratios. Chimerism was determined by quantifying the amount of *Ldlr*^{-/-} DNA in samples from 70 µl peripheral blood. To standardize for the amount of input DNA, the non-relevant *p50* gene was quantified. Samples were assayed in duplicate on a 7900HT Real-Time PCR System by using 25ng DNA, PowerSybr Green mastermix (4329001 and 4368708, both Applied Biosystems, Foster City, CA, USA), according to the manufacturers instructions.

Ldlr^{-/-} specific primers are: forward 5'-GCTGCAACTCATCCATATGCA-3' and reverse 5'-GGAGTTGTTGACCTCGACTCTAGAG-3'.

p50-specific primers are: forward 5'-ACCTGGGAATACTTCATGTGACTAA-3' and reverse 5'-ACACCAGAAGTCCAGGATTATAGC-3'.

A standard curve was generated by plotting the mean Δ Ct ($Ct\ p50 - Ct\ Ldlr^{-/-}$) against the logarithm of the percentage *Ldlr*^{-/-} and calculation of a regression line. Chimerism was calculated from the percentage of *Ldlr*^{-/-} DNA in the blood samples (representing the remaining recipient bone marrow), determined by applying the mean delta Ct of the sample to the standard curve.

Measuring aminotranferases

The level of aminotransferases ALT and AST in plasma of each individual mouse was measured by using the Reflotron-system and the test strips for ALT and AST measurments (Roche Diagnostics, Almere, The Netherlands), according to the manufacturers instructions. Shortly, for each aminotransferase 32 µl plasma was loaded on the appropriate test strip and inserted into the machine. After two minutes the results were shown on the display.

Measuring auto-antibody titers against modified LDL

Specific antibody titers against modified LDL in plasma were determined as described elsewhere^{21,22}. Plasma was serially diluted and antibody binding measured by chemiluminescent enzyme linked immunosorbent assay (ELISA). A titer was defined as the reciprocal of the maximal dilution at which binding of the secondary antibody was twice as high as the background binding.

Measuring oxysterols

The oxysterols 24S- and 27-hydroxycholesterol were measured after extraction from plasma as their trimethylsilyl ethers by highly specific and sensitive gas chromatography-mass spectrometry as described previously^{23,24}. Deuterium labeled 24- and 27-hydroxycholesterol were used as internal standards as an isotope dilution method.

Statistical analysis

Data was statistically analyzed by performing two-tailed non-paired t-tests using GraphPad Prism, version 4.03 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com) for comparing *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice for each diet group. One-way ANOVA test was used for comparing the different time points of high fat feeding within the same acceptor mice. Data were expressed as the mean $\pm$ SEM and considered significant at $p < 0.05$. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 resp.

Results

Plasma and liver lipid levels in *Msr1*^{+/+}/*Cd36*^{+/+} - and *Msr1*^{-/-}/*Cd36*^{-/-} transplanted mice

To establish the role of *Msr1* and *Cd36* on diet-induced NASH, *Ldlr*^{-/-} mice were transplanted with *Msr1*^{+/+}/*Cd36*^{+/+} and *Msr1*^{-/-}/*Cd36*^{-/-} bone marrow. Chimerism determination revealed an engraftment efficiency of 99%. After a recovery period of 9 weeks, mice received a HFC diet for either 7 days or 3 months. Chow fed mice that were sacrificed after 9 weeks recovery served as control groups. The body weights were not significantly different between all the groups (data not shown). As expected, HFC feeding increased plasma triglycerides (TG) and total cholesterol (TC) levels in a time-dependent manner in both transplanted groups after periods of 7 days and 3 months (Figure 4.1A,B) (TG: $p = 0.0003$ for *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice and $p = 0.0007$ for *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice; TC: $p < 0.0001$ for both *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice). Surprisingly, *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice displayed lower TG levels after 3 months of HFC diet than *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice ($p = 0.009$). A similar trend was observed for plasma cholesterol after 3 months of HFC diet, however, this did not reach statistical significance ($p = 0.08$). No effect on free fatty acid (FFA) levels was detected (Figure 4.1C).

Both short- and long-term HFC feeding resulted in the development of equal levels of steatosis in the two transplanted groups, with increasing levels after 7 days and 3 months of HFC diet compared to chow fed mice (time effect of TG, TC and FFA:

$p < 0.0001$ for both $Msr1^{+/+}/Cd36^{+/+}$ -tp and $Msr1^{-/-}/Cd36^{-/-}$ -tp mice). Neither hepatic TG levels, nor hepatic TC and FFA differed between the two transplanted groups (Figure 4.1D-F). Scoring of Haematoxylin-Eosin (HE)- and oil red O stained (ORO) liver sections by a trained pathologist confirmed this observation (data not shown).

Figure 4.1 Plasma and liver lipid levels. (A-C) Plasma total triglycerides (TG), total cholesterol (TC) and free fatty acids (FFA) after chow, 7 days, and 3 months on a HFC diet. (D-F) Liver TG, TC and FFA after chow, 7 days and 3 months on a HFC diet. *Significantly different from age-matched $Msr1^{+/+}/Cd36^{+/+}$ -tp group.

Lower levels of hepatic inflammation and apoptosis in the $Msr1^{-/-}/Cd36^{-/-}$ transplanted mice

To determine whether combined deletion of *Msr1* and *Cd36* on bone marrow cells affected the level of hepatic inflammation, liver sections were stained for inflammatory cell markers. For both short- and long-term HFC feeding, counting of Mac-1 positive cells (infiltrated macrophages and neutrophils) and NIMP positive cells (specifically neutrophils) revealed that hepatic inflammation was increased compared to chow ($p < 0.0001$ for both Mac1 and NIMP in both $Msr1^{+/+}/Cd36^{+/+}$ -tp and $Msr1^{-/-}/Cd36^{-/-}$ -tp mice), but was significantly lower in the $Msr1^{-/-}/Cd36^{-/-}$ -tp mice compared to $Msr1^{+/+}/Cd36^{+/+}$ -tp mice (Mac1: $p = 0.009$ (7d), $p = 0.05$ (3m); Nimp: $p = 0.02$ (7d), $p = 0.007$ (3m)) (Fig. 2A+B). Moreover, when present, the positive cells were more clustered in the $Msr1^{+/+}/Cd36^{+/+}$ -tp mice (Figure 4.2D+E). The number of CD3+ cells (T-cells) was also elevated upon HFC diet ($p < 0.0001$ for both $Msr1^{+/+}/Cd36^{+/+}$ -tp and $Msr1^{-/-}/Cd36^{-/-}$ -tp mice).

tp mice), and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice showed less positive T-cells after 3 months of HFC diet compared to *Msr1*^{+/-}/*Cd36*^{+/-}-tp mice ($p=0.0008$) (Figure 4.2C).

To further define the differences in inflammation between the two groups, we determined the expression of several genes known to be involved in inflammation. Table 1 shows that expression of tumor necrosis factor (*Tnf*) was significantly lower in the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice after 3 months on a HFC diet compared to *Msr1*^{+/-}/*Cd36*^{+/-}-tp mice ($p=0.02$), confirming the histological data. Despite a trend towards reduced expression of the pro-inflammatory cytokine interleukin 6 (Il-6), it was not significantly changed. The expression of both toll like receptors 2 and 4 (*Tlr-2* and *Tlr-4*), two well-known receptors involved in NASH, was lower in the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice after 7 days on a HFC diet compared to *Msr1*^{+/-}/*Cd36*^{+/-}-tp mice ($p=0.03$; $p=0.004$ resp.), but not after 3 months. Moreover, the expression levels of the anti-inflammatory nuclear receptors *Ppar-α* and $-γ$ were higher in the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice after 3 months HFC diet than in mice with *Msr1*^{+/-}/*Cd36*^{+/-} bone marrow ($p=0.001$; $p=0.03$ resp.) (Table 4.2). Together these data are consistent with a reduction in inflammation in mice lacking hematopoietic expression of *Cd36* and *Sr-α*.

Figure 4.2 Parameters of hepatic inflammation and apoptosis. (A-C) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP) and T cells (CD3+), respectively, and counted. (D+E) Representative pictures of Mac-1 staining (x200 magnification) after 7 days of HFC feeding in *Msr1*^{+/-}/*Cd36*^{+/-}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice, respectively. (F) Apoptosis was quantified by cleaved caspase 3 staining. *Significantly different from age-matched *Msr1*^{+/-}/*Cd36*^{+/-}-tp group. *, ** and *** indicate $p<0.05$, 0.01 and 0.001 resp.

The death of KCs and hepatocytes by apoptosis is thought to give rise to larger regions of liver damage. To test whether there was a relationship between inflammation and apoptosis in these mice, liver sections were stained for cleaved caspase 3 staining. After 3 months of HFC feeding, a significant decrease in cleaved caspase 3 positive inflammatory cells and, to a lesser extent, in hepatocytes was observed in livers of *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice (p=0.04) (Figure 4.2F).

The presence of elevated transaminases like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in plasma is considered to be a sensitive indicator of liver damage. In the present study, the plasma levels of these liver enzyme levels increased with the length of time of HFC feeding (ALT: p=0.0017 for *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice and p=0.019 for *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice; AST: p=0.024 for *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice and p=0.12 for *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice). However, neither ALT nor AST levels was significantly different between *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice (data not shown).

Table 4.2 Gene expression analysis of inflammatory related genes

Gene	Chow			7d HFC			3m HFC		
	<i>Msr1</i> ^{+/+} / <i>Cd36</i> ^{+/+}	<i>Msr1</i> ^{-/-} / <i>Cd36</i> ^{-/-}	p-value	<i>Msr1</i> ^{+/+} / <i>Cd36</i> ^{+/+}	<i>Msr1</i> ^{-/-} / <i>Cd36</i> ^{-/-}	p-value	<i>Msr1</i> ^{+/+} / <i>Cd36</i> ^{+/+}	<i>Msr1</i> ^{-/-} / <i>Cd36</i> ^{-/-}	p-value
<i>Tnf</i>	1 (+0.12)	1.01 (+0.15)	0.97	1.36 (+0.29)	0.86 (+0.11)	0.12	6.44 (+0.97)	3.71 (+0.42)	0.02*
<i>Il6</i>	1 (+0.17)	0.89 (+0.13)	0.61	1.80 (+0.31)	1.29 (+0.19)	0.17	0.49 (+0.11)	0.44 (+0.08)	0.10
<i>Tlr-2</i>	1 (+0.10)	0.86 (+0.09)	0.31	1.38 (+0.24)	0.77 (+0.07)	0.03*	2.12 (+0.26)	1.95 (+0.23)	0.64
<i>Tlr-4</i>	1 (+0.08)	0.78 (+0.05)	0.04*	1.23 (+0.08)	0.91 (+0.02)	0.004**	2.30 (+0.16)	1.92 (+0.02)	0.07
<i>Ppar-α</i>	1 (+0.07)	0.78 (+0.06)	0.06	1.23 (+0.06)	1.15 (+0.06)	0.37	1.19 (+0.06)	1.49 (+0.04)	0.001**
<i>Ppar-γ</i>	1 (+0.25)	0.99 (+0.20)	0.97	3.85 (+0.65)	2.58 (+0.48)	0.13	11.15 (+1.09)	18.97 (+1.98)	0.007***

Gene expression analysis of four well-known inflammatory markers: tumor necrosis factor (*Tnf*), interleukin 6 (*Il6*), toll-like receptors 2 and 4 (*Tlr-2* and *-4*) and two anti-inflammatory markers, peroxisome proliferator-activated receptors α and γ (*Ppar-α* and *-γ*), respectively. Data were set relative to the *Msr1*^{+/+}/*Cd36*^{+/+}-tp group on chow diet. *Significantly different from age-matched *Msr1*^{+/+}/*Cd36*^{+/+}-tp group. *, ** and *** indicate p<0.05, 0.01 and 0.001 resp.

Hepatic fibrosis is less pronounced in *Msr1*^{-/-}/*Cd36*^{-/-} transplanted mice compared to *Msr1*^{+/+}/*Cd36*^{+/+} transplanted mice

Hepatic fibrosis is viewed as one of the advanced consequences of NASH. After 3 months of HFC diet, *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice showed less hepatic fibrosis than *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice as evidenced by collagen staining with Sirius Red (Figure 4.3A-C). Collagen content was lower in livers of *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice and was primarily localized near vessels of periportal and centrolobular regions. Gene expression of four well-known fibrogenic-related genes in liver confirmed the findings of the Sirius Red staining. After 3 months of HFC diet, expression levels of these genes were increased compared to chow and 7 days of HFC diet. Comparing *Msr1*^{-/-}/*Cd36*^{-/-}-tp

mice with *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice after 3 months of HFC feeding revealed a significant decrease in fibrosis (i.e. collagen type 1 $\alpha 1$ (*Col1a1*) ($p=0.005$), matrix metalloprotease 13 (*Mmp-13*) ($p=0.05$), tissue inhibitor of metalloproteinase 1 (*Timp-1*) ($p=0.002$) and transforming growth factor β (*Tgf- β*) ($p=0.01$)) (Figure 4.3D).

Figure 4.3 Parameters of hepatic fibrosis. (A-B) Representative pictures (magnification x200) of Sirius Red positive sections after 3 months of HFC diet in (A) *Msr1*^{+/+}/*Cd36*^{+/+}-tp and (B) *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice. (C) Quantification of sirius red staining after 3 months of HFC diet. Livers were quantified as minimally, mildly or moderately positive for collagen. (D) Gene expression analysis of *Col1a1*, *Mmp-13*, *Timp-1* and *Tgf- β* . Data were set relative to the *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice on chow diet. *Significantly different from age-matched *Msr1*^{+/+}/*Cd36*^{+/+}-tp group. * and ** indicate $p<0.05$ and 0.01 resp.

No difference in foamy appearance of Kupffer cells between the models

Despite the absence of the two major modified lipoprotein receptors in macrophages, scoring of HE-stained (data not shown) and CD68 (macrophage marker which stains KCs) positive sections did not reveal a reduction in the size or presence of foamy KCs in *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice (Figure 4.4A-C). Moreover, a fluorescent double staining with CD68 and filipin (cholesterol marker) showed that the cholesterol content inside KCs increased with the size of the KCs upon HFC diet and that there were no differences in cholesterol content between KCs of *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice (data not shown).

Figure 4.4 Foamy appearance of Kupffer cells. (A-C) Representative pictures (magnification x200) after (A) chow, (B) 7 days and (C) 3 months of HFC diet with *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice, respectively. (D) Gene expression analysis of macrophage and Kupffer cell marker *Cd68*, ATP-binding cassette transporters *Abca1* and *Abcg1*. Data were set relative to the *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice on chow diet. *Significantly different from age-matched *Msr1*^{+/+}/*Cd36*^{+/+}-tp group. *, ** and *** indicate p<0.05, 0.01 and 0.001 resp.

Cd68 expression was comparable in livers of both groups after HFC feeding (Figure 4.4D). Notably, the expression levels of the ATP-binding cassette transporter A1 (*Abca1*) in liver revealed a significant decrease after 3 months on a HFC diet in the *Msr1^{-/-}/Cd36^{-/-}*-tp mice compared to *Msr1^{+/+}/Cd36^{+/+}*-tp mice ($p=0.02$). The ATP-binding cassette transporter G1 (*Abcg1*) showed the same trend after long-term HFC feeding (Figure 4.4D). Moreover, there were already basal differences between *Msr1^{+/+}/Cd36^{+/+}*-tp and *Msr1^{-/-}/Cd36^{-/-}*-tp mice for expression of *Cd68*, *Abca1* and *Abcg1* in total liver. Furthermore, gene expression analysis in whole liver revealed no compensation by other scavenger receptors (data not shown).

Msr1^{-/-}/Cd36^{-/-} transplanted mice show less lipid oxidation compared to *Msr1^{+/+}/Cd36^{+/+}* transplanted mice

To obtain an indirect measure of the degree of lipid oxidation, IgM and IgG auto-antibodies against malondialdehyde (MDA)-LDL and copper-oxidized (Cu-Ox)LDL were measured in plasma of transplanted animals. For each time point, IgM auto-antibody levels to MDA-LDL and Cu-OxLDL, which are thought to be to a large extent natural antibodies²⁵ and to be inversely correlated with cardiovascular diseases^{21,26}, showed significantly higher levels in *Msr1^{-/-}/Cd36^{-/-}*-tp mice compared to *Msr1^{+/+}/Cd36^{+/+}*-tp mice (MDA-IgM: $p=0.001$ (chow), $p=0.01$ (7d), $p<0.0001$ (3m); Cu-Ox-IgM: $p=0.006$ (chow), $p=0.02$ (7d), $p=0.0008$ (3m)) (Figure 4.5A,B). Immunoglobulin (Ig) G auto-antibody levels to MDA-LDL were significantly decreased in the *Msr1^{-/-}/Cd36^{-/-}*-tp mice after HFC feeding, indicating a less pronounced oxidative stress-mediated immune response ($p=0.09$ (chow), $p=0.006$ (7d), $p=0.03$ (3m))(Figure 4.5C). The IgG levels against Cu-OxLDL were not significantly different after HFC diet, but showed a basal difference between *Msr1^{+/+}/Cd36^{+/+}*-tp and *Msr1^{-/-}/Cd36^{-/-}*-tp mice (Figure 4.5D). In line with the protective pattern of auto-antibodies against oxLDL, two well-known oxysterols in plasma, 27- and 24S-hydroxycholesterol, were significantly lower after 3 months HFC diet in *Msr1^{-/-}/Cd36^{-/-}*-tp mice compared to *Msr1^{+/+}/Cd36^{+/+}*-tp mice ($p=0.02$, $p=0.008$ resp.), indicating less oxidation of cholesterol in these animals (Figure 4.5E). Similar to the decreased oxidation processes in these mice, the number of myeloperoxidase (MPO)-positive cells, which can generate a variety of reactive oxygen species, was significantly lower in the livers of *Msr1^{-/-}/Cd36^{-/-}*-tp mice compared to *Msr1^{+/+}/Cd36^{+/+}*-tp mice ($p=0.003$ (7d), $p=0.05$ (3m)) (Figure 4.5F).

Figure 4.5 Parameters of oxidative damage. (A-D) IgG and IgM auto-antibody titers to MDA-LDL and CuOx-LDL in plasma respectively. (E) Oxysterols in plasma: 27- and 24S-hydroxycholesterol. (F) Scoring of MPO-positive cells in liver sections. *Significantly different ($p < 0.05$) from aged matched *Msr1^{+/+}/Cd36^{+/+}-tp* group.

Discussion

Our study shows for the first time the involvement of macrophage SRs in early and advanced stages of NASH. Reconstitution of *Ldlr^{-/-}* mice with bone marrow from mice lacking both *Msr1* and *Cd36* significantly reduced hepatic inflammation, lipid oxidation and fibrosis without affecting steatosis. These novel observations support an analogy between mechanisms involved in the progression of liver inflammation and atherosclerosis.

Kupffer cells initiate early hepatic inflammation by scavenging modified lipoproteins

Currently, the risk factors that drive hepatic inflammation during the progression to NASH are largely unknown. We have previously shown that hyperlipidemic mice are more sensitive to developing early, diet-induced NASH⁸. In the current study, we demonstrate that the increased sensitivity of these mice to develop NASH is linked to expression of SR-A and CD36 on Kupffer cells.

The role of macrophage SRs CD36 and SR-A in atherogenesis has been intensively investigated. Uptake and internalization of modified LDL by SRs is believed to constitute one of the major pathways for foam cell formation in vivo. However, recent studies have revealed a wide spectrum of scavenger receptor functions, including the activation of signal transduction pathways regulating inflammation, apoptotic cell clearance, chemo-attraction and angiogenesis, which may also contribute to atherogenesis²⁷⁻²⁹. The current paradigm suggests that SR-mediated uptake of oxidized lipoproteins by macrophages sets off a cascade of pro-inflammatory events leading to the initiation of the inflammatory response. In line with this view, we have observed that upon targeted deletion of *Cd36* and *Msr1* in macrophages, the hepatic inflammatory response was dramatically decreased in a mouse model of NASH, as indicated by a decreased number of inflammatory cells and *Tnf*. In line with this observation, deletion of both *Msr1* and *Cd36* in mice lacking *ApoE* was shown to reduce aortic expression levels of several chemokines and cytokines including *Tnf*¹⁹. Therefore, the effect that we have observed in the livers of *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice is likely to be systemic and not restricted to the liver. Similarly, it was described that combined deletion of *Cd36* and *Msr1*, and even deletion of *Cd36* alone, resulted in decreased macrophage content, expression levels of serum pro-inflammatory cytokines and aortic chemokine expression^{18,19,30,31}. Moreover, CD36 has been shown to facilitate TLR signaling in response to both oxLDL and diacylglyceride via TLR4/TLR6 and TLR2/6, respectively, and thereby trigger the innate host response^{17,31-34}. Thus, similar innate immune signaling pathways initiated by SRs may also be activated during steatosis and these may promote inflammation associated with NASH. Notably, while most of the previous reports regarding the role of these SRs in inflammation were performed using the complete knock-out animals in an *apoE*^{-/-} background, our current study was performed in *Ldlr*^{-/-} mice transplanted with bone marrow from mice lacking *Msr1* and *Cd36*. Our approach has the advantage of being independent of the mixed genetic background of the mice which may have confounded previous studies, and it has also allowed us to specifically examine the contribution of SRs mediated by macrophages on hepatic inflammation.

The decreased fibrosis and expression of fibrogenic-related genes detected in the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice after 3 months of HFC diet was also associated with reduced levels of apoptosis. The effect on apoptosis may be attributed to the dramatic

reduction in expression of *Tnf* which is known to activate the expression of many pro-inflammatory cytokines and to induce apoptosis³⁵. There is considerable evidence contributing to the pathophysiologic concept that excessive apoptosis in the liver acts as a pro-inflammatory and pro-fibrogenic trigger³⁶⁻³⁸. The reduced level of apoptosis could therefore further contribute to the decreased development of fibrosis in the livers of the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice. In addition, Jaeschke and collaborators demonstrated that hepatocyte apoptosis is a potent stimulus for neutrophil infiltration and endotoxin-induced liver injury^{39,40}. These findings are in line with the finding that apoptosis correlates with enhanced myeloid cell infiltration and hepatic fibrosis in our models. Beside the reduced expression levels of inflammatory genes, levels of the anti-inflammatory nuclear receptors *Ppar-α* and γ were significantly higher in the livers of the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice after 3 months. The recognition that, in addition to their anti-inflammatory response, PPARs are also associated with decreased liver fibrogenesis and tumorigenesis is relevant⁴¹. Thus, the increase in hepatic expression of these nuclear receptors in the *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice may further contribute to the improvement of liver pathology in these mice.

Inflammatory response in liver is attributed to lipid oxidation rather than to foam cell formation

Whereas our original hypothesis was that the inflammatory response in livers of hyperlipidemic mice would be related to foam cell formation⁸, our data demonstrate a link between hepatic inflammation and LDL oxidation, rather than total lipid uptake. KCs lacking both *Cd36* and *Msr1* were increased in size throughout the duration of the diet, but were not significantly different from control macrophages. Although cholesterol content inside KCs was not different between *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice, gene expression analysis of *Cd68*, *Abca1* and *Abcg1* in liver showed already some basal differences. However, it is unlikely that these minor changes in gene expression are associated with a reduced efflux of cholesterol out of KCs via these ATP binding cassettes.

MSR1 and CD36 have been shown to be critical contributors to modified lipoprotein uptake in macrophages in vitro¹⁴. In addition, it has been shown that a specific CD36-dependent signaling pathway initiated by oxLDL is necessary for foam cell formation in vitro and in vivo¹⁷. However, hyperlipidemic *Cd36*^{-/-}/*apoE*^{-/-} and *Msr1*^{-/-}/*apoE*^{-/-} mice develop abundant foamy macrophages in atherosclerotic plaques, indicating that lipid uptake by intimal macrophages can occur in the absence of CD36 and MSR1¹⁸. In line with these observations, it has also been shown that loss of both CD36 and MSR1 activity reduces atherosclerotic lesion complexity and inflammation in particular, without abrogating foam cell formation in *apoE*^{-/-} mice¹⁹.

It is important to note that there is still no clear evidence establishing that lipids that generate foam cells in vivo are derived from oxidized lipoproteins. In fact, there is considerable evidence that LDL-derived lipids can enter macrophages via SR-independent pathways⁴². Native LDL, which is present in much higher concentrations in plasma compared to modified/oxidized LDL, has been reported to be internalized via macropinocytosis of extracellular fluid⁴³. In addition, aggregated or enzymatically modified forms of LDL can also be internalized by macrophages. It is therefore possible that while the total amounts of lipids in the hepatic macrophages from the *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice are identical, the lipids may be qualitatively different or the mechanism of internalization may trigger more inflammatory pathways.

The reduced inflammatory response in the livers of the mice with targeted deletions of the two SRs was associated with diminished cholesterol oxidation and oxidative stress. This effect was also characterized by decreased hepatic MPO in *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice compared to *Msr1*^{+/+}/*Cd36*^{+/+}-tp mice. MPO is secreted by neutrophils and monocytes that generate many oxidants, which can initiate the oxidation of LDL. Previously, it has been shown that CD36 is the major receptor for LDL modified by monocyte-generated reactive nitrogen species⁴⁴. These data suggest the existence of a defensive feedback mechanism to reduce levels of oxidation when CD36 expression is low. In line with the decreased levels of MPO in the liver, we observed significant differences in levels of IgM and IgG auto-antibodies against modified LDL in plasma of both transplanted groups. IgM auto-antibodies to MDA-LDL and Cu-OxLDL are increased in *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice, suggesting decreased consumption of these IgM, which have been suggested to be natural antibodies to a large part²⁵. In literature, it has been shown that IgM auto-antibodies to MDA-LDL were inversely related with atherosclerosis^{21,26}. Interestingly, an IgG immune response induced by lipid peroxidation products correlates with hepatic inflammation during alcoholic steatohepatitis (ASH) and the progression to advanced fibrosis during non-alcoholic steatohepatitis (NAFLD)^{45,46}. Moreover, a number of studies have shown that the levels of IgG auto-antibodies are correlated with increased risk for cardiovascular diseases^{47,48}. Surprisingly, the differences in levels of these auto-antibodies between *Msr1*^{+/+}/*Cd36*^{+/+}-tp and *Msr1*^{-/-}/*Cd36*^{-/-}-tp mice were already observed at basal levels. The generation of natural antibodies is known to occur in the complete absence of external antigenic stimulation, and therefore it is not surprising that these antibodies are present already before the initiation of the HFC diet. Importantly, the basal differences between recipients of the two genotypes suggest that MSR1 and/or CD36 are critically involved in the generation of these antibodies. Thus, our data further add to the breadth of scavenger receptor functions, in particular the activation of additional immune responses with analogous specificity. Since this effect is likely to be systemic and not restricted to the liver, our observations regarding lipid oxidation are of general importance and also relevant to the field of atherosclerosis.

Conclusion

Our study demonstrates that the SRs MSR1 and CD36 play an important role during early and advanced stages of NASH. Further studies are necessary to investigate the exact contribution of each of these receptors in order to dissect the molecular mechanisms involved in diet-induced NASH. Our data also establish the hyperlipidemic mice models as a tool to investigate the advanced stages of NASH in a physiological context and provide a mechanism for the initiation and progression of the disease in these models. Finally, this study demonstrates for the first time an analogy between mechanisms for NASH and atherosclerosis. Thus, the focus on the liver as a crucial driver of inflammation in the metabolic syndrome is expected to provide alternative strategies leading to new therapies for preventing atherosclerosis. This study also provides new evidence for the close and complex link between lipid metabolism and inflammation, as manifested in cardiovascular diseases.

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Chapter 5

CD36 and SR-A contribute similarly and independently
to non-alcoholic steatohepatitis in hyperlipidemic
mice

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Abstract

Background & aims

The major features of non-alcoholic steatohepatitis (NASH) are steatosis and inflammation, which can further progress into fibrosis and cirrhosis. Recently, we demonstrated that combined deletion of the two main scavenger receptors, Cd36 and macrophage scavenger receptor 1 (Msr1), which are important for the uptake of modified cholesterol-rich lipoproteins, reduced the progression of NASH. So far, the individual contributions of these receptors to NASH and the intracellular mechanisms by which they contribute to inflammation have not been established. We hypothesize that CD36 and MSR1 contribute similarly and independently to the progression of NASH.

Methods

Ldlr^{-/-} mice were lethally irradiated and transplanted with wild-type (Wt), *Cd36*^{-/-} or *Msr1*^{-/-} bone marrow and fed a Western diet for 3 months.

Results

Cd36^{-/-} and *Msr1*^{-/-} transplanted (tp) mice showed a similar reduction in hepatic inflammation and fibrosis compared to Wt-tp mice. In plasma, the protective IgM auto-antibodies were increased in both *Cd36*^{-/-} and *Msr1*^{-/-}-tp mice compared to Wt-tp mice. While the total amount of cholesterol inside KCs was similar in all groups, KCs of Wt-tp mice showed increased lysosomal cholesterol accumulation, whereas the *Cd36*^{-/-} and *Msr1*^{-/-}-tp mice showed increased cytoplasmic cholesterol accumulation. Moreover, lysosomal enzyme activity in the livers of both *Cd36*^{-/-} and *Msr1*^{-/-}-tp, but not in Wt-tp mice, was disturbed.

Conclusion

CD36 and MSR1 contribute similarly and independently to the progression of NASH in the presence of hyperlipidemia. Internalization of modified lipids by these two receptors leads to abnormal cholesterol trafficking in KCs and consequently disturbed lysosomal function and hepatic inflammation. These data provide a new basis for the prevention and treatment of NASH.

Introduction

Non-alcoholic steatohepatitis (NASH) is increasingly recognized as a major health burden in developed countries, with a prevalence of 2-3% in the general population and up to 37% of the morbidly obese population of Western countries¹. It is characterized by hepatic lipid accumulation (steatosis) combined with inflammation. While steatosis itself is generally considered reversible, the presence of inflammation can lead to a further progression of NASH, resulting in liver fibrosis, cirrhosis and eventually liver failure and hepatocellular carcinoma². The inflammatory response in the liver represents a key step in NASH development as it contributes to further damage such as cirrhosis, liver failure, and hepatocellular carcinoma.

We have previously shown that hyperlipidemic mice fed a Western diet show an early onset of hepatic inflammation, which is associated with bloated Kupffer cells (KCs). These KCs resemble the foam cells of atherosclerotic lesions³. The current view favours a model where scavenger receptors (SR) mediate the uptake of oxidized lipoproteins by macrophages, leading to foam cell formation and initiation of the inflammatory response. In line with this view, we have already shown that the selective deletion of scavenger receptors *Msr1* and *Cd36* from the haematopoietic cells of low density lipoprotein (LDL) receptor-deficient (*Ldlr*^{-/-}) mice attenuates hepatic inflammation⁴. However, while hepatic inflammation, lipid oxidation and liver fibrosis were significantly reduced, these mice showed no difference in foamy KCs. Similarly, it has also been shown that combined deletion of both CD36 and MSR1 reduces atherosclerotic lesion complexity and inflammation in particular, without abrogating foam cell formation in *apoE*^{-/-} mice⁵. Thus, in contrast to the current view, these observations suggest that scavenger receptors modulate inflammation without altering total lipid accumulation in macrophages. Likewise, there is a growing appreciation of the numerous similar but independent functions of each one of these scavenger receptors in addition to lipoprotein uptake, including their role in inflammatory signal transduction and tissue homeostasis⁶. Currently, the individual contributions of these receptors to NASH and the intracellular mechanisms by which they contribute to inflammation have not yet been established. Based on these different functions in inflammatory signal transduction, we hypothesized that CD36 and MSR1 contribute similarly and independently to the progression of NASH.

Under normal conditions, lipoproteins which are endocytosed by KCs, will initially be directed to the lysosomes, where these lipoproteins are hydrolyzed by lysosomal enzymes and transferred into the cytoplasm. However, it was shown that the uptake of oxLDL by macrophages *in vitro* is associated with lysosomal trapping⁷. Several lines of evidence indicate an association between lysosomal cholesterol accumulation and inflammation⁸⁻¹¹.

In order to investigate whether a macrophage specific deletion of each scavenger receptor individually can affect hepatic inflammation, *Ldlr*^{-/-} mice were lethally irradiated and transplanted with bone marrow from Wt, *Msr1*^{-/-} or *Cd36*^{-/-} mice and fed a high-fat-high-cholesterol (HFC) diet for 3 months. We found that CD36 and MSR1 contribute in a similar manner to the progression of NASH in the presence of high levels of plasma-modified lipoproteins. The effect of these receptors on inflammation is likely modulated via disturbed cholesterol trafficking inside KCs and increased lysosomal cholesterol accumulation.

Materials and methods

Mice

Female *Ldlr*^{-/-} mice were lethally irradiated and transplanted with Wt, *Cd36*^{-/-} and *Msr1*^{-/-} bone marrow as described previously⁴. After a recovery period of 9 weeks, the mice were given a HFC diet for 3 months (n=9 Wt-tp mice, n=8 for both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice). The collection of blood and specimens, the biochemical determination of lipids in plasma and the liver, liver histology, RNA isolation, cDNA synthesis and qPCR, aminotransferases, oxysterols and auto-antibody titers against modified LDL were extensively described previously⁴.

Lipid analysis

Liver lipid analysis: approximately 50 mg of frozen liver tissue was homogenized for 30 s at 4800 rpm in a closed tube with 1.0 mm glass beads and 1.0 ml SET buffer (sucrose 250 mM, EDTA 2 mM and Tris 10 mM). Complete cell destruction was achieved via two freeze-thaw cycles and two passes through a 27-gauge syringe needle. The protein content was measured via the BCA method (Pierce, Rockford, USA). Cholesterol, triglycerides and free fatty acid levels were measured using enzymatic colour tests (cholesterol CHOD-PAP, Roche, Basel, Switzerland; TG GPOtrinder, Saint Louis, USA; NEFAC, ACS-ACOD, Wako Chemicals, Neuss, Germany) according to the manufacturers' protocols on a Benchmark 550 micro-plate reader (Bio-Rad, Hercules, USA).

Plasma lipid analysis: blood collected from the tail vein was centrifuged for 3 min at 3500 rpm and the plasma supernatant was transferred to a fresh tube. Plasma cholesterol, triglycerides and free fatty acids were measured using the same enzymatic colour tests described above, with a Benchmark 550 micro-plate reader (Bio-Rad, Hercules, USA).

Liver histology

Frozen liver sections (7 μm) were fixed with acetone and blocked against endogenous peroxidase by incubation with 0.25% of 30% H_2O_2 for 5 min. The primary antibodies used were against infiltrated macrophages (macrophage marker, Mac-1), CD68 Kupffer cells (CD68 marker, FA11), T cells (T-cell marker, KT3), fibroblasts (fibroblast marker, ERTR7) and neutrophils (neutrophil marker, NIMP). For NIMP staining, no amplification step was needed as a secondary antibody, α -rat-PO, was used. Prior to incubation with the first antibody, the slides were incubated with 4% fetal calf serum (FCS), 1 $\times$ PBS plus an amplification step: 1:5 Avidin D Block solution (ABC kit, Vector Laboratories, USA). Primary antibodies were dissolved in 4% FCS, 1 $\times$ PBS plus the first antibody (1:1000 for Mac-1, 1:200 for NIMP, 1:100 for CD68 and 1:5 for CD3) plus an amplification step: 1:5 Biotin Block solution (ABC kit, Vector Laboratories, USA). Incubation with the second antibody for α -rat-BIO at 1:300 (1:100 for α -rat-PO) was carried out in 4% FCS, 2% normal mouse serum (NMS) and 1 $\times$ PBS. Afterwards, the slides were washed and incubated for an additional amplification step in 1 $\times$ PBS plus 1:50 Avidin D solution and 1:50 Biotin solution (ABC kit, Vector Laboratories, USA). Then, 13-amino-9-ethylcarbazole (AEC) was applied as the colour substrate using an AEC kit (2% buffer, 3% AEC, 2% H_2O_2 in demi water) and Haematoxylin for nuclear counterstaining. The sections were enclosed with Faramount aqueous mounting medium.

Paraffin-embedded liver sections (4 μm) were stained with Haematoxylin-Eosin (Haematoxylin, Klinipath, Duiven, the Netherlands and Eosin, Sigma-Aldrich, Saint Louis, USA) after deparaffination in xylol and dehydrated with 90%, 70% and 50% ethanol. For Sirius Red staining, the liver sections were first fixed in Susa fixative after deparaffination. Subsequently, the sections were stained with Sirius Red (Sigma-Aldrich, Saint Louis, USA), washed with 0.01 M HCl and dehydrated. The sections were mounted with Entellan.

Pictures were taken using a Nikon DMX1200 digital camera and ACT-1 v2.63 software. Positively stained cells were counted in six microscopic views (magnification $\times 200$) and were recorded as cells/ mm^2 . The staining of CD68 was scored as follows: mild foamy appearance (score 1), moderate (score 2) and severe foamy appearance (score 3). The Sirius Red and ERTR7 stainings were scored as follows: no fibrosis (score 1), mild (score 2), moderate (score 3) and severe fibrosis (score 4). Scores for the level of fibrosis were given both around and in between blood vessels, and the mean was calculated.

Electron microscopy

A detailed overview about the (post)fixation, embedding, cutting and type of electron microscope was described previously³. Scoring of the electron microscopy (EM) pictures was performed by a trained specialist. Fifty Kupffer cells per mouse were

analysed and scored. The Kupffer cells were divided into three different categories, i.e., primary lysosomes, secondary fatty lysosomes and cytoplasmic fat accumulation and the percentage of each subtype was calculated. Furthermore, the secondary fatty lysosomes were scored from 0 to 3 for their fat content, where a score of 0 indicated not positive and a score of 3 indicated extreme fat accumulation inside the lysosomes of the KCs. The same scoring index was used for the cytoplasmic fat accumulation inside Kupffer cells.

Cathepsin D enzyme-linked immunosorbent assay

Plasma cathepsin D levels were determined by the Cathepsin D enzyme-linked immunosorbent assay according to the manufacturers' protocol (Uscn Life Science Inc, Wuhan, China). The plasma samples required a 50-fold dilution.

Statistical analysis

The data were statistically analysed by performing a one-way ANOVA test with a Bonferroni post-test using GraphPad Prism for comparing Wt-tp, *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice with each other. The data were expressed as the mean ± SEM and considered significant at $p < 0.05$. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001, respectively.

Results

Inflammation and fibrosis are reduced in *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice

To determine whether both *Cd36* and *Msr1* are independently involved in diet-induced hepatic inflammation, *Ldlr*^{-/-} mice were transplanted with *Cd36*^{-/-} or *Msr1*^{-/-} bone marrow and fed a HFC diet for 3 months. Scoring of the liver sections stained for inflammatory cell markers revealed that infiltrated macrophages and neutrophils were significantly decreased in the livers of mice transplanted with both *Cd36*^{-/-} and *Msr1*^{-/-} bone marrow compared to Wt- transplanted (tp) mice (Figure 5.1A+B+D). The same decrease was seen after staining with an antibody against myeloperoxidase (MPO), which is an oxidative stress/neutrophil marker (Figure 5.1C).

After 3 months of HFC diet, both *Cd36*^{-/-}- and *Msr1*^{-/-}-tp mice showed less hepatic fibrosis than Wt-tp mice as evidenced by collagen staining with Sirius Red (Figure 5.1E+F). Collagen content was lower in livers of *Cd36*^{-/-}- and *Msr1*^{-/-}-tp mice and was primarily localized near vessels of periportal and centrilobular regions. In conclusion, *Cd36*^{-/-}- and *Msr1*^{-/-}-tp mice have decreased hepatic inflammation and fibrosis after 3 months of HFC feeding compared to Wt-tp mice.

Figure 5.1 Parameters of inflammation. (A-C) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP) and MPO, and positively stained cells were counted as cells/mm². (D) Representative pictures of Mac-1 staining (x200 magnification) after 3 months of HFC feeding in Wt-tp, Cd36^{-/-}-tp and Msr1^{-/-}-tp mice. (E) Representative pictures (magnification x200) of Sirius Red positive sections after 3 months of HFC diet in Wt-tp, Cd36^{-/-}-tp and Msr1^{-/-}-tp mice (F) Quantification of Sirius Red staining after 3 months of HFC diet. Livers were quantified as minimal, mild or moderate positive for collagen. * Significantly different from Wt-tp group. * and ** indicate $p < 0.05$ and 0.01 , respectively.

The reduced inflammatory response in Cd36^{-/-}-tp and Msr1^{-/-}-tp mice is not correlated with lipid levels

Liver lipid levels were analysed in order to investigate the amount of steatosis present in these mice. Liver triglycerides (TG), free fatty acids (FFA) and total cholesterol were

all significantly higher in the *Msr1*^{-/-}-tp mice compared to Wt-tp mice. The amount of cholesterol esters (CE) in the liver was elevated in both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice. Desmosterol, a cholesterol synthesis marker, was increased in the *Msr1*^{-/-}-tp mice compared to Wt-tp mice, whereas the cholesterol absorption marker cholestanol was lower in *Msr1*^{-/-}-tp mice compared to Wt-tp and *Cd36*^{-/-}-tp mice (Table 5.1). Nevertheless, Oil red O staining revealed no differences between any of the groups (data not shown). Thus, the observed differences found in hepatic steatosis between Wt-tp, *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice were mild and not consistent.

Table 5.1 Liver and plasma lipid related parameters.

	Wt-tp mice	<i>Cd36</i> ^{-/-} -tp mice	<i>Msr1</i> ^{-/-} -tp mice
Liver			
Triglycerides (μg)	0.38 (±0.02)	0.40 (±0.03)	0.47 (±0.02) *
Free fatty acids (μg)	0.17 (±0.003)	0.19 (±0.006)	0.20 (±0.01) *
Cholesterol (μg)	0.08 (±0.0038)	0.10 (±0.0062)	0.11 (±0.0071) **
Lathosterol (ng/mg)	21.8 (±1.76)	19.71 (±2.44)	19.10 (±2.55)
Desmosterol (ng/mg)	16.11 (±1.06)	14.44 (±0.85)	12.62 (±0.92) *
Cholestanol (ng/mg)	93.03 (±8.42)	97.33 (±6.38)	126.7 (±11.22) *, #
Cholesterol esters (μg/mg)	14.37 (±1.58)	21.2 (±1.88) *	20.99 (±1.27) **
Free cholesterol (μg/mg)	8.63 (±0.28)	9.00 (±0.23)	8.16 (±0.36)
Plasma			
Triglycerides (mM)	1.49 (±0.16)	1.29 (±0.14)	1.27 (±0.18)
Free fatty acids (mM)	0.58 (±0.04)	0.59 (±0.06)	0.45 (±0.03) *
Cholesterol (mg/dl)	1052 (±113.1)	1210 (±37.76)	1097 (±69.45)
Lathosterol (mg/dl)	0.31 (±0.03)	0.24 (±0.04)	0.17 (±0.03) **
Desmosterol (mg/dl)	0.77 (±0.06)	0.77 (±0.08)	0.52 (±0.06) *, #
Cholestanol (mg/dl)	3.03 (±0.31)	4.34 (±0.13) **	4.60 (±0.14) **
7α-OH cholesterol (ng/ml)	1.50 (±0.13)	1.02 (±0.13) *	0.92 (±0.03) **
24-OH cholesterol (ng/ml)	448.8 (±46.85)	506.5 (±28.77)	505.6 (±41.62)
27-OH cholesterol (ng/ml)	504 (±59.81)	504.8 (±42.63)	455 (±46.12)
MDA-LDL IgM (RLU)	86348 (±4857)	89405 (±6189)	113896 (±2889) ***,###
CuOx-LDL IgM (RLU)	31987 (±6331)	62903 (±11844) *	102371 (±6341) ***,###
T15id+ (IgM) (RLU)	3726 (±395.2)	9000 (±1815) *	19079 (±3412) **, #
MDA-LDL IgG (RLU)	42367 (±4502)	37223 (±4371)	28978 (±2180) *
CuOx-LDL IgG (RLU)	1406 (±247.3)	1221 (±185.2)	2182 (±170.7) *, ###
ALT (U/l)	61 (±9.35)	58.43 (±3.40)	65.5 (±3.53)
AST (U/l)	129.5 (±13.77)	79 (±14.3) *	84 (±6.52) *
AST / ALT	2.25 (±0.38)	1.52 (±0.15)	1.28 (±0.07) *

*Significantly different from Wt-tp group. *, ** and *** indicate p<0.05, 0.01 and 0.001 resp. # Significantly difference between *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice. # and ## indicate p<0.05 and 0.01 respectively.

Plasma TG and total cholesterol were similar in all transplanted groups. Plasma FFA was significantly higher in *Msr1*^{-/-}-tp mice compared to Wt-tp mice. Although the total amount of cholesterol did not differ between the groups, the cholesterol synthesis markers lathosterol and desmosterol were significantly higher in the plasma of *Msr1*^{-/-}-tp mice compared to Wt-tp mice. Moreover, the cholesterol absorption marker cholestanol was significantly higher in both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice. Plasma levels of 7 α -hydroxy (OH) cholesterol were increased in both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice, while the other oxysterols (24-OH and 27-OH cholesterol) were not different from Wt-tp mice. Overall, these plasma lipid data were inconclusive and did not reveal any major differences between Wt-tp, *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice.

Immunoglobulin M auto-antibody levels against MDA-LDL and Cu-OxLDL, and T15id+ IgM, which are natural antibodies known to decrease atherogenesis, showed significantly higher levels in *Msr1*^{-/-}-tp mice compared to Wt-tp mice and *Cd36*^{-/-}-tp mice. The Cu-OxLDL and T15id+ IgM auto-antibodies were also elevated in *Cd36*^{-/-}-tp mice compared to Wt-tp mice. Immunoglobulin (Ig) G auto-antibody levels against MDA-LDL, which have been suggested to be positively correlated with increased risk for cardiovascular diseases, were significantly decreased in *Msr1*^{-/-}-tp mice compared to Wt-tp mice, indicating a less pronounced oxidative stress-induced immune response. In contrast, the IgG levels against Cu-OxLDL were increased in *Msr1*^{-/-}-tp mice compared to Wt-tp mice and *Cd36*^{-/-}-tp mice, suggesting a T-cell independent IgG3 respons. Thus, both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice have an increased protective phenotype compared to Wt-tp mice.

The plasma transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are considered as sensitive indicators of liver damage. Although the ALT levels were equal in all transplanted groups, the AST levels were significantly lower in *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice. Moreover, the ratio of AST/ALT was lower in *Msr1*^{-/-}-tp mice compared to Wt-tp mice. In general, these data indicate that both *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice were protected from further liver damage.

No difference in the foamy appearance of Kupffer cells

The staining of CD68 (macrophage marker) revealed no differences in the size or presence of KCs between Wt-tp, *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice (Figure 5.2A-C, respectively). Moreover, the mRNA expression level of *Cd68* showed similar results as seen in the histological figures of CD68 staining (data not shown).

Figure 5.2 CD68 staining of Kupffer cells in liver of (A) Wt-tp, (B) *Cd36*^{-/-}-tp and (C) *Msr1*^{-/-}-tp mice.

Wt-tp mice have increased lysosomal cholesterol storage inside the KCs compared to *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice

To determine whether the reduced inflammatory response in *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice was associated with decreased lysosomal fat accumulation, detailed analyses of the KCs were performed using electron microscopy (EM) (Figure 5.3A-C). Interestingly, lipid distribution inside these cells revealed a clear difference between Wt-tp and *Cd36*^{-/-}-tp / *Msr1*^{-/-}-tp mice (Figure 5.4A). The Wt-tp mice showed increased lysosomal cholesterol storage inside the KCs compared to *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice, whereas the latter two had increased cytoplasmic fat storage compared to Wt-tp mice (Figure 5.4B+C). Moreover, plasma levels of the lysosomal enzyme Cathepsin D were significantly higher in *Cd36*^{-/-}-tp and *Msr1*^{-/-}-tp mice compared to Wt-tp mice (Figure 5.4D). In conclusion, lysosomal function is disturbed in *Cd36*^{-/-}-tp / *Msr1*^{-/-}-tp mice compared to Wt-tp mice.

Figure 5.3 Electron microscopic views of Kupffer cells (K) after 3 months of a high fat diet. (A) The Kupffer cell in the liver of Wt-tp mice shows several fatty lysosomes (*). Primary lysosomes (#) and secondary lysosomes without fat accumulation (+) can be seen as well. The Kupffer cells in the liver of (B) *Cd36*^{-/-}-tp and (C) *Msr1*^{-/-}-tp mice contain massive cytoplasmic lipid accumulation (=). H: hepatocyte; +: secondary lysosomes without lipid inclusions.

Figure 5.4 Scoring of electron microscopic views. (A) Percentage of KCs with primary lysosomes, secondary fatty lysosomes and cytoplasmic fat accumulation. (B+C) Arbitrary scoring of secondary fatty lysosomes and cytoplasmic fat accumulation inside the Kupffer cells. (D) Plasma levels of Cathepsin D. *Significantly different from Wt-tp group. * and ** indicate $p < 0.05$ and 0.01 , respectively.

Discussion

Our study showed for the first time that the uptake of modified lipids *in vivo* leads to lysosomal fat accumulation in KCs. By specifically inactivating one of the two predominant scavenger receptors on macrophages, we established that the contribution of each one of these two receptors to hepatic inflammation is similar. Internalization of lipids by these receptors leads to abnormal levels of cholesterol in lysosomes and increased inflammation.

CD36 and MSR1 contribute in a similar manner to the progression of NASH

Currently, the risk factors that drive hepatic inflammation during the progression of NASH are largely unknown. We previously showed that hyperlipidemic mice are more sensitive to developing early diet-induced NASH³. We also demonstrated that the increased sensitivity of these mice towards developing NASH is related to the expression of *Msr1* and *Cd36*⁴.

Scavenger receptors MSR1 and CD36 have been identified as the principal receptors responsible for the uptake of modified lipids and cholesterol into macrophages¹². Scavenger receptor MSR1 was found to account for the majority (80%) of the macrophage uptake of acetylated low density lipoproteins (acLDL), but it also binds

oxidized LDL (oxLDL), albeit with lower affinity. Scavenger receptor CD36 binds oxLDL rather than acLDL, and it is involved in oxLDL-induced JNK activation. While the relative affinities of MSR1 and CD36 to modified and oxidized lipids were different, the effects on inflammation in our study were similar. These results are surprising, since it was found that cultured macrophages incubated with oxLDL showed lysosomal cholesterol accumulation while incubation with acLDL did not lead to lysosomal cholesterol accumulation but rather to cytoplasmic cholesterol storage inside macrophages⁷. Thus, it is possible that in contrast to the *in vitro* data, the affinity of both receptors towards oxLDL *in vivo* is similar. Alternatively, it is possible that the markedly increased levels of oxLDL in the plasma of *Ldlr*^{-/-} mice fed the HFC diet exceeded the maximal amount of cholesterol that can be taken up by KCs and trapped inside their lysosomes. The scavenger receptors CD36 and MSR1 differed from each other not only in their relative affinities to modified and oxidized lipids, but also in their signalling pathways and other important functions triggered by the binding and uptake of modified LDL, such as innate immune responses, cellular adhesion, and the phagocytosis of apoptotic cells⁶. These functions may also be involved in the development of NASH. The similarity in the degree of inflammation between the two models suggests that this inflammation is a consequence of the two receptors sharing similar modified lipid internalization mechanisms, rather than each receptor having its own specialized functions. In line with this hypothesis, previous studies with *Msr1* and *Cd36*-knockout mice showed that disruption of each receptor pathway partially inhibited the uptake of modified LDL in macrophages and retarded atherosclerosis progression in a similar manner¹³⁻¹⁵. All together, this study provides evidence of a central contribution of both of these receptors to the pathogenesis of NASH. Further investigations are necessary to determine the exact interaction between CD36 and MSR1 upon the internalization of lipids.

Hepatic inflammation is correlated with increased lysosomal cholesterol accumulation

Interestingly, it was shown that lysosomal cholesterol accumulation *in vitro* was associated with increased pH inside lysosomes¹⁶, which can lead to partial lysosomal enzyme inactivation and inflammatory response. Similarly, it was shown that in foam cells of advanced atherosclerotic plaques, cholesterol is not transferred into the cytoplasm but rather accumulates in the lysosomes of the macrophages¹⁷. Several lines of evidence further indicate an association between lysosomal cholesterol accumulation and inflammation: first, lysosomal acid lipase (LAL) is the essential enzyme for the hydrolysis of triglycerides and cholesteryl esters in lysosomes. Similar to our observations in hyperlipidemic mice, a deficiency of this enzyme was found to lead to lysosomal cholesterol accumulation and inflammation⁸. Furthermore, the macrophage-specific expression of human *LAL* was found to correct inflammation in *Lal*^{-/-} mice⁸. Second, lysosomes have been assigned a central role in many processes

involving tissue injury and inflammation¹⁸. Finally, it was recently demonstrated that modified LDL has the potential to damage lysosomal membranes and thereby prime cells for inflammation¹⁹.

Since the liver plays a key role in the clearance of circulating cholesterol carried in lipoproteins, disturbed cholesterol clearance is expected to have a significant effect on liver function. Indeed, in mice with mutations in Niemann-Pick type C (*Npc*) 1 and 2, proteins which facilitate the movement of cholesterol from the lysosomes to the cytoplasmic compartment, lysosomal cholesterol content correlates with liver damage²⁰. Similarly, patients with NPC disease commonly have a very poorly functioning liver function, as about 10% of these patients dies from liver failure²¹⁻²³. Our data suggest that inflammation could play a major role in NPC-associated liver dysfunction. Indeed, previous studies showed that oxLDL causes an accumulation of the NPC1 protein in the Golgi apparatus. This accumulation appears to be linked to the inability of the cells to remove lysosomal cholesterol. Moreover, increased lysosomal cholesterol accumulation was shown to induce autophagic stress in *Npc*^{-/-} mice²⁴. Since disturbed autophagy promotes inflammation via activation of inflammasomes²⁵, it is likely that increased lysosomal cholesterol accumulation is associated with the activation of inflammasomes. In line with this hypothesis, oxLDL was shown to prime cells for inflammation via the activation of NLRP3 inflammasomes¹⁹.

Taken altogether, our novel observations point towards the abnormal cholesterol metabolism and trafficking in KCs as a central event in the pathogenesis of NASH, and thus they provide new bases for the prevention and treatment of NASH.

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Chapter 6

Myeloperoxidase promotes progression of non-alcoholic steatohepatitis in mice

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Submitted

Abstract

Background and objective

Chronic inflammation and oxidative stress play fundamental roles in the pathogenesis of non-alcoholic steatohepatitis (NASH). Previously, we reported that myeloperoxidase (MPO), an aggressive oxidant-generating neutrophil enzyme, is associated with NASH severity in man. We now investigated the hypothesis that MPO plays an active role in the development of NASH.

Methods

Low-density lipoprotein receptor-deficient mice with an MPO-deficient hematopoietic system (*Ldlr*^{-/-}/*Mpo*^{-/-} mice) were generated by bone-marrow transplantation and compared with *Ldlr*^{-/-}/*Mpo*^{+/+} mice after induction of NASH by high-fat feeding for 8 weeks. Parameters of lipid accumulation and metabolism, MPO activity, inflammation, and fibrosis were studied using plasma, liver and adipose tissue samples.

Results

High-fat feeding caused a 4-fold induction of liver MPO in *Ldlr*^{-/-}/*Mpo*^{+/+} mice which was associated with hepatic sequestration of MPO-positive neutrophils and high levels of nitrotyrosine, a marker of MPO activity. Importantly, high-fat feeding to *Ldlr*^{-/-}/*Mpo*^{-/-} mice resulted in markedly reduced hepatic neutrophil and T-lymphocyte infiltration ($p < 0.05$), and strong down regulation of pro-inflammatory genes such as TNF- α and IL-6 ($p < 0.05$, $p < 0.01$) in comparison with *Ldlr*^{-/-}/*Mpo*^{+/+} mice. Visceral adipose tissue inflammation was markedly reduced as well, with a complete lack of macrophage crown-like structures in *Ldlr*^{-/-}/*Mpo*^{-/-} mice. Next to the generalised reduction of inflammation, liver cholesterol accumulation was significantly diminished in *Ldlr*^{-/-}/*Mpo*^{-/-} mice ($p = 0.01$). Moreover, MPO deficiency attenuated the development of hepatic fibrosis as evident from reduced hydroxyproline levels ($p < 0.01$).

Conclusions

MPO is a driving factor in the development of NASH, supporting an important new role for neutrophils in the pathogenesis of metabolic liver disease.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the developed world. It encompasses a spectrum of hepatic disorders characterised by triglyceride accumulation in the absence of excess alcohol consumption. The spectrum ranges from benign steatosis without inflammation to steatosis with a necro-inflammatory component, which is defined as non-alcoholic steatohepatitis (NASH), and may have associated fibrosis. Progression of NASH potentially leads to liver cirrhosis, liver failure, and hepatocellular carcinoma¹.

Although the pathogenic mechanisms underlying the development of NASH are still obscure, insulin resistance, sustained inflammation, and oxidative stress are thought to be major factors². Insulin resistance is primarily responsible for the accumulation of hepatic triglycerides³. NASH progression is associated with innate immune activation, which contributes to hepatocyte damage and fibrosis in various ways². For example, Kupffer cell depletion ameliorates various histopathological aspects of NASH⁴. Furthermore, we have recently found that activation of the complement system, an ancient branch of the innate immune system, is associated with NASH severity.⁵ In addition, neutrophil accumulation is a prominent feature of the inflammation observed in NASH^{6,7}. These phagocytes are notorious for their ability to induce tissue damage through generation of aggressive oxidants⁸.

One of the principal mechanisms by which activated neutrophils produce cytotoxic compounds is through the release of myeloperoxidase (MPO), a heme protein that constitutes up to 5% of total neutrophil protein content. MPO reacts with H₂O₂ formed by the respiratory burst to catalyse formation of hypochlorous acid (HOCl) and other oxidizing species⁹. HOCl, in turn, can damage both lipids and proteins, leading to the formation of oxidised lipids and cross-linked proteins^{10,11}. Furthermore, MPO catalyses nitration of tyrosyl groups in proteins¹², which is associated with various diseases and occurs in human NAFLD^{7,13}.

Importantly, increased MPO activity has previously been suggested to promote lipid peroxidation in steatotic livers⁶, a process involved in the progression of simple steatosis to steatohepatitis¹⁴. Recently, we obtained additional evidence implicating MPO in the progression of NASH by showing that the liver of patients with NASH contained increased numbers of MPO-positive neutrophils and Kupffer cells in comparison with patients showing simple steatosis. Moreover, this was accompanied by accumulation of HOCl-modified proteins and nitrated proteins, and associated with increased hepatic CXC chemokine expression⁷.

Next to its ability to induce tissue damage, MPO can also directly regulate inflammatory pathways and processes involved in fibrosis. For example, MPO induces

neutrophil activation by binding to CD11b/CD18 integrins¹⁵, and prolongs neutrophil lifespan by delaying apoptosis¹⁶. Furthermore, MPO promotes TNF- α release from macrophages, and enhances their cytotoxicity¹⁷. In addition, MPO-derived HOCl causes fragmentation of the extracellular matrix¹⁸, which is known to result in activation of hepatic stellate cells, a critical step in the development of hepatic fibrosis¹⁹. Additional pro-fibrotic effects of MPO are related to the inactivation of matrix metalloproteinases²⁰ and the generation of oxidised lipids which also increase matrix protein synthesis by stellate cells²¹.

All in all, there is compelling evidence to suggest that MPO plays a crucial role in the pathogenesis of NASH by affecting inflammation, oxidative stress, and fibrogenesis. We now report on studies with NASH-prone²² low-density lipoprotein receptor-deficient mice (*Ldlr*^{-/-} mice) transplanted with *Mpo*^{-/-} or *Mpo*^{+/+} bone marrow. Our data demonstrate that *Mpo* deficiency attenuates hepatic cholesterol accumulation, inflammation, and fibrosis in response to a high-fat diet, indicating a key role of MPO in metabolic liver disease.

Experimental procedures

Mice

Twelve weeks old female *Ldlr*^{-/-} (Jackson Laboratory, Bar Harbor, Maine) and *Mpo*^{-/-} or *Mpo*^{+/+} mice²³, on a C57BL/6 background, were randomly assigned to the *Ldlr*^{-/-}/*Mpo*^{-/-} (n=9) or the *Ldlr*^{-/-}/*Mpo*^{+/+} (n=9) group. First, *Ldlr*^{-/-} mice were subjected to 10 Gy of radiation. The following day, bone marrow was flushed from femurs and tibias of *Mpo*^{-/-} and *Mpo*^{+/+} mice and 10⁷ bone marrow cells were injected into the tail vein of recipient *Ldlr*^{-/-} mice to repopulate their hematopoietic system. One *Ldlr*^{-/-}/*Mpo*^{+/+} mouse did not survive after bone marrow transplantation. After 10 weeks recovery to allow for Kupffer cell repopulation, steatohepatitis was induced by feeding the mice a diet containing 17% casein, 0.3% DL-methionine, 34% sucrose, 14.5% cornstarch, 0.2% cholesterol, 5% cellulose, and 21% butter for 8 weeks (Scientific Animal Food and Engineering, Villemoisson-sur-orge, France). Mice were maintained in an accredited animal facility on a 12 hour light/dark schedule (07:00-19:00 hours) and fed ad libitum. Animals from each group were euthanised alternately after 4hrs fasting starting at 11:00 on two consecutive days. All experiments were approved by the Committee for Animal Welfare of the Maastricht University.

Tissue specimens

Mice were sacrificed by CO₂ asphyxiation followed by removal of liver and mesenteric adipose tissue. Tissues were divided into pieces and snap-frozen in liquid nitrogen for

real-time reverse transcriptase polymerase chain reaction (RT-PCR), ELISA, and lipid analysis. A second part was fixed with formalin and embedded in paraffin for histopathology and immunohistochemistry. The third part was snap-frozen in 2-methylbutane after embedding in Tissue-Tek OCT (Sakura Finetek, Zoeterwoude, the Netherlands).

Lipid analysis

Tail vein blood was collected after 4 hours fasting in glass capillaries coated with heparin and diethyl p-nitrophenyl phosphate. Blood was obtained alternately from wild type and *Mpo*^{-/-} transplanted mice at 11:00. Plasma and liver triglyceride and cholesterol levels were measured using the GPO-PAP kit (Roche, Basel, Switzerland). Approximately 50 mg of frozen liver tissue was homogenised in 1 ml SET buffer (250 mM Sucrose, 2 mM EDTA, 10 mM Tris) followed by two freeze-thaw cycles and three times passing through a 27-gauge needle. Protein content was measured by the BCA method (Pierce, Rockford, IL).

Histology and immunohistochemistry

Paraffin-embedded sections were cut and stained with haematoxylin and eosin for histopathological analysis and with Sirius red to study collagen distribution. Frozen liver sections were immersed in a solution of 3g Oil Red O/L isopropanol (Sigma-Aldrich, Zwijndrecht, the Netherlands) to stain neutral lipids or in 100 µg/ml filipin (Sigma-Aldrich) to stain free cholesterol. Immunohistochemical MPO staining was performed as previously described⁷. To identify granulocytes, T-lymphocytes, and Kupffer cells/macrophages, frozen liver or visceral adipose tissue sections were fixed in acetone and stained using rat-anti-mouse Ly-6G (clone NIMP-R14, Hycult Biotechnology, Uden, the Netherlands), rat-anti-mouse CD3 (clone KT3, Thermo Scientific, Etten-Leur, the Netherlands), rat-anti-mouse Mac-1 (clone M1/70, BD Biosciences, Breda, the Netherlands), or rat-anti-mouse CD68 (clone FA11, Hycult Biotechnology) following a similar protocol. For quantification, positive cells in six 200x fields were counted in a blinded fashion and were noted as cells per mm². MPO foci were defined as aggregation of >2 MPO positive cells.

Quantitative real-time PCR

Total RNA isolation, reverse transcription, and real-time PCR was performed as previously described⁷. Relative gene expression was normalised against cyclophilin A and β-actin gene expression.

Myeloperoxidase and nitrotyrosine ELISA

Plasma and liver MPO and liver nitrotyrosine were measured using sandwich ELISA according to the manufacturer's protocol (Hycult Biotechnology). Samples were analysed in duplicate in the same run. The intra-assay coefficient of variance was <10%.

Hydroxyproline assay

Hydroxyproline content of proteins was measured after acid hydrolysis with 6M HCl during 24 hours. Amino acid analysis was performed as recently described²⁴. Briefly, samples were introduced into a tandem mass spectrometer using UPLC. Amino acids were measured in multiple reaction mode in ESI-positive mode. The mass transition 131.75>85.9 was used for the identification of hydroxyproline. Stable isotope-labelled asparagine was used as internal standard.

Statistics

Data are represented as mean±SEM. Differences between groups were analysed using the Mann Whitney test, or one-way ANOVA with Dunnett's test for multiple comparison. Statistical analyses were performed using Graphpad Prism 5.02 for Windows (Graphpad Software, San Diego, CA). A p-value <0.05 was considered statistically significant.

Results

Strong induction of hepatic MPO in *Ldlr*^{-/-} mice after high-fat feeding

High-fat feeding of hyperlipidemic *Ldlr*^{-/-} mice is associated with elevated plasma MPO levels²⁵. Furthermore, we previously found that wild type and particularly *Ldlr*^{-/-} mice show hepatic upregulation of *Mpo* expression in response to high-fat feeding^{22,25}. These interesting observations triggered us to assess the effects of a high-fat diet on liver MPO protein content. We first studied the livers of *Ldlr*^{-/-} mice, prone to the development of NASH, that were fed a diet containing 21% fat and 0.2% cholesterol for three weeks²². Remarkably, high-fat feeding caused a 3.7-fold increase of liver MPO protein in *Ldlr*^{-/-} mice (p<0.01; Figure 6.1A), in line with our previous studies^{22,26}. This was associated with substantial infiltration of MPO-positive neutrophils into the liver (Figure 6.1B). These neutrophils frequently assembled into aggregate structures around hepatocytes with macrovesicular steatosis, resembling the crown-like macrophage structures found in adipose tissue of obese animals. Thus, hepatic lipid accumulation triggered by a high-fat diet is associated with liver neutrophil infiltration leading to increased hepatic MPO levels.

Figure 6.1 Strong high-fat diet-induced induction of MPO in the liver. (A) Total liver MPO content of *Ldlr*^{-/-} mice as assessed by ELISA was almost four-fold increased by three weeks of high-fat feeding (19±1 vs. 68±10 ng/μg protein, $p < 0.01$; $n = 6$ in both groups). (B) MPO immunostaining reveals infiltration of neutrophils into the liver of *Ldlr*^{-/-} mice after three weeks of high-fat feeding (100x magnification). Many neutrophils are organised into aggregates predominantly surrounding steatotic hepatocytes (right panel; 200x magnification).

Reduced MPO-mediated nitrotyrosine in *Ldlr*^{-/-}/*Mpo*^{-/-} mice on high-fat diet

The accumulation of MPO in the liver upon high-fat feeding suggested that MPO, which is known to cause tissue damage in several chronic inflammatory disorders⁹, might be causally involved in the pathogenesis of NASH. To further examine the role of MPO in NASH, we performed bone marrow transplantation experiments that resulted in the generation of combined *Mpo*^{-/-}, *Ldlr*^{-/-} mice (*Ldlr*^{-/-}/*Mpo*^{-/-}) mice. *Ldlr*^{-/-}/*Mpo*^{+/+} and *Ldlr*^{-/-}/*Mpo*^{-/-} mice had a similar body weight, and weight gain was indistinguishable between the groups during the eight weeks of high-fat feeding (*Ldlr*^{-/-}/*Mpo*^{+/+} from 19.9±0.4 to 21.8±0.3 g, *Ldlr*^{-/-}/*Mpo*^{-/-}: from 20.0±0.2 to 21.9±0.7 g).

After eight weeks of high-fat feeding, MPO plasma levels in the *Ldlr*^{-/-}/*MPO*^{+/+} mice were 324 ng/ml whereas plasma from *Ldlr*^{-/-}/*Mpo*^{-/-} animals contained only 22 ng/ml of MPO ($p < 0.01$), which is probably attributable to a minor proportion of *MPO*^{+/+} host stem cells that survived irradiation. Similar to plasma MPO, liver MPO was very significantly reduced in the *Ldlr*^{-/-}/*Mpo*^{-/-} group after high-fat feeding ($p < 0.001$; Figure 6.2B), further confirming successful bone marrow transplantation. In addition, immunohistochemical staining of liver sections of *Ldlr*^{-/-}/*Mpo*^{-/-} revealed a strongly reduced number of MPO-positive foci in comparison with *Ldlr*^{-/-}/*Mpo*^{+/+} mice ($p < 0.001$; Figure 6.2C).

Figure 6.2 Severely reduced hepatic MPO and MPO-derived nitrated proteins in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks of high-fat feeding. (A) Plasma MPO levels of *Ldlr*^{-/-}/*Mpo*^{+/+} mice are much higher (324±52 ng/ml) than those of *Ldlr*^{-/-}/*Mpo*^{-/-} animals, in which MPO is almost absent (22±7 ng/ml, $p < 0.01$). (B) Important reduction of high-fat diet-induced liver MPO in *Ldlr*^{-/-}/*Mpo*^{-/-} as compared to *Ldlr*^{-/-}/*Mpo*^{+/+} mice (8±0.5 vs. 29±2 ng/μg protein, $p < 0.01$). (C) Strongly reduced number of MPO-positive foci in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} vs. *Ldlr*^{-/-}/*Mpo*^{+/+} mice after high-fat feeding (0.08±0.03 vs. 1.03±0.16 foci/mm², $p < 0.01$). (D) Hepatic levels of nitrotyrosine, a marker of MPO activity, are markedly reduced in *Ldlr*^{-/-}/*Mpo*^{-/-} animals on a high-fat diet in comparison with *Ldlr*^{-/-}/*Mpo*^{+/+} mice (126±7 vs. 149±9 mmol/μg protein, $p = 0.02$).

To investigate if the marked reduction of hepatic MPO in *Ldlr*^{-/-}/*Mpo*^{-/-} mice translated into diminished generation of MPO-mediated cytotoxic products, we studied hepatic levels of nitrotyrosine, a protein modification that is generated as a result of MPO activity at sites of inflammation, and that was previously shown to accumulate in NAFLD^{7,12,13}. As expected, mice in the *Ldlr*^{-/-}/*Mpo*^{-/-} group displayed significantly reduced levels of nitrotyrosine in the liver, indicating reduced MPO activity (Figure 6.2D; $p < 0.05$).

Mpo deficiency leads to reduced liver cholesterol but does not affect hepatic steatosis

Next, the effects of *Mpo* deficiency on the development of hepatic steatosis were examined. It was previously established that *Ldlr*^{-/-} mice readily accumulate triglycerides in the liver in response to the diet used in the present study²². Oil red O staining of liver sections did not reveal obvious differences with respect to size, distribution, or extent of lipid accumulation between *Ldlr*^{-/-}/*Mpo*^{+/+} mice and *Ldlr*^{-/-}/*Mpo*^{-/-} mice (Figure 6.3A). In line with this, biochemical analysis revealed a similar liver triglyceride content in *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} animals ($p = 0.24$; Figure 6.3B). Plasma levels of triglycerides were also similar in *Ldlr*^{-/-}/*Mpo*^{+/+} and *Ldlr*^{-/-}/*Mpo*^{-/-} mice after the high-fat diet period (Figure 6.3C). In contrast, both plasma cholesterol and hepatic cholesterol levels were significantly lower in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after the high-fat diet as compared with the *Ldlr*^{-/-}/*Mpo*^{+/+} animals (33.5±1.2 vs. 39.5±1.9 mM; $p = 0.01$, and 0.072±0.005 vs. 0.090±0.004 μg/μg protein; $p = 0.01$, respectively; Figure 6.3D,E).

Figure 6.3 Decreased cholesterol accumulation in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice. (A) Representative Oil red O stainings of liver sections of *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} mice fed a high-fat diet for 8 weeks, showing comparable lipid accumulation (100x magnification). (B) Similar hepatic triglyceride levels in *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} mice after 8 weeks of high-fat feeding (0.31 ± 0.02 vs. 0.35 ± 0.02 $\mu\text{g}/\mu\text{g}$ protein, $p=0.24$). (C) Plasma triglyceride levels are similar in *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} animals after high-fat feeding (1.50 ± 0.09 vs. 1.64 ± 0.09 mmol/l, $p=0.42$). (D) High-fat feeding results in higher plasma cholesterol levels in *Ldlr*^{-/-}/*Mpo*^{-/-} animals as compared with *Ldlr*^{-/-}/*Mpo*^{-/-} mice (33.5 ± 0.1 vs. 39.5 ± 2.0 mmol/l, $p=0.02$). (E) Diet-induced liver cholesterol accumulation is reduced in *Ldlr*^{-/-}/*Mpo*^{-/-} mice compared with *Ldlr*^{-/-}/*Mpo*^{+/+} animals (0.072 ± 0.004 vs. 0.090 ± 0.004 $\mu\text{g}/\mu\text{g}$ protein, $p=0.01$). (F) Hepatic mRNA expression of key enzymes in cholesterol metabolism is not altered in *Ldlr*^{-/-}/*Mpo*^{-/-} mice, whereas scavenger receptor expression is significantly reduced (*Sr-b1* 1.7-fold, $p<0.01$; *Cd36* 1.4-fold, $p<0.01$).

The differences in hepatic cholesterol accumulation do not appear to be due to altered cholesterol synthesis, since hepatic gene expression of the two master regulators of cholesterol synthesis, *Srebp1* and *Srebf2*, was comparable in *Ldlr*^{-/-}/*Mpo*^{+/+} and *Ldlr*^{-/-}/*Mpo*^{-/-} animals ($p=0.89$, $p=0.32$, respectively; Figure 6.3F). Expression of hydroxymethylglutaryl-CoA reductase (*Hmgcr*), the key rate-limiting enzyme in cholesterol synthesis, was also not significantly different between the groups ($p=0.42$; Figure 6.3F). Interestingly, however, expression of *Sr-b1* and *Cd36*, two important proteins involved in the uptake of oxidised cholesterol, was significantly reduced in the *Ldlr*^{-/-}/*Mpo*^{-/-} group ($p<0.01$, $p<0.01$, respectively; Figure 6.3F). This may indicate reduced internalization of the oxidised cholesterol fraction in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice.

Generalised attenuation of high-fat diet-induced liver inflammation in *Ldlr*^{-/-}/*Mpo*^{-/-} mice

Active MPO has powerful pro-inflammatory effects, partly attributable to the generation of oxidised cholesterol^{10,27}. Therefore, we next sought to establish the effect of MPO deficiency on hepatic inflammation following high-fat feeding. The cellular nature of the inflammation was investigated by immunohistochemical analysis of Mac-1, Ly-6G, and CD3, markers of Kupffer cells/macrophages, neutrophils, and T-lymphocytes, respectively. Interestingly, the number of neutrophils and T-lymphocytes was significantly reduced in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice ($p<0.05$, $p<0.05$, respectively; Figure 6.4A). Moreover, whereas the number of Kupffer cells appeared to be similar in the two groups, quantitative PCR analyses indicated that mRNA expression of *Cd68*, which is associated with macrophage activation, and of Mac-1, a migratory marker expressed by monocytes, was diminished in the *Ldlr*^{-/-}/*Mpo*^{-/-} animals ($p<0.05$, $p<0.05$, respectively; Figure 6.4b). In line with the reduced *Cd68* expression, hepatic monocyte chemoattractant protein-1 (*Mcp-1*) mRNA expression was significantly lower in *Ldlr*^{-/-}/*Mpo*^{-/-} mice ($p<0.01$; Fig.4b).

Since Kupffer cells/macrophages can adopt different phenotypes that may direct pro- or anti-inflammatory responses, we also performed gene expression analyses of phenotype markers. Surprisingly, hepatic expression levels of several markers considered anti-inflammatory such as *Jag-1*, *Emr1*, and *Dectin-1* were lower in the *Ldlr*^{-/-}/*Mpo*^{-/-} group ($p<0.05$, $p<0.01$, $p<0.01$, respectively; Figure 6.4C). On the other hand, expression of *Il-10*, an important cytokine with anti-inflammatory properties, was higher ($p<0.05$; Figure 6.4C). Importantly, additional quantitative PCR analyses showed that the expression of several pro-inflammatory genes that have previously been implicated in the pathogenesis of NASH was consistently strongly reduced in the *Ldlr*^{-/-}/*Mpo*^{-/-} animals (Fig.4d). For example, tumor necrosis factor- α (*Tnf- α*) and *Il-1 α* mRNA expression were almost two-fold lower in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice compared with *Ldlr*^{-/-}/*Mpo*^{+/+} mice ($p<0.05$, $p<0.01$, respectively). In addition, hepatic

Il-6 expression tended to be reduced in *Ldlr*^{-/-}/*Mpo*^{-/-} mice relative to *Ldlr*^{-/-}/*Mpo*^{+/-} mice, although the difference was not statistically significant. Taken together, these results show that MPO plays an important role in high-fat diet-induced inflammation of the liver, affecting inflammatory cell recruitment, phenotype, and cytokine/chemokine expression.

Figure 6.4 General reduction of diet-induced hepatic inflammation in *Ldlr*^{-/-}/*Mpo*^{-/-} mice. (A) Significant reduction of hepatic Ly-6G⁺ neutrophils and CD3⁺ T-lymphocytes in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks of high-fat feeding (Ly-6G: 36.7±2.6 vs. 47.8±3.1 cells/mm², *p*=0.03; CD3: 49.1±4.2 vs. 62.9±5.0 cells/mm², *p*=0.04). (B) Reduced hepatic expression of *Cd68* (1.3-fold, *p*<0.05), *Mac-1* (2-fold, *p*<0.05), and *Mcp-1* (2.5-fold, *p*<0.01), genes associated with macrophage activation and attraction, in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks of high-fat feeding. (C) Gene expression analysis of genes characteristic of anti-inflammatory M2 macrophages/Kupffer cells indicates that steatohepatitis induced by 8 weeks of high-fat feeding is not associated with a particular macrophage/Kupffer cell phenotype in *Ldlr*^{-/-}/*Mpo*^{-/-} mice. (D) Hepatic pro-inflammatory cytokine expression is substantially reduced in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks high-fat diet (*Tnf-α* 1.8-fold, *p*=0.03, *Il-1α* 1.6-fold, *p*<0.01, *Il-6* 1.3-fold, *p*=0.67).

Reduced high-fat diet-induced adipose tissue inflammation in *Ldlr*^{-/-}/*Mpo*^{-/-} mice

The pathogenesis of NASH is known to be mediated by cross-talk between inflamed adipose tissue and the liver²⁸. More specifically, adipocytes can secrete inflammatory mediators or 'adipokines' that interact with liver cells, such as adiponectin. In order to investigate the potential contribution of adipose tissue-derived factors to the reduced hepatic inflammation in *Ldlr*^{-/-}/*Mpo*^{-/-} mice, several inflammatory parameters were investigated in adipose tissue. First of all, Ly-6G staining revealed an absence of

neutrophils in visceral adipose tissue in both groups, apart from occasional positive cells inside blood vessels (data not shown). Next, visceral adipose tissue was stained for the macrophage marker CD68. High-fat diet-induced obesity is characterised by marked infiltration of macrophages into adipose tissue, where they frequently organize into so-called ‘crown-like structures’ surrounding dead adipocytes. Interestingly, adipose tissue of *Ldlr*^{-/-}/*Mpo*^{-/-} mice was completely devoid of such crown-like structures, whereas they were readily identifiable in adipose tissue of *Ldlr*^{-/-}/*Mpo*^{+/+} mice (Figure 6.Aa). Quantitative PCR analysis of adipose tissue *Mac-1* expression, another macrophage marker, was in line with these results, showing a marked reduction in *Ldlr*^{-/-}/*Mpo*^{-/-} animals ($p < 0.05$; Figure 6.5B). Similarly, expression of *Mcp-1*, a potent chemo-attractant for macrophages, was strongly reduced in the *Ldlr*^{-/-}/*Mpo*^{-/-} group ($p < 0.05$; Figure 6.5B). Furthermore, adipose tissue expression of the pro-inflammatory adipokines *leptin* and *Tnf- α* was lower in *Ldlr*^{-/-}/*Mpo*^{-/-} animals, whereas expression of adiponectin, which has anti-inflammatory properties, was higher (Figure 6.5C). Thus, reduced adipose tissue inflammation may contribute to the attenuation of diet-induced inflammation in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice.

Figure 6.5 Reduced diet-induced adipose tissue inflammation in *Ldlr*^{-/-}/*Mpo*^{-/-} mice. (A) Lack of high-fat diet-associated macrophage ‘crown-like structures’ in visceral adipose tissue of *Ldlr*^{-/-}/*Mpo*^{-/-} mice as revealed by CD68 immunostaining (200x magnification). (B) Adipose tissue mRNA expression of the macrophage marker *Mac-1* and the macrophage chemokine *Mcp-1* is significantly lower in *Ldlr*^{-/-}/*Mpo*^{-/-} mice fed a high-fat diet for 8 weeks ($p < 0.05$). (C) Reduced adipose tissue expression of the adipokines *leptin* and *Tnf- α* in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks of high-fat feeding, whereas expression of *adiponectin* is increased.

Decreased liver fibrosis in *Ldlr*^{-/-}/*Mpo*^{-/-} mice

Progression of NAFLD, mediated by sustained inflammation, ultimately results in the development of hepatic fibrosis. Since MPO exerts strong effects on various mechanisms involved in fibrogenesis and has been implicated in pro-fibrotic states in various other chronic inflammatory conditions, we next evaluated the extent of fibrosis in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} mice. Sirius red staining of liver sections indicated mild fibrosis but did not reveal obvious differences in collagen content or distribution between *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} mice (Figure 6.6A). However, quantitative biochemical analysis of the collagen/elastin content in liver homogenates as determined by hydroxyproline quantity revealed a clearly lower amount in *Ldlr*^{-/-}/*Mpo*^{-/-} mice ($p < 0.01$), indicating that their liver was less fibrotic (Figure 6.6B).

Figure 6.6 Attenuation of liver fibrosis in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after 8 weeks high-fat diet. (A) Sirius red staining does not reveal major differences with respect to collagen content or distribution between *Ldlr*^{-/-}/*Mpo*^{+/+} and *Ldlr*^{-/-}/*Mpo*^{-/-} mice (200x magnification). (B) The amount of hydroxyproline, a protein modification specifically present in collagen and elastin, is significantly lower in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice in comparison with *Ldlr*^{-/-}/*Mpo*^{+/+} mice (3.3 ± 0.2 vs. 5.1 ± 0.2 nmol/mg tissue, $p < 0.01$). (C) *Ldlr*^{-/-}/*Mpo*^{-/-} mice display reduced expression of collagen type I (*Col1a1*), *Pai-1*, and *Timp1*, genes associated with hepatic fibrosis.

This was further substantiated by the fact that hepatic gene expression of collagen 1A1 was lower in the *Ldlr*^{-/-}/*Mpo*^{-/-} group ($p < 0.05$; Figure 6.6C). Moreover, mRNA levels of PAI-1, an important regulator of hepatic fibrosis, were significantly reduced in these animals ($p < 0.01$; Figure 6.6C). Expression of tissue inhibitor of metalloproteinase 1 (*Timp1*), which inhibits resolution of liver fibrosis, tended to be lower as well ($p = 0.15$; Figure 6.6C). Overall, these data indicate that MPO promotes the progression of NAFLD towards more advanced stages with fibrosis.

Figure 6.7 Schematic overview of the possible roles of MPO in the pathogenesis of NASH, integrating current data and literature. High fat feeding leads to infiltration of the liver by activated MPO-secreting neutrophils. Active MPO generates HOCl and NO₂ radicals, causing oxidation and nitration of proteins and lipids, thereby promoting inflammation and creating a vicious cycle. Similarly, increased diet-induced MPO activity in adipose tissue initiates local inflammation, leading to macrophage infiltration and adipokine secretion profiles that promote hepatic inflammation and fibrosis. In addition, MPO-generated oxidants contribute to fibrosis by activating stellate cells and extracellular matrix synthesizing and degrading enzymes both directly and indirectly.

Discussion

Hepatic inflammation is one of the defining criteria in the diagnosis of NASH, and primarily characterised by the abundant presence of neutrophils²⁹. Neutrophils are equipped with formidable enzyme systems that generate factors with a high potential of causing tissue damage, most prominently represented by MPO. The results of the

present study point to a central role for MPO in the development of NASH by increasing the severity of its hallmark characteristics lipid accumulation, inflammation, and fibrosis.

The effect of MPO deficiency on plasma lipid levels and inflammation has been studied before in the context of atherosclerosis, another chronic inflammatory condition^{25,30}. In line with our findings, plasma triglyceride levels were comparable between *Ldlr*^{-/-}/*Mpo*^{-/-} and *Ldlr*^{-/-}/*Mpo*^{+/+} mice, whereas plasma cholesterol was lower in mice lacking MPO. We now report that hepatic cholesterol levels are also reduced in *Ldlr*^{-/-}/*Mpo*^{-/-} mice after high-fat feeding. There are several mechanisms by which MPO could affect cholesterol levels in plasma and liver. MPO inhibits cholesterol efflux from lipid-laden macrophages by oxidizing apoA-I in HDL³¹. MPO is also able to oxidize other apolipoproteins including apoB, an important component of LDL³². This may alter interaction with hepatocyte receptors and contribute to dyslipidemia by affecting clearance. Furthermore, formation of nitrotyrosine has been reported on apoB-100, leading to enhanced uptake of cholesterol-containing LDL by macrophages³³. In view of mounting evidence that Kupffer cells acquire characteristics similar to lipid-laden macrophages/foam cells in response to high-fat feeding^{4,22}, reduced hepatic cholesterol in *Ldlr*^{-/-}/*Mpo*^{-/-} mice may be related to diminished formation of foamy Kupffer cells.

Regardless of the mechanism, the reduced cholesterol levels in *Ldlr*^{-/-}/*Mpo*^{-/-} animals are significant in light of recent data indicating that cholesterol plays a pivotal role in the induction of early inflammation in NASH^{22,34}. In this context, scavenging of oxidised cholesterol/lipoprotein particles by Kupffer cells and hepatocytes may be an initiating factor. Uptake of oxidised LDL/HDL is mediated by scavenger receptors, and is associated with chronic inflammation³⁵. Of note, scavenger receptor expression is regulated by oxidised LDL through a positive feedback loop³⁶. The reduced scavenger receptor expression that we found in the liver of *Ldlr*^{-/-}/*Mpo*^{-/-} mice may therefore be indicative of lower oxidised LDL levels, consistent with lower MPO activity. Future studies on the levels of oxidised cholesterol in both plasma and liver are required to further define the mechanisms involved.

In addition to the pro-inflammatory effects related to cholesterol accumulation and modification, MPO can promote inflammation in various other ways. First, MPO-mediated generation of HOCl and NO₂ radicals directly results in chlorination and nitration of proteins and nucleic acids¹⁰ reflecting cellular damage, a potent inducer of inflammation. The fact that hepatic nitrotyrosine levels were lower in *Ldlr*^{-/-}/*Mpo*^{-/-} mice suggests that MPO-mediated nitration of proteins may indeed contribute to high-fat diet-induced hepatic inflammation. Second, MPO and MPO-derived HOCl activate NF-κB signalling and increase TNF-α production by leukocytes^{15,17,37}. This is consistent with our observation of lower hepatic and adipose tissue *Tnf*-α expression in the

Ldlr^{-/-}/*Mpo*^{-/-} mice. Importantly, pro-inflammatory TNF- α signalling appears to be a key factor in the progression of NAFLD³⁸. Third, MPO activity is linked to the initiation of lipid peroxidation, which is a prominent characteristic of fatty livers, promoting activation of stellate cells and attraction of inflammatory cells^{39,40}. Indeed, the substantial reduction of hepatic neutrophils and T-lymphocytes in *Ldlr*^{-/-}/*Mpo*^{-/-} mice provides supporting evidence for an important role of MPO-mediated lipid peroxidation in chemo-attraction of leukocytes in NASH.

Interestingly, whereas we could not detect neutrophils in adipose tissue, we did observe reduced numbers of adipose tissue macrophages in *Ldlr*^{-/-}/*Mpo*^{-/-} mice. This is in line with recent data indicating that high-fat diet-induced infiltration of macrophages into adipose tissue is preceded by neutrophil infiltration⁴¹. Moreover, lipid peroxidation is known to be markedly elevated in adipose tissue of obese mice⁴². Hence, our findings suggest that the reported early diet-induced sequestration of neutrophils in adipose tissue may promote lipid peroxidation via MPO-dependent mechanisms. Furthermore, accumulation of oxidised lipids in adipose tissue is associated with dysregulated adipokine expression⁴², which is in line with our data on leptin and adiponectin expression. Importantly, reduced adiponectin and increased leptin secretion by adipose tissue promotes lipid accumulation, inflammation, and fibrogenesis in the liver⁴³.

Next to dysregulated adiponectin and leptin expression, numerous other factors modulated by MPO and MPO-derived products affect the development of fibrosis. For example, MPO-generated oxidants activate matrix metalloproteinases⁴⁴, while inhibiting protease inhibitors such as TIMP1⁴⁵. These actions are thought to suppress fibrosis. In contrast, high levels of MPO-derived HOCl can also inactivate matrix metalloproteinase 7²⁰, thereby promoting fibrosis. Furthermore, MPO-related lipid peroxidation products stimulate stellate cell synthesis of type I collagen, the major collagen of the fibrotic liver²¹, which expression was significantly reduced in the *Ldlr*^{-/-}/*Mpo*^{-/-} mice. Finally, HOCl enhances the proteolytic susceptibility of matrix proteins⁴⁶ and fragments the extracellular matrix¹⁸, which is associated with stellate cell activation as well. Our data indicate that *in vivo*, the pro-fibrotic effects of MPO outweigh anti-fibrotic processes in the context of NASH, and provide strong evidence that MPO is an important factor with respect to fibrogenesis in NASH.

The clinical impact of these findings is evident since human NAFLD is associated with high numbers of MPO-expressing cells and accumulation of HOCl-modified and nitrated proteins^{6,7,13}. Furthermore, there is strong evidence for increased oxidative stress and extensive lipid peroxidation in human NASH^{6,14,40}. In this regard it is also important to note that in comparison to the mouse, human blood contains 5-7 times more neutrophils with a longer half-life, each containing about 10-fold more MPO^{9,47}. As such, it is likely that the contribution of MPO to the progression of NAFLD in man is

even more pronounced. Moreover, high and sustained MPO activity has been shown to result in widespread oxidative DNA damage⁴⁸, which is associated with the ultimate and most devastating complication of the progression of NASH, hepatocellular carcinoma^{40,49}.

In conclusion, we have shown for the first time that MPO-deficiency diminishes high-fat diet-induced NASH by reducing hepatic cholesterol accumulation, inflammation, and fibrosis (Figure 6.7). The key role of MPO in the progression of NASH makes the enzyme a particularly attractive target for treatment¹⁰. Furthermore, our data indicate a general role for MPO in the chronic low-grade inflammation associated with obesity, and therefore argue for a re-evaluation of the role of neutrophils and their cytotoxic products in the pathogenesis of metabolic disease.

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Chapter 7

CYP27: a novel player in diet-induced non-alcoholic steatohepatitis

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This chapter includes confidential information

Abstract

Background

Non-alcoholic steatohepatitis (NASH) is a disorder that is characterized by fat accumulation in the liver or steatosis combined with hepatic inflammation. While steatosis itself is generally considered benign and reversible, the presence of inflammation can lead to further damage to the liver. Recently, we demonstrated a clear association between hepatic inflammation and lysosomal cholesterol accumulation inside Kupffer cells (KCs). 27-hydroxycholesterol (27HC), a derivate of cholesterol formed by CYP27, was previously shown to mobilize cholesterol from the lysosomes to the cytoplasm of the cell *in vitro*. Therefore, we hypothesize that CYP27 can redirect the intracellular cholesterol pool from the lysosomes to the cytoplasm of the KCs *in vivo*, thereby reducing hepatic inflammation.

Methods

To investigate the role of 27HC in NASH, *Ldlr*^{-/-} mice were transplanted with bone marrow from mice lacking *Cyp27*. Control mice were transplanted with wild-type (Wt) bone marrow. After a recovery period of 9 weeks, the mice were put on chow or a high fat, high cholesterol (HFC) diet for 3 months.

Results

As expected, electron microscopy analysis of KCs revealed increased lysosomal fat accumulation in *Cyp27*^{-/-}-tp mice compared to Wt-tp mice after 3 months of HFC diet. Additionally, we observed an increased amount of cholesterol precipitation inside the lipid droplets of *Cyp27*^{-/-}-tp mice. Liver histology demonstrated that *Cyp27*^{-/-}-tp mice had increased hepatic inflammation compared to Wt-tp mice as indicated by the elevated numbers of infiltrated macrophages, neutrophils and T-cells. These findings were confirmed by hepatic expression of *Tnf*, *Il-6* and *Il-1b*.

Conclusion

In the present study, we demonstrated the potential of CYP27 to modulate the intracellular cholesterol distribution inside KCs *in vivo* and to affect hepatic inflammation.

Introduction

Metabolic syndrome is one of the leading disease states in developed countries and its incidence is closely related to the incidence of other diseases, such as insulin resistance and obesity. Non-alcoholic steatohepatitis (NASH) is a disorder that is known as the hepatic component of metabolic syndrome. It is characterized by fat accumulation in the liver (steatosis) and hepatic inflammation. While steatosis itself is generally considered a rather benign and reversible condition, the presence of inflammation in a fatty liver is the key feature of NASH that preludes further disease progression and allows more advanced stages of the disease to develop, such as fibrosis, cirrhosis or hepatocellular carcinoma, leading to the need for liver transplantation^{1,2}. Therefore, knowledge regarding the intracellular mechanisms that trigger inflammation during NASH is of utmost importance. We have previously shown that targeted deletion of the two main scavenger receptors (CD36 and SR-A), which are responsible for the uptake of modified lipoproteins, sets off a cascade of pro-inflammatory events leading to the initiation of the inflammatory response in *Ldlr*^{-/-} mice³. Importantly, the reduction in inflammation was associated with reduced lysosomal cholesterol accumulation and increased cholesterol in the cytoplasm of KCs. However, a causal link between lysosomal cholesterol accumulation in KCs and hepatic inflammation has not yet been established.

Under normal conditions, lipoproteins circulating in the blood will be endocytosed and initially directed to lysosomes, where the lipoproteins are hydrolyzed by lysosomal enzymes and transferred into the cytoplasm⁴. However, similar to our observations in the *Ldlr*^{-/-} mice, in foamy macrophages of inflamed atherosclerotic plaques, cholesterol is not transferred into the cytoplasm, but rather accumulates in the lysosomes of the macrophages⁵. Accumulation of cholesterol inside lysosomes is also a key feature in lipid storage disease that occurs due to a deletion in Niemann-Pick type C (NPC1), a key protein involved in the transition of cholesterol from the lysosomes to the cytoplasm. In cells with a deletion in Niemann-Pick type C (NPC1), the production of 27-hydroxycholesterol (27HC), the major oxysterol found in human circulation, by the mitochondrial enzyme CYP27, is severely blunted. Notably, the lysosomal cholesterol pool in NPC1^{-/-} cells is dramatically reduced upon incubation with 27HC^{6,7}. Thus, 27HC has been shown to reduce lysosomal cholesterol accumulation *in vitro*.

The aim of the current study was to investigate whether mobilization of intracellular cholesterol inside the KCs from lysosomes to the cytoplasm can reduce hepatic inflammation *in vivo*. Therefore, we generated bone-marrow chimeras using *Ldlr*^{-/-} mice as the host and bone marrow cells from *Cyp27*^{-/-} mice. We hypothesized that decreased levels of 27HC inside KCs will inhibit the transition of cholesterol from the lysosomes to the cytoplasm, and thereby increase hepatic inflammation. In agreement with our hypothesis, *Cyp27*^{-/-} transplanted (-tp) mice showed increased lysosomal

cholesterol accumulation in KCs compared to mice transplanted with bone marrow from wild type (Wt) C57BL/6J. These observations therefore indicate that 27HC also modulates lysosomal cholesterol accumulation *in vivo*. In addition, the increased lysosomal cholesterol accumulation in these mice was associated with increased inflammation, suggesting a causal role for lysosomal cholesterol accumulation in hepatic inflammation.

Materials and methods

Mice, diet and bone marrow transplantation

Mice were housed under standard conditions and given free access to food and water. Experiments were performed according to Dutch laws, approved by the Committee for Animal Welfare of Maastricht University. Ten-week-old female *Ldlr*^{-/-} mice were put in filter-top cages one week before transplantation. They received acidified water supplemented with neomycin (100 mg/l) and polymyxin B sulphate (60,000 U/l). One day before transplantation, the mice were irradiated with a lethal dose of 10 Gray röntgen. The bone marrow of four female wild-type and four female *Cyp27*^{-/-} littermates was collected and pooled. Bone marrow cells were derived by flushing the femur and tibia of the mice with PBS. Each irradiated *Ldlr*^{-/-} mouse received bone marrow cells via injection in the tail vein. After a recovery period of 9 weeks, mice were given normal chow or an HFC diet, containing 21% butter and 0.2% cholesterol, for 3 months. Blood was collected at the start of HFC feeding and on the day of sacrifice. After a 4-hour fast, blood samples were taken from the tail vein, collected in glass capillaries and coated with heparin and diethyl p-nitrophenyl phosphate. Subsequently, mice were sacrificed by cervical dislocation. Tissues were isolated and snap-frozen in liquid nitrogen and stored at -80 °C or fixed in 4% formaldehyde/PBS.

Plasma and liver lipid analysis

Frozen liver tissue was homogenized for 30 s at 5000 rpm in a closed tube with 1.0 mm glass beads and 1.0 ml SET buffer (Sucrose 250 mM, EDTA 2 mM and Tris 10 mM). Complete cell destruction was performed by two freeze-thaw cycles and three passes through a 27-gauge syringe needle and a final freeze-thaw cycle. Both plasma and liver lipid levels were measured using enzymatic colour tests according to the manufacturers' protocols. Protein content was measured using the BCA method. Measurements were made on a Benchmark 550 Micro-plate reader.

RNA isolation and quantitative PCR

Total RNA was isolated from cells obtained from liver and bone marrow from the four different groups. RNA clean-up was performed using the Qiagen RNeasy Mini Kit

(74104, Qiagen, Venlo, The Netherlands). Quality and quantity were determined using the Agilent2100 Bioanalyzer and RNA 6000 NanoLabChip (5065-4476, AgilentTechnologies, Amstelveen, The Netherlands) according to the manufacturers' protocols. Total RNA (500 ng) from the cells of each individual mouse sample was converted into first strand cDNA with the iScript cDNA synthesis kit (170-8891, Bio-Rad, Hercules, CA, USA). The changes in gene expression of inflammatory markers were determined by quantitative PCR on a Bio-Rad MyIQ with IQ5 v2 software (Bio-Rad, Hercules, CA, USA) using IQ SYBR Green Supermix with fluorescein (170-5006CUST, Bio-Rad, Hercules, CA, USA) and 10 ng of cDNA. For each gene, a standard curve was generated with a serial dilution of a cDNA pool. To standardize for the amount of cDNA, Cyclophilin A (Ppia) was used as the reference gene. Primer sets for the selected genes were developed using Primer Express v2.0 (Applied Biosystems, Foster City, CA, USA) with default settings. Data from qPCR were analysed according to the comparative Ct method.

Liver histology

Frozen liver sections (7-micrometres thick) were fixed in acetone and stained with the marker for infiltrated macrophages Mac1 (M1/70), the neutrophil marker NIMP, the T-cell marker KT3 and the Kupffer cell marker CD68 (FA11). After fixation, these sections were blocked with H₂O₂ to minimize background colouring. Prior to incubation with the first antibody, slides were incubated with 4% fetal calf serum (FCS)/ 1xPBS + 1:5 Avidin D Block solution (ABC kit). For incubation with the first antibody, we used 4% FCS/ 1xPBS + 1st antibody + 1:5 Biotin Block solution (ABC kit). Incubation with the second antibody (α -rat-BIO) was carried out in 4% FCS/ 2% normal mouse serum (NMS)/ 1xPBS. After this, the slides were washed and incubated in 1xPBS + 1:50 Avidin D solution + 1:50 Biotin solution (ABC kit). Then staining was carried out using the AEC kit (2% buffer/ 3% AEC/ 2% H₂O₂ in demi water) and Haematox. Pictures were taken with a Nikon DMX1200 digital camera and ACT-1 v2.603 software from Nikon Corporation. Immune cells were counted in six 200x microscopic views and were recorded as cells/mm². Additionally, Oil Red O staining was performed to investigate the amount of lipids present in the livers. Frozen liver sections were fixed in 4% formalin and stained in Oil Red O working solution (filtrate from stock solution: 250 mg Oil Red O powder in 50 ml 60% triethylphosphate). Further staining was performed using Haematox before mounting with glycerol jelly (10% glycerol in PBS).

Electron microscopy pictures were also taken to explore the intracellular fat distribution in Kupffer cells in the different transplanted groups. For this, liver sections were first perfused with glutaraldehyde. When perfusion was complete, the tissue was kept in glutaraldehyde fixative. Then, liver sections were washed in cacodylate-buffer to remove the glutaraldehyde fixative and post-fixed in 1% osmium tetroxide for 1 hour. After this fixation, the osmium fixative was washed off using buffer before the

liver sections were placed in alcohol (70%–100%). Further preparative steps were then possible.

Acid phosphatase determination

Activity of acid phosphatase was measured using the acid phosphatase assay kit (Cayman Chemical Company, Ann Arbor, Michigan, USA), according to the manufacturers' protocols.

In vitro murine macrophage culture

Bone marrow cells were isolated from femurs and tibiae of Wt- and *Cyp27^{-/-}*-tp mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, The Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine 2 mM (all GIBCO Invitrogen, Breda, The Netherlands) (R10) supplemented with 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously⁸. Cells were treated for 24 h with 25 µg/ml oxidized and acetylated LDL (Intracel, Frederick, United States).

Statistical analysis

The data were analysed using Graphpad Prism 4.0.3. Groups were compared using the unpaired t-test. The data were expressed as the mean and standard error of the mean and were considered significantly different from those obtained from the WT transplanted mice at $p \leq 0.05$. Significant results for *Cyp27^{-/-}*-tp animals on HFC were always based on comparison with WT transplanted mice on the HFC diet (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Results

Upon HFC feeding, plasma lipids levels are lower in *Cyp27^{-/-}*-tp mice compared with Wt-tp mice

To investigate the role of *Cyp27* in diet-induced NASH, *Ldlr^{-/-}* mice were transplanted with *Cyp27^{+/+}* (Wt) and *Cyp27^{-/-}* bone marrow. After a recovery period of 9 weeks, mice received a chow or HFC diet for 3 months. The body weights did not differ significantly between groups (data not shown). Three months of HFC feeding resulted in the development of equal levels of steatosis in the two transplanted groups. Neither hepatic triglyceride (TG) levels, nor hepatic cholesterol or free fatty acids (FFA) differed (Figure 7.1A). Additionally, Oil Red O staining showed no difference in the amount of

liver lipids in Wt- and *Cyp27^{-/-}*-tp mice (Figure 7.1B). In general, both groups developed equal amounts of hepatic steatosis.

The HFC diet increased plasma TG, cholesterol and FFA levels in both transplanted groups after 3 months (Figure 7.1C). Surprisingly *Cyp27^{-/-}*-tp mice displayed lower lipid levels after 3 months of HFC diet than Wt-tp mice. There was no difference between the two models after 3 months on chow diet.

Cholesterol synthesis is decreased in *Cyp27^{-/-}*-tp mice

The cholesterol synthesis markers lathosterol and desmosterol (ratio to total cholesterol) were decreased in both the liver and plasma of *Cyp27^{-/-}*-tp mice compared to Wt-tp mice on the HFC diet (Figure 7.2). The cholesterol absorption markers campesterol, sitosterol and cholestanol did not differ between *Cyp27^{-/-}*-tp and Wt-tp mice (data not shown). These findings are in line with the decreased total plasma cholesterol levels observed in *Cyp27^{-/-}*-tp mice on the HFC diet.

Kupffer cells of *Cyp27^{-/-}*-tp mice on the HFC diet have a less foamy appearance

To determine whether there was a difference in the foamy appearance of KCs, scoring of CD68 positive (a macrophage marker that stains KCs) sections revealed a clear difference between Wt- and *Cyp27^{-/-}*-tp mice. As expected, the KCs of Wt-tp mice on the HFC diet were extremely foamy, whereas the KCs of *Cyp27^{-/-}*-tp mice were much less foamy after 3 months on the HFC diet (Figure 7.3A–E).

Increased lysosomal cholesterol accumulation in Kupffer cells of *Cyp27^{-/-}*-tp mice

Electron microscopy was performed to explore the intracellular cholesterol distribution inside KCs. As already demonstrated in Figure 7.3, Wt-tp mice showed many more lipid droplets inside KCs compared to *Cyp27^{-/-}*-tp mice fed on the HFC diet (Figure 7.4A+B). Surprisingly, *Cyp27^{-/-}*-tp mice showed increased lysosomal cholesterol accumulation on the HFC diet (Figure 7.4C+D), although this was less foamy compared to Wt-tp mice. Moreover, the lipid droplets inside the KCs of *Cyp27^{-/-}*-tp mice were much bigger compared to the total size of the KC and they showed more cholesterol precipitation compared to Wt-tp mice. These changes in intracellular lipid distribution were accompanied by reduced acid phosphatase (AP) activity in the plasma of *Cyp27^{-/-}*-tp mice compared to Wt-tp mice on the HFC diet (Figure 7.4E).

Figure 7.1 Liver and plasma lipid levels. (A) Liver triglycerides (TG), cholesterol and free fatty acids (FFA) after chow and 3 months of HFC diet. (B+C) Oil Red O staining after 3 months of HFC diet in Wt and Cyp27^{-/-}-tp mice, respectively. (D) Plasma triglycerides (TG), cholesterol and free fatty acids (FFA) after chow and 3 months of HFC diet. (***: p<0.001)

Figure 7.2 Cholesterol synthesis markers in liver and plasma. The ratio of cholesterol synthesis markers lathosterol and desmosterol to total cholesterol in Wt-tp and Cyp27^{-/-}-tp mice (**: p<0.01)

Figure 7.3 Foamy appearance of Kupffer cells. (A–D) Representative pictures (magnification x200) after (A+B) chow and (C+D) 3 months of HFC diet in Wt and *Cyp27*^{-/-}-tp mice, respectively. (E) Scoring of CD68 positive area. * indicates $p < 0.05$.

Cyp27^{-/-}-tp mice demonstrate increased inflammation when fed a HFC diet

To investigate whether the deletion of *Cyp27* from all haematopoietic cells affects inflammation, liver sections were stained for several markers of inflammation. Staining with antibodies against infiltrated macrophages (Mac1), neutrophils (NIMP) and T-cells (KT3) revealed a significant increase in inflammation in livers of *Cyp27*^{-/-}-tp mice compared to Wt-tp mice (Figure 7.5A). Moreover, when present, the Mac1 positive cells were more clustered in *Cyp27*^{-/-}-tp mice compared to Wt-tp mice (Figure 7.5B+C). In line with these results, blood monocytes and T-cells were also increased in *Cyp27*^{-/-}-tp mice on the HFC diet compared to Wt-tp mice, while the granulocytes did not differ (data not shown). To further define the differences in hepatic inflammation between the two models, gene expression analysis was performed. Several

inflammatory genes showed increased expression in *Cyp27^{-/-}*-tp mice compared to Wt-tp mice on the HFC diet. As shown in Figure 7.5E, the gene expression of tumour necrosis factor (*Tnf*) and interleukin 6 and 1 beta (*Il6*, *Il1b*) was significantly increased in *Cyp27^{-/-}*-tp mice compared to Wt-tp mice, confirming the histological data. In conclusion, *Cyp27^{-/-}*-tp mice have increased hepatic inflammation compared to Wt-tp mice on the HFC diet.

Figure 7.4 Electron microscopy of foamy Kupfer cells. Representative electron microscopy pictures of Kupfer cells from (A+B) Wt- and (C+D) *Cyp27^{-/-}*-tp mice after 3 months of HFC diet, respectively. (E) Activity of acid phosphatase in plasma of Wt- and *Cyp27^{-/-}*-tp mice. * indicates $p<0.05$.

Figure 7.5 Parameters of hepatic inflammation. (A) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP) and T cells (CD3+), respectively, and counted. (B+C) Representative pictures of Mac-1 staining (x200 magnification) after 3 months of HFC feeding in Wt- and *CYP27*^{-/-}-tp mice, respectively. (D) Gene expression analysis of tumour necrosis factor (*Tnf*), interleukin 6 (*Il6*) and interleukin 1b (*Il1b*), respectively. Data were relative to Wt-tp mice on chow diet. * and ** indicate $p < 0.05$ and 0.01 , respectively

Increased *Lxr* expression in *Cyp27*^{-/-}-tp mice

To investigate the effect of the deletion of *Cyp27* on cholesterol metabolism and cholesterol efflux, the gene expression of the nuclear receptor Liver X receptor (*Lxr*) and its downstream target genes adenosine triphosphate (ATP)-binding cassette transporter A1 (*Abca1*) and G1 (*Abcg1*) was analysed. The expression of *Lxrα*, together with the two cholesterol efflux transporters *Abca1* and *Abcg1* was increased in *Cyp27*^{-/-}-tp mice compared to Wt-tp mice after 3 months of HFC diet (Figure 7.6). Moreover, the HFC diet increased the expression of all three genes.

Figure 7.6 Gene expression analysis of LXR and the related target genes. Data were relative to Wt-tp mice on chow diet. * indicates $p < 0.05$.

Increased liver damage in *Cyp27^{-/-}-tp* mice fed the HFC diet

Elevated alanine aminotransferase (ALT) levels in plasma are considered to be sensitive indicators of liver damage. In the present study, plasma ALT levels were increased after 3 months of HFC diet in *Cyp27^{-/-}-tp* mice compared to Wt-tp mice (Figure 7.7A). In line with these findings, hepatic fibrosis was also increased in *Cyp27^{-/-}-tp* mice compared to Wt-tp mice, as demonstrated by gene expression analysis and collagen staining with Sirius Red (Figure 7.7B–D). Hepatic gene expression of *Tgfb* was increased after 3 months of HFC diet in *Cyp27^{-/-}-tp* mice compared to Wt-tp mice (Figure 7.7B). A similar trend was observed for the expression of *Col1a1* after 3 months of HFC diet; however, this did not reach statistical significance ($p = 0.08$) (Figure 7.7C). After HFC feeding, the collagen content around the periportal and centrilobular regions of the liver was more pronounced in the *Cyp27^{-/-}-tp* mice compared to the Wt-tp mice. The same was observed for the collagen content in between these regions (Figure 7.7D). In conclusion, *Cyp27^{-/-}-tp* mice had increased liver damage compared to Wt-tp mice when fed the HFC diet.

After loading with modified LDL, bone marrow derived macrophages of *Cyp27^{-/-}-tp* mice also have increased lysosomal cholesterol accumulation and crystallization

Bone marrow derived macrophages (BMM) of Wt-tp and *Cyp27^{-/-}-tp* mice were loaded with modified cholesterol to mimic foam cell formation *in vitro*. After 24 hours, lysosomes of *Cyp27^{-/-}-BMM* had increased levels of lysosomal cholesterol accumulation in the form of cholesterol crystals, while this effect was not as pronounced in Wt-BMM (Figure 7.8A+B). Moreover, gene expression analysis in *Cyp27^{-/-}-BMM* demonstrated higher levels of *Tnf* and *Mcp1* after loading compared to Wt-BMM. Furthermore, mRNA expression levels of *Mcp1*, *Il1b* and *Tlr4* after LPS stimulation were also higher in *Cyp27^{-/-}-BMM* compared to Wt-BMM, indicating a more pronounced inflammatory response (data not shown). In conclusion, lysosomal cholesterol accumulation in *Cyp27^{-/-}-BMM* was associated with an increased inflammatory response.

Figure 7.7 Liver damage and hepatic fibrosis. (A) Aminotransferase (ALT) levels in plasma. (B+C) Gene expression analysis of *Tgfb* and *Col1a1*, respectively. Data were relative to the Wt-tp mice on chow diet. (D) Quantification of Sirius Red staining after being fed the HFC diet. Livers were quantified as minimally, mildly or moderately positive for collagen around and in between the blood vessels of the liver. * indicates $p < 0.05$

Discussion

Despite much effort, the mechanisms explaining the cause of inflammation in the aetiology of NASH are unknown. We have previously shown the importance of dietary cholesterol for the development of hepatic inflammation. Here, we show that the actual trigger for the development of inflammation is not the level of cholesterol within the plasma and KCs, but rather the disturbed intracellular cholesterol distribution inside the KCs.

In the present study, Cyp27^{-/-}tp mice demonstrated increased lysosomal cholesterol accumulation when fed the HFC diet, compared to Wt-tp mice. Several lines of evidence further indicate an association between lysosomal cholesterol accumulation and inflammation. Lysosomal acid lipase (LAL) is essential for the hydrolysis of triglycerides and cholesteryl esters in lysosomes. Similar to our observations in hyperlipidaemic mice, a deficiency of this enzyme leads to lysosomal cholesterol accumulation and inflammation in multiple organs⁹. Aberrant expression levels of multiple inflammatory cytokines and chemokines were monitored in association with

Lal^{-/-} macrophages. Moreover, administration of exogenous LAL to *Ldlr*^{-/-} mice on an HFC diet led to significant reductions in inflammation and steatosis in the livers of these mice¹⁰. Furthermore, lysosomes have been assigned a central role in many processes involving tissue injury and inflammation¹¹. Finally, it was recently demonstrated that modified LDL has the potential to damage lysosomal membranes¹². Endocytosed oxLDL partially inactivates lysosomal enzymes and destabilizes the acidic vacuolar compartment, causing relocation of lysosomal enzymes to the cytosol¹³ and activation of the NLRP3 inflammasome¹⁴.

Figure 7.8 Electron microscopy of bone marrow derived macrophages after loading with modified LDL. (A) Representative picture of a bone marrow derived macrophage of a Wt-tp mouse. (B) Representative picture of a bone marrow derived macrophage of a *Cyp27*^{-/-}-tp mouse.

LXRs together with other members of the nuclear receptor superfamily contribute to cellular cholesterol homeostasis by regulating genes that contribute to the storage, transport and catabolism of cholesterol¹⁵. In this manner, cytotoxicity due to excess cholesterol accumulation in macrophages can be prevented. In response to cholesterol loading in macrophages, 27HC has been proposed to be an important physiological LXR ligand^{16,17}. Interestingly, 27HC is found in significantly high amounts in foam cells of atherosclerotic plaques, where it is thought to eliminate the excess cholesterol by stimulating reverse cholesterol transport via LXR and inhibiting cholesterol synthesis and uptake via SREBP¹⁸. Similarly, it has been reported that in *Npc1*^{-/-} macrophages, 27HC deficiency is responsible for decreased LXR activation, thereby impairing cholesterol efflux and reduced ABCA1 protein expression^{7,19,20}. Nevertheless, we observed increased expression of *Lxr*, *Abca1* and *Abcg1* along with a reduction in the size of the KCs, indicating the opposite effect. A possible explanation for the decreased foamy appearance of KCs in *Cyp27*^{-/-}-tp mice is the increased expression of other cholesterol derivatives that serve as LXR ligands and therefore increased cholesterol efflux and decreased foam cell formation.

In humans, *Cyp27A* deficiency leads to cerebrotendinous xanthomatosis (CTX). CTX is associated with the accumulation of cholesterol and cholestanol in many organs. However, *Cyp27*^{-/-} mice were reported to lack the classic symptoms of CTX²¹. They had either normal plasma lipid levels, similar to CTX patients^{21,22}, or were hyperlipidemic²³. These findings are supported by our data, as we found that plasma cholesterol levels did not differ between *Cyp27*^{-/-}-tp mice compared to Wt-tp mice on a normal diet. However, after feeding with the HFC diet, cholesterol synthesis and plasma cholesterol levels were dramatically decreased in *Cyp27*^{-/-}-tp mice compared to Wt-tp mice. These findings indicate that the HFC diet induced the differences in plasma lipid levels between the two transplanted models. Similar to our observations, Zhang *et al.* demonstrated that in *Npc1*^{-/-}-tp *Ldlr*^{-/-} mice, serum cholesterol and triglycerides were reduced after feeding an HFC diet in the presence of increased aortic atherosclerosis²⁴. Nevertheless, despite the improved cholesterol metabolism in *Cyp27*^{-/-}-tp mice, hepatic inflammation was still increased. Thus, the observed inflammation in *Cyp27*^{-/-}-tp occurs despite the reduction in cholesterol levels within the KCs and the plasma.

Frolov *et al.* found that treatment of *Npc*^{-/-} fibroblasts with 27HC corrected the NPC phenotype, lowering the accumulation of free cholesterol in the lysosomes⁷. One possible explanation for this phenomenon is that the added oxysterol eliminates the excess cholesterol in the cytoplasm and therefore cholesterol is transferred from lysosomes to the cytoplasm to maintain homeostasis. However, intriguing recent findings that 27HC binds to the N-terminal luminal loop of NPC1 also suggest a direct molecular link between oxysterols and NPC function²⁵. Along with the increased lysosomal cholesterol storage in *Cyp27*^{-/-}-tp mice on the HFC diet, electron microscopy

revealed increased lysosomal damage, as indicated by the disturbed lysosomal membranes and cholesterol precipitation. Moreover, in our *Cyp27^{-/-}* cultured bone marrow macrophages, we observed increased lysosomal cholesterol accumulation in the form of cholesterol crystals, and increased inflammation upon loading with modified cholesterol. Recently, it was shown that lysosomal damage and crystallization could activate the NALP3 inflammasome and subsequently lead to inflammation^{14,26,27}. According to these studies, cholesterol-induced inflammasome activation may represent an important link between cholesterol metabolism and inflammation. In the present study, lysosomal cholesterol accumulation and destabilization was associated with increased hepatic inflammation. These data provide a mechanism by which lysosomal cholesterol accumulation can act as a trigger for hepatic inflammation.

To date, it has not been clear why not all individuals with hepatic steatosis develop inflammation. Our results indicate that lysosomal cholesterol accumulation in KCs is likely to be an important trigger for hepatic inflammation. Moreover, our discovery points towards the potential for 27HC to be used as a novel therapy for NASH.

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Chapter 8

Pneumococci: a new treatment for non-alcoholic steatohepatitis?

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This chapter includes confidential information

Abstract

Background

Non-alcoholic steatohepatitis (NASH) is a chronic liver disease that can progress from lipid accumulation, to liver inflammation and ultimately to cirrhosis. Recently, we demonstrated that the deletion of both scavenger receptors (SRs) CD36 and SR-A from haematopoietic cells reduced hepatic inflammation during NASH. In addition to the uptake of modified lipoproteins, CD36 and SR-A are involved in other functions that can also trigger the inflammatory response. Therefore, the actual trigger for SR activation during NASH is not clear. We hypothesized that hepatic inflammation is triggered by uptake of oxLDL by Kupffer cells (KCs). Increased levels of autoantibodies to epitopes of oxidized LDL (oxLDL) have been shown to block uptake of oxLDL by macrophages. Many autoantibodies against oxLDL share complete genetic and structural identity with antibodies from infectious pathogens, such as *S. pneumoniae*.

Approach

To investigate whether autoantibodies against oxLDL have a protective phenotype in the liver after high fat high cholesterol (HFC) feeding, female *Ldlr*^{-/-} mice were immunized for 9 weeks with heat-inactivated pneumococci. The mice received HFC diet during the last 3 weeks of the immunization protocol.

Results

Immunization of *Ldlr*^{-/-} mice with heat-inactivated pneumococci led to a reduction in hepatic inflammation, as shown by reduced macrophage, neutrophil and T-cell infiltration, and reduced gene expression of *Tnf*, *Il-6*, *Il-1b* and *Mcp1*. No decrease in steatosis was observed. In addition, pneumococcal immunization reduced the total plasma cholesterol level and fibrosis-related gene expression. CD68 positive liver sections and electron microscopy revealed a reduction in the size of foamy KCs in immunized *Ldlr*^{-/-} mice. Moreover, the KCs showed less lysosomal cholesterol accumulation and crystallization compared to the non-immunized mice on the HFC diet.

Conclusion

These results indicate that oxidized cholesterol plays an important role in diet-induced NASH and that pneumococcal immunization could lead to a vaccination protocol towards the therapy of NASH in humans.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is a condition ranging from benign lipid accumulation in the liver (steatosis) to steatosis combined with inflammation. The latter is referred to as non-alcoholic steatohepatitis (NASH). NASH is viewed as the hepatic component of metabolic syndrome. Estimates from the USA are that 5.7% to 17% of all adults have NASH, while 17% to 33% of Americans have NAFLD^{1,2}. As obesity and insulin resistance reach epidemic proportions in industrialized countries, the prevalence of both NAFLD and NASH is increasing and is therefore considered to be a major health hazard³. Steatosis alone is considered a relatively benign and reversible condition. However, the transition towards NASH represents a key step in the pathogenesis, as it sets the stage for further damage to the liver, such as fibrosis, cirrhosis and liver cancer. While the mechanisms leading to steatosis are well described, little is known about the actual risk factors that drive hepatic inflammation during the progression to NASH. Consequently, therapeutic options are poor. Therefore, knowledge about the events that lead to hepatic inflammation is of great importance for the diagnosis and treatment of NASH.

Recently, many observations have indicated the importance of dietary cholesterol in NASH⁴⁻⁸. We have previously shown that hepatic inflammation is correlated with the appearance of foamy Kupffer cells (KCs) (the resident macrophages of the liver), analogous to foamy macrophages in atherosclerosis⁵. Cholesterol-loaded foamy cells are known to be formed *in vitro* by uptake of modified (i.e. acetylated, oxidized) cholesterol-rich low density lipoproteins (LDL) via scavenger receptors. Foam cells secrete a variety of molecular mediators that can further evoke the inflammatory response⁹. To study the role of foamy KCs in hepatic inflammation, we transplanted donor bone marrow from mice lacking scavenger receptors (CD36 & SR-A) to hyperlipidemic recipient mice. These animals showed significantly reduced hepatic inflammation¹⁰. The reduction in inflammation was associated with reduced lysosomal cholesterol accumulation and increased cholesterol in the cytoplasm of KCs. In addition to uptake of modified lipids, scavenger receptors are involved in many other pathways that can directly affect inflammation. These pathways include cellular adhesion, innate immune responses and phagocytosis of apoptotic cells¹¹. Based on the analogy between mechanisms for atherosclerosis and NASH, it is likely that uptake of oxLDL into KCs, rather than other pathways, is the actual trigger for scavenger-receptor-mediated inflammation. Therefore, we hypothesized that hepatic inflammation is triggered by uptake of oxLDL by KCs.

It has recently been shown that the levels of IgM autoantibodies to modified LDL were inversely correlated with atherosclerosis^{12,13}. These natural antibodies (Nabs) arise spontaneously without prior infection or immune exposure and mainly consist of the IgM isotope. These IgM Nabs are of B-1 cell origin, which are known to provide a first

line defense against bacterial and viral pathogens¹⁴. A growing body of evidence supports the notion of oxLDL being recognized as an antigen. Upon oxidation of LDL, reactive oxidation products from phospholipids retain the intact phosphorylcholine (PC) headgroup. These PC headgroups represent the so-called 'oxidation-specific' epitopes and are found on the outer side of the membrane, triggering an innate immune response¹⁵. A panel of monoclonal autoantibodies directed to epitopes of oxLDL was cloned from the spleen of *apoE*^{-/-} mice¹⁶. In particular, one immunodominant clonotypic set of IgM autoantibodies was identified, EO6, which bears the T15 idiotype and which was shown to specifically bind to the PC moiety of oxidized PC-containing phospholipids, such as those present in oxLDL¹⁵. Interestingly, EO6 antibodies were found to be identical to the natural T15 antibodies derived from B-1 cells. These T15 antibodies, which are known to protect mice against *Streptococcus pneumoniae* infections, recognize the same epitope present on oxLDL since the cell wall of this bacterium contains the same PC group. Accordingly, by immunizing *Ldlr*^{-/-} mice with *S. pneumoniae*, higher titers of serum anti-oxLDL antibodies were measured. In addition, increased IgM immune complexes with apoB-containing particles were found in the plasma of these *Ldlr*^{-/-} mice immunized with *S. pneumoniae*, suggesting that IgM Nabs bind oxLDL *in vivo*¹⁷. These findings support the hypothesis that some anti-oxLDL antibodies are directed to the PC group present on oxLDL, possibly inhibiting the uptake of oxLDL by macrophage scavenger receptors, such as CD36.

The aim of the study was to determine whether oxLDL is causally involved in the pathogenesis of NASH. To increase the serum anti-oxLDL antibody levels, the immune system of mice was boosted with heat-inactivated pneumococci and afterwards NASH was induced by a HFC diet. According to our hypothesis, immunized *Ldlr*^{-/-} mice showed reduced hepatic inflammation compared to non-immunized mice, indicating the induction of a protective immune response after immunization with heat-inactivated pneumococci. Thus, these data clearly demonstrate the importance of oxLDL in the pathogenesis of NASH.

Materials and methods

Preparation of immunogen

For immunization, the heat-inactivated R36A strain of *Streptococcus pneumoniae* (Birmingham, Alabama) was used, still bearing the PC headgroup epitope similar to oxLDL. Colonies of the R36A strain were harvested at mid log phase after incubation at 37°C on blood agar plates and transferred to Todd-Hewitt plus 0.5% yeast broth. The mid log phase is characterized by an OD value of 0.425 to 0.45 at 600 nm. *S. pneumoniae* was heat-inactivated at 60°C for 30 min, after which no colonies of this suspension were detected on blood agar plates. For freezer stocks, small aliquots of *S.*

pneumoniae at mid log density were harvested and suspended in Todd-Hewitt plus 80% sterile glycerol and stored at -80°C .

Mice, immunization and diet

Ldlr^{-/-} mice on a C57Bl/6 background were housed under standard conditions and had access to food and water ad libitum. Experiments were performed according to Dutch laws, approved by the Animal Experiment Committee of Maastricht University. The immunization protocol started in 12-week-old female mice, fed a normal chow diet. Mice were divided into four groups (n=10 for each group) and received 10^8 CFU of pneumococcal immunogen emulsified in 200 μl sterile 0.9% NaCl for the primary subcutaneous immunization, after which three intraperitoneal booster immunizations were administered at two-week intervals. The control group received a NaCl injection only. After immunization, the mice were given normal chow, forming the control group, or a high fat cholesterol diet (HFC diet), forming the experimental group, for 3 weeks. Blood from the tail vein was collected after the dietary period and mice were then sacrificed by cervical dislocation. Liver tissue was isolated and snap-frozen in liquid nitrogen and stored at -80°C or fixed in 4% formaldehyde/PBS.

Gene expression analysis

Total RNA was isolated from mouse liver tissues by homogenizing frozen liver tissues together with 1.0 ml Tri Reagent (Sigma Aldrich, Saint Louis, USA) and 1.0 mm of glass beads in a closed tube for 30 s at 4800 rpm. After centrifugation with the addition of 200 μl chloroform, an aqueous phase was visible and was transferred to a fresh tube. Isopropanol (0.5 ml) was added and upon another centrifugation step, RNA was precipitated. The RNA pellet was washed by adding 1.0 ml 70% ethanol. After centrifugation, the supernatant was removed and the pellet was dissolved in an appropriate volume of DEPC sterile H_2O . All the materials used were RNase free and the samples were placed on ice during the procedure. Afterwards the RNA concentration and RNA quality were determined on a NanoDrop ND-1000 spectrophotometer.

Total RNA (500 ng) from each individual mouse was converted into first strand complementary DNA (cDNA) with an iScript cDNA synthesis kit (Bio-Rad, Hercules, USA). Subsequently, changes in the gene expression of inflammatory markers were determined by quantitative PCR (qPCR) on a Biorad MyIQ with IQ5 v2.1 software using IQ SensiMix™ SYBR with Fluorescein (Quantace, London, UK) or on an Applied Biosystems 7900HT with SensiMix™ SYBR (Quantace, London, UK), which is capable of screening more samples during one single run, and 10 ng of cDNA. For each gene, a standard curve was generated with a serial dilution of a liver cDNA pool with a known concentration. To standardize for the amount of cDNA, Cyclophilin A was used as the reference gene. Primer sets for the selected gene were developed with Primer Express

v2.0 (Applied Biosystems, Foster City, USA) using default settings. Data from qPCR were analysed according to the comparative Ct quantities.

Lipid analysis

Liver lipid analysis: approximately 50 mg of frozen liver tissue was homogenized for 30 s at 4800 rpm in a closed tube with 1.0 mm glass beads and 1.0 ml SET buffer (sucrose 250 mM, EDTA 2 mM and Tris 10 mM). Complete cell destruction was achieved by two freeze-thaw cycles and two passes through a 27-gauge syringe needle. Protein content was measured via the BCA method (Pierce, Rockford, USA). Cholesterol, triglycerides and free fatty acids levels were measured with enzymatic colour tests (cholesterol CHOD-PAP, Roche, Basel, Switzerland; TG GPOtrinder, Saint Louis, USA; NEFAC, ACS-ACOD, Wako Chemicals, Neuss, Germany) according to the manufacturers' protocols and measured on a Benchmark 550 micro-plate reader (Bio-Rad, Hercules, USA). Plasma lipid analysis: blood collected from the tail vein was centrifuged for 3 min at 3500 rpm and plasma supernatant was transferred to a fresh tube. Plasma cholesterol, triglycerides and free fatty acids were measured with the same enzymatic colour tests described above using a Benchmark 550 micro-plate reader (Bio-Rad, Hercules, USA).

Liver histology

Frozen liver sections (7 μ m) were fixed with acetone and blocked against endogenous peroxidase by incubation with 0.25% of 30% H₂O₂ for 5 minutes. Primary antibodies were used against infiltrated macrophages (macrophage marker, Mac-1), CD68 Kupffer cells (CD68 marker, FA11), T cells (T cell marker, KT3), fibroblasts (fibroblast marker, ERTR7) and neutrophils (neutrophil marker, NIMP). For NIMP staining no amplification step was needed as a secondary antibody, α -rat-PO, was used. Prior to incubation with the first antibody, slides were incubated with 4% fetal calf serum (FCS), 1x PBS plus an amplification step: 1:5 Avidin D Block solution (ABC kit, Vector Laboratories, USA). Primary antibodies were dissolved in 4% FCS, 1x PBS + 1st antibody (1:1000 for Mac1, 1:200 for NIMP, 1:100 for CD68 and 1:5 for CD3) plus an amplification step: 1:5 Biotin Block solution (ABC kit, Vector Laboratories, USA). Incubation with the 2nd antibody 1:300 for α -rat-BIO (1:100 for α -rat-PO) was carried out in 4% FCS, 2% normal mouse serum (NMS) and 1x PBS. Afterwards the slides were washed and incubated for an additional amplification step in 1x PBS + 1:50 Avidin D solution + 1:50 Biotin solution (ABC kit, Vector Laboratories, USA). Then, 13-amino-9-ethylcarbazole (AEC) was applied as the colour substrate using an AEC kit (2% buffer, 3% AEC, 2% H₂O₂ in demi water) and haematoxylin for nuclear counterstaining. Sections were enclosed with Faramount aqueous mounting medium.

Paraffin-embedded liver sections (4 μ m) were stained with Haematoxylin-Eosin (Haematoxylin, Klinipath, Duiven, The Netherlands and Eosin, Sigma-Aldrich, Saint

Louis, USA) after deparaffination in xylol and dehydrated with 90%, 70% and 50% ethanol respectively. For Sirius Red staining the liver sections were first fixed in Susa fixative after deparaffination. Subsequently, sections were stained with Sirius Red (Sigma-Aldrich, Saint Louis, USA), washed with 0.01 M HCl and dehydrated. Sections were mounted with Entellan.

Pictures were taken with a Nikon Digital Camera DMX1200 and ACT-1 v2.63 software. Positive stained cells were counted in six microscopic views (magnification 200x) and were recorded as cells/mm². CD68 staining was scored as follows: mild foamy appearance (score 1), moderate (score 2) and severe foamy appearance (score 3). The Sirius Red and ERTR7 staining was scored as follows: no fibrosis (score 1), mild (score 2), moderate (score 3) and severe fibrosis (score 4). Scores for the level of fibrosis were given both around and in between blood vessels, and the mean was calculated.

For electron microscopy, liver sections were first perfused with glutaraldehyde. When perfusion was complete, tissue was stored in glutaraldehyde fixative. Then, liver sections were washed in cacodylate buffer to remove the glutaraldehyde fixative followed by post-fixation in 1% osmium tetroxide for 1 hour. After this fixation, the osmium fixative was washed off using buffer before the liver sections were placed in alcohol (70%–100%). Further steps in the preparative procedure were then followed.

Statistical analysis

The data were statistically analysed by performing two-tailed non-paired *t*-tests using GraphPad Prism, version 4.03 for Windows. Data were expressed as the mean $\pm$ SEM and considered significant at $p < 0.05$. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively.

Results

Increased IgM antibody titers against modified LDL after immunization with heat-inactivated pneumococci

Immunization of *Ldlr*^{-/-} mice with heat-inactivated pneumococci resulted in a strong increase in IgM titers to oxLDL, which was not observed in non-immunized *Ldlr*^{-/-} mice (Figure 8.1A). Only weak, although significant IgG responses were observed, consistent with previous reports that pneumococcal immunizations induce a thymus-independent type-2 response that is highly specific for phosphorylcholine (Figure 8.1B).

To define the effect of immunization on the general health condition, relative weight gain as well as liver weight were monitored. The percentage of relative weight gain was higher in immunized *Ldlr*^{-/-} mice fed on the HFC diet compared to non-immunized mice. The relative weight gain did not differ between immunized and non-immunized mice fed on chow (Figure 8.1C). The liver weights were calculated relative to the total body weight. On the chow diet, relative liver weight was significantly lower in immunized *Ldlr*^{-/-} mice compared to non-immunized mice. On the HFC diet, no differences in relative liver weights were observed between immunized and non-immunized mice (Figure 8.1D).

Figure 8.1 Pneumococcal immunization induces anti-oxLDL antibodies. (A+B) Antibodies against IgM and IgG respectively. (C) Percentage of relative weight gain. (D) Liver weight relative to total body weight. (*, ** and ***: $p < 0.05$; 0.01; 0.001 respectively)

No difference in liver lipid levels between immunized and non-immunized *Ldlr*^{-/-} mice after 3 weeks of HFC diet

To investigate liver lipid levels in hyperlipidemic mice with or without immunization, biochemical assessment of liver cholesterol, triglycerides (TG) and free fatty acids (FFA) was carried out (Figure 8.2A). After 3 weeks on the HFC diet, a clear increase in all liver lipid levels was observed compared to mice on a chow diet. Liver lipid levels did not differ between immunized and non-immunized *Ldlr*^{-/-} mice on the HFC diet. For those on the chow diet, there was a small increase in liver lipid levels after immunization when compared to non-immunized *Ldlr*^{-/-} mice. Oil red O staining confirmed the biochemical liver lipid measurements (Figure 8.2B–E).

Figure 8.2 Liver lipid levels. (A) Liver cholesterol, triglycerides (TG) and free fatty acids (FFA) after chow and 3 weeks of HFC diet. (B–E) Oil red O staining after 3 weeks of HFC diet in non-immunized (B+D) and immunized (C+E) *Ldlr*^{-/-} mice after chow (B+C) and 3 weeks of feeding on the HFC diet (D+E), respectively. * Significantly different from the mice on chow diet. (*, ** and ***: $p < 0.05$; 0.01 ; 0.001 respectively)

Decreased plasma cholesterol in immunized *Ldlr*^{-/-} mice on the HFC diet compared to control mice

The effect of immunization on plasma lipids was assessed by measuring the levels of plasma cholesterol, triglycerides (TG) and free fatty acids (FFA). After feeding on the HFC diet, a significant increase was observed for all plasma lipids compared to mice on a chow diet. Plasma cholesterol was reduced in immunized *Ldlr*^{-/-} mice compared to non-immunized mice on the HFC diet. Plasma TG and FFA did not differ between the models following the HFC diet. On chow diet, plasma lipid levels did not differ between the models (Figure 8.3).

Figure 8.3 Plasma lipid levels. Plasma cholesterol, triglycerides and free fatty acids after chow and 3 weeks of HFC diet in non-immunized and immunized $Ldlr^{-/-}$ mice.

Decreased hepatic inflammation in $Ldlr^{-/-}$ mice immunized with heat-inactivated pneumococci

To determine whether immunization of $Ldlr^{-/-}$ mice with heat-inactivated pneumococci affects hepatic inflammation, liver sections were stained for the inflammatory cell markers Mac-1 (infiltrated macrophages and neutrophils), NIMP (neutrophils) and CD3 (T cells). As shown in Figure 8.4A, the amount of infiltrated macrophages, neutrophils and T cells was lower in immunized $Ldlr^{-/-}$ mice compared to non-immunized mice after feeding on the HFC diet. Moreover, the HFC diet induced a significant increase in the number of neutrophils in immunized chow-fed mice compared to non-immunized mice. Representative histological pictures of the Mac-1 staining for all four experimental groups are shown in Figure 8.4B-E. To further confirm the reduced hepatic inflammation in immunized $Ldlr^{-/-}$ mice on the HFC diet, liver gene expression analysis showed a significant decrease in the inflammatory markers tumour necrosis factor (*Tnf*), interleukin-1beta (*Il-1b*), interleukin 6 (*Il-6*) and monocyte chemo-attractant protein-1 (*Mcp1*) in immunized $Ldlr^{-/-}$ mice on the HFC diet compared to non-immunized mice (Figure 8.4F). However, hepatic inflammation in $Ldlr^{-/-}$ mice on the HFC diet after immunization was still higher than chow-fed immunized mice according to the inflammatory markers *Tnf*, *Il-1b* and *Mcp1*.

Figure 8.4 Parameters of hepatic inflammation. (A) Liver sections were stained for infiltrated macrophages and neutrophils (Mac-1), neutrophils (NIMP) and T cells (CD3), respectively, and counted. (B+E) Representative pictures of Mac-1 staining (200x) after feeding on the (B+C) chow and (D+E) HFC diet in (B+D) non-immunized and (C+E) immunized *Ldlr*^{-/-} mice, respectively. (F) Gene expression analysis for tumour necrosis factor (*Tnf*), interleukin 6 (*Il6*) and β (*Il1b*) and monocyte chemoattractant protein 1 (*Mcp1*). * Significantly different from the mice on chow diet. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively.

After 3 weeks of HFC diet, expression of fibrosis-related genes is reduced in immunized *Ldlr*^{-/-} mice compared to non-immunized *Ldlr*^{-/-} mice

Fibrosis is considered to be an advanced stage of NASH. Collagen staining (Sirius Red) (Figure 8.4A) was performed to determine the degree of fibrosis. No differences were observed between the experimental groups after 3 weeks of HFC diet (Figure 8.5A). Gene expression analysis for collagen type 1A1 (*Col1A1*) and transforming growth factor beta (*Tgf-β*) indicated that the mRNA levels of these genes were lower in immunized mice compared to non-immunized mice on the HFC diet (Figure 8.5B).

Figure 8.5 Parameters of hepatic fibrosis. (A) Quantification of sirius red (collagen) after 3 weeks of HFC diet. Livers were quantified as minimal, mild or moderate positive for collagen around and in between the blood vessels of the liver. (B) Gene expression analysis of the fibrosis markers, collagen (*Col1a1*) and transforming growth factor beta (*Tgf-β*). * Significantly different from the mice on chow diet. *, ** and *** indicate $p < 0.05$, 0.01 and 0.001 respectively.

Decreased foamy appearance of Kupffer cells in immunized *Ldlr*^{-/-} mice on the HFC diet

Immunohistochemistry for CD68 was performed to establish the foamy appearance of KCs. Scoring of the CD68 positive sections revealed a reduction in the size of foamy KCs in immunized *Ldlr*^{-/-} mice compared to non-immunized mice on the HFC diet (Figure 8.6A+C-F). Gene expression of *Cd68* was reduced in the immunized *Ldlr*^{-/-} mice compared to non-immunized mice on the HFC diet (Figure 8.6B). No differences in *Cd68* expression were observed between the immunized and non-immunized group fed a chow diet. Electron microscopy of KCs confirmed the differences in size of the KCs between immunized and non-immunized *Ldlr*^{-/-} mice, and showed that the non-immunized mice had more lysosomal cholesterol accumulation and crystallization compared to immunized mice on the HFC diet (Figure 8.7).

Figure 8.6 Foamy Kupffer cells. (A) Liver sections were stained for CD68 (Kupffer cells) and scored for the level of foamy appearance: 1 (mild foamy appearance) to 3 (severe foamy appearance). Mean scores were calculated from six microscopic views. (B) Gene expression analysis of the Kupffer cell activation marker, *Cd68*. (C-F) Representative images of liver sections stained for CD68 for the *Ldlr*^{-/-} mice on a chow diet with and without immunization and for the *Ldlr*^{-/-} mice on the HFC diet with and without immunization respectively at a 200x magnification. * Significantly different from the mice on chow diet. * and *** indicate $p < 0.05$ and 0.001 respectively.

Figure 8.7 Electron microscopy of foamy Kupffer cells. Acid phosphatase staining indicating the lysosomes of the Kupffer cells in (A) non-immunized and (B) immunized *Ldlr*^{-/-} mice on the HFC diet.

Discussion

Until now, the actual risk factors that drive hepatic inflammation during the progression to NASH have been unknown. However, by increasing serum anti-oxLDL antibody levels with heat-inactivated pneumococci, hepatic inflammation was dramatically decreased. These data point towards oxLDL as a trigger for hepatic inflammation. Furthermore, our data suggest that pneumococcal immunization could lead to a vaccination protocol towards the therapy of NASH in humans.

Scavenger-receptor-mediated uptake of oxidized lipoproteins by macrophages sets off a cascade of pro-inflammatory events leading to the initiation of the inflammatory response. OxLDL is phagocytosed by macrophages via binding of the oxPC molecules present in oxLDL to macrophage scavenger receptors, and changes the macrophages into foam cells^{18,19}. Previously, we have shown that both CD36 and SR-A play an important role in diet-induced NASH²⁰. Targeted deletion of both SRs on haematopoietic cells demonstrated reduced hepatic inflammation during NASH, despite unchanged total cellular cholesterol and foam cell formation¹⁰. Since scavenger receptors have a wide spectrum of functions²¹⁻²³, it was not clear whether the uptake of modified lipoproteins is the actual trigger for hepatic inflammation during NASH.

Our novel data demonstrate that inflammation is reduced in the livers of heat-inactivated pneumococci-immunized mice. These results are in line with Binder *et al*, who demonstrated decreased atherosclerotic lesion formation after pneumococcal immunization¹⁷. The inflammatory process associated with atherosclerotic plaque formation is linked to the cytotoxicity and macrophage chemo-attractivity of oxLDL. Moreover, oxLDL is thought to be an atherogenic factor due to its localization in macrophage-derived foam cells accumulated in human atherosclerotic lesions^{18,19,24}. In a similar study, *apoE*^{-/-} mice were immunized with PC, one of the epitope of anti-oxLDL autoantibodies present on the outer membrane of oxLDL, but also *S. pneumoniae*. After PC immunization, an increase in anti-oxLDL autoantibodies was observed together with a reduction in atherosclerotic lesions, suggesting that these specific autoantibodies against oxLDL prevented uptake of oxLDL by macrophages²⁵. Therefore, our data provide evidence towards the similarity of mechanisms causing atherosclerosis and NASH. This proposed protective mechanism for heat-inactivated R36A was shown to be related to inhibition of the binding and uptake of oxLDL to macrophages *in vitro*^{14,17,26,27}. Thus, the reduced inflammation in mice in which the scavenger receptors on haematopoietic cells had been deleted is likely to be related to the reduced uptake of oxLDL into KCs.

Interestingly, plasma cholesterol levels were significantly reduced in our immunized *Ldlr*^{-/-} mice. However, it is the first time that immunization with heat-inactivated pneumococci is causing such a dramatic reduction in plasma cholesterol levels. The formation of immune complexes may already have protective properties by retaining lipoproteins containing both LDL and oxLDL particles largely in the circulation. Whether this would promote clearance is questionable, as recent studies by Reardon *et al* showed that the apparent clearance rates of infused oxLDL did not differ between immunocompetent *apoE*^{-/-} mice and *apoE*^{-/-} mice that lacked any antibodies as a result of an additional deficiency of recombinase activating gene-2²⁸. However, plasma cholesterol levels have been shown to be an important cause in the development of hepatic inflammation⁵. Therefore, it is likely that the protective effect of these

antibodies *in vivo* is also partly modulated via a reduction in plasma cholesterol levels, by a mechanism that is still unclear.

As NASH patients are often associated with high levels of lipid peroxidation products such as those present in oxLDL, it has been suggested that the elevated levels of lipid peroxidation might make an important contribution to the pathogenesis of NASH^{29,30}. Ikura *et al.* demonstrated that oxidized phosphatidylcholines (oxPC) were found predominantly in steatotic hepatocytes and macrophages/KCs and were more abundant in NAFLD/NASH livers than in normal control livers³¹. As oxidative stress has been implicated as a key factor contributing to hepatic injury in patients with NASH^{32,33}, vitamin E therapy of non-diabetic NASH patients was associated with a significantly higher rate of improvement in NASH³⁴. Moreover, Gupte *et al* demonstrated that the combination of aging and metabolic stress appears to induce mitochondrial dysfunction and impaired anti-oxidant responses, resulting in excess oxidative stress and tissue injury³⁵.

Although hepatic inflammation was significantly lower in our immunized *Ldlr*^{-/-} mice on the HFC diet, steatosis was not affected, suggesting that steatosis and inflammation are two unconnected events. This observation contradicts the proposed classic 'two-hit' model, according to which liver steatosis itself is the causal factor of inflammation. Similarly, our group has previously shown that hyperlipidaemic mouse models develop hepatic inflammation after only 2 days of HFC diet, even without the presence of steatosis³⁶. These data are in line with others that demonstrated that TG do not play the key role as a first hit in NASH^{37,4,38}. As fibrosis is one of the later consequences of NASH, we investigated the effect of immunization with heat-inactivated pneumococci on hepatic fibrosis. Gene expression of fibrosis-related genes was decreased, yet not confirmed by Sirius Red and ERT7 staining. This is probably due to the short time period of 3 weeks on the HFC diet, as *Ldlr*^{-/-} mice only develop fibrosis after 3 months on a mild atherogenic diet³⁹. However, we report for the first time that uptake of oxLDL is associated with liver fibrosis *in vivo*. In line with these observations, a study by Kang *et al* demonstrated that oxLDL can activate hepatic stellate cells *in vitro*^{40,41}. These findings indicate a crucial role for oxLDL in the fibrogenic process.

In the present study, immunization of HFC-fed *Ldlr*^{-/-} mice with a pneumococcal vaccine induced high oxLDL-specific IgM antibodies. Although hepatic inflammation was reduced, immunized mice had a significantly higher percentage of total body weight gain compared to non-immunized mice after feeding on the HFC diet. These findings contrast with those of Binder *et al.*¹⁷ and indicate a dissociation between hepatic inflammation and weight gain. In general, obesity is associated with increased circulating inflammatory mediators and increased macrophage accumulation⁴²⁻⁴⁴. Therefore, it is unclear why immunized *Ldlr*^{-/-} mice on the HFC diet have increased body weight compared to non-immunized mice.

As expected, immunized mice on the HFC diet showed decreased foamy KCs compared to non-immunized mice. This reduction in size is probably due to the decreased plasma cholesterol levels, as the size of the foamy KCs is not always correlated with the inflammatory state of the liver^{5,20}. Therefore, the intracellular distribution of cholesterol rather than the total amount of cholesterol present in the KCs, is the actual trigger for hepatic inflammation. In oxLDL-loaded macrophages, most of the cholesterol appears to be trapped in lysosomes. Enrichment of macrophages with oxLDL promotes lysosomal cholesterol accumulation by both sequestration of free cholesterol and inhibition of cholesterylester hydrolysis in oxLDL particles. Therefore, oxLDL-loaded macrophages mimic two important features of foam cells: lysosomal lipid accumulation and irreversible sequestration. A consequence of oxLDL loading is that the cholesterol within the lysosomes appears to be trapped and is not available for efflux or extralysosomal metabolism⁴⁵⁻⁴⁷. Moreover, lysosomal overloading with oxLDL will lead to the formation of cholesterol monohydrate crystals within lipid-filled lysosomes⁴⁸. In line with these findings, we showed that after immunization with heat-inactivated pneumococci, KCs were less foamy, had less lysosomal cholesterol accumulation and therefore also less crystallization. Our data indicate that the increased lysosomal cholesterol accumulation, together with the crystallization, is linked to hepatic inflammation.

To date, no therapy for NASH is available. Our novel data clearly suggest that future research should focus on oxLDL as a trigger for NASH. Moreover, these data clearly demonstrate the potential of pneumococcal immunization to be used as a novel tool for the prevention and therapy of NASH.

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Chapter 9

General discussion

Currently, it is still not clear why patients with simple steatosis do not always progress into NASH. In the present thesis, we aimed to establish the mechanisms by which hepatic inflammation is triggered during NASH. The major findings of the studies presented in this thesis will be described in the context of our current knowledge and some suggestions for further research will be given.

Central role for Kupffer cells in NASH

Kupffer cells are the resident macrophages of the liver and constitute approximately 35% of the hepatic non-parenchymal cells in normal adult mice¹. Their anatomical position in the sinusoidal spaces of the liver permits them to act as immune sentinels for portal (gut) and systemic circulations. Their proximity to parenchymal cells presents opportunities for them to influence glucose and lipid metabolism and potentially to respond to parenchymal cell-derived signals. The activation of KCs can best be described as a wide spectrum of gradually differing alterations of the KC phenotype, resulting from a complex interplay between various activators and signalling pathways². The KCs contribute to the pathogenesis of various liver diseases, including non-alcoholic fatty liver diseases. Despite the fact that KCs comprise 10% of all liver cells, we have demonstrated their importance in NASH by performing bone marrow transplantations where the exact contributions of infiltrating inflammatory cells and of activated KCs were identified. In all of the studies where a bone marrow transfer was used to modulate KC function, we were able to clearly demonstrate remarkable effects on hepatic inflammation, indicating the importance of KCs in NASH. In line with this conclusion, it was shown that the removal of KCs has substantial effects on the innate immune response in the liver. For example, KC inactivation or depletion can prevent the development of steatosis and inflammation^{3,4}. In choline-deficient-diet-induced fatty liver, KC depletion reduced liver IL12 expression, suggesting that KCs are important cellular sources of IL12 in the liver in NAFLD. In addition, IL12 promotes the production of hepatic Th1-associated cytokines and is involved in hepatic NKT cell depletion⁵.

Next to IL12, activated KCs can also produce TNF. While visceral adipose tissue can also contribute to hepatic TNF, KCs are the primary source of hepatic TNF, especially in obese human subjects⁶. Indeed, in the present thesis, we observed a considerably reduced hepatic TNF expression in most studies where hepatic inflammation was reduced. The activation of KCs can also trigger liver damage by increasing the release of lipid peroxidation products and cytokines⁷, which generate hepatic oxidative stress. Furthermore, fibrosis, which is one of the later stages of NASH, has also been shown to be related to the activation of KCs, producing TGF- β ⁸. Finally, NASH patients show often high levels of lipid peroxidation products such as oxLDL^{9,10}, further supporting the idea that activated KCs are important in NASH. Moreover, we also proved that

MPO, an aggressive oxidant-generating neutrophil enzyme, plays an important role in the progression of NASH (Chapter 6). In conclusion, these data indicate the major contribution of KCs to NASH and the potential of KCs to be used as a target for cell-specific therapy.

Recently, a study by Kinoshita *et al.* divided mouse KCs into two subpopulations: a CD68+ subset with phagocytic activity and a CD11b+ subset with cytokine-producing capacity¹¹. It was found that CD68 participates in the endocytosis of LDL and it may also be involved in the phagocytosis of other antigens. The CD11b+ KCs produce a large amount of pro-inflammatory cytokines that can activate other phagocytes/lymphocytes and, consequently, activated neutrophils and CD68+ KCs might effectively kill bacteria. A similar study reported mouse KC heterogeneity in terms of two groups: bone marrow-derived macrophages and resident hepatic ones¹². The former group appears to be CD11b+ KCs and the latter group consists of CD68 cells. These data are in line with the heterogeneity in KC that was observed in our hyperlipidemic mouse models, where CD11b+ cells (CD11b staining) were the inflammatory macrophages elevated upon HFC feeding and the CD68+ cells (CD68 staining) were the resident phagocytic cells that became swollen upon HFC feeding. Moreover, we observed an increase in size rather than an increase in the total number of CD68+ KCs. These observations demonstrate the central role of macrophage (CD11b staining) infiltration into the liver during the pathogenesis of NASH.

Two-hit model: steatosis is dissociated from hepatic inflammation

The “two-hit” model is still the dominant paradigm for understanding the etiology of NASH¹³. According to this model, hepatic steatosis is the critical first hit, thereby sensitizing the liver to further insults, collectively called the second hit. Consequently, the development of steatosis is generally considered as a prerequisite for further progression of the disease and liver damage. Until now, hepatic inflammation has always been associated with increased liver lipid levels and lipids are also viewed as a cause by generating ROS during oxidation¹⁴⁻¹⁷. However, recent reports have raised doubts about steatosis as a precondition for the development of inflammation during NASH progression. Mari *et al.* reported that free cholesterol (FC) accumulation, but not the accumulation of triglycerides (TG) or free fatty acids (FFA), plays a key role as a first hit agent since FC sensitizes hepatocytes to TNF- and Fas-induced apoptosis and NASH¹⁸. Further, the benign accumulation of liver TG was confirmed by Yamaguchi *et al.*, who demonstrated that TG themselves are not hepatotoxic, but protect the hepatocytes from lipotoxicity by buffering the accumulation of FFA¹⁹. In a model of diet-induced obesity, mice showing an overexpression of diacyl glycerol acyltransferase 1 (DGAT1), which catalyses the final step in triglyceride synthesis, in

adipocytes and macrophages were protected from macrophage activation and their accumulation in white adipose tissue. In addition, these mice were also protected from systemic inflammation and insulin resistance²⁰. Inhibition of triglyceride synthesis via DGAT2 antisense oligonucleotides improves liver steatosis but worsens liver damage, also suggesting that the accumulation of liver triglycerides could be a protective mechanism¹⁹. In line with these observations, we have previously shown that hyperlipidemic mouse models develop NASH after only 2 days on an HFC diet. Inflammation developed alongside, rather than subsequent to, steatosis²¹. Additionally, male hyperlipidemic mice failed to develop steatosis after 7 days of HFC feeding, whereas they did develop an inflammatory response in the liver, thereby completely disconnecting hepatic inflammation from steatosis. Moreover, steatosis was not associated with the reduction in hepatic inflammation by the deletion of scavenger receptors (Chapters 4 and 5), MPO (Chapter 6), immunization with heat-inactivated pneumococci (Chapter 9) and the increased inflammatory response by deletion of CYP27 (Chapter 7). Furthermore, normolipidemic mice failed to develop hepatic inflammation, although they developed equal levels of steatosis (Chapter 5). These studies clearly demonstrate the dissociation of hepatic steatosis and inflammation. Moreover, steatosis cannot be regarded as a pathology or disease in many instances, but rather as a physiological response to increased calorific consumption²². In human epidemiological studies, clinical data on the sequence of events during NASH progression are scarce, and therefore a direct correlation between steatosis and inflammation has not really been established. The concept of ‘multiple parallel hits’ might reflect more precisely the current knowledge of this metabolic disease and could explain why this disease can also occur in rather lean subjects²³.

Central role for cholesterol in NASH

Thus far, we could not establish a direct link between steatosis and the development of hepatic inflammation. Alternatively, we did find a correlation between hepatic inflammation, dietary cholesterol and plasma total cholesterol levels (Chapter 2). Moreover, additional data from our group indicated a prominent role of cholesterol metabolism during the development, inhibition and reversal of hepatic inflammation in NASH²⁴. Together, these findings indicate that plasma cholesterol, and not steatosis, determines the inflammatory response in the liver. After feeding hyperlipidemic mice a high fat diet low in cholesterol, plasma VLDL cholesterol levels were lowered and prevented the development of hepatic inflammation (Chapter 2). Moreover, after inhibiting the aggressive oxidant-generating neutrophil enzyme MPO, which is responsible for the oxidation of cholesterol, we demonstrated that hepatic inflammation was reduced (Chapter 6). Furthermore, Matsuzawa *et al.* reported that cholesterol can induce oxidative damage to the liver and that a high fat component in the diet further aggravates oxidative stress and NASH, possibly by inducing insulin

resistance and down-regulating the genes for antioxidant enzymes¹⁴. Therefore, these findings indicate that plasma cholesterol plays an important role in the inflammatory process in the liver. In humans, only a few studies have shown a correlation between LDL-conjugated dienes (as markers of oxLDL) and hepatic inflammation and fibrosis²⁵. The levels of LDL, possibly in a modified form, were also found to be increased in NASH patients²⁶. Moreover, NASH patients show often high levels of lipid peroxidation products such as oxLDL^{9,10}. All of these data confirm the clinical relevance of elevated cholesterol levels, possibly in the modified form, in the progression to NASH. Of note, in Chapter 3, we demonstrated that the inflammatory response in *APOE2ki* mice was abolished after 3 months on the HFC diet, although plasma cholesterol levels were still elevated. These data suggest that cholesterol mainly plays a role in the initiation of hepatic inflammation.

Cholesterol-loaded foamy Kupffer cells in NASH: does size really matter?

In the search for the molecular mechanism by which plasma cholesterol induces hepatic inflammation, we observed that KCs became increased in size, rather than in number, after hyperlipidemic mouse models were fed a high fat diet (Chapters 2 and 3). The observed bloated KCs, resembling foam cells in atherosclerotic plaques, have also been identified in other studies in the sinusoidal space in the liver^{27,28}. Strikingly, omitting cholesterol from the diet in these mice resulted in a dramatic inhibition of hepatic inflammation and the disappearance of these foamy KCs (Chapter 2). Therefore, the hypothesis was that due to the increased residence time of lipoproteins in the plasma of the hyperlipidemic animals, these lipoproteins are more prone to modification (e.g. becoming oxidized) and are subsequently scavenged by KCs in the liver. Modified lipoproteins and lipoprotein aggregates are internalized by macrophages via a scavenger receptor (SR) pathway. The SRs were initially described in cultured macrophages where they mediate cholesterol uptake from modified lipoproteins, determining the formation of lipid-loaded macrophages that resemble the foam cells present in atherosclerotic lesions²⁹⁻³¹. Like typical macrophages, KCs also express SRs and are thus capable of taking up modified lipoproteins³². Whereas clear effects of SR-A and CD36 on cholesterol uptake and foam cell formation were found *in vitro*³³, the results *in vivo* are less consistent. Findings in mouse models of atherosclerosis lacking one or both of these SRs ranged from reduced foam cell formation and atherosclerosis to no effect or to greater levels of foam cell formation and increased atherosclerosis³⁴⁻³⁶. The reasons for these discrepancies are unknown. It is important to note that there is still no clear evidence to show that the lipids which generate foam cells *in vivo* are derived from oxidized lipoproteins. In fact, there is considerable evidence that LDL-derived lipids can enter macrophages via SR-independent pathways, such as macropinocytosis^{37,38}. Therefore, it is possible that

although the total amount of lipids inside the KCs is identical, the lipids may be qualitatively different or the mechanism of internalization may trigger more inflammatory pathways. Our experiments with hyperlipidemic mice lacking one or both of these SRs support this concept, as we observed no correlation between the uptake of modified lipids and the size of the Kupffer cells (Chapters 2 and 5). Instead, we observed a correlation between the uptake of modified lipids and inflammation. In line with our observations, there is a growing appreciation of the numerous functions that SRs can play in addition to lipoprotein uptake, including their roles in inflammatory signal transduction and tissue homeostasis. Thus, in contradiction to the current view, our data indicate that not all foamy macrophages are inflammatory. This new concept may explain, to some extent, the controversial data in the atherosclerosis field regarding the role of SRs in atherosclerosis.

When the lipoproteins are taken up by the KCs, they are initially directed to the lysosomes where they are hydrolysed by lysosomal enzymes and transferred into the cytoplasm. However, in foam cells of advanced atherosclerotic plaques, cholesterol is not transferred into the cytoplasm but rather it accumulates in the lysosomes of the macrophages. In line with these observations, it was shown that the uptake of oxLDL *in vitro* is also associated with lysosomal trapping³⁹. Several lines of evidence indicate an association between lysosomal cholesterol accumulation and inflammation⁴⁰⁻⁴³. Accordingly, we hypothesized that scavenger receptor-mediated uptake of modified cholesterol is associated with reduced lysosomal cholesterol accumulation and, therefore, with less hepatic inflammation. In Chapters 4 and 5, KCs deficient for *Cd36*, *Msr1* or both showed reduced hepatic inflammation upon HFC feeding, despite unchanged total cellular cholesterol and foam cell formation. Remarkably, this reduction in inflammation was associated with reduced lysosomal cholesterol accumulation and increased cytoplasmic cholesterol accumulation inside the KCs. In Chapter 7, we performed a bone marrow transfer from mice lacking *Cyp27* into *Ldlr*^{-/-} mice. Deletion of *Cyp27* in the haematopoietic system resulted in significantly increased levels of lysosomal cholesterol accumulation in KCs, associated with increased inflammation. Interestingly, KCs in these mice were less foamy compared to those in the control mice. These data suggest that lysosomal cholesterol accumulation plays an important role in the development of hepatic inflammation. Besides this, the intracellular cholesterol distribution inside KCs seems to be a key factor in hepatic inflammation, rather than their foamy appearance.

It is known that the inflammatory process associated with atherosclerotic plaques is linked to the cytotoxicity and macrophage chemo-attractivity of oxLDL. Moreover, oxLDL is thought to be an atherogenic factor due to its localization in the macrophage-derived foam cells accumulated in human atherosclerotic lesions⁴⁴⁻⁴⁶. As NASH patients are often associated with high levels of lipid peroxidation products such as oxLDL, it has been suggested that the elevated levels of lipid peroxidation might make an

important contribution to the pathogenesis of NASH^{9,10}. In addition, increased MPO activity has previously been suggested to promote lipid peroxidation and it is also involved in the progression of NASH^{47,48} (Chapter 6). To investigate our hypothesis that hepatic inflammation is triggered by oxLDL, *Ldlr*^{-/-} mice were immunized with heat-inactivated pneumococci. We demonstrated that upon immunization, *Ldlr*^{-/-} mice showed reduced hepatic inflammation when fed an HFC diet, suggesting the induction of a protective immune response after immunization with heat-inactivated pneumococci and a causal role of oxLDL during diet-induced NASH.

NASH and atherosclerosis are two sides of the same coin

Currently, the importance of NASH and its strong relationship with the metabolic syndrome has stimulated interest in the possible role of NASH in the development of cardiovascular diseases (CVD). Overall, NASH patients have a higher risk of death than the general population, mainly due to CVD or liver-related causes⁴⁹. The close interrelationships between NASH, insulin resistance and the metabolic syndrome make it extremely difficult to dissect any cause-effect relationship leading to increased CVD risk. NASH might act as a stimulus for increasing whole-body insulin resistance and dyslipidaemia further, leading to accelerated atherosclerosis⁵⁰. Chronic inflammation is also a possible underlying mechanism linking NASH and atherosclerosis. Interleukin 6 and TNF are the major stimuli responsible for the increased hepatic production of C-reactive protein (CRP), fibrinogen, plasminogen activator inhibitor-1 (PAI-1) and other acute-phase proteins. It has been shown that levels of fibrinogen, PAI-1 and CRP, which are all CVD risk factors, are increased in NASH and correlate with histological severity, independently of the traditional risk factors and components of the metabolic syndrome⁵¹⁻⁵⁴. Moreover, NASH could also be linked to accelerated atherogenesis through the presence of abnormal lipoprotein metabolism. The magnitude of postprandial triglyceride and oxLDL responses after oral fat load tests has been shown to correlate with the severity of the liver histopathology⁵⁵⁻⁵⁹. In studying NASH, we used genetically modified animals that are also commonly used in atherosclerosis research. NASH was induced after only a few days of HFC feeding in these hyperlipidemic mice, whereas atherosclerosis only develops after a few weeks of HFC feeding, indicating that NASH develops prior to atherosclerosis. Therefore, we believe that NASH is not merely a marker but also an early mediator of atherosclerosis. In addition to the common mechanisms of these diseases, the current therapy options for both atherosclerosis and NASH are similar and include lipid lowering and anti-inflammatory drugs. In line with this concept, we also used fibrates (a PPAR alpha agonist, commonly used for atherosclerosis) as a tool for decreasing hepatic inflammation²⁴. In conclusion, our data points towards NASH as an early event in cardiovascular disease and to the liver as an important organ for studying the complex link between the lipid metabolism and inflammation manifested in the metabolic syndrome.

Clinical implications of the results

Diagnosis

At present, the available non-invasive markers for steatosis include a set of clinical symptoms and combinations of blood tests, such as ALT, AST and alkaline phosphatase liver enzymes, albumin, transaminases and gamma-glutamyl transferase. Although several of these markers are useful in the diagnostic evaluation of a patient with steatosis, they lack the specificity and sensitivity required for distinguishing NASH from simple steatosis⁶⁰. This situation represents a key clinical problem, as patients with NASH need close monitoring and frequent follow-ups.

The identification of cholesterol as a potential risk factor for the development of NASH may contribute to the regular criteria of the MetS for identifying patients who are at risk and who are in need of a liver biopsy, in combination with the current features of MetS. In addition to some of the existing clinical studies that reported correlations between cholesterol and hepatic inflammation and/or fibrosis^{25,26,61}, the value of plasma cholesterol as a diagnostic predictor of hepatic inflammation needs to be investigated in greater detail and in human subjects. In the present thesis, we showed that oxLDL plays an important role in the development of hepatic inflammation. As NASH patients are often associated with high levels of lipid peroxidation products such as oxLDL, elevated levels of lipid peroxidation products should be monitored in human subjects as they might have an important contribution to the pathogenesis of NASH^{9,10}. Since levels of 27HC were inversely correlated with hepatic inflammation and plasma lipid levels (Chapter 7), it is suggested that the level of 27HC in the plasma should be monitored as a possible biomarker of hepatic inflammation. The role of other oxysterols in relation to hepatic inflammation during NASH should also be further characterized. Furthermore, naturally occurring IgM autoantibody responses to modified LDL were also found to be inversely correlated with NASH (Chapters 4, 5 and 8), indicating their potency as non-invasive biomarkers of hepatic inflammation. Disturbed lysosomal function can also be reflected in the rest of the body, as we demonstrated that plasma acid phosphatase levels are dramatically reduced upon lysosomal cholesterol loading (Chapter 7). Therefore, other lysosomal enzymes could also be measured as potential markers for NASH. As a result, all of the abovementioned markers should be further investigated in human subjects. In conclusion, the data of this thesis suggest that modified cholesterol, 27HC, plasma levels of lysosomal enzymes and the naturally occurring IgM autoantibody responses, either alone or in combination with each other, could be used as non-invasive markers for the detection of NASH.

Therapy

Until now, the treatment options for NASH were very poor. Due to the fact that the mechanisms which trigger hepatic inflammation are unknown and that in the 'two hit' model steatosis is the first hit, steatosis is the main focus for therapy at the moment. Since simple hepatic steatosis is a benign process in the majority of patients, the pathogenesis of NASH might be different and therefore needs different treatment.

As plasma cholesterol was identified as a possible cause of hepatic inflammation in the present thesis, the existing cholesterol-lowering drugs may be effective for treating the inflammatory component of NASH, at least. Recent studies that used the HMG-coA reductase inhibitor atorvastatin showed significant improvements or even normalization in serum aminotransferase and lipid/cholesterol levels, as well as improvements in hepatic inflammation in patients with NASH⁶²⁻⁶⁴. Other reports also demonstrated the effectiveness and safety of atorvastatin in NASH patients with hyperlipidaemia^{62,65}. Similar results were obtained by a small pilot study using another HMG-coA reductase inhibitor, pravastatin, but without improvements in the fibrosis score⁶⁶. Moreover, a cumulative histopathological follow-up study evaluated the effect of statin therapy on histological outcomes in NASH patients demonstrated a significant reduction in liver steatosis at follow-up⁶⁷. In addition to their cholesterol-lowering abilities, statins possess direct anti-inflammatory effects, as was shown in *apoE*^{-/-} mice, in which cholesterol levels do not respond to statin treatment⁶⁸. Current evidence suggests that statin therapy has pleiotropic effects on the vascular endothelium, inflammation and plaque stability that extend beyond cholesterol reduction and may benefit patients regardless of their cholesterol level⁶⁹.

As TNF is one of the most important cytokines in the pathogenesis of NASH, anti-inflammatory drugs directed towards it would be of great importance in treating NASH. Pentoxifylline has recently emerged as point of new interest because of its TNF inhibitor property. Patients with NASH, who were treated with pentoxifylline for 6 to 12 months, showed improved ALT levels, HOMA-IR, steatosis, lobular inflammation and fibrosis⁷⁰⁻⁷². However, a reduction in ALT levels was not observed in a placebo-controlled trial of NASH patients treated with pentoxifylline in combination with exercise and diet for 3 months⁷³. As no biopsies were taken in this study, the conclusions should be drawn carefully, as plasma ALT levels are not specific or sensitive enough to be used as a marker for hepatic inflammation. Treating steatotic animals with resveratrol, which is another anti-inflammatory constituent acted partly mediated through a decrease in TNF production, demonstrated a reduction in hepatic steatosis and ALT levels⁷⁴.

The administration of 27HC can also be a potential target in treating NASH, as we showed that endogenous 27HC plays an important role in NASH (Chapter 7). Additional research is needed to prove the beneficial effects of exogenous 27HC on NASH *in vivo*.

Furthermore, the potential of other oxysterols with similar effects and anti-oxidants should also be further investigated. Pneumococcal immunization can be used as a vaccination protocol towards the therapy of NASH as we showed that pneumococcal immunization of hyperlipidemic mice led to a reduction in hepatic inflammation. Since infections with *S. pneumoniae* are common, and since pneumococcal vaccines are increasingly used, it will be important to determine the potential impact of induced anti-phosphorylcholine responses to NASH in humans. Therefore, these observations in hyperlipidemic mice need to be further investigated in order to assess the influence of pneumococcal infections on NASH, since phosphorylcholine-based immunization strategies may have therapeutic potential for ameliorating NASH, as well as other inflammatory diseases in which oxidized phospholipids are generated. In general, therapy should not focus on steatosis itself, but rather on cholesterol and its modifications. Therefore, the data in this thesis suggest that lipid lowering drugs, antioxidants, 27HC and pneumococcal immunization could be used as potential therapies for NASH. Also, Kupffer cells should be the main target for future therapies of NASH.

Novel findings of this thesis

Currently, the molecular mechanisms by which hepatic inflammation is triggered during NASH are unknown. Consequently, hepatic inflammation cannot be determined in a non-invasive way and therapy options for NASH are very poor. In the present thesis, we used hyperlipidemic mice to investigate the pathogenesis of NASH. The novel findings of the studies presented in this thesis are listed below.

Mechanisms and mouse models:

- The infiltration of macrophages into the liver, along with activation of KCs, is a primary event in the pathogenesis of NASH.
- Dietary cholesterol, rather than liver steatosis, is a risk factor for the early inflammatory response in the hyperlipidemic models of NASH.
- Long-term-high-fat feeding alone is not sufficient for development of the later consequences of NASH in all hyperlipidemic mouse models. The *Ldlr*^{-/-} mouse is the most suitable model for studying advanced NASH.
- Scavenger receptor expression (both CD36 and SR-A) by KCs in the liver increases the risk of NASH in the presence of high levels of plasma-modified lipoproteins.
- An HFC diet leads to increased hepatic MPO activity, promoting the progression of NASH.
- The intracellular distribution of cholesterol inside the KCs is an indicator of hepatic inflammation, rather than the size of foamy KCs.
- Lysosomal cholesterol accumulation is associated with hepatic inflammation and lysosomal enzymes are potential markers for diagnosing NASH.
- CYP27 can modulate lysosomal cholesterol accumulation inside KCs and also hepatic inflammation.
- Hepatic inflammation is triggered by the uptake of oxLDL into KCs. Naturally occurring IgM autoantibodies can bind to oxLDL and thereby prevent the harmful effects of oxLDL.

List of suggested non-invasive markers for the diagnosis of NASH:

- (Modified) Cholesterol
- MPO
- 27HC
- Levels and activity of lysosomal enzymes
- Naturally occurring IgM antibodies.

List of possible therapy options for NASH:

- Lipid lowering drugs
- Anti-oxidants
- 27HC
- Pneumococcal immunization

Summary of the main findings of this thesis (A) Under normal conditions, circulating cholesterol is taken up by Kupffer cells (KCs) and initially directed to lysosomes. After hydrolysis by lysosomal enzymes, free cholesterol (FC) is transferred into the cytoplasm. There, FC can be converted into cholesterol esters (CE), excreted from the cell by efflux mechanisms, or converted into 27-hydroxycholesterol (27-HC) by CYP27. The latter one can enhance the transition of cholesterol from the lysosomes to the cytoplasm via unknown mechanisms. (B) OxLDL forms immune complexes (IC) with IgM autoantibodies or it can be taken up by scavenger receptors CD36 and SR-A on the KCs. OxLDL is then directed to the lysosomes of the KCs, where it will accumulate via unknown mechanisms. Therefore, oxLDL cannot be converted into FC and it cannot leave the lysosomes; thus, it will trigger an inflammatory response.

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Summary

Summary

In **Chapter 1**, NASH was introduced as the hepatic manifestation of the metabolic syndrome. It is characterized by lipid accumulation (steatosis) in combination with hepatic inflammation. Currently, NASH is thought to develop via the ‘two-hit’ model. According to this hypothesis, hepatic steatosis is the critical first hit, thereby sensitizing the liver for further insults, collectively called the second hit. However, it is still not clear why not every patient with hepatic steatosis will progress further to develop NASH. Although several processes have been identified that occur during the development of liver inflammation, the exact trigger(s) that induces liver inflammation and worsens disease outcome remains to be identified. Consequently, hepatic inflammation cannot be determined in a non-invasive way and therapy options for NASH are very poor. In the present thesis, we used hyperlipidemic mouse models to establish the mechanisms by which hepatic inflammation is triggered.

In **Chapter 2**, we hypothesized that steatosis is not mandatory for the development of hepatic inflammation. We showed that dietary cholesterol, rather than liver steatosis, is a risk factor for the early inflammatory response in the hyperlipidemic models of NASH. Moreover, we described for the first time how KCs in mice fed an HFC diet have a bloated ‘foamy’ appearance. These KCs are full with cholesterol and resemble the foam cells in atherosclerotic plaques. These findings indicate that dietary cholesterol, possibly in the form of modified plasma lipoproteins, is an important risk factor for the progression to hepatic inflammation in diet-induced NASH.

In **Chapter 3**, we hypothesized that long-term HFC feeding in hyperlipidemic mice would induce the advanced stages of NASH, such as fibrosis. Therefore, *APOE2ki* and *Ldlr*^{-/-} mice were put on a HFC diet for 3 months. Hepatic inflammation was only sustained in *Ldlr*^{-/-} mice, whereas it was completely abolished in *APOE2ki* mice. Moreover, *Ldlr*^{-/-} mice developed hepatic fibrosis after a prolonged period of HFC feeding. We concluded that the *Ldlr*^{-/-} mouse model is the most suitable model for studying the later consequences of NASH.

In **Chapter 4**, we hypothesized that a decreased uptake of modified lipoproteins by KCs would lead to a reduced inflammatory response in the liver. Therefore, we investigated the role of two scavenger receptors, CD36 and MSR1, which play an important role in the uptake of modified lipoproteins in NASH. For this purpose, we transplanted *Ldlr*^{-/-} mice with bone marrow from *Cd36*^{-/-}/*Msr1*^{-/-} mice and fed the mice a HFC diet. Hepatic inflammation, apoptosis and fibrosis were all reduced in these *Cd36*^{-/-}/*Msr1*^{-/-} transplanted mice compared to control mice. Surprisingly, the effect on hepatic inflammation was independent of the foamy KCs. These data suggest that scavenger receptor expression by KCs in the liver increases the risk of NASH in the presence of high levels of plasma-modified lipoproteins.

In **Chapter 5**, we hypothesized that the scavenger receptors CD36 and MSR1 contribute similarly and independently to the progression of diet-induced NASH. The *Ldlr*^{-/-} mice were depleted from their haematopoietic system and were transplanted with bone marrow from mice lacking either *Cd36* or *Msr1*. After 3 months of HFC feeding, we found that CD36 and MSR1 contributed in a similar manner to the progression of NASH in the presence of high levels of plasma-modified lipoproteins. Moreover, we found a clear association between lysosomal cholesterol accumulation inside the KCs and hepatic inflammation. We concluded that the effect of both receptors on hepatic inflammation is modulated via disturbed intracellular cholesterol distribution.

In **Chapter 6**, we hypothesized that MPO, an aggressive oxidant-generating neutrophil enzyme which can also lead to the oxidation of cholesterol, plays an active role in the development of NASH. By transplanting *Mpo*^{-/-} bone marrow into *Ldlr*^{-/-} mice, we demonstrated that *Mpo* deficiency strongly reduced hepatic inflammation and fibrosis. Moreover, visceral adipose tissue inflammation was also reduced in mice lacking *Mpo*. Collectively, this chapter supports an important new role for neutrophils in the pathogenesis of NASH by affecting important aspects such as inflammation, oxidative stress, and fibrogenesis.

In **Chapter 7**, we hypothesized that CYP27 is able to redirect the intracellular cholesterol pool from the lysosomes to the cytoplasm of the KCs *in vivo*, thereby reducing hepatic inflammation. CYP27 is a P450 enzyme, responsible for the conversion of cholesterol into 27-hydroxycholesterol (27HC), a well-known oxysterol. Previously, 27HC was shown to reduce lysosomal cholesterol accumulation *in vitro*. In this chapter, *Ldlr*^{-/-} mice were depleted from *Cyp27* in their hematopoietic system by bone marrow transplantation. We demonstrated that *Cyp27*^{-/-} transplanted mice show increased lysosomal cholesterol accumulation and hepatic inflammation. Therefore, we concluded that CYP27 can modulate lysosomal cholesterol accumulation inside KCs, and thereby also hepatic inflammation.

In **Chapter 8**, we hypothesized that hepatic inflammation is triggered by uptake of oxLDL by KCs. Therefore, we immunized *Ldlr*^{-/-} mice with heat-inactivated pneumococci, as antibodies against these bacteria have the same epitope as naturally occurring antibodies against oxidized LDL. We demonstrated that hepatic inflammation and plasma cholesterol levels were significantly decreased after treating *Ldlr*^{-/-} mice with these heat-inactivated pneumococci. We concluded that pneumococcal immunization could lead to a therapeutic vaccination protocol towards the prevention of NASH.

Finally, **Chapter 9** discusses the major findings of this thesis, places them in the context with the current state of this field and addresses the clinical implications of the results.

Samenvatting

Samenvatting

In **hoofdstuk 1** werd NASH geïntroduceerd als de hepatische manifestatie van het metabool syndroom. NASH wordt gekenmerkt door een ophoping van vet (steatose) in combinatie met ontsteking in de lever. Momenteel wordt er vanuit gegaan dat NASH zich ontwikkelt via het "twee-hit"-model. Volgens deze hypothese is hepatische steatose de kritieke eerste stap, waardoor de lever gevoelig wordt gemaakt voor verdere schade. Het is echter de vraag waarom niet iedere patiënt met leversteatose uiteindelijk NASH zal ontwikkelen. Er zijn al verschillende processen geïdentificeerd die een rol spelen tijdens leverontsteking, maar de exacte oorzaak van deze ontsteking moet nog geïdentificeerd worden. Bijgevolg gebeurt de diagnose van leverontsteking op een niet-invasieve manier bepaald worden en is er nog geen therapie voor NASH beschikbaar. In dit proefschrift hebben we gebruik gemaakt van hyperlipidemische muismodellen om de mechanismen die leverontsteking veroorzaken vast te stellen.

In **hoofdstuk 2** hadden we de hypothese dat steatose geen vereiste is voor de ontwikkeling van leverontsteking. We hebben aangetoond dat cholesterol een risicofactor is voor de initiatie van de ontstekingsreactie in de hyperlipidemische modellen voor NASH. Bovendien hebben we voor de eerste keer beschreven dat de KCs na een HFC dieet een opgeblazen 'schuimig' uiterlijk hebben en op schuimcellen in atherosclerotische lesies lijken. Deze bevindingen geven ook aan dat cholesterol afkomstig uit het dieet, waarschijnlijk in de vorm van gemodificeerde plasma lipoproteïnen, een belangrijke risicofactor is voor de progressie tot leverontsteking tijdens NASH.

In **hoofdstuk 3** werd de hypothese gesteld dat een langdurig HFC dieet in hyperlipidemische muismodellen de latere gevolgen van NASH, zoals fibrose, zal induceren. Voor deze hypothese te testen werden *APOE2ki* en *Ldlr*^{-/-} muizen gedurende 3 maanden op een HFC dieet gezet. Leverontsteking was enkel nog aanwezig in de *Ldlr*^{-/-} muizen, terwijl deze volledig was verdwenen in *APOE2ki* muizen. Bovendien ontwikkelden de *Ldlr*^{-/-} muizen ook leverfibrose na deze lange periode van HFC dieet. We besluiten dat de *Ldlr*^{-/-} muizen het meest geschikte model is om de latere gevolgen van NASH te bestuderen.

In **hoofdstuk 4** hadden we de hypothese dat een verminderde opname van gemodificeerde lipoproteïnen door KCs zou leiden tot verminderde leverontsteking. Daarom onderzochten we de rol van twee scavenger receptoren, CD36 en MSR1, die een belangrijke rol spelen in de opname van gemodificeerde lipoproteïnen. Voor dit doel hebben we *Ldlr*^{-/-} muizen getransplanteerd met beenmerg van *Cd36*^{-/-}/*Msr1*^{-/-} muizen en voedde de muizen een HFC dieet. Leverontsteking, apoptose en fibrose waren verlaagd in deze *Cd36*^{-/-}/*Msr1*^{-/-} getransplanteerde muizen in vergelijking met de controle muizen. Verrassend was dat het effect op leverontsteking onafhankelijk was van de schuimige KCs. Deze gegevens suggereren dat scavenger receptor expressie door KCs in de lever het risico voor NASH verhogen.

In **hoofdstuk 5** hypotheesden we dat de scavenger receptoren CD36 en MSR1 een gelijkaardige bijdrage leveren aan de progressie van dieet-geïnduceerde NASH. *Ldlr*^{-/-} muizen werden van hun hematopoietische systeem ontzien en werden getransplanteerd met beenmerg van *Cd36*^{-/-} en *Msr1*^{-/-} muizen. Na 3 maanden van HFC dieet hebben we aangetoond dat zowel CD36 als MSR1 bijdragen aan NASH en onafhankelijk zijn van elkaar. Bovendien hebben we een duidelijk oorzakelijk verband gevonden tussen de lysosomale stapeling van cholesterol in de KCs en leverontsteking. We concluderen dat het effect van beide receptoren op leverontsteking wordt gemoduleerd via verstoorde intracellulaire cholesterol verdeling in de KCs.

In **hoofdstuk 6** werd de hypothese gesteld dat MPO, een agressief oxidantengenererend neutrofiel enzym, wat bovendien ook de oxidatie van cholesterol kan veroorzaken, een actieve rol speelt in de ontwikkeling van NASH. Door het uitvoeren van een beenmergtransplantatie in *Ldlr*^{-/-} muizen uit te voeren met *Mpo*^{-/-} beenmerg, hebben we aangetoond dat *Mpo*-deficiëntie leverontsteking en fibrose sterk vermindert. Bovendien was de viscerale vetweefselontsteking ook verminderd in deze *Mpo*^{-/-} getransplanteerde muizen. Daarom kunnen we besluiten dat er in de pathogenese van NASH een belangrijke nieuwe rol voor neutrofielen aanwezig is, door het beïnvloeden van belangrijke aspecten zoals ontsteking, oxidatieve stress, en fibrogenese.

In **hoofdstuk 7** hadden we de hypothese dat CYP27 het intracellulaire cholesterol in de KCs van de lysosomen naar het cytoplasma zou kunnen verplaatsen, waardoor leverontsteking zou verminderen. CYP27 is een P450-enzym, verantwoordelijk voor de omzetting van cholesterol in 27-hydroxycholesterol (27HC), een bekende oxysterol. Voorheen is *in vitro* al aangetoond dat 27HC de lysosomale stapeling van cholesterol kan doen verminderen. In dit hoofdstuk werden *Ldlr*^{-/-} muizen getransplanteerd met *Cyp27*^{-/-} beenmerg. We toonden aan dat *Cyp27*^{-/-} getransplanteerde muizen een verhoogde lysosomale cholesterol stapeling hadden, in combinatie met verhoogde leverontsteking. We concluderen dat CYP27 de lysosomale stapeling van cholesterol in KCs kan moduleren en daarmee ook de leverontsteking.

In **hoofdstuk 8** hypotheesden we de opname van oxLDL door KCs leverontsteking veroorzaakt. Daarom hebben we *Ldlr*^{-/-} muizen geïmmuniseerd met hitte-geïnactiveerd pneumococci, omdat deze hetzelfde epitoom hebben als natuurlijk voorkomende antilichamen tegen geoxideerd LDL. We hebben aangetoond dat zowel leverontsteking als plasma cholesterol waarden waren afgenomen na de behandeling van *Ldlr*^{-/-} muizen met deze hitte-geïnactiveerd pneumococci. Daarom besluiten we dat een vaccinatie met hitte-geïnactiveerde pneumococci kan dienen als preventie van NASH.

Tot slot werden in **hoofdstuk 9** de belangrijkste resultaten van deze thesis besproken in het licht van de huidige stand van zaken. Daarenboven werden de mogelijke klinische toepassingen en gevolgen toegelicht.

Dankwoord

Dankwoord

Eindelijk is het zover, mijn boekje is af 😊. Naast het harde werk, was het ook een periode van plezier en mooie ervaringen. Ik heb veel bijgeleerd en ben ook enorm trots op mijn werk. Dit heb ik natuurlijk niet enkel aan mezelf te danken, maar aan iedereen die mij de afgelopen periode op de een of andere manier gesteund of geholpen heeft, zowel in als naast het lab.

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student bij ons in de groep geweest en het was altijd zeer fijn om met u samen te werken. Ook grappig dat we vroeger al bij elkaar op school hebben gezeten en al tegen elkaar hebben gevoetbald, de wereld is precies toch klein. Ik ben blij dat ge mij gaat 'opvolgen' en ben er zeker van dat ge dat goed gaat doen. Luran, Nadine, Ine, Marten en Emiel, de macrofaag-AIO's, het was zeer aangenaam om met jullie samen te werken. Jullie zijn een leuke bende, waar ik ook veel plezier mee heb beleefd. Jullie waren niet enkel collega's maar ook vrienden.

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Veerle

Curriculum Vitae

Curriculum Vitae

Veerle Bieghs was born on February 21st, 1985 in Leut, Belgium. In 2003, she acquired her high school diploma at the Stedelijke Humaniora Dilsen, Belgium. She started her bachelor education in molecular life sciences at the transnational University Limburg (tUL; campus Hasselt, Belgium) in September 2003 and finalized her bachelor study in 2006. Subsequently, she participated in the master of “clinical molecular life sciences” at Hasselt University, Belgium. For this degree, she performed an internship of 6 months at the department of General Surgery, Maastricht University, where she investigated the role of inflammation and oxidative damage in morbidly obese persons and graduated with distinction in July 2007.

In August 2007, she started her PhD program at the department of Molecular Genetics within the school of Health, Medicine and Life Sciences, under supervision of Dr. Ronit Shiri-Sverdlov and Prof. Dr. Marten Hofker. The topic of her research was the unraveling of molecular pathways that are responsible for the initiation of NASH. During this time, she mentored several master students during their internships. She also actively attended international and national conferences, among which the European Lipoprotein Club (ELC), European Association for the study of the Liver (EASL), The International Conference on the Bioscience of Lipids (ICBL), the Deuel Conference on Lipids, the Dutch Atherosclerosis Society (DAS), Nederlandse Lipoproteinen Conferentie (NLC) and the Dutch Liver Science Retraite (DLSR). During these conferences, she was able to present her research both orally and by poster presentation. She obtained the first prize for best poster presentation at the 12th DAS symposium in Ede 2009. In addition, she achieved a full bursary and a waived registration bursary for the EASL Special Conference on NAFLD/NASH and related metabolic disease in Bologna, Italy and the EASL Monothematic Conference entitled “Signaling in the Liver”, Amsterdam, respectively. Moreover, she received a travel award at the Deuel Conference on Lipids in Napa, California.

From January 2011 until present, she continues her work at the department of Molecular Genetics in Maastricht, since she obtained funding from Maastricht University (Kootstra Talented fellowship). This gave her the opportunity to investigate the role of oxidized cholesterol in NASH in more detail and in human subjects.

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- Bieghs V, Vlassaks E, Custers A, Van Gorp PJ, Gijbels MJ, Bast A, Bekers O, Zimmermann LJ, Lütjohann D, Voncken JW, Gavilanes AW, Kramer BW, Shiri-Sverdlov R. Chorioamnionitis induced hepatic inflammation and disturbed lipid metabolism in fetal sheep. *Pediatr Res*. 2010 Aug 16.
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- Rensen R, Bieghs V, Arfianti E, Bakker J, Shiri-Sverdlov R, Greven JW, Buurman J. Myeloperoxidase promotes progression of non-alcoholic steatohepatitis in mice. Submitted
- Bieghs V, van Gorp PJ, Wouters K, Marion Gijbels MJ, Lütjohann D, Staels B, van Bilsen M, Hofker MH, Shiri-Sverdlov R. The LDL receptor knock-out mouse is a physiological model particularly vulnerable for studying inflammation in non-alcohol steatohepatitis. Submitted
- Bieghs V, van Gorp PJ, Verheyen F, Hendriks T, Gijbels MJ, Leitersdorf E, Hofker MH, Lütjohann D, Shiri-Sverdlov R. CYP27: a novel player in diet-induced non-alcoholic steatohepatitis. In preparation.
- Bieghs V, Verheyen F, van Gorp PJ, Walenbergh S, Gijbels MJ, Hofker MH, Binder C, Shiri-Sverdlov R. Pneumococci: a new treatment for non-alcoholic steatohepatitis? In preparation.

Oral presentations

- December, 2010: Masterclass Ben Suratt (Maastricht)
Title: Non-alcoholic steatohepatitis: cholesterol and Kupffer cells as partners in crime.
- October, 2010: Dutch Liver Science Retraite (Nederlandse Vereniging voor Hepatologie), Westerbork, the Netherlands
Title: CYP27: a novel key player during diet-induced NASH
- September, 2010: Nederlandse Lipoproteinen Conferentie (NLC), Leiden, the Netherlands
Title: Are all hyperlipidemic mice suitable for NASH?
- September, 2010: Seminar Genetics and Cell Biology, Maastricht University Medical centre (MUMC)
Title: CYP27: a novel key player during diet-induced NASH
- September, 2010: European Lipid Conferene (ELC), Tutzing, Germany
Title: CYP27: a novel key player during diet-induced NASH
- September, 2010: Masterclass Jonathan Smith (Maastricht)
Title: Role of Scavenger Receptor A and CD36 in diet-induced non-alcoholic steatohepatitis
- June, 2010: Masterclass Siamon Gordon (Maastricht)
Title: Role of Scavenger Receptor A and CD36 in diet-induced non-alcoholic steatohepatitis
- March, 2010: Dutch Atherosclerosis Society (DAS), Ede, the Netherlands
Title: Role of Scavenger Receptor A and CD36 in diet-induced non-alcoholic steatohepatitis
- December, 2009: Liverday, Maastricht University Medical centre (MUMC)
Title: Hepatic inflammation: are all hyperlipidimic models the same?
- October, 2009: Seminar Genetics and Cell Biology, Maastricht University Medical centre (MUMC)
Title: Relationship between Kupffer cells and liver inflammation
- July, 2009: Liverday, Maastricht University Medical centre (MUMC)
Title: Relationship between Kupffer cells and liver inflammation
- February, 2009: Liverday, Maastricht University Medical centre (MUMC)
Title: Modulating liver inflammation: a crucial role for cholesterol
- May 2008: Seminar Genetics and Cell Biology, Maastricht University Medical centre (MUMC)
Title: Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of non-alcoholic steatohepatitis.
- May 2008: NUTRIM meeting, Maastricht University Medical centre (MUMC)
Title: Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of non-alcoholic steatohepatitis.

Poster presentations

- March, 2011: Deuel Conference on Lipids, Napa, California.
Title: CYP27: a novel player in diet-induced non-alcoholic steatohepatitis.
- March, 2011: Deuel Conference on Lipids, Napa, California.
Title: CD36 and SR-A contribute similarly and independently to non-alcoholic steatohepatitis in hyperlipidemic mice.
- January, 2011: Plenary meeting, Department of Genetics and Cell Biology, Maastricht University Medical centre (MUMC).
Title: CYP27: a novel player in hepatic inflammation.
- November, 2010: NUTRIM day, Maastricht University Medical centre (MUMC).
Title: CYP27: a novel player in hepatic inflammation.
- September, 2010: European Lipid Conferene (ELC), Tutzing, Germany.
Title: Are all hyperlipidemic mice suitable for NASH?
- February, 2010: EASL Monothematic Conference entitled "Signaling in the Liver".
Title: Combined deletion of Scavenger Receptor A and CD36 in hematopoietic cells decreases diet induced non-alcoholic steatohepatitis in hyperlipidemic mice.
(Awarded with a waived registration bursary)
- January, 2010: Plenary meeting, Department of Genetics and Cell Biology, Maastricht University Medical centre (MUMC).
Title: Combined deletion of Scavenger Receptor A and CD36 in hematopoietic cells decreases diet induced non-alcoholic steatohepatitis in hyperlipidemic mice.
- November, 2009: NUTRIM day, Maastricht University Medical centre (MUMC).
Title: Combined deletion of Scavenger Receptor A and CD36 in hematopoietic cells decreases diet induced non-alcoholic steatohepatitis in hyperlipidemic mice.
- September, 2009: EASL Special Conference on NAFLD/NASH and related metabolic disease, Bologna, Italy.
Title: Differences in diet induced hepatic inflammation between LDL receptor knock-out and APOE2 knock-in mouse models. **(Awarded with a full bursary)**
- March, 2009: Dutch Atherosclerosis Society (DAS), Ede, the Netherlands
Title: Combined deletion of Scavenger Receptor A and CD36 in hematopoietic cells decreases diet induced non-alcoholic steatohepatitis in hyperlipidemic mice.
(Awarded with a poster price)
- February, 2009: Plenary meeting, Department of Genetics and Cell Biology, Maastricht University Medical centre (MUMC); NUTRIM day, Maastricht University Medical centre (MUMC).
Title: Differences in diet induced hepatic inflammation between LDL receptor knock-out and APOE2 knock-in mouse models
- August, 2008: The International Conference on the Bioscience of Lipids (ICBL), Maastricht, the Netherlands.
Title: Differences in diet induced hepatic inflammation between LDL receptor knock-out and APOE2 knock-in mouse models

- March, 2008: Dutch Atherosclerosis Society (DAS), Ermeloo, the Netherlands
Title: Lowering plasma VLDL inhibits the development of liver inflammation in hyperlipidemic mice

Fellowships / Awards

- Travel award at the Deuel Conference on Lipids (2011), Napa, California
- Kootstra talent fellowship (2010).
- Waived registration bursary for the EASL Monothematic Conference entitled "Signaling in the Liver" (2010), Amsterdam, the Netherlands.
- Full bursary for the EASL Special Conference on NAFLD/NASH and related metabolic disease, Bologna, Italy (2009).
- Poster award at the Dutch Atherosclerosis Society (DAS) in the lipid metabolism and metabolic diseases section, Ede, the Netherlands (2009).

Patent applications

- Method for treating fatty liver diseases, in particular nonalcoholic steatohepatitis (EP10172304.7).
- Method for diagnosing fatty liver diseases, in particular non-alcoholic steatohepatitis (EP10172295.7).
- Method for treating liver inflammation, fibrosis and non-alcoholic steatohepatitis (EP11155267.5)

List of abbreviations

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A	ABC	Adenosine Triphosphate binding cassette
	ACAA	Acetyl coA acyltransferase
	acLDL	Acetylated low density lipoprotein
	ACOX	Acyl CoA oxidase
	ADFP	Adipose differentiation related protein
	ALT	Alanine transaminase
	APO	Apolipoprotein
	AST	Aspartate aminotransferase
	ATF	Activating transcription factor
	AU	Arbitrary unit
B	BMT	Bone marrow transplantation
	BD	Beta-defensin
C	Cat	Catalase
	Cav	Caveolin
	CETP	Cholesteryl ester transfer protein
	CM	Chylomicron
	CMR	Chylomicron remnant
	COL	Collagen
	COX	Cytochrome oxidase
	CPT	Carnitine acyl-carnitine translocase
	CROT	Carnitine octanoyltransferase
	CuOx	Copper-oxidized
	CVD	Cardiovascular disease
	CYP	Cytochrome P450
E	Ech1	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase
	EMR	Erythromycin resistance
	ER	Endoplasmatic reticulum
F	FA	Fatty acid
	FABP	Fatty acid binding protein
	FAS	Fatty acid synthase
	FASL	Fatty acid synthase ligand
	FD	Familial dysbetalipoproteinemia
	FFA	Free fatty acid
	FH	Familial hypercholesterolemia
	FXR	Farnesoid X receptor
G	GC-MS	Gas Chromatography mass spectrometry
	GSTA	Glutathione S-transferase
H	Hadha	Hydroxyacyl-CoA-dehydrogenase/3-ketoacyl-CoA thiolase enoyl-CoA hydratase, alpha subunit

	HC	Hydroxy-cholesterol
	HDL	High density lipoprotein
	HE	Heamatoxin/Eosin
	HFC	High fat high cholesterol
	HFnC	High fat no added cholesterol
	HLP	Hyperlipoproteinemia
	HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
	HMOX	Hema oxygenase
	HOCl	Hypochlorous acid
I	ICAM	Intracellular adhesion molecule
	IDL	Intermediate density lipoprotein
	IFN	Interferon
	Ig	Immunoglobulin
	IKKb	IkB kinase beta
	IL	Interleukin
	Insig	Insulin induced gene
	IR	Insulin resistance
	Itgam	Integrin alpha M
J	JNK	C-Jun N-terminal kinase
K	KC	Kupffer cell
	Ki	Knock-in
	Ko	Knock-out
L	LDL	Low density lipoprotein
	LDLR	Low density lipoprotein receptor
	LipC	Lipin C
	LOX	Lectin like oxidized low density lipoprotein receptor
	LPL	Lipoprotein lipase
	LPS	Lipopolysacharice
	LRP	Low density lipoprotein receptor related protein
	LSEC	
	LXR	Liver X receptor
M	M6prbp1	Mannose-6-phosphate receptor binding protein 1
	MCD	Methionine choline-deficient
	MCP	Monocyte chemotractant protein
	MDA	Malondialdehyde
	MetS	Metabolic syndrome
	MIP	Major intrinsic protein
	MMP	Matrix metallo proteinase
	modLDL	Modified low density lipoprotein
	MPO	Myeloperoxidase
	MRI	Magnetic resonance imaging

	MSR	Macrophage scavenger receptor
	MTTP	Microsomal triglyceride transfer protein
N	NAFLD	Non-alcoholic fatty liver disease
	NASH	Non-alcoholic steatohepatitis
	NFkB	Nuclear factor kappa B
	NPC	Niemann pick type C
O	ORO	Oil Red O
	oxLDL	Oxidized low density lipoprotein
P	PAI	Plasminogen activator inhibitor
	PAS	Periodic acid Schiff
	PC	Phosphorycholine
	POR	P450 oxidoreductase
	PPAR	Peroxisome proliferating activated receptor
	PPIA	Cyclophilin A
	PSOX	Receptor for phosphatidylcholine and oxidized low density lipoprotein
	PUFA	Poly unsaturated fatty acid
Q	QPCR	Quantitative polymerase chain reaction
R	ROS	Reactive oxygen species
S	SAA	Serum amyloid A
	SCD	Stearoyl-CoA desaturase
	Scarb1	Scavenger receptor class B type 1
	SOCS	Suppressor of cytokine signaling
	SR	Scavenger receptor
	SRBI	Scavenger receptor B type I
	SREBP1c	Sterol regulatory element binding protein 1c
	STAT	Signal transducers and activators of transcription
T	TC	Total cholesterol
	TG	Triglycerides
	TGF	Transforming growth factor
	TIMP	Tissue inhibitors of metalloproteinases
	TLR	Toll like receptor
	TNF	Tumor necrosis factor
	-tp	transplanted
	TZD	Thiazolidinediones
V	VCAM	Vascular cell adhesion molecule 1
	VEGFA	Vascular endothelial growth factor A
	VLDL	Very low density lipoprotein
	VLDLR	Very low density lipoprotein receptor
W	WT	Wild type